The time of the quantitative biology in cell level: Increasing stomatal density to an intermediate level through over-expressing *FSTOMAGEN* improved the biomass of plants

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Abstract

Stomata are the pores on plant surface, and these tiny pores are responsible for the flow of gas between plants and atmosphere. Increased stomatal density through genetical manipulation has been shown to accompany improved leaf photosynthetic rate. However, whether increasing stomatal density by genetical manipulation could improve plant biomass remains unknown.

The Arabidopsis transgenic lines with increased stomatal density were acquired through over-expressing a gene (the homologs of *STOMAGEN*, *FSTOMAGEN*, the homologs in *Flaveria*). The biomass of Arabidopsis transgenic lines with stomatal density raised to intermediate-level increased. Compared to the lines with lowest stomatal density, the biomass of the transgenic lines with exorbitant stomatal density showed no difference, even decrease. There was a positive correlation between biomass and water content, supporting that biomass is affected by water content, and excess stomata do not benefit the improvement of biomass due to the excessive loss of water. During the earlier stage of growth, the leaf growth rate and leaf area of the lines with increased stomatal density was faster and larger respectively, which was in agreement with the notion that more stomata indicated faster CO_2 assimilation and then faster accumulation of biomass.

Results indicated that finely tuning stomatal density could improve Arabidopsis biomass by genetically manipulating *FSTOMAGEN.* The function of *STOMAGEN* family is extremely conserved in evolution, thus it is promising that this genetically modified strategy to increase biomass could be applied in the other species, and furthermore benefit the reduction of the concentration of atmosphere CO_2 and the production.

Introduction

Stomata are the tiny pores on the surface of plants. They are the passages for the transfer of gas (e.g. CO_2, H_2O, O_2) between plants and atmosphere. Passing stomata, a large amount of water loses from plant and spreads into atmosphere. Therefore, stomata is a part that controls the water status of plants. Besides, stomata also participate in the photosynthesis, since the entrance of CO_2 into plants requires stomata. Stomata are responsible for the control of the amount of carbon in plants. In addition to these two principal functions, stomata also play an important role in many biological processes.

The mechanism of stomatal development has been well studied, and many genes related to stomatal development have been found (Pillitteri and Torii 2012; Zoulias et al. 2018). *EPF* family contain a series of genes which encode signal peptides(Takata et al. 2013; Hunt and Gray 2009; Hara et al. 2007; Sugano et al. 2010; Kondo et al. 2010; Hunt et al. 2010). For these genes, more focuses are put on *EPF1*, *EPF2* and *STOMAGEN*. The genes in EPF family *EPF1/2* negatively regulate the development of stomata, and *EPFL9* (*STOMAGEN*) positively regulate this development(Hara et al. 2007; Hunt and Gray 2009; Sugano et al. 2010; Kondo et al. 2010;

Hunt et al. 2010). The *EPF1/2* mutant and overexpression lines have the changed stomatal density in multiple species(Wang et al. 2016; Hughes et al. 2017; Caine et al. 2018), and *STOMAGEN* mutant and overexpression lines also have the changed stomatal density in various species(Lu et al. 2019), suggesting that these genes in *EPF* family are functionally conserved during evolution. The changes in these genes could affect the stomatal density, therefore many works change stomatal conductance through genetically manipulating these genes, and thus change transpiration and photosynthetic rate(Tanaka et al. 2013; Franks et al. 2015). Therefore, in theory, the changes in stomatal density likely cause the consequences that plant biomass is changed in these genetical engineering processes. Increased photosynthetic rate caused by raised stomatal density likely leads to increased plant biomass. However, work shows that the overexpression of *STOMAGEN* can't increase the biomass of Arabidopsis, though stomatal density of the line with overexpression of *STOMAGEN* dramatically increases(Tanaka et al. 2013). The possible reason for this increased biomass might be the overmuch stomata in the transgenic lines, since it is possible that overmuch stomata will result in the excessive loss of water in plants and consume the excessive loss of energy which participates in stomatal movement and development. Therefore, this study proposes the notion that moderately increasing stomatal density improve the biomass. Recently, stomatal density modified through genetical engineering has been raised a finely tuned level, influencing the drought resistant(Nicholas G. Karavolias 2023). In this study, they found only the lines with moderately decreased stomatal density could improve the drought resistant without decreased stomatal conductance, carbon assimilation and thermoregulation(Karavolias et al. 2023). This study supports the notion that changing stomatal density to a moderate level could be advantage than an overmuch level. Currently, whether increasing stomatal density to an intermediate level by genetically manipulating could increase photosynthetic rate remain unknown.

In this study, we checked (*FSTOMAGEN*) overexpression lines with different stomatal density in Arabidopsis. Our hypothesis is that stomatal density is increased to an intermediate level by genetical manipulation could accompany the increased biomass of Arabidopsis.

Methods

1, The building of construct

We over-expressed the functional region of *FSTOMAGEN* (the homologs of *STOMAGEN* in *Flaveria*) into Arabidopsis(Zhao et al. 2022). Specific speaking, the 35S promoter was used to drive the overexpression of the functional region of *FSTOMAGEN*. The functional region of *FSTOMAGEN* was acquired through the amplification from cDNA of *F.rob*. The signal peptide of the gene in the lines used in this study was also amplificated from *F. rob*. These molecular fragments were installed in the pCAMBIA-3300 carrier through homologous recombination(Vazyme). The pCAMBIA-3300 carrier was transformed into colon bacillus. Then, the carrier was transformed into agrobacterium GV3101(veidi). Transgenic lines were acquired by flower-dipping method. The transgenic lines were selected with 2000-diluted Basta. T2 generation of transgenic Arabidopsis was performed for subsequent experiment.

2, Growth condition

For the experiment, at the beginning, the transgenic Arabidopsis lines were grown under about 100 PPFD. After 19 days, the transgenic Arabidopsis lines were transferred into about 300 PPFD. Then, these transgenic Arabidopsis lines were transferred into about 500 PPFD.

3, The calculation of water content

The relative water content was calculated with following equation:

(The fresh weight – The dry weight)/The fresh weight

4, The photoing of Arabidopsis

After transfer the plants to soil, waiting 7 days and then photoing plants, and the interval of the photoing was two days. The photoing was performed by Cannon EOS 1500D camera (Canon Inc., Japan)

Results

1, The transgenic lines with moderately increased stomatal density showed increased rosette area and biomass.

The 9 Arabidopsis transgenic lines with the overexpression of the gene (*FSTOMAGEN*, the homologs in *Flaveria* of *STOMAGEN*) were acquired. Our work showed that stomatal density in these lines increased stepwise, and there was a positive relationship between stomatal density and expression level(Zhao et al. 2022). Besides, the work from others also indicates that the expression level of *STOMAGEN* among the different lines with the overexpression is positively corelated with stomatal density(Sugano et al. 2010).

Markedly, the differences in the total leaf area between these transgenic lines were observed (Fig 1A). Compared with other lines, two lines (11 and 12 line) had apparently increased total leaf area (Fig 1A). This encouraged us to test the biomass of these transgenic Arabidopsis lines. In consistent with total leaf area, there was a variance in biomass between these lines. The changed trend in fresh and dry biomass, accompanied by the stepwise increase in stomatal density, was first rise and then decrease (Fig 2A, B). Compared with the line with lowest stomatal density (8 line), the medians of the fresh and dry biomass in 11 and 12 lines were higher (Fig 2A, B). Particularly, compared with 8 line, the dry and fresh biomass of 12 line significantly differed (Supplementary file). The difference in dry biomass between 11 and 8 lines was in the margin of statistic test (Supplementary file). The stomatal density in 30 lines and 9 lines were extremely high among all these lines, but the biomass in 30 lines was significantly lower than that in 8 line and there is no significant difference in biomass between 9 lines and 8 lines (Supplementary file).

Three level in biomass was apparently observed (Fig 2C), and stomatal density gradually increased. Therefore, the lines were divided into three groups to analyze the difference (Fig 2C). The results showed that there was a significant difference in biomass between lower level and intermediate level, or intermediate level and higher level, whether dry weight or fresh weight (Fig 2C). There was no significant difference in biomass between lower level and higher level (Fig 2C).

2. There was a positive correlation between biomass and water content in different transgenic lines of Arabidopsis.

In the late stage of growth, the rosette of Arabidopsis in some lines turned to purple, brown and dried-up (Supplementary figure 2). Since these transgenic lines had increased stomatal density, the change in rosette might be caused by the excessive loss of water. Additionally, photosynthesis could be significantly affected by the water content of plants. Therefore, we tested the water content of these transgenic plants. We found indeed that the lines with increased stomatal density had lower water content (Fig 3A, supplementary file). Especially, the water content of the 30 lines accompanied with overmuch stomatal density was extremely low (Fig 3A), and the purple and brown of the leaf for this line were most apparent (Supplementary figure 2). More important, the leaf of this line became withered. These results indicated that these transgenic lines experienced water limitation during the growth.

The decreased water content in plants probably decrease the photosynthetic rate, and then decrease the biomass of plants. Therefore, we tested the relation between biomass and the water content of plants. Results showed that there was a positive relationship between dry weight and water content ($y=-0.8444+1.2575x$, $R^2=0.3038$, $P=0.0052$) (Fig 3B).

3. The leaf area and leaf growth rate of the transgenic lines with increased stomatal density were increased in the earlier period.

We observed the leaf growth rate of these transgenic lines during the earlier period. We found both the leaf area and leaf growth rate of these transgenic lines increased stepwise as stomatal density increase during this period (Fig 4A, B). Especially, because the stepwise increase in leaf growth rate (differential coefficient in the Figure 4C), This result accorded to the theory that increased stomatal density induce increased photosynthetic rate and furthermore increased biomass accumulated rate (leaf growth rate almost equal biomass accumulated rate). The increased biomass accumulated rate caused the final difference in the biomass and leaf area (Fig 1,4). It is worth to note that the pattern of growth rate after 20 days was not similar to the period before this day (Fig 4B). There was a large difference in growth rate between these lines from 15 to 20 days (Fig 4B).

Discussion

1) Appropriately increase stomatal density could increase plant biomass

Increasing stomatal conductance could improve photosynthetic rate. Therefore, it is promising to improve plants biomass through increasing stomatal conductance. Enhanced stomatal conductance could be achieved by increased stomatal density or increased stomatal aperture. In this study, we focused on the increase in stomatal density by genetically manipulating the expression of the gene belonging to *Flaveria* (the homolog of *STOMAGEN*, the homolog is from *Flaveria* genus). Our results showed that the lines with moderately increased stomatal density had higher biomass as compared to the lines with lower stomatal density (Fig 2A, B). In agreement with other work(Tanaka et al. 2013), our work also found that the biomass of the line with extremely increased stomatal density didn't change, certain line even decreased (Fig 2A, B). The reason for this phenomenon might be that the overmuch

stomata largely enhance transpiration, and the amount of the lost water largely exceed the amount of the absorbed water through root. This causes a consequence that plants are at the status with an extreme lack of water (Fig 3A). The extreme lack of water will cause the two occasions that restrict photosynthesis: 1, inducing the decrease in stomatal conductance; 2, weaken the photosynthetic biochemical reaction. Therefore, the growth of the plants was restricted and the biomass was decreased. The notion that the decreased biomass was caused by the decreased water content in this study was supported by (Fig 3B). There was a positive correlation between biomass and water content (Figure 3B), suggesting that decrease in water content weakened the growth of plants and the accumulation of biomass.

Though the trouble that decreased water content might accompany the lines with moderately increased stomatal density (Fig 2A), the effect that decreased water reduce the growth of plants might be relatively weak, since the biomass of moderately increased stomatal density was increased (Fig 2A, B). The extent of the decrease in water content is not reach the bound where photosynthesis is mightily reduced.

Another reason for the decreased biomass in the lines with extremely increased stomatal density should be that energy was overly consumed in the stomatal development and movement in the lines with overmuch stomata. The overly consumption in energy reduced the biomass which should be increased in the lines with overmuch stomata.

Our work has showed that the substitution of signal peptide did not affect the function that regulates stomatal density (Supplementary figure 1), indicating the combination of the functional region and the signal peptide does not affect stomatal density. The function of the functional region is independent from the function of the signal peptide. The function of the signal peptide should be restricted in the accurate location of the gene. Therefore, many types of signal peptide that can accurately locate the gene may be able to replace the own signal peptide of the gene.

2) Stomata have a large potential to become the crucial model in the age of quantitative biology of cell level.

Genetic engineering in biological science has long been a hotspot. However, many studies focus on the quality in the biological process. Overly changes in the biological process would cause the consequence that is not expected. The results for the lines with overmuch stomata in this study can be an example for this claim (Fig 2A, B). Moderate extent in the engineering of biological process is required.

Our study was an initiating work in the research area that study on quantity in cell level to regulate plants morphology by genetically engineering. In particular, it was the first time that study showed that the lines with moderate stomatal density indeed are able to possess the expected biological process, i.e. increased biomass. There are some limitation for this study. In this study, we used the 35S promoter to launch the over-expression of *FSTOMAGEN*, and selected the lines with different expression. This will encounter multiple troubles. For example, engineering in this study introduced the exogenous gene into the plants. Second, selecting the lines with moderate expression will consume much time. Recently, a large number of biological techniques has been developed, representing that the approach to manipulate molecular level has been becoming various and abundant. Among these approaches, the representative widely known is the crispr-cas9. Directly editing the genome

to produce the various expression of the gene could be a better strategy.

The molecular mechanism of stomatal development has been well studied. Besides, stomata are convenient to observe and count, and many software tools for counting stomata has been developed in recent years(Fetter et al. 2019). More crucially, the reduced atmosphere CO_2 and increased biomass through increasing stomatal density could enable us attach importance to this research. Therefore, stomata could be an ideal model for the quantity biology in cell scale.

3) Increasing stomatal density to a moderate level through genetically engineering can benefit the reduction of the concentration of CO_2 and production.

The genes in regulating stomatal development are functionally conserved across different species, and many genes found in Arabidopsis are also work in regulating stomatal development in other species(Yin et al. 2017; Lu et al. 2019; Wang et al. 2016; Caine et al. 2018; Hughes et al. 2017). This means that we can modify stomatal density in these species through genetically manipulating the homologs of the genes in regulating stomatal development, which is similar to the experiment conducted in this study.

The increase in the concentration of atmosphere CO_2 could raise the temperature, and this could affect and change global environment. The accelerated increase in the concentration of atmosphere CO_2 could accelerate the change of global climate and environment. This may cause the extreme and rare climate and environment, and induce the natural hazard like drought and flood. Therefore, exploring the approach to handle the increase in the concentration of atmosphere CO_2 is necessary. This study provides insights into the approach. Our study indicated that moderately increased stomatal density could improve the biomass of plants, denoting that increased stomatal density could improve the net CO_2 assimilation rate of plants and enhance the capacity of plants to absorb CO_2 from external environment. Because the function of genes in regulating stomatal development found in Arabidopsis is conserved in other species, and these species are important for the ecology. It is likely that increasing stomatal density to a moderate level in these species could significantly improve the biomass and benefit the decrease in concentration of atmosphere CO_2. Besides, the increased biomass of plants can be used for human. For example, in the industrial and manufacturing sectors, the increased biomass can be used to produce some productions that need cellulose.

4) An example for the combination between genetic engineering and the controlling of environment

The traits or characteristics of species enable plants to adapted to certain environment, and this is done by long-term evolution. But in current age, the changes in environment become faster. A faster strategy is needed to meet the current changes. Our study suggested that these goals could be reached by manipulating in the molecular levels. The increased stomatal density enables plants to adapted to grow in the environment with sufficient water and high light intensity. In addition to adapt to the natural environment, in future, the environment (sufficient water and high light intensity) in the intelligent plant factory will be suitable to the increased stomatal density. In theory, the characteristics are more complex (For example, biomass), the modification from the single gene used by genetic engineering

will be more difficult to enable plants having the expected characteristics. Our study proposed that the cooperation between the genetic engineering and the controlling of environment could solve this trouble. Additionally, the current goal of the genetic engineering is to find the genes that function in most, changed and even all environments. This seems not realistic. Although there probably are few genes can reach this goal, most genes do not accord to this characteristic. This largely restricts the application and the development of genetic engineering and molecular experiment. It is believed that most genes have their roles, and when in corresponding environment, they can show their function in regulating the traits. Though there are some pseudogenes, these genes may perform a role in certain conditions. In this study, the biomass of Arabidopsis was increased under a higher light intensity. Although we didn't perform the experiment under lower light intensity, increasing stomatal density should not improve the biomass since the photosynthetic biochemistry is limited. Therefore, this study is a valuable example and provides the new insights for these kinds of studies.

Acknowledgement

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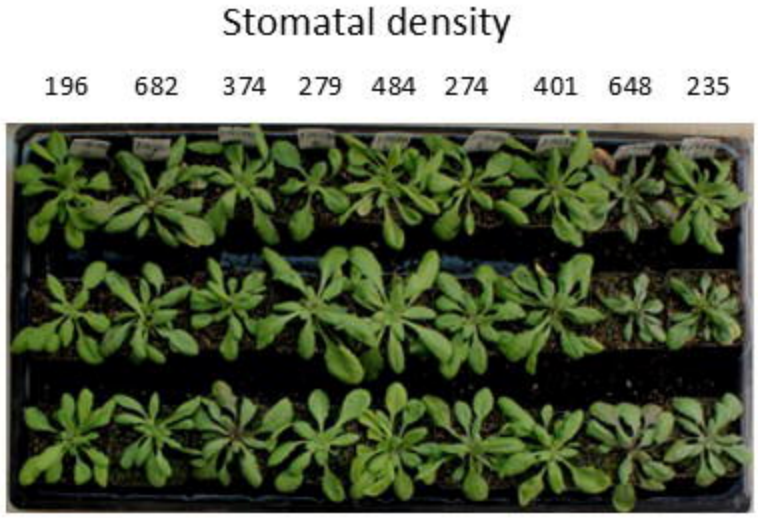
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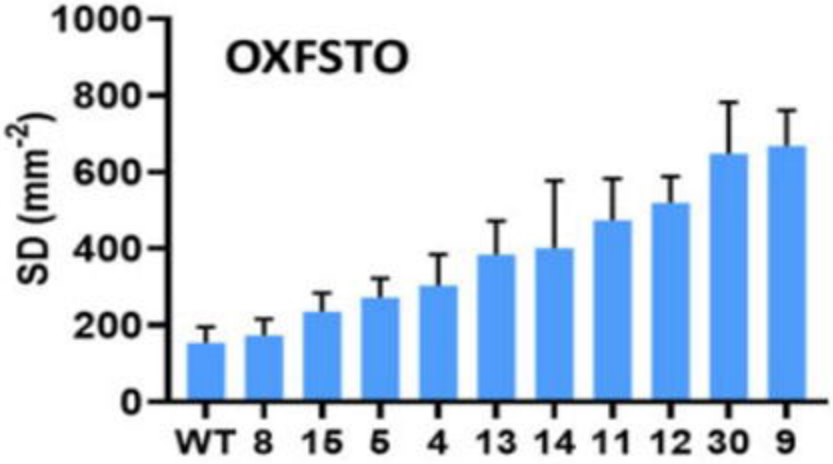
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600
400
200
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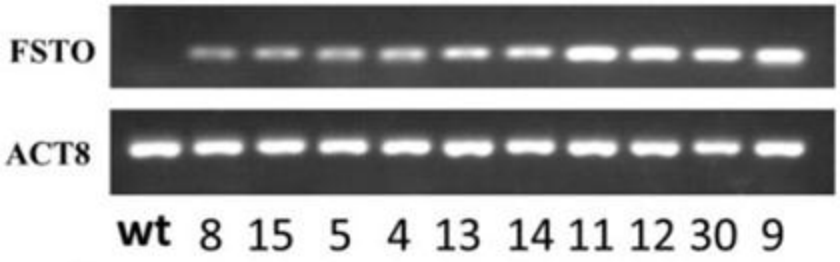
WT 8 15 5 4 13 14 11 12 30 9

C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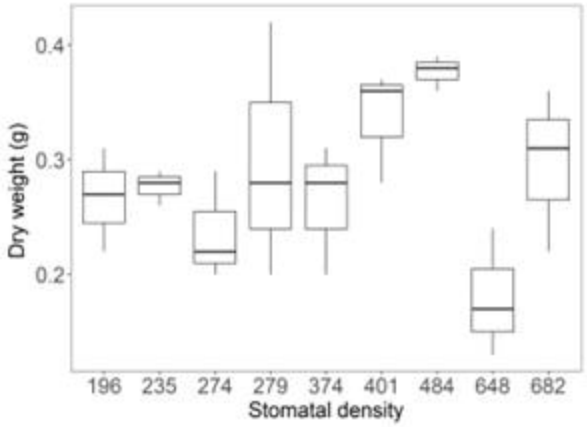
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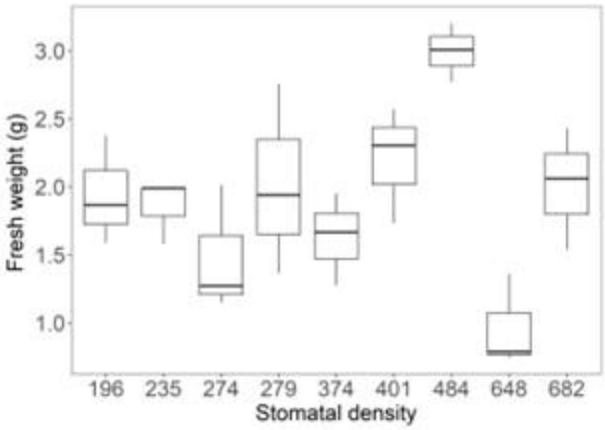
Stomatal density

B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1.0
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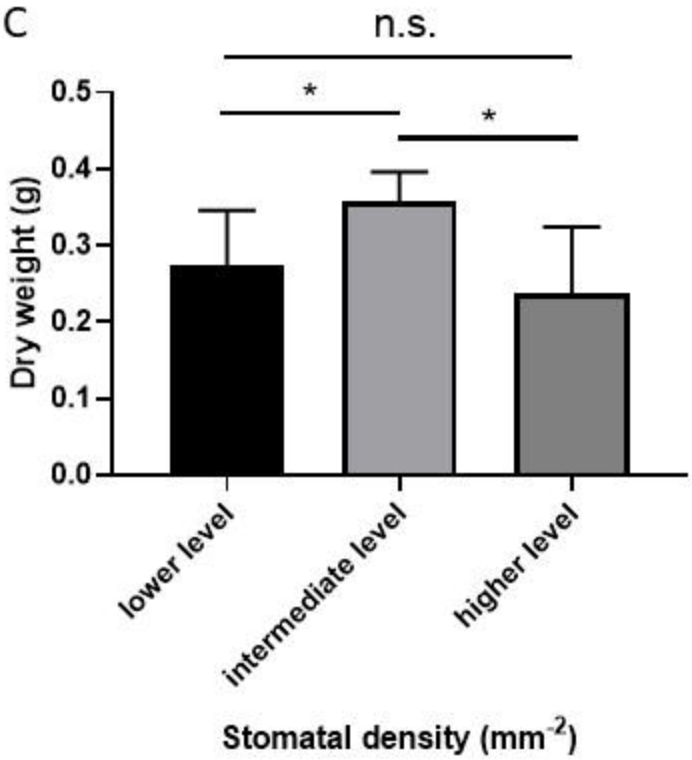
Stomatal density

C


n.s.
*
*
Dry weight (g)
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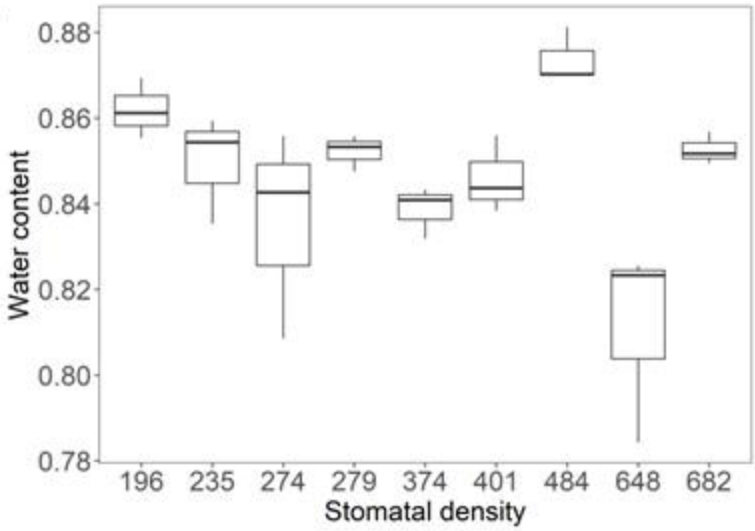
lower level
intermediate level
higher level
Stomatal density (mm-2)

A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Water content
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374
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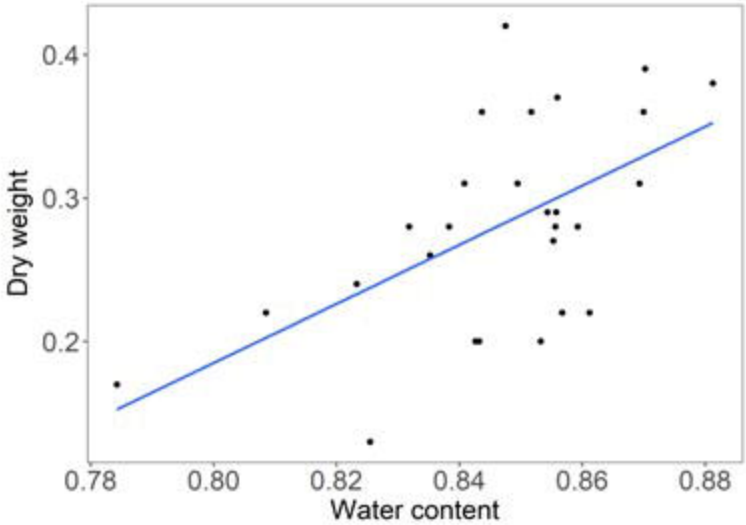
682
Stomatal density

B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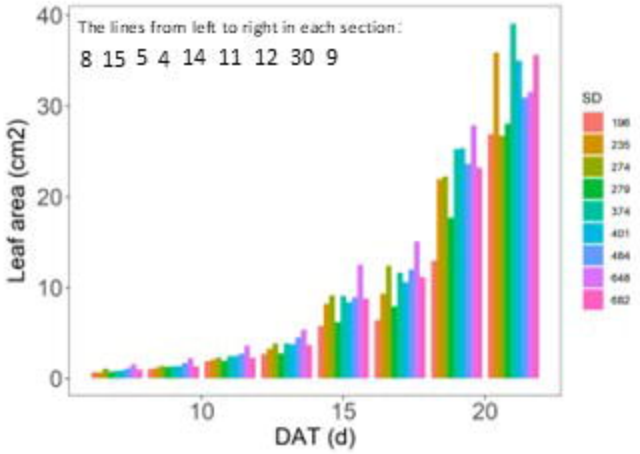
0.88
Water content

A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8 15 5 4 14 11 12 30 9
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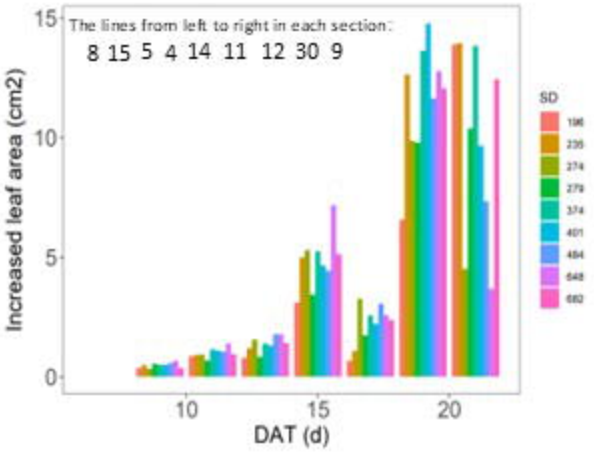
DAT (d)
SD
196
235
274
279
374
401
484
648
682

B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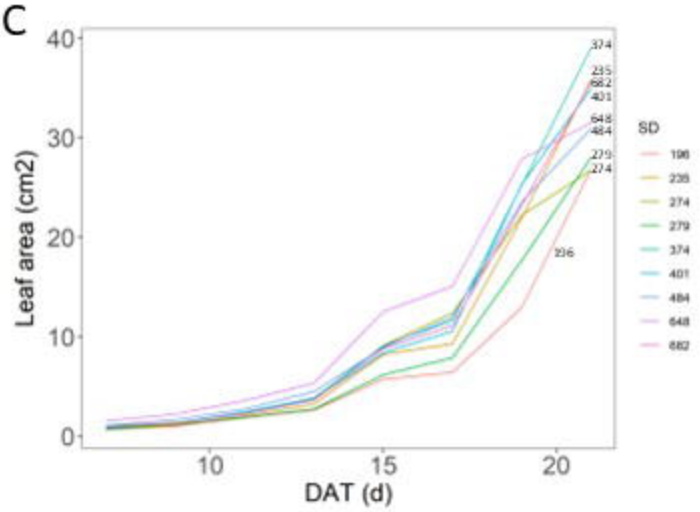
484
648
682

C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DAT (d)
SD
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274
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484
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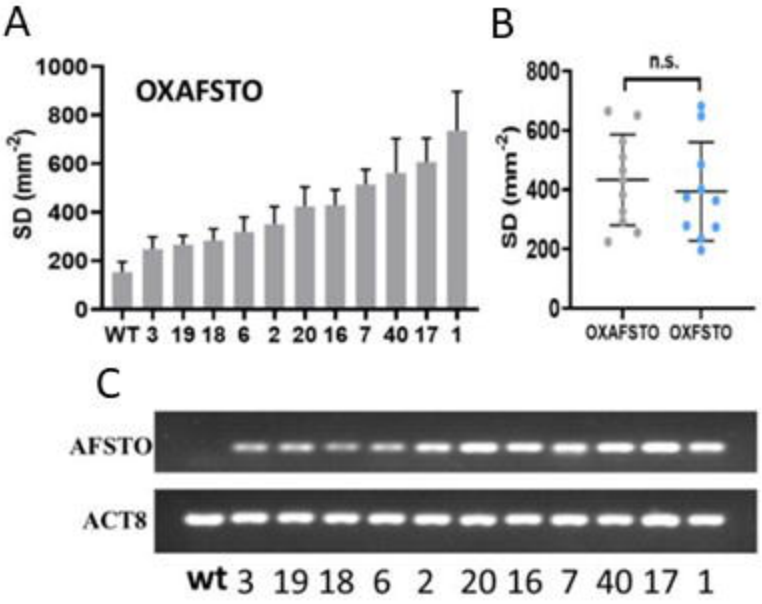
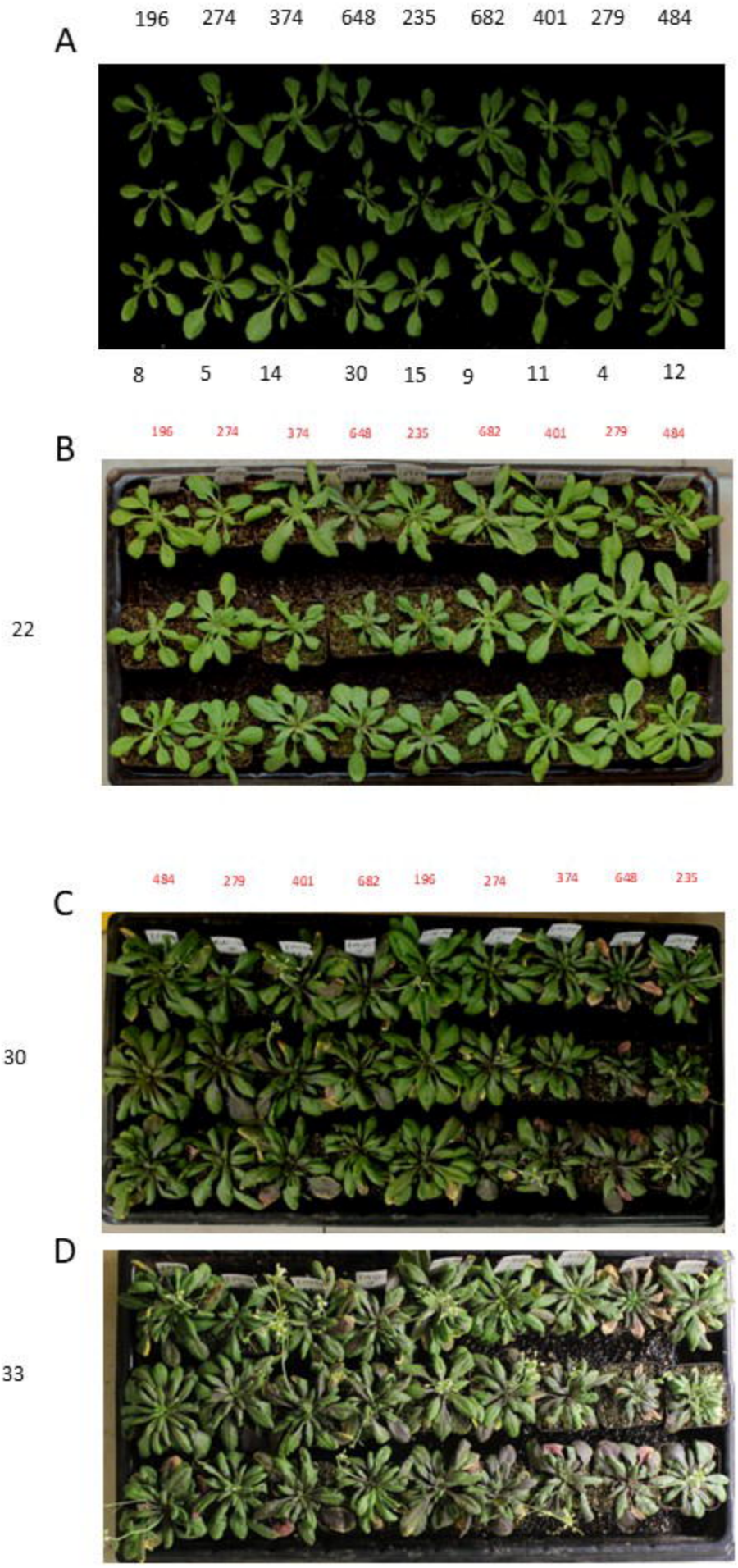
682



Supplementary figure 1



Supplementary figure 2