The physiological and molecular response of *StCDF* antisense lncRNA to drought stress in *Solanum tuberosum*

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Abbreviations

AA Amino acids
ABA Abscisic acid
Bp Base pair
CDF Cycling Dof Factor
CK Cytokinin
CO Constans
DOF DNA binding with one finger
ES Epidermal cell size
FKF1 Flavin-Binding Kelch Repeat F-Box
FT Flowering locus T
GA Gibberellin
GI Gigantea
G_s Stomatal conductance
LD Long days
LncRNA Long non-coding RNA
MES 2-(N-morpholino) ethanesulfonicacid
RNAi RNA interference
SD Short days
SD Stomata density
SI Stomatal index
SS Stomatal size
WT Wild type

Abstract

Potato (*Solanum tuberosum*) is one of the most important crops in the word. Potato originates from the Andean regions of South America and evolved short-day-dependent tuber formation as a reproductive strategy. The transition from vegetative to reproductive growth is controlled by photoperiodic information via the circadian rhythm. In previous studies, we have identified the central regulator called *StCDF1*. This gene belongs to the DOF (DNA-binding with one finger) transcription factors family and it controls tuberization and plant life cycle. Apart from the wild type (WT) allele (*StCDF1.1*) that regulates tuberization under short day conditions, there are two mutated alleles which induce tuberization under long day conditions; StCDF1.2 with a 7bp insertion and StCDF1.3 with an 865bp insertion. Both StCDF1.2 and StCDF1.3 have a damaged regulatory domain and increased stability during the day. In addition, we have detected the presence of a 200bp lncRNA we have named *StFLORE*, transcribed in an opposite orientation covering the second exon of the StCDF1 coding region. However, in *StCDF1.3*, *StFLORE* is unlikely to be functional since the large insertion displaced *StFLORE*.

In previous observations, the *StCDF1* knock down plants were highly tolerant to drought and the *StFLORE* expression was enhanced under drought condition in these plants. These preliminary results show a potential link between *StCDF1-StFLORE* locus and drought tolerance. In potato breeding, reducing water loss by having lower value of transpiration is considered to be a beneficial trait in responding to drought. In this study, we further look into how different combinations of StCDF1 alleles and different expression levels of StCDFs affect transpiration related stomatal characteristics, such as stomatal ABA responsiveness, stomatal size, stomatal density, etc. By calculating stomatal conductance (g_s) to estimate transpiration level, we found that *StCDF1* knock down plants have a very low calculated g_s value and this result is in agreement with the drought tolerance phenotype. Moreover, overexpressing StCDF1 leads to an abscisic acid (ABA) insensitive stomata phenotype and this result provides a possible explanation for the negative effect of StCDF1 in drought response. In this study, we also examined the *StFLORE* expression level in both control and ABA treatment conditions. As expected, the ABA treatment enhanced StFLORE expression and this result delivered the message that StFLORE has a positive effect on drought response under the influence of ABA.

Key words: *Solanum tuberosum*, Potato, CDF1, *StFLORE*, Drought Tolerance, Tuberization, Transpiration.

1. Introduction

Potato (*Solanum tuberosum*) is the fourth largest food crop, following rice, wheat, and corn (Alva, Fan, Qing, Rosen, & Ren, 2011). The total world potato production had achieved 375 million metric tons (MMT) at 2014 (FAOSTAT, 2014), and this number has not changed much today. The previous studies indicate the increasing aridity has become one of the most threatening factors to crops productivity (Godfray *et al.* 2010), especially for potato. Despite the fact that potato stands out for its high water-use efficiency, it is also highly sensitive to the drought stress. In 2010, the severe summer drought resulted in 30% (around 7 million metric tons) loss in potato production in Russia (GAIN, 2010). With the increasing uncertainty as a result of climate change, this situation could reappear in many other countries in the future. Potato's drought sensitivity is thought to result from the regulation of transpiration from leaves and shallow root system. The preliminary data in our research group indicates that the factors regulating tuber formation are closely connected to the regulation of drought susceptibility and tolerance.

Transpiration is the essential process by which water is moved through plant from roots to leaves, where water will be released to the atmosphere via stomata (Lloyd, 1908; Fanourakis *et al.*, 2014). In plants, stomata control the water evaporation and the gas exchange, that is essential for photosynthesis. Under non-drought conditions, high light intensity and high humidity lead to negative cell's electrical potential, increasing in the stomatal aperture (Christodoulakis, Menti & Galatis, 2002). However, under drought condition, to prevent water loss by transpiration, plants accumulate a stress-response hormone called abscisic acid (ABA) that induces the rapid closing of stomata by regulating Ca^{+} and K^{+} channels (Cai *et al.,* 2017).

The variation in stomatal anatomical features and stomatal closing ability influences transpiration in a diverse way. Recent research indicates that lower stomatal density and larger stomatal size had reduced transpiration, and increased tolerance to limited water availability (Doheny-Adams *et al*.,2012). For stomatal closing ability, the genotype was recognized as the major role in stomatal response to drought (Skirycz and Inze, 2010), but the genetic variation of stomatal closing ability hasn't been explored much yet (Fanourakis *et al.*, 2014). In potato, several genes have been reported to be modulated under drought stress (Obidiegwu, Jones, & Prashar, 2015). The recently unpublished studies by J. Abelenda *et al*. demonstrated that silencing the gene *StCDF1* increase drought stress (personal communication) by modulating stomatal anatomical features and stomatal closing ability.

The stomatal anatomical features are highly depending on the development of stomata which involved complicated genes and several plant hormones. The initiation of stomata has been known that multiple plant hormones involved in this regulation. Both auxin and ABA has been reported that they can negatively effect on stomatal density in *Arabidopsis* (Tanaka, Nose, Jikumaru & Kamiya, 2013; Saibo, *et al.,* 2003). However, gibberellin (GA) treatment in *Arabidopsis* and

enhanced cytokinin (CK) signalling in tomato increase the stomatal density (Saibo, *et al*., 2003; Farber, Attia & Weiss, 2016). The knowledge about stomatal size regulation is still quite limited. By studying the stomatal formation in *Arabidopsis thaliana*, this process involves at least one asymmetric division as well as a single symmetric division (Qi, & Torii, 2018). Some researchers suggest the stomatal and epidermal cells enlargement can be result from endoreduplication (Kondorosi, Roudier, & Gendreau, 2000; Tanaka, Nose, Jikumaru & Kamiya, 2013).

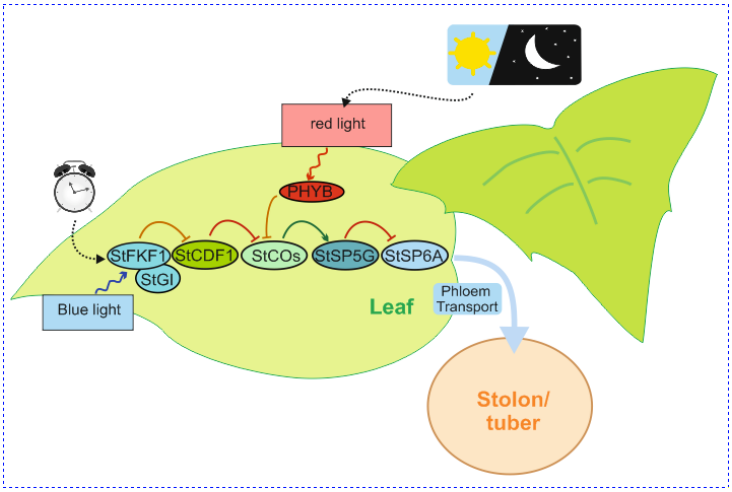

Figure1. Simplified visualization of the tuberisation pathway. Under short days, StGI and StFKF1 do not bind to StCDF1, resulting in transport of StSP6A from the stolon to the tubers.

In higher plants, DNA-binding with one finger (DOF) type transcription factors are involved in many regulatory processes, such as flowering time and seed maturation (Corrales *et al*., 2017). Among all DOF factors, there is a family of DOF factors in *Arabidopsis* whose transcription fluctuated under the constant light condition and they are known as CYCLING DOF FACTORS (CDF1-5) (Fornara *et al.* 2009). In potato, a CDF-homologue called *StCDF1* is located on Chromosome 5 that was identified as an indirect inducer of tuberization (Kloosterman *et al*. 2013). Under short-day conditions, StCDF1 is not degraded because of the lack of StGI-StFKF1 complex which normally binds StCDF1 third domain tagging it for ubiquitination and degradation by proteasome. Therefore, under inductive short-day conditions, StCDF1 remains available to repress *StCO* (*CONSTANS*) gene expression, promoting an indirectly increasing of *StSP6A* expression giving to initiation of tuberization (Navarro *et al*. 2011; Abelenda, Navarro, & Prat, 2014; Navarro, Cruz-Oró, & Prat, 2015) (figure1).

Allelic variation at the StCDF1 locus also results in natural variation in the time point of tuber formation. The wild-type allele which results in late tuberisation under long-day conditions has been called StCDF1.1. Additional early alleles (*StCDF1.2* and *StCDF1.3*) code for truncated variants of the StCDF1 protein that prevent the binding of the FKF1 protein, thereby evading the normal degradation of the protein resulting in constitutive repression of *StCO* gene expression leading to day-length independent tuberization. Due to their early phenotype, these two alleles have been

widely selected and applied in the modern potato breeding and cultivation. However, the current results from our research group, show that the homozygous diploid potatoes for *StCDF1.3* allele grow unhealthily and weakly even under normal condition. Furthermore, the heterozygous diploid potatoes with single copy of *StCDF1.3* allele show significant less tolerance to drought (figure 2.).

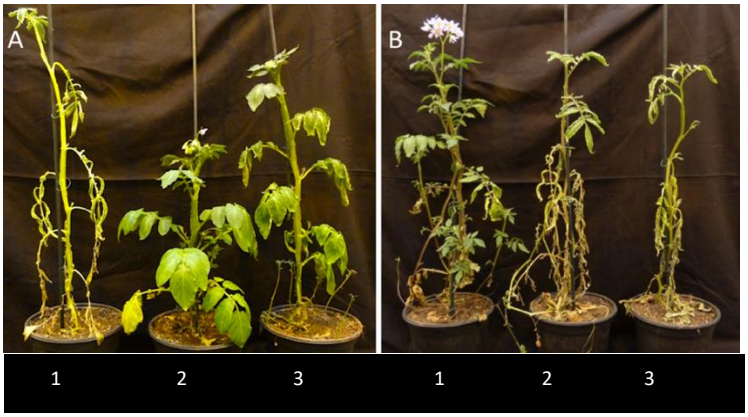

Figure2. The drought response in different plant line with different combination of *StCDF1* and *StFLORE*. A) 1; Heterozygous diploid potato carrying *StCDF1.2/1.3* alleles (drought sensitive), 2, 3; two independent *StCDF1*: RNAi knockdown plant with elevated *StFLORE1* expression (drought tolerant). B) 1; diploid homozygous StCDF1.1, wild type (drought tolerant) 2; diploid StCDF1.1/1.3 heterozygote (moderately drought sensitive).3; StCDF1.3 homozygote (very drought sensitive).

In *Arabidopsis*, the *CDF* genes have been shown coding transcription factor for the photoperiodic flowering-time control (Imaizumi *et al,* 2005). Recently, a long non-coding transcript (lncRNA) covering the 3'- half of the *CDF5* gene was mapped and named as *FLORE.* In potato, we have also detected the presence of a 200bp lncRNA we have called *StFLORE1*, transcribed in an opposite orientation from the 3'-UTR of *StCDF1* gene extending into the first exon. Moreover, experiments from our laboratory indicate that plants with silenced *StCDF1* have not only enhanced drought tolerance but also remarkable increased expression of *StFLORE1* (Bachem *et al*. unpublished data).

As mentioned above, the earliness phenotypes of *StCDF1.2* and *StCDF1.3* are result from mutations. In the case of *StCDF1.2* there is a premature stop codon in the second exon due to a 7-base-pair (bp) insertion, whereas *StCDF1.3* has a larger insertion of 865bp at the same location that results in an additional 22 amino acids (AAs) extension of the *StCDF1* protein (Kloosterman *et al.,* 2013) (figure 3). The insertion in the *StCDF1.3* allele displaces the *StFLORE1* transcript, which is likely to render it non-functional, while in the *StCDF1.2* allele the 7bp insertion is less likely to have a significant effect on the *StFLORE1* transcript. In a comparison of heterozygous 1.1/1.3 with 1.1/1.2 diploid potatoes, the latter allelic configuration was shown to have larger size of stomata with lower density and these stomatal phenotypes seems related with its lower susceptibility to drought stress. That delivered important message about it is possible for *StFLORE* involved in the regulation of drought response. However, without more genetic modified plants, the unique gene structure of this locus makes it very hard to analysis the individual effect of *StFLORE*.

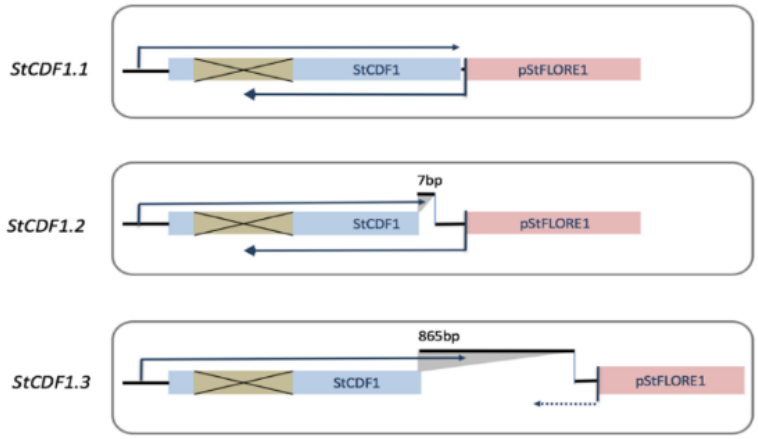

Figure 3. Structure of the three different alleles of the *StCDF1* gene. Blue box: *StCDF1* coding region, grey triangles: insertions, red box: *StFLORE1* promoter, green: intron, arrows: transcripts (dotted indicates non-functional transcript)

In *Arabidopsis* CDF proteins appear to be largely functionally redundant (Fornara *et al*. 2009). In potato, *StCDF1* appears to be a master transcription factor that not only regulates CONSTANS genes but also supresses the expression of *StCDF2* and *3* while not effecting expression of *StCDF4 & 5*. At the same time, over expression of *StCDF3* was shown to also lead to early tuberization indicating a degree of functional overlap. Moreover, only *StCDF1* and *StCDF4* genes appear to have a corresponding lncRNA. Together, these data indicate that there are a complex set of regulatory networks between *CDF* genes and their associated lncRNA transcripts.

Base on this background information, the formulated research questions are:

1. How does *StCDF1-StFLORE* affect drought response in potato?
2. How different combinations of different *StCDF1* allelic variations' affect stomata phenotypes?
3. Which gene give the main influence on drought response, *StCDF1* or *StFLORE*?

2. Materials and Methods

In this study, three experiments have been done. The first experiment was done under the non-drought condition, as a baseline experiment to observe the phenotypic differences of stomata in genotypes with different variations of *StCDF1* alleles and transgenic genotypes with over-expression or knock down of CDFs genes listed below. Then, both transgenic plants and non-transgenic plants leave samples were exposed to ABA treatment. The third experiment is designed to test the *StCDF1* and related genes expression level response to ABA treatment *in vitro*. In experiment 4, the plants we used for experiment 1 will be put under drought condition to determine the macroscopic and microscopic response to drought stress.

2.1 Available plant lines

In this experiment, plant lines 1-8 were grown. From each plant line, 6 replicates were prepared. Thus, 60 plants will be used in this experiment in total. First, plants were grown on MS medium for 2 weeks and then were transferred to the greenhouse conditions which was conducted at 25 °C and 20% relative humidity.

Table 1: Non-GMO lines analysed for non-drought treatment and drought tolerance

Number	Name	Ploidy	*CDF1* alleles	*StFLORE* expected
1	CE3027	Diploid	1.1/1.1	Two copies
2	CE605	Diploid	1.1/1.2	Two copies
3	CE630	Diploid	1.1/1.3	One copy
4	RHXE_30	Diploid	1.3/1.3	No
5	CE3130	Diploid	1.2/1.3	No

Table 2: GMO lines analysed for non-drought treatment and drought tolerance

Number	Name	Ploidy	Background Genotype	*StFLORE* expected
6	CE3130 RNAi CDF1	Diploid	1.2/1.3	One copy
7	35S CDF1.2 CE3027_4	Diploid	1.1/1.1	Two copies
8	35S CDF3 CE3130_5	Diploid	1.2/1.3	One copy

2.2 Experiment 1: non-drought comparison

2.2.1 Stomatal size and density.

Stomatal density and size were measured in 9 potato plants lines after 4 weeks of being grown in the greenhouse by using the terminal leaf leaflet of the mature leaf closest to the apical zone.

The sampling preparation was obtain according to Pei, Kuchitsu,Ward, Schwarz, and Schroeder (1997), briefly, cleared epidermal peels from transparent nail polish was applied in the abaxial leaflet to obtain an imprint of the epidermal surface that was examined under the microscope using 40X magnification (Olympus Provis AX70). The lecture was made in 10 rectangular fields of view for 5 replicates by selecting randomly 40 stomates per line (40 stomatasx9 lines, N=360).

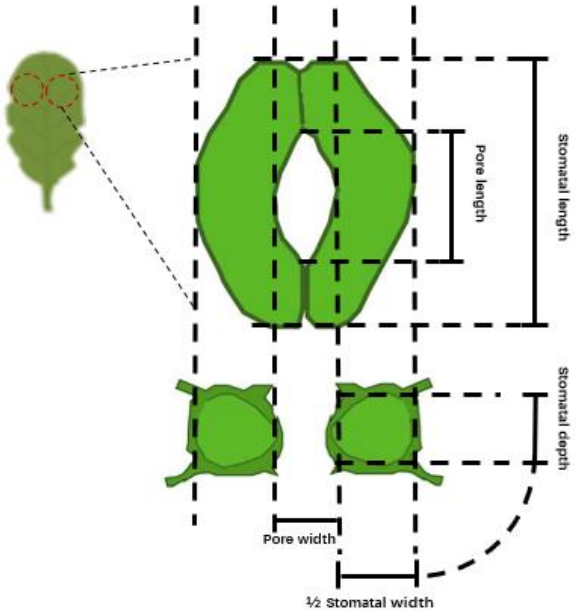

Figure 4. Measurement parameters of stomata.

The stomata density (SD) and stomatal index (SI) was calculate as follow:

$$\mathrm{SD}\ \ (mm^{-2}) \ \ = \frac{N^{\circ}\ \mathrm{of}\ stomatas}{field\ area\ (0.0314\ mm2)}$$

$$\mathrm{SI}\ (\%) = \frac{stomatal\ density}{Stomatal\ density + epidermal\ cell\ density} \times 100\%$$

Whereas for the stomatal size (SS) and epidermal cell size (ES) we calculated:

$$\mathrm{SS}\ \ (mm^2) \ \ = \pi \times \frac{stomatal\ length}{2} \times \frac{stomatal\ width}{2}$$

$$\mathrm{ES}(mm^2) = \frac{field\ area\ (0.0314\ mm^2) - (\overline{\mathrm{SS}} \times \overline{\mathrm{SD}})}{1 - SI}$$

It is important to mention that with the purpose of measuring the stomata size properly we decided to force stomatal closure for all the 9 lines evaluated by using a buffer 50Mm ABA MES/KOH buffer (50Mm ABA 5mM KCl,10mM MES(2-(N-morpholino) ethanesulfonicacid), 50µM CaCl2, pH 6.15) reported by Desikan *et al*,.2008 and Eisele *et al*, 2016.

2.2.2 ABA response in stomates by using calculated stomatal conductance.

To estimate the ABA response in stomates, at least 6 leaf samples from each plant line were collected in the morning. As figure 5 shown, first each leaf sample was incubated in Petri dishes with MES/KOH buffer (5mM KCl,10mM MES(2-(N-morpholino) ethanesulfonicacid), 50µM CaCl2, pH 6.15; (Desikan *et al,*.2008; Eisele, Fäßler, Bürgel, & Chaban, 2016) for two hours to force stomata opening, and after that it was cut along the midrib. Half of one leaflet was treated for 30 minutes with ABA MES/KOH buffer (10µM ABA, MES/KOH buffer) to induce stomata closure and the other half was used as a control (only treat with MES/KOH buffer, Mock). At least 30 stomata were obtained from each line

(30 stomates x 8 lines, N=240). The length and width of the stomata aperture was examined under the micro-scope using 40X magnification and measured by image J program.

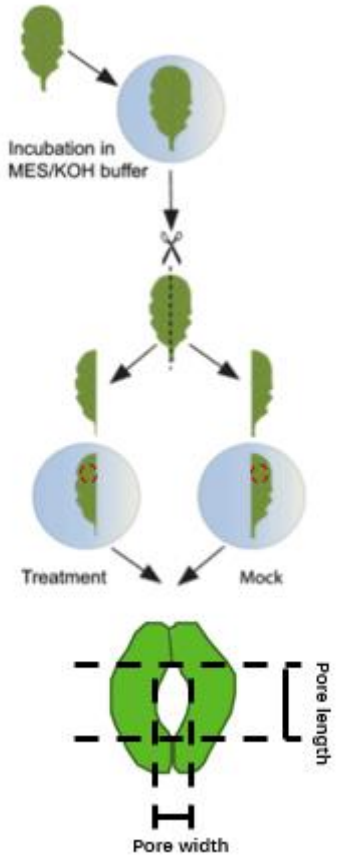

Figure 5. Stomatal conductance measurement.

The pore aperture ratio between ABA treatment and MOCK treatment indicate the sensitivity of stomata to ABA. Therefore, Δpore aperture, was calculated by following equation:

$$\Delta pore\ aperture\ ratio = \frac{pore\ aperture\ area\ in\ ABA\ treatment}{pore\ aperture\ area\ in\ MOCK\ treatment} \times 100\%$$

In order to have general view of gas change and water vapor, the g_s was calculated for abaxial leaf surface under both MOCK and ABA treatment conditions, based on the following equation (Franks and Farquhar, 2001).

$$g_S = \frac{(diffusion\ coefficien\ t) \times (stomatal\ density) \times (pore\ area)}{(molar\ volume\ of\ air) \times [(pore\ depth) + \pi/2 \times \sqrt[2]{(pore\ area/\pi)}]}$$

The mean molar volume of air was considered 0.024 $m^3 mol^{-1}$, and the effective diffusion coefficient for water vapor in air was 2.43×10^{-5} $m^2\ s^{-1}$ at 21 °C, according to Jones (1992). Stomatal pore depth was considered to be equal to the guard cell width (i.e. stomatal width/2), assuming that guard cells inflate to a circular cross-section (Franks & Beerling 2009; Franks & Farquhar 2007).

2.3 Experiment 2: drought comparison

After experiment 1, 8 plant lines (40 plants in total) grown for another 3 weeks under non-drought condition before drought treatment. The drought treatment continued 3 weeks. After 3 weeks drought treatment, the IR camera (FLIR one Pro IOS) used to measuring leaves temperature for each plant line and the temperature difference recorded in photos.

2.4 Experiment 3: Gene expression of StFLORE1 under ABA treatment.

Because RNAi CDF1 transgenic plants were the candidates to be more drought tolerant, we decided to test the StFLORE1 expression under ABA treatment for RNAi CDF1 3027, RNAi CDF1 CE3130 and include CE3027 and CE3130 as controls. Plants were grown under the same conditions in climate chamber and after 2 weeks, 6 apexes per each genotype were cut and planted in plates containing MS medium (0.5×MS, 1% sucrose, and 0.8% agar) for 7 days. Thereafter, the ABA treatment was imposed, in which 3 replicates per each genotype were transfer to MS with ABA (50μM) and others 3 were transfer to new MS media without ABA for 4 hours at 22°C with a 16-h light/8-h dark cycle. After treatment, the roots and apex were collected and store at -80 °C.

Table 3: plant lines analyzed for gene expression under ABA treatment

Name	Ploidy	CDF1 alleles	StFLORE expected
CE3027	Diploid	1.1/1.1	Yes
CE3130	Diploid	1.2/1.3	No
CE3130 RNAi	Diploid	1.2/1.3	Yes
CE3027 RNAi	Diploid	1.1/1.1	Yes

Briefly, total RNA was extracted using the RNeasy Plant Mini Kit from Qiagen, with the corresponding protocol. RNA was treated with DNAse I (Invitrogen) to eliminate residual DNA. After DNAse treatment, cDNA was performed from 1ug RNA template using Invitrogen SuperScript III (Appendix X) and diluted 1:10. Quantitative real-time PCR was conducted using iQ SYBR Green supermix (Bio-rad) in a Bio-Rad iCycleriQ machine.

Table 4: Primers used for making cDNA for the *StFLORE1*

target	Name of the primer	Sequence
CDF1	cDNAr1CDF	GACGTGATTGTAGTTACTAACC
CDF1	cDNAr2CDF	TGGGACGTAAAGACGTTCTC
EF3	RT_REV_349 REV	CGTTGGTGAATGCGGCAGTAGG
NAC	NAC_reverse	TCCATGATAGCAGAGACTA

For Real Time PCR, a master mix contained 5uL 2X iQ SYBR GREEN, 1uL forward primer (3uM), 1uL reverse primer (3uM) and 3 uL cDNA template into a final volume of 10uL was prepared. Thermocycling conditions were 95C for 3 minutes,40 cycles of 95C for 30 seconds and 60C for 30

seconds. The primers used for the expression analysis are listed in Table X. We tested NAC and ELF3 as housekeeping, but finally we kept ELF3 as a housekeeping control gene because the expression was more homogenous.

Table 5: Primers used for qPCR analysis of *StFLORE1*

target	Name of the primer	Sequence
StFLORE1	qPCRrFCDF	TGCAGACTCGTCGATTGAAC
	qPCRrRCDF	GAGTGCCTTTTCCTCACTCG
NAC	NAC_forward	ATATAGAGCTGGTGATGACT
	NAC_reverse	TCCATGATAGCAGAGACTA
ERF3	ERF3_forward	
	ERF3_reverse	

For the data analysis an RGE expression was calculated by the following formula according to Livak and Schmittgen, 2001.

$$RGE = 2^{Cq_{NAC} - Cq_{GOI}}$$

2.5 Statistical analyses

Data analysis was performed using the IBM SPSS software (version 22; https://www.ibm.com/analytics/spss-statistics-software). The stomatal size, stomatal density, stomatal index, stomatal ABA responsiveness and calculated stomatal conductance (g_s) data were subjected to an analysis of variance homogeneity to determine if its distribution was normal. Since the data presented non-normal distribution we applied non-parametric test by using Kruskal-Wallis Test. The differences among the genotypes were found through Fisher's LSD test. The differences were considered significant for a value of $P \leqslant 0.05$.

3. Results

3.1 Stomatal ABA responsiveness

We have shown that overexpressing *StCDF3* and *StCDF1*.2 in potato induces earliness phenotype. While knocking down *StCDF1* gene expression by RNAi delays tuberisation, these transgenics also show an enhanced tolerance to drought. However, no clear link has been established between drought responsiveness and StCDF proteins. Since stomatal opening is linked to transpiration and stomatal opening is regulated by the plant hormone ABA, we examined ABA responsiveness in three transgenic lines (RNAi *CDF1* CE3130, 35S *CDF3* CE3130 and 35S *CDF1.2* CE3027) and their background lines (CE3130 and CE3027). These plants all have either different allelic variants of *StCDF1* or have transgenes with altered *StCDF1* or *StCDF3* expression.

In this study, the ABA responsiveness is represented by the pore aperture ratio between ABA treatment and control treatments (see Material & Methods). A lower value of aperture ratio indicates a more rapid stomatal response to ABA treatment. The *StCDF1* knock-down in CE3130 (1.2/1.3) plants had reduced pore aperture ratio compared to CE3130 (1.2/1.3) plants (figure 6 A). This result suggests the more rapid drought response in RNAi *CDF1* CE3130. However, overexpressing *StCDF3* in CE3130 did not seem to affect ABA responsiveness, since the pore aperture ratio of 35S *CDF3* CE3130 did not show a significant difference with CE3130. We conclude that, StCDF3 may not be directly involved in regulating stomatal movements.

Overexpressing *StCDF1.2* in CE3027 resulted in a very high pore aperture ratio (>1; figure 6 B). This indicates the stomata in this line seem to barely respond to the stomatal closing signal and may need a longer time or more ABA to trigger stomatal closure.

Although both StCDF1 and StCDF3 show tuberization inducing ability, they work differently in regulating stomatal ABA response. Unlike StCDF3, overexpressing *StCDF1.2* seems to have a strong negative effect on stomatal ABA responsiveness. In a previous study, the StCDF1 had been proposed to have general negative effect on drought response in potato (Bachem et al., unpublished data). Based on this study, the negative effect of StCDF1 may relate to stomatal insensitivity to ABA.

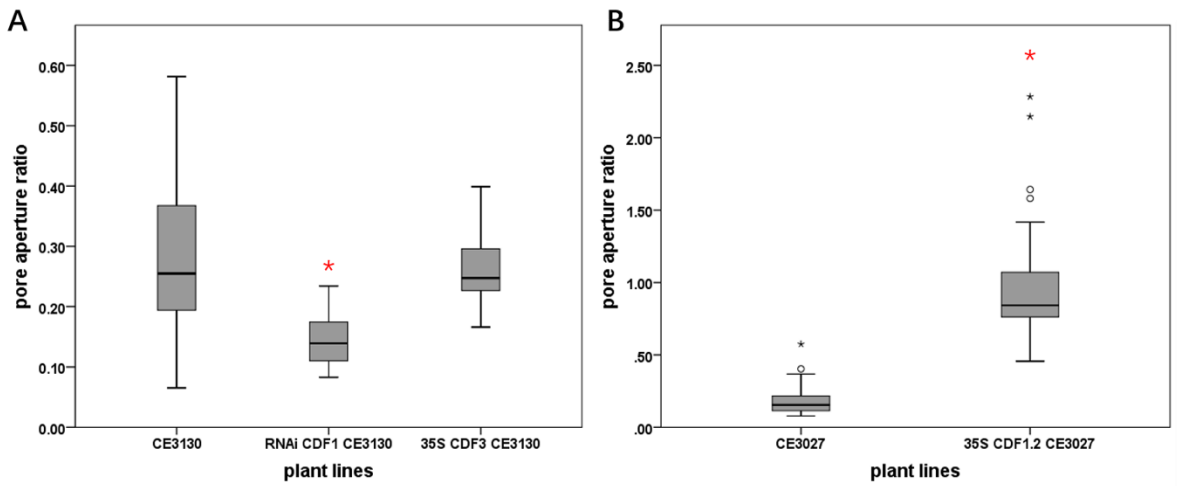

Figure 6. Pore aperture ratio in different plant lines with different combination of *StCDF1* and *StFLORE*. (A) Pore aperture ratio in CE3130 (1.2/1.3), RNAi *CDF1* CE3130, and 35S *CDF3* CE3130 plants. (B) Pore aperture ratio in CE3027 (1.1/1.1) and 35S CDF1.2 CE3027 plants. Red star mark (*/**) represents a statistically significant difference ($p<0.05$).

3.2 *In vitro*, *StFLORE* has a function in response to ABA treatment.

In potato the *StCDF1* locus encodes a long non-coding RNA transcribed in an antisense orientation called *StFLORE.* We have shown that multiple DOF binding sites present in the *StFLORE* promoter make it likely that this transcript is regulated by StCDF1. The upregulation of *StFLORE* in RNAi *CDF1* CE3130 is a confirmation of this hypothesis (Bachem *et al*, unpublished data). As described above, the stronger stomatal ABA response was only observed in RNAi *CDF1* CE3130. Therefore, to gain an insight into whether the stronger stomatal responsiveness to ABA in RNAi *CDF1* CE3130 relates to *StFLORE*, the expression level of *StFLORE* under control and ABA treatment (50 μM) for 4 hours exposition was examined in plants of 1 weeks old in MS medium in apex and root samples.

Our results showed that RNAi *CDF1* CE3130 has dramatically increased *StFLORE* expression under ABA treatment in both apex and root samples (figure 7). Therefore, it may be that *StFLORE* expression is induced by ABA and we infer it may be related to the stomatal responsiveness as showed in the 3.1 results. In contrast, CE3130 wild-type plants did not show any change in *StFLORE* expression under ABA treatment, which also agrees with our results in 3.1, that its stomata do not respond to ABA treatment as fast as RNAi *CDF1* CE3130.

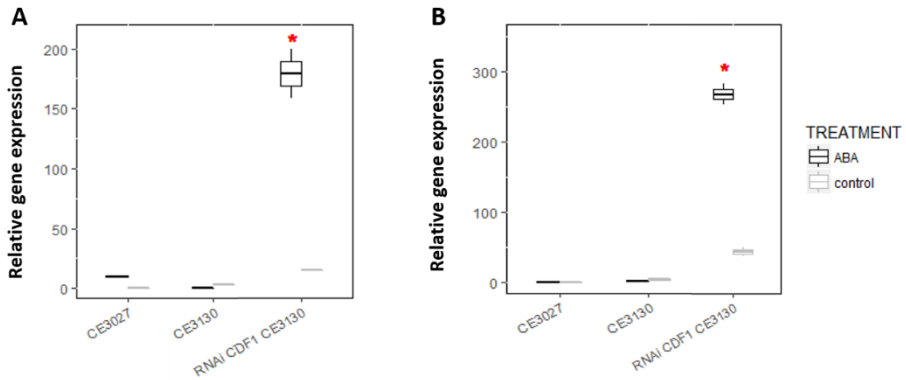

Figure 7. Relative gene expression of *StFLORE1* of *in vitro*. A) gene expression of StFLORE1 in leaves. The comparisons were made between ABA treatment (50 μM) and Control. B) gene expression of StFLORE1 in roots of in vitro plants. The comparisons were made between ABA treatment (50 μM) and Control. Red star marks (*/**) represent a statistically significant difference ($p<0.05$).

3.3 Stomatal phenotype under non-drought conditions.

3.3.1 Stomata size and density.

Stomatal size and density have always been recognized as two important values for estimating stomatal conductance and plant performance under drought. Plants with larger stomata and lower stomatal density have been considered to have a "water saving" stomatal phenotype (Doheny-Adams *et al*.,2012). So far, no link has been found between *StCDF1* and stomatal phenotype. In this study, we did a further investigation on stomatal phenotype among 8 different genotypes to discover if *StCDF1-StFLORE* locus has an effect on these stomatal characters (see figure 8).

Unfortunately, no significant difference was found between the early varieties and late varieties. Only CE605 (harboring *StCDF1.1/1.2* alleles) stands out among all non-transgenic plant lines for its larger stomatal size and lower density (figure 9, A-B).

The stomatal characteristics of RNAi *CDF1* CE3130 fit with previous expectations. Knocking down *StCDF1* in CE3130 background showed increased stomatal size and reduced stomatal density (figure 9, C-D). However, the most dramatic stomatal enlargement and reduced density were found in the two overexpression lines (figure 9, C-F). Both of these two plant lines increase their stomata size to almost twice that of their background plants and have half of their background plants' stomatal density. There was no significant difference in stomatal size and density between 35S *CDF3* CE3130 and 35S CDF1.2 CE3027.

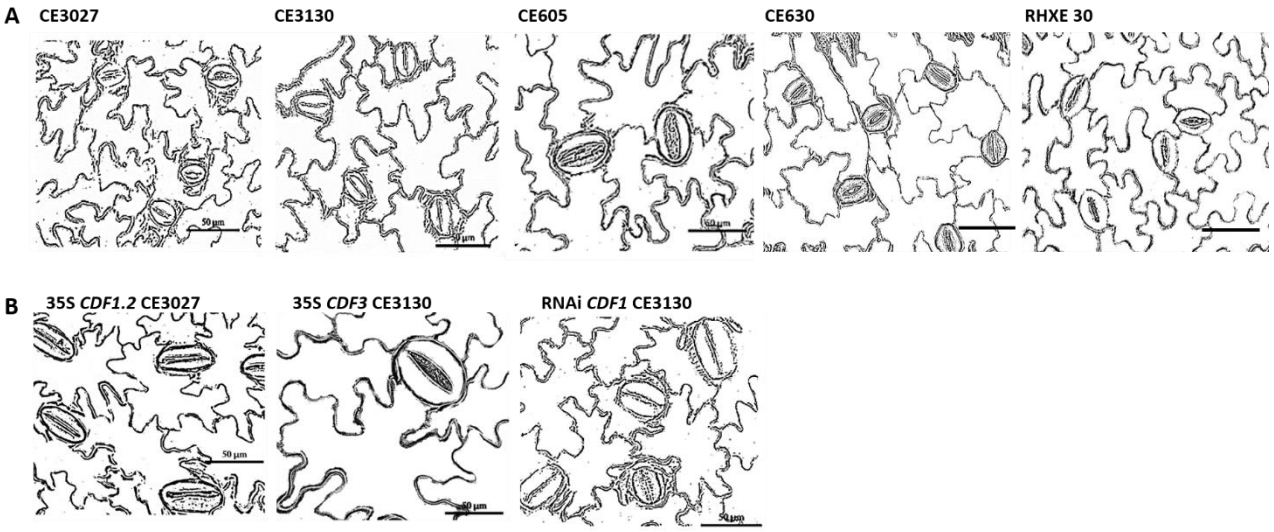

Figure 8. Leaf abaxial surface of transgenic lines and the non-transgenic lines under a magnification of 40X. (A) From right to left are CE3027 (1.1/1.1), CE3130 (1.2/1.3), CE605 (1.1/1.2), CE630 (1.1/1.3) and RHXE30 (1.3/1.3). (B) From right to left are 35S CDF1.2 CE3027, 35S CDF3 CE3130, RNAi *CDF1* CE3130. All the photos were taken with a scale bar = 50 μm

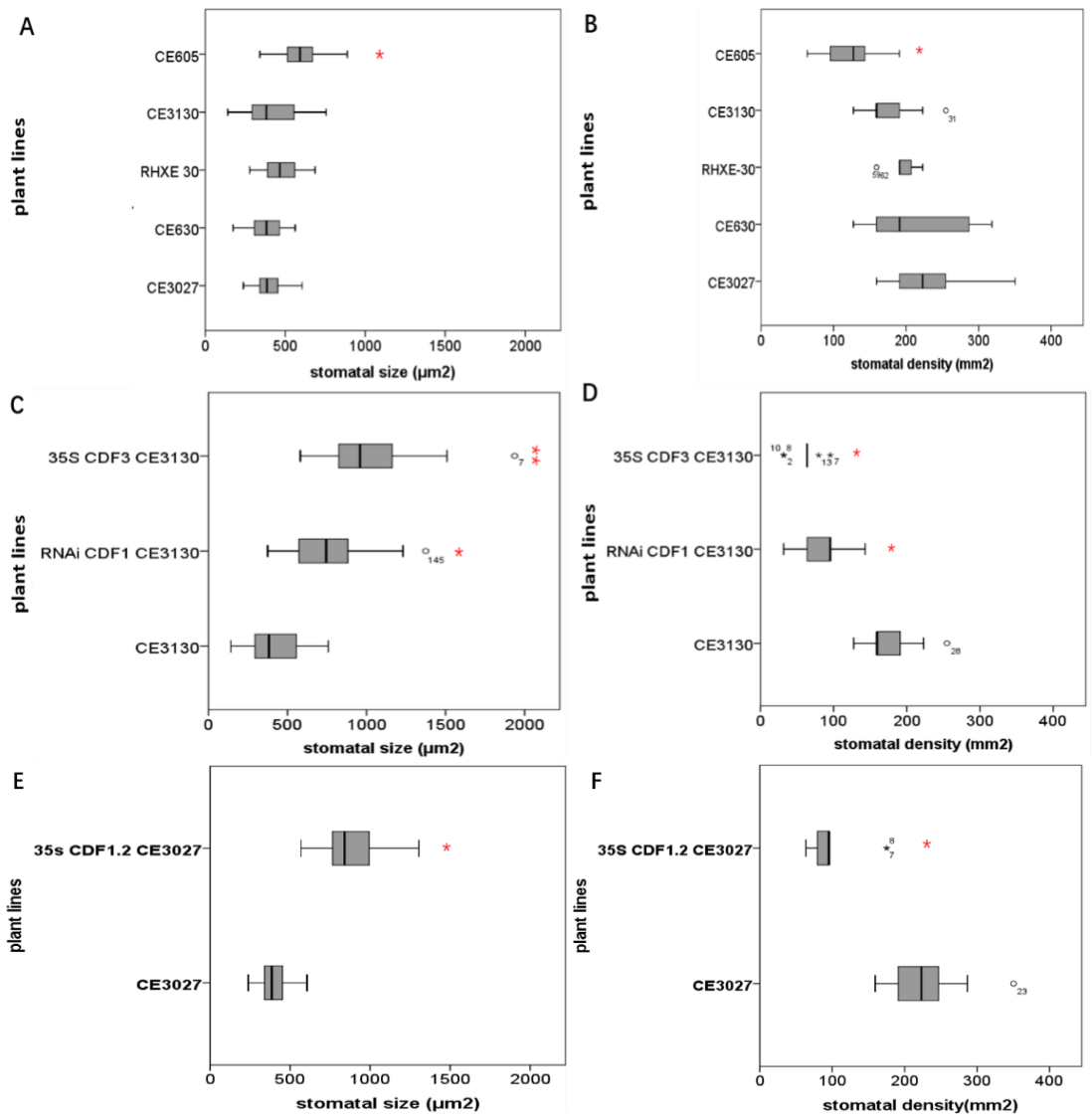

Figure 9. stomatal size and density in different plant lines with different combinations of *StCDF1* and *StFLORE*. **(**A-B) stomatal size and density in non-transgenic plants lines: CE605 (1.1/1.2), RHxE30(1.3/1.3), CE3130 (1.2/1.3), CE630(1.1/1.3), and CE3027 (1.1/1.1); C-D) stomatal size and density CE3130 background plants; red star mark (*/**) represents the significant difference level.

The transgenic plant lines delivered a clear message that StCDFs are involved in stomatal phenotypes regulation. Unlike stomatal ABA response, overexpressing *StCDF1.2* and *StCDF3* seem to act similarly in inducing stomatal enlargement and reducing stomatal density. However, the increased stomatal size and reduced stomatal density has also been found in RNAi *CDF1* CE3130. That suggests there may be more than one way to obtain the “water saving” stomatal features. For CE605, more sublines are required in future research to exclude the additional effects of genetic background.

3.3.2 Stomata index and pavement cell enlargement

In chapter 3.3.1, lower stomatal density was observed in several plant lines. The similar pattern of increasing stomatal size and decreasing density in 35S *CDF3* CE3130 and RNAi *CDF1* CE3130 lead us to look into additional stomatal characteristics. Considering the decrease of stomatal density could result from less stomatal formation, epidermal cell expansion or other reasons, we further measured stomatal index (formula can be found in Material & Method) in three transgenic lines (35S *CDF3* CE3130, RNAi *CDF1* CE3130 and 35S *CDF1.2* CE3027) and their background plant lines (CE3130 and CE3027).

Surprisingly, only RNAi *CDF1* CE3130 has a reduced stomatal index (figure 10, A). This result also delivers a clear message that less stomata were formed in RNAi *CDF1* CE3130 plants. However, the 35S *CDF3* CE3130 did not have a lower stomatal index, it suggests again that the upregulation of *StFLORE* may be involved in this decrease, but not StCDF3.

In order to clearly show the ratio between stomata and pavement cell (stomatal density: pavement cell density), we made a chart (figure 10, B). We found that indeed, RNAi *CDF1* CE3130 has the lowest stomatal formation, since per 1 stoma it has 4.44 pavement cells in a field area whereas the other lines, including the overexpression lines, have fewer pavement cells (around 3.3) per 1 stomata (figure 10, B). Therefore, the lower stomatal density of the two overexpressed lines did not result from less stomatal formation.

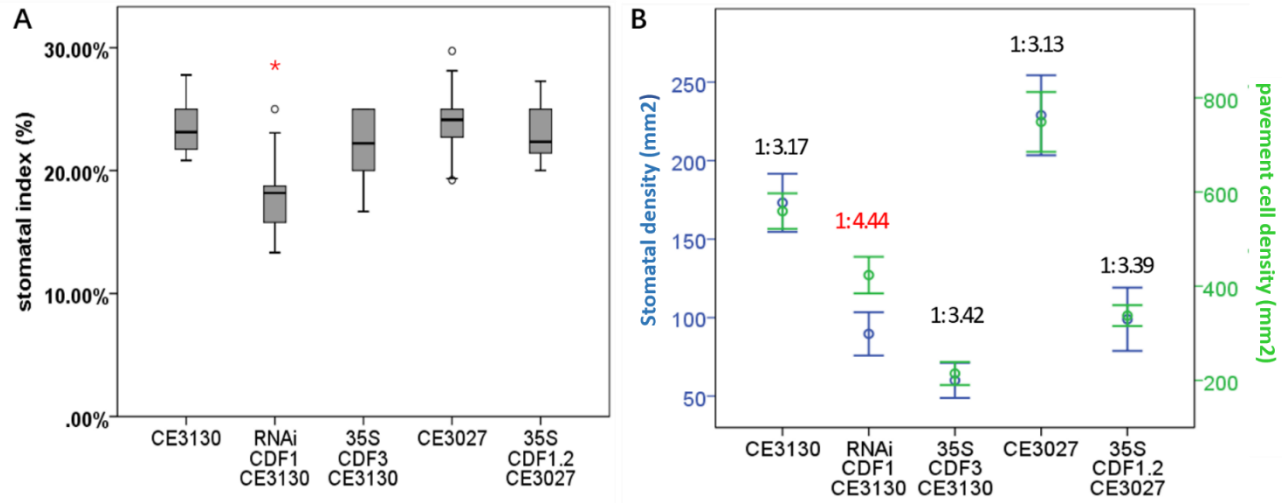

Figure 10. Stomatal index in different plant lines with different combination of *StCDF1* and *StFLORE*. (A) stomatal index in CE3130 (1.2/1.3), RNAi CDF1 CE3130, 35S CDF3 CE3130, CE3027 (1.1/1.1) and 35S CDF1.2 CE3027 plant lines. (B) stomatal density and pavement cell density in E3130 (1.2/1.3), RNAi CDF1 CE3130, 35S CDF3 CE3130, CE3027 (1.1/1.1) and 35S CDF1.2 CE3027 plant lines. Green data point represents pavement cell density; Blue data point represent stomatal density; The number above data point is stomatal density: pavement cell density ratio; Error bar = confident interval (95%) (n=15) * / red numbers represent the significant difference; The comparisons are crossing plants' genetic backgrounds.

In figure 8, beside the stomatal size, we found that pavement cell size also showed various size differences among different genotypes. Therefore, we calculated pavement cell size of the same 5 plant lines in this experiment and compared it with stomatal size based on stomatal morphological data in this study. From figure 11 (A-B), we show that both pavement cell size and stomatal size in 35S *CDF3* CE3130 plants were almost as twice as large as CE3130 plants. This pattern can also be found in 35S *CDF1.2* CE3027 plants and its background plants. Therefore, it seems like the stomata and pavement cells in these two overexpressing lines co-enlarged with each other, leading to the very low stomatal density. Notably, the calculated pavement cell size of RNAi *CDF1* CE3130 is slightly but insignificantly larger than CE3130. This result further explained that the lower stomatal density in RNAi *CDF1* CE3130 results from different reasons than the other two overexpression lines.

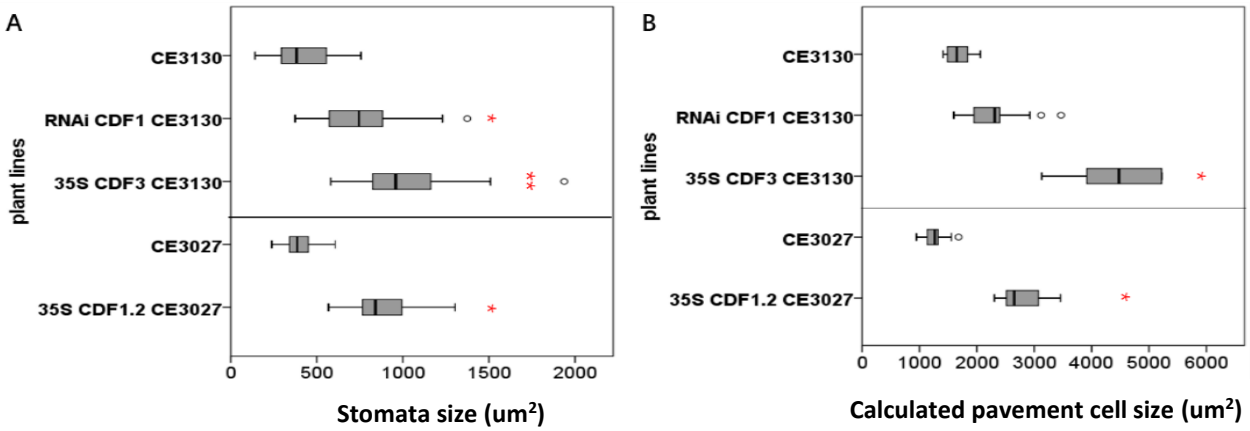

Figure 11. enlargement of stomata and pavement cell in different plant lines with different combination of *StCDF1* and *StFLORE*. (A) stomatal size in CE3130 (1.2/1.3), RNAi CDF1 CE3130, 35S CDF3 CE3130, CE3027 (1.1/1.1) and 35S CDF1.2 CE3027 plant lines; (B) calculated pavement cell size in CE3130 (1.2/1.3), RNAi CDF1 CE3130, 35S CDF3 CE3130, CE3027 (1.1/1.1) and 35S CDF1.2 CE3027 plant lines; The comparisons are not crossing plants' genetic backgrounds. red star marks (*/**) represent the significance level.

3.4 Calculated stomatal conductance (g_s)

After gathering several stomatal morphological parameters, we were able to estimate the transpiration level by calculating stomatal conductance (g_s). To obtain the transpiration level under different conditions, we calculated stomatal conductance under both control and ABA treatment conditions. The differences between control and treatment are significant in CE3130(1.2/1.3), RNAi *CDF1* CE3130, 35S *CDF3* CE3130 and CE3027(1.1/1.1) and the calculated stomatal conductance did not significantly change in 35S *CDF1.2* CE3027 plants. These results consistent with the results of pore aperture ratio (in chapter 3.1).

Under ABA treatment, only RNAi *CDF1* CE3130 plants had significant lower value than other plant lines. The low calculated stomatal conductance of RNAi *CDF1* CE3130 was very close to zero (figure 12). This result indicated these plants had very low transpiration under ABA condition, which agrees with their drought tolerance phenotype (figure 14). In control condition, 3 transgenic lines (RNAi *CDF1* CE3130, 35S *CDF3* CE3130 and 35S *CDF1.2* CE3027) had lower calculated stomatal conductance values. Since the calculated g_s was mainly based on stomatal size and density, these three transgenic lines "water saving" (larger stomata and lower density) stomatal phenotype did help to reduce calculated g_s value under control condition. However, having low transpiration under drought condition (ABA treatment) seems to be more dependent on the high stomatal ABA response.

Notably, the CE3027 shows better drought tolerance than CE3130 in previous observations, but this difference was not seen in calculated g_s. However, this calculated g_s was only estimated by using morphological characteristics.

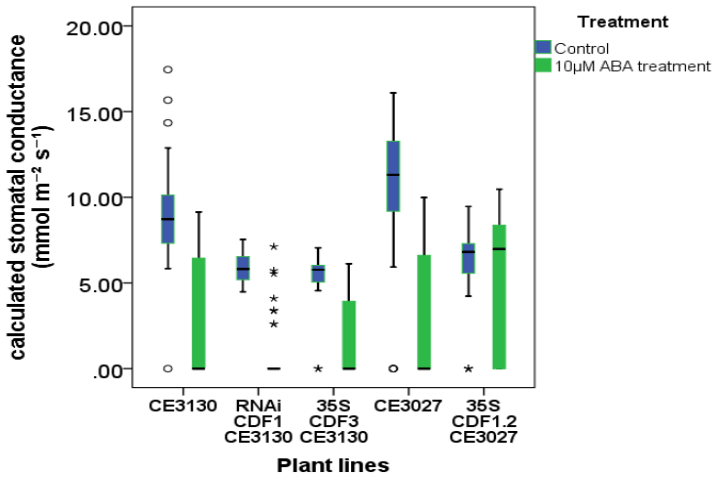

Figure 12. Calculated stomatal conductance in different plant lines with different combination of *StCDF1* and *StFLORE*. The Calculated stomatal conductance in CE3130 (1.2/1.3), RNAi *CDF1* CE3130, 35S *CDF3* CE3130, CE3027 (1.1/1.1) and 35S *CDF1.2* CE3027 plant lines.

3.5 Phenotypic response to drought

To have a better view of the performance of selected genotypes under drought condition, we examined 8 genotypes (table 1-2) under 3 weeks drought treatment. The plants' performance under drought and control treatments were tested and recorded in photos. Among the non-transgenic plant lines, the CE3027(1.1/1.1) plants and CE605(1.1/1.2) plants stand out for their better drought tolerance. Both of these two lines had no significant difference between control treatment and drought treatment (figure 13, A-B). It seems like the 7bp insertion in *StCDF1.2* did not affect drought sensitivity much. Unlike StCDF1.2, the heavily truncated allele *StCDF1.3* seems to have a more negative effect in general. Although the RHXE 30 (1.3/1.3) plants also seem less affected by drought, the plants growth was largely repressed (figure 14, A-B). Even after almost 10 weeks growing in the glasshouse, the leaf size and plant height of RHXE-30 were far smaller than in other plant lines. The drought sensitivity of CE3130 (1.2/1.3) plants was very similar to CE630 (1.1/1.3) plants'. Both of these two *StCDF1.3* allele carriers' (CE3130 and CE630) plants died very quickly under drought condition.

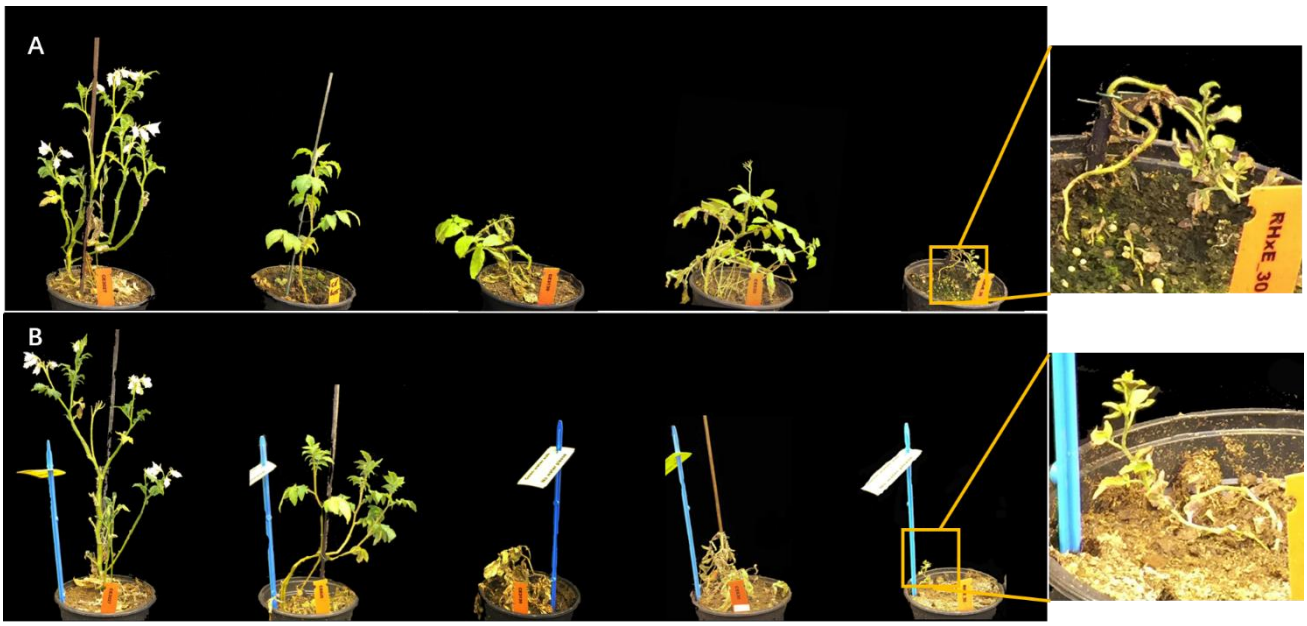

Figure 13. drought response in non-transgenic plant lines. (A) from left to right, the about 7 weeks old diploid potato plants CE3027(1.1/1.1), CE605(1.1/1.2), CE3130(1.2/1.3), CE630(1.1/1.3) and RHXE-30(1.3/1.3) was treated as control. The CE605(1.1/1.2) plants were 5 days younger than the other plants. The plant growth of RHXE-30 was strongly repressed and adaxial leaf side turns to purple even at young age. (B) from lift to right, the about 7 weeks diploid potato CE3027(1.1/1.1), CE605(1.1/1.2), CE3130(1.2/1.3), CE630(1.1/1.3) and RHXE-30(1.3/1.3) was treated under drought for 3 weeks.

Knocking down *StCDF1* in CE3130 plants improved the drought tolerance significantly (figure 14). The RNAi *CDF1* CE3130 plants also became taller than CE3130 (1.2/1.3) plants. However, overexpressing *StCDF3* in CE3130 results in the dwarf phenotype. Apart from that, the 35S *CDF3* CE3130 plants also seem to be more tolerant to drought than CE3130 plants. Unlike 35S *CDF3* CE3130 plants, overexpressing StCDF1.2 in CE3027 did not affect plants growth. Moreover, the 35S *CDF1.2* CE3027 plants had very similar drought response to CE3027 plants.

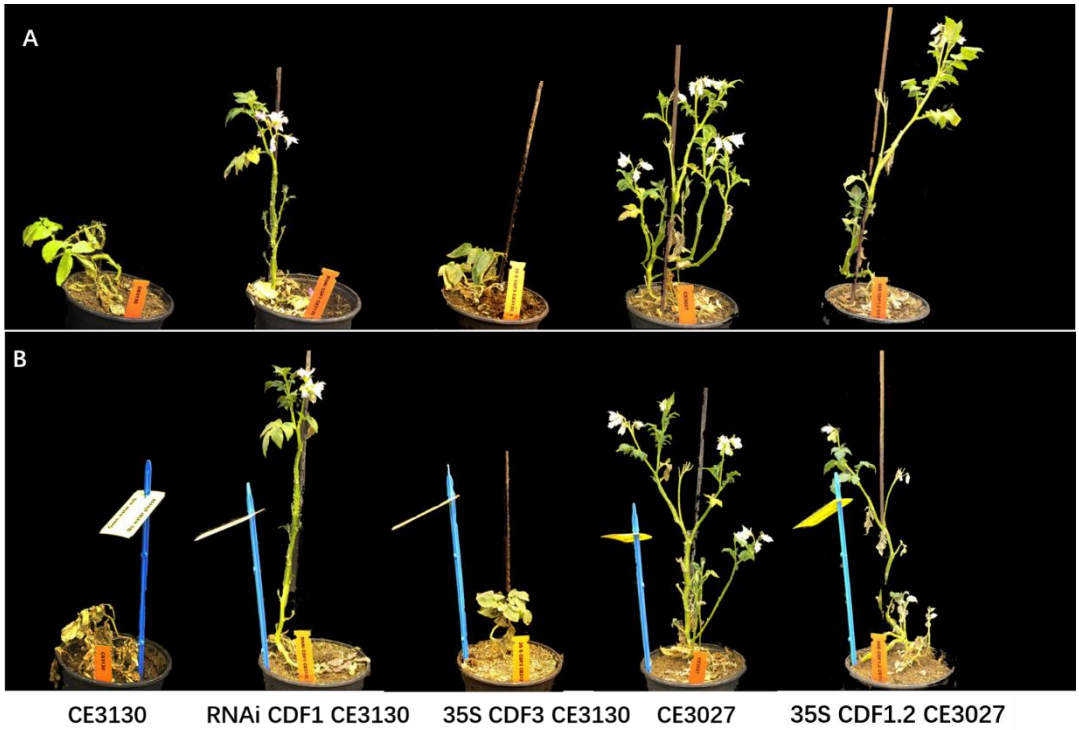

Figure 14. Drought response in transgenic plant lines and their background plants. (A) from lift to right, the about 7 weeks old diploid potato plants CE3130(1.2/1.3), RNAi CDF1 CE3130, 35S CDF3 CE3130, CE3027(1.1/1.1) and 35S CDF1.2 CE3027 were treated as control. (B) from lift to right, the about 7 weeks diploid potato CE3130(1.2/1.3), RNAi CDF1 CE3130, 35S CDF3 CE3130, CE3027(1.1/1.1) and 35S CDF1.2 CE3027 were treated under drought for 3 weeks.

In conclusion, the results indicate that the *StCDF1.3* can induce a negative effect on plant growth and drought tolerance. The *StCDF1.1* and *StCDF1.2* may have comparably slightly more positive effects on drought tolerance than *StCDF1.3*. Moreover, the high drought sensitivity in CE630 and CE3130 suggests the *StCDF1.3* allele is dominant over the *StCDF1.1* and *StCDF1.2* allele. Overexpressing *StCDF3* seems to be able to affect plant growth but also induce positive effect on drought response. Considering the previous results in this study, the improvement of drought response in 35S *CDF3* CE3130 may result from very low stomatal density.

Unfortunately, due to timing issues, plants were quite old (7 weeks old) before drought treatment. Therefore, some early genotype (CE3130, CE630) control plants shows less vigor and are unhealthy after another 3 weeks control treatment. The CE605 (1.1/1.2) plants in figure 14 were 9 weeks old (1 week younger than other plant), because we had to regrow them after roof leakage in the glasshouse destroyed the original CE605 plants.

4. Discussion

4.1 Novel functions of StCDFs in regulating plant drought tolerance

In previous study, we have found that knocking down *StCDF1* enhanced drought tolerance (Bachem *et al*. unpublished data). Therefore, we further look into how drought tolerance has been improved in this study. We examined 8 genotypes with different *StCDF1* alleles combination or different StCDFs expression levels. Our results showed transpiration related stomatal characters, such as stomatal size, stomatal initiation, and stomatal ABA responsiveness are significantly affected by the combinations of different *StCDF1* allelic variants or the expression level of StCDFs. These results suggest a clear participation of StCDFs in regulating transpiration in several ways to affect drought tolerance.

The well-documented function of CDF1 is regulating photoperiodic flowering-time in *Arabidopsis*, by affecting the diurnal rhythm of CONATANS (CO) expression and consequently the expression of FLOWERING LOCUS T (FT) (Fornara *et al*. 2009; Corrales *et al*, 2017). In potato, the homologs of FT did more than inducing flowering. It was shown that an FT homolog called *StSP6A* acts as a tuberization signal in potato, which regulated by two light responded proteins called StGI and StFKF1 via StCDFs and StCO (Abelenda, Navarro, & Prat, 2014).

Beside the reproduction, the circadian clock also responsible for regulating many other processes including stomatal movements (Hubbard, & Webb, 2011). In the day time, photosynthesis requires stomatal opening to assimilate carbon dioxide. In Arabidopsis, FT has been reported as a stomatal opening signal which regulated by CO (Kinoshita et al. 2011). CDF also involved in this regulation by mediating CO and FT expression. However, it is also possible that CDF more directly mediate stomatal movements. Several Dof binding sites have been found in the promoter of guard cell specific genes and these Dof binding motifs have been shown to be essential for their expression (Cominelli et al.,2011). Although Cominelli et al. suggested in their analysis that Arabidopsis CDFs were not involved in regulation of this stomatal specific MYB transcription factor, it should be noted that the quadruple CDF mutant line that this work was based on, remains considerably leaky with respect to StCDF4 & 5 expression (Dr. Fabio Fornara, personal communication). This finding suggest Dof protein may have direct effect on stomatal movements and CDFs still could be good candidates.

To adapt to drought, decreasing water loss while maintaining water uptake is necessary for plants. For potato, canopy temperature (transpiration) has been used as selection factor in breeding high drought tolerance variety (Prashar et al., 2013; Obidiegwu, Jones, & Prashar, 2015). Our results show that the combinations of different StCDF1 allelic variations result in diverse transpiration levels under ABA treatment. Furthermore, the low transpiration level plant lines are mostly in agreement with the plant lines which has better performance under drought treatment. Therefore,

here, we propose a novel function of StCDFs in regulating drought response by affecting transpiration.

4.2. StCDF1 has an antagonistic effect on stomatal ABA responsiveness

ABA is a stress-response hormone, which induces rapid stomatal closure. Under stress conditions, the stomatal ABA sensitivity has a crucial impact on minimizing water loss by transpiration (Lawson & Blatt, 2014). Our study demonstrates that StCDF1 has a general repressive effect on stomatal ABA responsiveness, and this effect was consistent in CE3130, RNAi *CDF1* CE3130, CE3027 and 35S *CDF1.2* CE3027. Moreover, the strong negative effect on stomatal ABA responsiveness induced by overexpressing *StCDF1.2* could not be induced by overexpressing *StCDF3* as same.

Stomatal opening and closure induced by different signals. As figure 15 shows, light induce stomatal opening and the stomatal opening could be repressed by ABA. However, overexpressing StCDF1.2 prevent stomata closure by ABA. A previous study shows a novel function in regulating stomatal movement by FT. This is a function that is independent of the regulating flowering and tuberization. Researchers nominated FT as a cell autonomously regulated timekeeper for regulating opening and closure of stomatal guard cells (Kinoshita et al. 2011). Their results suggest that FT provides a positive effect on stomatal opening via the activity of H+-ATPase in guard cells (figure 15). Based on our results, this model possibly also be applied to potato. However, we suggest that the central regulator is more likely to be *StCDF1*. In potato, the *StCDF3* has a redundant gene function for *StCDF1*. The StCDF3 also represses *StCO* and indirectly regulates expression of FT homologs' such as the tuberization signal *StSP6A.* However, overexpressing *StCDF3* in CE3130 (1.2/1.3) did not reduce the stomatal response to ABA as overexpressing StCDF1.2 in CE3027 did. Therefore, the stomatal

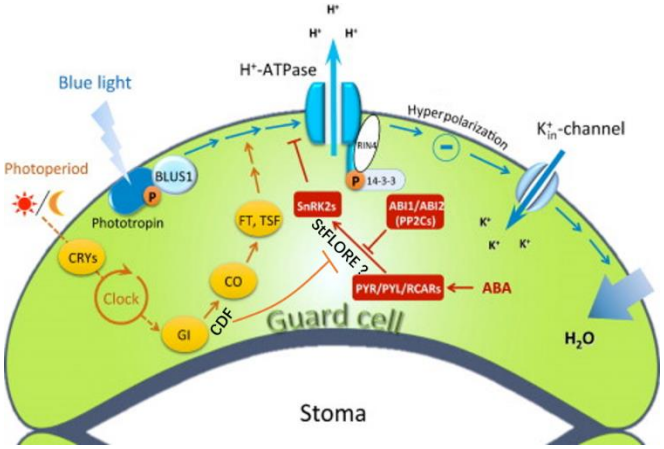

Figure 15. Overview of H+-ATPase-mediated stomatal movement. Transcriptional regulation of flowering is possibly involved in activation of the H+-ATPase to open stomata (orange arrows); ABA inhibition of the H+-ATPase to indcuce stomatal closure (red arrows). (Kinoshita et al. 2011; Wang, Y., Shimazaki, K. I., & Kinoshita, T. 2014)

opening signal could only be induced by StCDF1. Thus, we propose that *StCDF1* play a non-redundant role in regulating stomatal movement.

Moreover, both late genotypes ---- RNAi *CDF1* CE3130 and CE3027 had stomata that are very sensitive to ABA. In these plants, StCDF1 protein was expected to absent, due to RNAi or protein degradation. Our results suggest having less StCDF1 protein enhanced stomatal ABA sensitivity and is consistent with the discussion outlined above. It is interesting to note, that expression of the long non-coding RNA, *StFLORE1* that coincides with the StCDF1 locus, is increased in *StCDF1* RNAi knockdown in CE3130. This opens up the possibility that this lncRNA transcript may also have a role in stomatal ABA responsiveness regulation.

4.3 The role of *StFLORE* in drought response

Several natural antisense lncRNAs (NATs) have been reported to be involved in phytohormone-mediated response, *via* regulating gene expression at multiple levels (Wang *et al.*, 2017). Such regulation mechanisms include i) antisense lncRNAs that complex with other target transcripts (Csorba *et al.,* 2014). ii) act as a linker between proteins. iii) change chromatin configuration (Helliwell *et al.,* 2011) or modify miRNA expression (Pai *et al.* 2017) (see Figure 17). Another recent finding was an *Arabidopsis* lncRNA called *DRIR* which is also induced by ABA treatment allowing decreasing transpiration rate and the ability to up-regulate other genes related to drought stress such as aquaporins, *ARSK1* (root-specific kinase) and others (Qin *et al*., 2017). Moreover, a study from Cagirici in 2017, showed that in wheat, lncRNAs can mimic the role of a *miRNA1439* to increase aquaporin translation under drought stress. Since it is known that aquaporin is able to induce stomata closing under drought stress (Cagirici *et al*., 2017). These findings indicate firstly that lncRNAs activities may be complex and secondly that lncRNA regulations is frequenctly accociated with regulation of stress responses, light signaling and regulation of flowering (Wang et al., 2014)

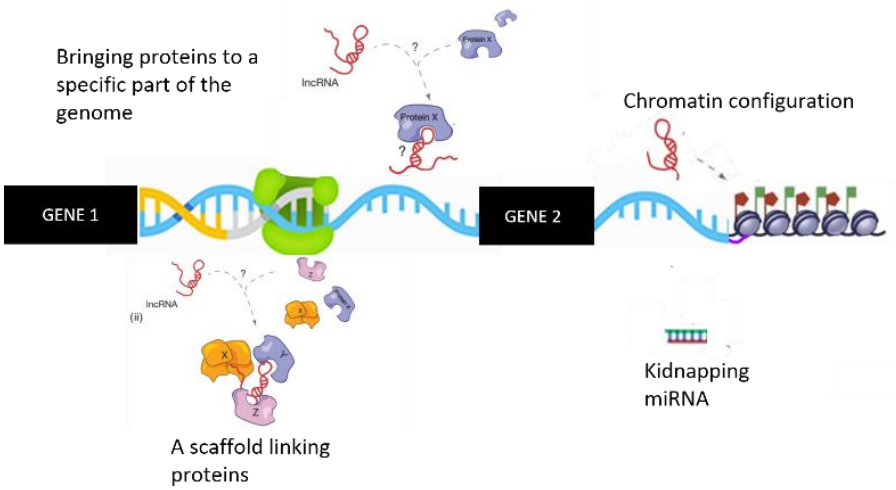

Figure 16. Different roles of antisense *lncRNA*s in stress response in plants.

In chapter 3.2, we described the major finding that *StFLORE* expression response to ABA treatment *in vitro* plants. In RNAi *CDF1* CE3130, ABA induced *StFLORE* expression in both root and apex, while root tissues had generally higher expression in both control and ABA conditions. Thus, it is possible that *StFLORE* transcript in both apex and root or mainly transcript in root and then being transport

to apex tissue. In previous study, we have found that StCDF1 seems able to repress *StFLORE* expression, probably by binding to the multiple $[A]_3G$ binding sites present in the promoter of this transcript (data not shown). In chapter 4.2, we discussed that the negative role played by StCDF1 in regulating stomatal response to ABA. Moreover, the ABA can enhance the StFLORE expression. It is possible that *StFLORE* was involved in inducing stomatal closure under influence of ABA, with StCDF1 protein acting as a repressor in this process.

Several models can be used to predict how *StFLORE* enhance stomatal ABA sensitivity. For example, it is possible that *StFLORE* act as a linker for recruiting other ABA response proteins to trigger stomatal closure ~~etc~~. However, the most urgent research question is whether *StFLORE* actually inducing stomatal closure or other drought tolerance phenotypes. Therefore, building up the construct that overexpressing *StFLORE* in CE3130 background is necessary for providing consistent evidence.

Notably, the ABA response has been found only in RNAi CDF1 CE3130 *in vitro* plants. We expected CE3027 should has similar response to ABA, since it supposes to have two copies of functional *StFLORE*. However, we did not observe the ABA response in CE3027. It is possible that due to the circadian regulation of all the genes and transcripts involved in this complex process, only a detailed time-course expression analysis will reveal the actual regulation mechanisms in all genotypes tested.

4.4 StCDFs affect stomatal features in multiple ways

4.4.1 Stomatal initiation

In this study, the reduced stomatal index has been found only in RNAi *CDF1* CE3130 and it suggest that, in addition to its roles in diurnal stomatal dynamics, StCDF1 may affect stomatal formation via

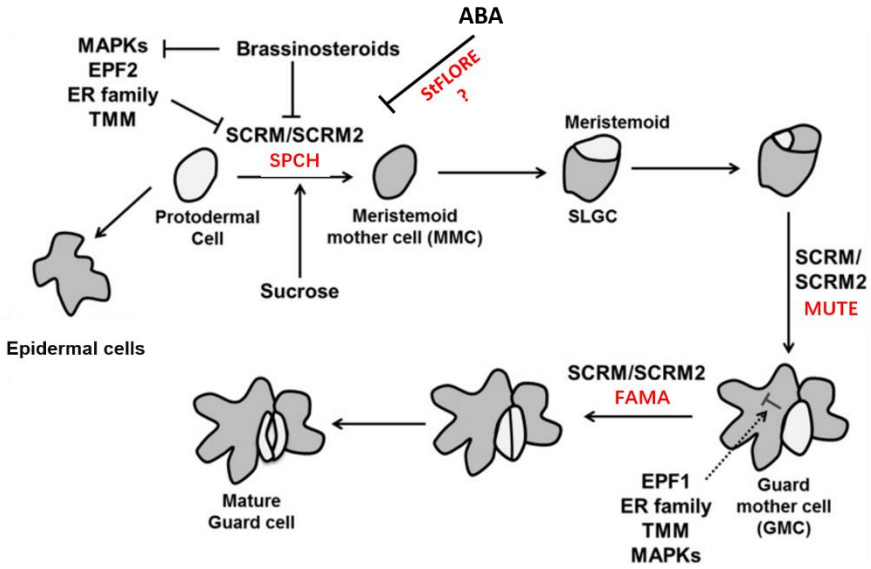

Figure 17. The control of stomatal development. Stomatal initiation and differentiation are determined by three essential transcription factors. SPCH. MUTE. and FAMA (Kalve. & Beemster. 2014).

regulating *StFLORE*. Based on the current knowledge of stomatal development, the reduced stomatal index is very likely to be a result of fewer protodermal cells transforming into meristemoid mother cells (MMC) (figure 18). In *Arabidopsis*, *SPEECHLESS* (*SPCH*) induces this transformation. In *SPCH* loss-of-function mutants, stomata initiation will be inhibited, and only epidermal pavement cells will develop (Lampard, MacAlister, & Bergmann, 2008; Bergmann, Lukowitz, & Somerville, 2004; Qi, & Torii, 2018). ABA has been shown to prevent stomatal initiation via prolonged expression of *SPCH* and *MUTE* (Tanaka *et al*., 2013; Le, *et al*., 2014). Since *StFLORE* response to ABA and this response has been found in leaves, it is possible that *StFLORE* participates in the prevention stomatal initiation. In future study, overexpressing *StFLORE* in CE3130 background would provide more insight of this potential regulation.

4.4.2 Epidermal cells expansion

Besides reducing stomatal index, cell expansion is another way to obtain lower stomatal density. We have found enlarged stomata and pavement cells in 35S *CDF1.2* CE3027 and 35S *CDF3* CE3130 plants. In both of the two overexpressing lines, epidermal cells are almost as twice as big in the wild-type backgrounds. Moreover, the stomatal densities in these lines is around half of their background plant lines. As mentioned in the introduction, larger stomatal size and lower stomatal density have the positive effect on reducing transpiration. Although overexpressing *StCDF3* and *StCDF1.2* can result in epidermal cell expansion (larger stomata, lower density) and indeed both

genotypes had a drought tolerance phenotype, the latter genotype lead to ABA insensitivity stomatal phenotype, while overexpressing StCDF3 lead to dwarf phenotype. Thus, overexpressing StCDFs seems induce epidermal cell expansion and it has positive effects in reducing transpiration, but the negative effects on plant fitness should also be noted.

However, the cell expansion in this study also showed that StCDFs may have more complicated functions other than regulating tuberization and transpiration. So far, not much single gene overexpression plant or single gene mutant were reported to have similar stomatal pattern like this. The most similar stomatal pattern we found is in polyploidy (figure 19) (Gu *et al*., 2016). Moreover, Tai and Gardi has also report similar pattern that Polyploid tomato had fewer and larger stomata and ordinary epidermal cells per leaf unit area than diploid tomato (Tal & Gardi, 1976). The polyploid epidermal cells expansion also has been linked with lower transpiration (Chen & Tang, 1945). Thus, it is like that StCDFs has diverse effects on plant development.

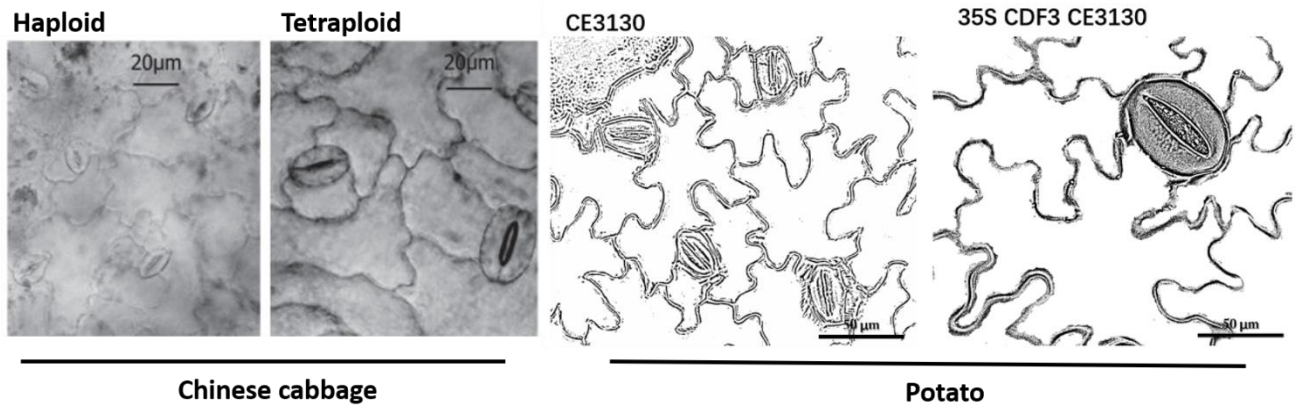

Figure 18. Epidermal cells expansion in Chinese cabbage and potato (Gu et al., 2016).

4.5 In potato, *StCDF1-StFLORE* connects tuberization and transpiration.

Potato has always been recognized as a drought sensitive crop (Porter *et al.,* 1999). The most common explanation for this characteristic has been its shallow and sparse root system, negatively affecting water uptake (Iwama and Yamaguchi, 2006; Jefferies, 1993). Apart from access to water resources in the soil, reducing water-loss though transpiration has been considered as another possible way to improve water stress resilience in potato (Wang and Clarke, 1993; Prashar *et al.,* 2013). In this study, the better phenotypic response to drought in different genotypes are mostly in agreement with the low calculated stomata conductance (g_s) genotypes. These results show that reducing transpiration is a promising way to improve drought tolerance in potato.

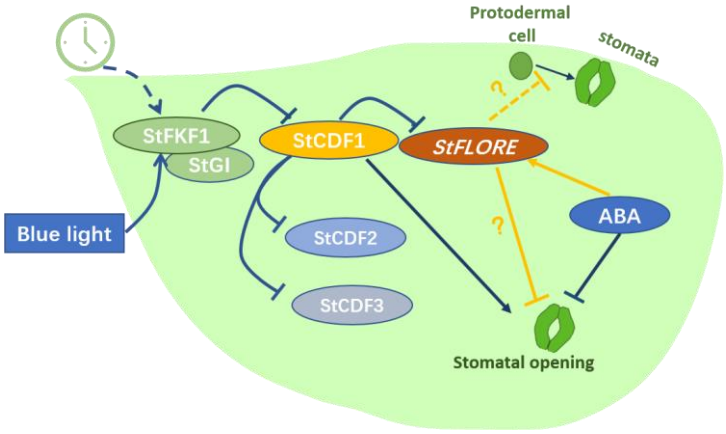

Figure 19. Proposed model on *StCDF1* cooperates with its long non-coding partner *StFLORE* in regulating drought response related stomatal characteristic. When StGI and StFKF1 do not bind to StCDF1, StCDF1 leads to stomatal opening (figure 6) and repress *StFLORE* expression (data not shown). The *StFLORE* expression could be induced by stress hormone ABA (figure 7) and it may have a beneficial effect on drought response related stomatal characteristics.

The StCDFs have been known for being an important mediator between the circadian clock and the tuberization signal (*StSP6A*) in recent years. Tuberization requires photosynthesis products and photosynthesis requires stomatal opening to obtain CO_2. Here, we have found the circadian clock genes *StCDFs* also mediate transpiration regulation. Based on data present in this work, we proposed a model of how the circadian clock is connected to transpiration related characteristics. (see figure 19).

As figure 15 shows, light drives StCDF1 to induce stomatal opening, while the stress hormone ABA represses the stomatal opening to reduce the water loss. The competition between these two pathways determines plant performance under drought condition. In our study, we found the *StCDF1-StFLORE* locus determines which pathway takes the priority under drought condition. These findings shed new light on the key role played by StCDFs in optimizing CO_2 gain against water loss in stressful environments.

Our results show the CE630 (1.1/1.3) and RHXE30 (1.3/1.3) plants were more sensitive to drought than others. Their stomatal also seems less sensitive to ABA than other genotypes (S2). As mentioned in the introduction, the 200bp insertion in *StCDF1.3* helps the protein escape from degradation to induce earliness. Considering StCDF1 can induce stomatal opening, the decreased

degradation of StCDF1.3 may lead to a higher transpiration rate. Moreover, the 200bp insertion in *StCDF1.3* also very likely results in non-functional *StFLORE.* Although the function of *StFLORE* is unclear, *StFLORE* is very likely to have positive effect on drought response which is regulated by the stress hormone ABA.

Whereas knocking down *StCDF1* in CE3130(1.2/1.3) and the CE3027(1.1/1.1) background plants resulted in a delayed tuberization, they also had better drought tolerance. In these two plants, StCDF1 protein levels were expected to be lower, due to RNAi or protein degradation. The delayed tuberization mechanism has been well explained by Kloosterman *et al*., 2013 and Navarro *et al*., 2011. Our results indicated StCDF1 also acts as a stomatal opening signal and fewer StCDF1 proteins lead to better stomatal sensitivity to ABA for reducing transpiration under stress condition. Beside the reduced stomatal opening signal, the upregulation of *StFLORE* in RNAi *CDF1* CE3130 may contribute to beneficial characteristics to drought response. Therefore, these genotypes are more tolerant to drought due to the higher priority of water conservation.

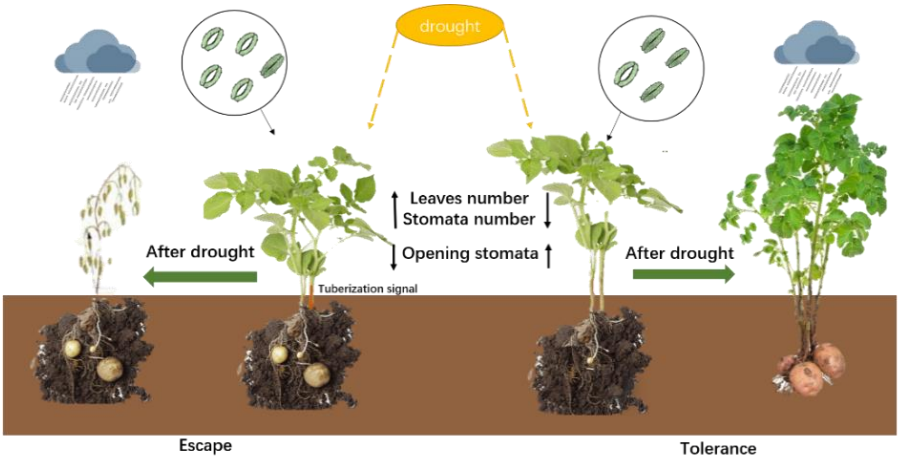

Figure 20. Drought response strategy in potato.

In conclusion, this study provides new information about the function *StCDF1* as a key factor that displays multiple roles related to plant responses to drought stress and the developmental program underlying the transition from vegetative to reproductive phase.

5.Conclusion

In this research, we proposed a model of how the circadian clock gene *StCDF1* is connected to transpiration related characteristics to influence drought response. In this model, StCDF1 has a negative effect on drought response by inducing an ABA insensitive stomata phenotype. The upregulated *StFLORE* expression under ABA treatment shows that *StFLORE* is likely to have a positive effect on drought tolerance under ABA influence. However, more research is needed to substantiate this model. For future research overexpressing *StFLORE* in CE3130 as well as CE3027 plants without a functional *StFLORE* promoter would be more than useful.

6. References

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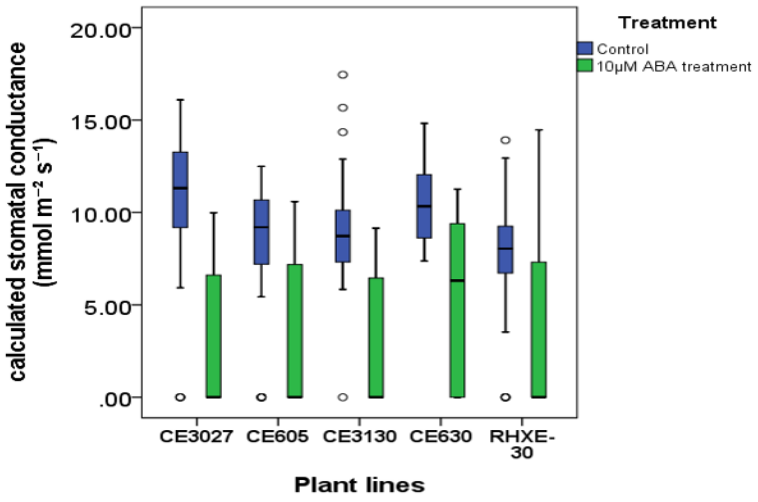
7. Supporting information

S1



S2