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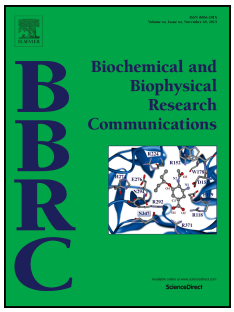

Modification of *AtGRDP1* gene expression affects silique and seed development in *Arabidopsis thaliana*

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Modification of *AtGRDP1* gene expression affects silique and seed development in *Arabidopsis thaliana*

Running head

The *AtGRDP1* gene deregulation alters the fruits

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Abstract

Glycine Rich Proteins (GRPs) are induced at different developmental stages and in specific plant tissues. Recently, we described a novel Arabidopsis gene encoding a short glycine-rich domain protein (AtGRDP1). This gene is involved in abiotic stress responsiveness; the *Atgrdp1*-null mutant seeds were more sensitive to stress, while the opposite phenotype was achieved by *AtGRDP1* overexpression. In this study, we analyzed the phenotype of the fruits produced by Arabidopsis *Atgrdp1* mutants and *35S*::*AtGRDP1* overexpression lines. Our analyses revealed important changes in silique length, seed number, seed weight and morphology in the analyzed lines. In particular, *Atgrdp1* mutant lines exhibited several defects including short siliques, a diminished number of seeds per silique, and reduction in seed size and weight as compared to Col-0. The overexpression of the *AtGRDP1* gene also generated phenotypes with alterations in size of silique, number of seeds per silique, and size and weight of seed. In addition, the expression analysis of *AtGRDP1* gene showed that it was expressed in floral and fruit organs, with the highest expression level in mature siliques. The alterations in the siliques and seeds traits in the *Atgrdp1* mutant line, as well as the phenotypes observed in *AtGRDP1* overexpression lines, suggest a role of the *AtGRDP1* gene in the Arabidopsis fruit development.

Key words: *AtGRDP1*; Glycine-rich domain protein; seeds; siliques.

Introduction

Seeds are important for plant reproduction, nourishment of the embryo, spread of plants, and in emergence and survival of seedlings [1]. Seeds with an appropriate height and width are a determinant factor of evolutionary fitness in plants, and are also an important agronomic trait in crop domestication [2]. Larger seeds ensure better seedling establishment under several stress conditions, whereas small-seeded plants produce large numbers of seeds resulting in a successful scattering [3,4].

In Arabidopsis, we recently reported a novel gene family "DUF1399-GRDP" composed of four members, two of which (*AtGRDP1* and *AtGRDP2*) were partially characterized by our group. The main feature of the both novel proteins is the presence of short glycine-rich domain, a domain of unknown function (DUF1399), and a potential RNA-binding domain [5,6]. Glycine-rich domain proteins (GRDPs) have been reported in other plants, such as Eucalyptus and common bean [5,6,7,8,9]. The initial characterization of *AtGRDP1* gene revealed an important role during abiotic stress tolerance [5]. Moreover, the overexpression of the *AtGRDP2* paralogue in Arabidopsis, lettuce and common bean resulted in salt tolerant phenotypes. *AtGRDP2* gene is mainly expressed in Arabidopsis floral organs; and its involvement in Arabidopsis development was evidenced through the analysis of *Atgrdp2* mutants and 35S::*AtGRDP2* overexpression lines [6].

Herein, we describe the phenotypes of the fruits generated in Arabidopsis loss-of-function *Atgrdp1* mutant and 35S::*AtGRDP1* overexpression lines. To this aim, we evaluated several parameters such as silique length, seed number per silique, seed weight and morphology. In

addition, we analyzed the transcript levels of *AtGRDP1* in floral and fruit organs at different developmental stages. Our data suggest that *AtGRDP1* gene is required for a normal silique and seed development.

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Materials and Methods

Plant material and growth conditions

In this study, we used Col-0 ecotype, *Atgrdp1-null* mutant and 35S::*AtGRDP1-3*, -5, and -*6* overexpression lines [5]. The Arabidopsis seeds were sterilized by using a 40 % (v/v) chlorine solution for 10 min, after seeds were washed six times with sterile distilled water. Seeds were then stratified on agar plates containing 0.5x Murashige and Skoog (MS) medium, pH 5.7, 0.5% (w/v) sucrose, and 1.2% (w/v) agar for 2 days at 4ºC [10]. MS plates were incubated in a growth chamber with a photoperiod of 16 h light (120 μmolm^{-2} s^{-1}) and 8 h darkness cycle, at a temperature of 22 ± 2ºC. In order to obtain the lot of seeds for all lines, seedlings were grown in plastic pots with a mixture of Sunshine Mix#3 commercial substrate and vermiculite (3:1) under environmental controlled conditions.

Stereoscopic and environmental scanning electron microscopy (eSEM) analysis

The immature siliques were collected from Col-0, *Atgrdp1*, 35S::*AtGRDP1-3*, *-5* and *-6* overexpression lines, and the valves were gently removed from siliques employing forceps and scalpel. Septum and seeds were observed with the help of stereomicroscope (MoticSMZ-143). The magnification for the images captured was 25x immature siliques. Three immature siliques of each line were analyzed, and a representative image was shown in each microphotographs.

For eSEM analysis, dried seeds for each line were glued onto pure carbon containing polymer films, and fixed onto sample holders. The seed coat morphology of Col-0, *Atgrdp1*-null mutant and 35S::*AtGRDP1-3*, *-5* and *-6* overexpression lines were observed, and the seed width and length were measured with a high-resolution scanning electron

96 microscope (eSEM/QUANTA 200 FEI, Low Vacuum/Water). Microphotographs were
97 taken with the eSEM (pressure chamber at 90 - 100 Pa and a voltage of 15.0 and 30.0 Kv).
98 The image analysis software was used to evaluate seed size. The morphology of the
99 embryos from Col-0, *Atgrdp1*-null mutant and 35S::*AtGRDP1-3*, *-5*, and *-6* overexpression
100 lines were evaluated by the eSEM analysis. Embryos were glued onto pure carbon, and
101 fixed on eSEM sample holders. Morphological seed assays were carried out using 15 seeds
102 of each genotype.
103
104 **Determination of silique length and seed number per silique of *AtGRDP1* mutants and**
105 **overexpression lines**
106 Silique length was evaluated in 35 day-old Col-0, *Atgrdp1*, 35S::*AtGRDP1-3*,
107 35S::*AtGRDP1-5*, and 35S::*AtGRDP1-6* plants. A total of twelve plants of each line were
108 used for silique and seeds counting (three siliques per plant). Siliques were analyzed at 8
109 days after flowering. Seed number per silique was recorded for each line. The silique length
110 was expressed in mm from 3 siliques per plant and twelve biological replicates of each line
111 ($n = 36$). The plot was expressed as number of seeds per silique.
112
113 **Determination of seed weight of *Atgrdp1* mutant and overexpression lines**
114 Seed weight was calculated in Col-0, *Atgrdp1*, 35S::*AtGRDP1-3*, 35S::*AtGRDP1-5* and *-6*
115 lines. We recorded a total of 500 seeds with three replicates of each line ($n = 3$). The
116 analyzed seeds were harvested at the same time. Weights and seed size were expressed in
117 mg and µm. All measurements were repeated at least three times with similar results.
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Quantification of *AtGRDP1* transcript in different floral organs and fruits

Buds, flowers, and siliques of 0.3-05 cm, 0.7-0.9 cm, 1-1.5 cm, and > 2 cm, and also mature siliques of 30-day-old plants were frozen in liquid nitrogen and processed to obtain a total RNA using the Concert Plant RNA Kit (Invitrogen, Carlsbad, USA). All possible remaining genomic DNA was removed by DNase Turbo (Ambion, Austin, TX, USA). Quantitative RT-PCR (qRT-PCR) analysis were performed with 30 ng of total RNA by the one-step assay using the iScriptTM One-Step RT-PCR kit with SYBR Green (Applied Biosystems, USA). All reactions were performed in 10 µl reaction mixture containing, 5 µl of Power SYBR Green RT-PCR Mix (2X), 200 nM of each oligonucleotide, and 0.08 µl of RT Enzyme Mix (125X), using the StepOne Real-Time PCR Detection System (Applied Biosystems). The thermal cycling conditions used were as reported by [5]. For each RNA sample, three biological replicates (n = 3) were analyzed with their respective technical replicates. The *ubiquitin 5* (*UBQ5*) gene from *A. thaliana* was used as expression control. The relative gene expression levels of *AtGRDP1* gene was presented as $2^{-\Delta Ct}$, where Δ Ct = $Ct_{AtGRDP1}$ - Ct_{UBQ5} [11]. The primers used for the *AtGRDP1* gene were: UTR3-F 5′AAATGGAGGCGGTTGCGGT3′ and UTR3-R 5′CAGATCCTCACAGTCTTTGGC3′; For the *UBQ5* gene loading the primers employed were UBQ5-F 5′TCGACGCTTCATCTCGTCCT3′ and UBQ5-R 5′CGCTGAACCTTTCCAGATCC3′. Experiments were repeated at least twice with similar results.

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144 **Statistical Analysis**

145 The data generated from silique and seed evaluation parameters were statistical
146 analyzed using One-way (ANOVA) in GraphPad Prism version 5.0b (GraphPad, San
147 Diego, California, USA) software. The significance of differences among samples was
148 determined with Tukey's post-test. The data are presented as the mean ± standard error
149 of the mean (SEM). Significant differences are represented with letters at ($P < 0.05$).

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Results

Evaluation of silique traits in *Atgrdp1*-null mutant and 35S::*AtGRDP1* overexpression lines

Different silique traits such as length and seeds per fruit were examined in 35 day-old *Atgrdp1* mutant and 35S::*AtGRDP1* overexpression lines. Our analyses revealed silique size alterations in the *Atgrdp1* loss-of-function mutant, displaying a 38.5% reduction in the silique length when compared to parental Col-0 siliques (Fig 1A, B). The 35S::*AtGRDP1* transgenic lines also showed differences in siliques. All overexpressing lines had smaller siliques than parental Col-0, observing a reduction of 29.4% in the 35S::*AtGRDP1-3* line, 22.4% in the 35S::*AtGRDP1-5* line, and 35.7% in the 35S::*AtGRDP1-6* line (Fig 1A, B).

Regarding the number of seeds per silique (Fig. 1C), the 35S::*AtGRDP1-5* line produced the highest number of seeds per silique (58.2 seeds); whereas, the *Atgrdp1* mutant (with 42.2 seeds), 35S::*AtGRDP1-3* line (with 45.7 seeds) and 35S::*AtGRDP1-6* (with 32.7 seeds) generated the lowest number of seeds per silique when compared with the parental Col-0 with 54.3 seeds/silique (Fig. 1C). In the Figure 1D, we showed stereomicroscope images of immature green siliques of all lines, where *Atgrdp1* mutant and 35S::*AtGRDP1* lines had reduced space among seeds probably due to shorter siliques. Moreover, *Atgrdp1* mutant and 35S::*AtGRDP1-3* and 35S::*AtGRDP1*-6 lines displayed aborted ovules (Fig. 1D).

Seed weight and morphology of *Atgrdp1*-null mutant and 35S::*AtGRDP1* overexpression lines

We evaluated the seed weight of the *Atgrdp1*-null mutant and *35S::AtGRDP1* overexpression lines. Seeds of *Atgrdp1*-null mutant plants were lighter than those of the parental Col-0 plants; these mutant seeds were 16% lighter than Col-0 seeds. Conversely, we found that the 35S::*AtGRDP1-3* and 35S::*AtGRDP1-6* overexpression lines generated seeds with higher weight than Col-0, achieving 11.5 and 22%, respectively (Fig. 2A). In the case of 35S::*AtGRDP1-5* line produced seeds whose mean weight values were similar to Col-0 (Fig. 2A). Additionally, an environmental scanning electron microscopy (eSEM) analysis was carried out to observe changes among the seeds from Col-0, *Atgrdp1*-null mutant and 35S::*AtGRDP1* overexpression lines. Parameters evaluated were seed shape and size (Fig. 2B, C). Consistent with seed weight data, *Atgrdp1*-null mutant seeds were smaller and disclosed a round-shape. These small seeds displayed 20% reduction in width and 15% in length versus Col-0. Interestingly, seeds of 35S::*AtGRDP1* overexpression lines exhibited altered seed width and length; the biggest differences were observed in 35S::*AtGRDP1-6* seeds (Fig. 2B). In addition, we observed that the surface of the seed testa in the *Atgrdp1*-null mutant and 35S::*AtGRDP1* overexpression line displayed variations in the columella shape compared to Col-0 seeds (Fig. 2C).

Expression profile of *AtGRDP1* gene in Arabidopsis floral organs and fruits

AtGRDP1 organs-specific expression pattern was evaluated by qRT-PCR in different floral and siliques stages of 30 days-old Arabidopsis ecotype Col-0 plants. The floral organs analyzed were buds, and flowers; the fruits were immature developing siliques in four sizes 0.3-0.5 (stage 1), 0.7-0.9 (stage 2), 1-1.5 (stage 3) and 2 cm (stage 4) and mature siliques

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(stage 5) (Fig. 3). We observed that *AtGRDP1* expression was weaker in buds and siliques (stages 1, 2 and 3) than in flowers. In contrast, the highest *AtGRDP1* expression levels were found in the siliques (stage 4) and in mature siliques (stage 5) (Fig. 3).

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Discussion

The *Atgrdp1* mutant displayed several phenotypes in siliques and seeds; particularly, this mutant line produced less seeds, which in turn were smaller and round-shape in comparison to the parental (Col-0) seeds. These results are in agreement with the shorter siliques and with aborted ovules observed in the *Atgrdp1* mutant line. Apparently, only seed size was affected, since the embryos of the *Atgrdp1* mutant were not smaller than the embryos of the parental Col-0. This could suggest a deregulation during seed formation in the absence of *AtGRDP1* gene, resulting in a smaller seed and less seed number/silique. Previously, we reported that during germination under different abiotic stress inductors such as salt and osmotic treatments, the *Atgrdp1*-null mutant line showed a significant decrease in the rate of germination [5]; accordingly, the *Atgrdp1* mutant small seeds are more vulnerable to abiotic stress.

Variations on seeds and siliques were also observed when the overexpression lines (*35S::AtGRDP1-3*, *-5*, and *-6*) were analyzed. All overexpression lines of *AtGRDP1* gene had smaller siliques than the parental Col-0. The *35S::AtGRDP1-3* and -6 showed a lower number of seed per silique, since they possessed aborted ovules. On the other hand, *35S::AtGRDP1-5* was the line that presented highest number of seeds per silique, even more than Col-0, showing a reduced space among seeds. Interestingly, the 35S::*AtGRDP1-6* generated the biggest seeds; this transgenic line also had the highest expression rate of the *AtGRDP1* gene of the three overexpression lines [5].

We found that the *AtGRDP1* gene was expressed during flower and fruit development, with the highest expression being in >2cm siliques and mature siliques. Accordingly, there are

reports that canonical Glycine Rich Proteins (GRPs) are expressed in floral organs and related to correct development of reproductive structures [12]. In Arabidopsis, it has been reported that *AtGRP1* and *AtGRP2* transcripts are abundant in flowers tissues [13]. Another characterized GRP, the AtOGB3 that exhibit an oleosin domain, is required for pollen hydration [14]. Additionally, immature seeds, particularly in cells of the globular, heart shaped, and torpedo embryos showed the high GUS activity in spatial analysis of the *AtGRP2* expression pattern [15]. Recently, our research group reported the characterization of Arabidopsis Glycine Rich Domain Protein (*AtGRDP2*) gene, paralogue of *AtGRDP1* gene, put in evidence the high expression levels of *AtGRDP2* in buds, flowers and immature siliques [6]. These data showed that both glycine-rich domain and glycine rich proteins are expressed in plant reproductive tissues. All these data suggest a role of the *AtGRDP1* gene in the silique and seed development.

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Legend to figures

Figure 1. Silique morphology of *Atgrdp1*-null mutant, and 35S::*AtGRDP1* overexpressing lines. (A) Silique phenotype of 35-days-old Col-0, *Atgrdp1* and 35S::*AtGRDP1* overexpression lines grown under long day conditions. The scale bar corresponds to 0.5 cm. (B) Measure of length of mature seeds were represented graphically (C). The seed number per mature silique was represented graphically. The measures were carried out using three siliques per plant, and twelve plants per line were analyzed (n = 36). Error bars denote ± SE and different letters are used to indicate significant differences among lines according to the One-way ANOVA analysis and Tukey's multiple comparison tests ($P < 0.05$). (D) Representative images of a cut section in green silique from 35-day-old Col-0, *Atgrdp1*-null mutant, and 35S::*AtGRDP1* overexpression plants grown under long day conditions. The yellow arrows indicate the abortive events in silique. The scale bar corresponds to 600 µm.

Figure 2. Seed morphology of *Atgrdp1* mutant line and 35S::*AtGRDP1* overexpressing lines. (A) Weight of seeds (mg) from the Col-0, *Atgrdp1*-null mutant, and 35S::*AtGRDP1* over-expression lines. Error bars represent the means ± SE (n = 500) with three replicates. (B) Width and length of seeds (µm) from the Col-0, *Atgrdp1*-null mutant, and 35S::*AtGRDP1* overexpression lines. Error bars represent the means ± SE (n = 15) with three replicates. Different letters indicate significant differences between the Col-0, *Atgrdp1*-null mutant, and 35S::*AtGRDP1* overexpression lines. One-way ANOVA thought Tukey's multiple comparisons test was used to analyze the data ($P < 0.05$). (C) Scanning electron micrographs showing: whole seed, scale bar corresponds to 200 µm; seed coat,

scale bar corresponds to 50 µm; seed coat detail, zoom 30x from micrograph of seed coat; and embryos, scale bar corresponds to 300 µm of Col-0, *Atgrdp1* mutant line, and 35S::*AtGRDP1* overexpression lines.

Figure 3. **Expression profile of *AtGRDP1* gene in floral and fruits organs.** (A) Estimation of *AtGRDP1* transcript in flowers and fruits of 30 day-old Arabidopsis Col-0 plants by qRT-PCR. Buds, flowers, immature developing siliques in four sizes 0.3-0.5 (1), 0.7-0.9 (2), 1-1.5 (3) and 2 cm (4) and mature siliques (5) were analyzed. The *A. thaliana ubiquitin5* (*UBQ5*) gene was used as loading control, and the relative gene expression levels of *AtGRDP1* gene were presented as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{AtGRDP1} - Ct_{UBQ5}$. Bars represent the means ± SE ($n = 3$). Different letters indicate significant differences at ($P < 0.05$) analyzed with One-way ANOVA thought to Tukey's multiple comparison test.

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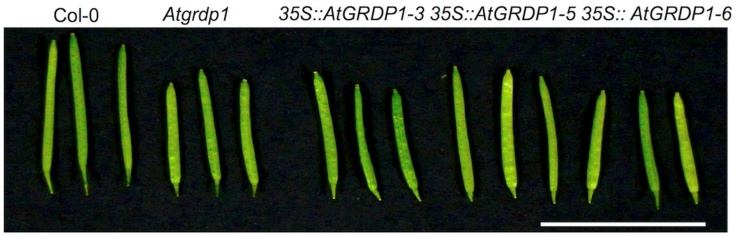

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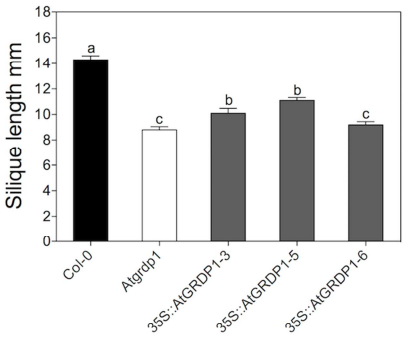

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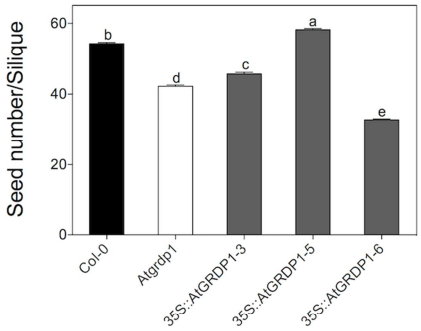

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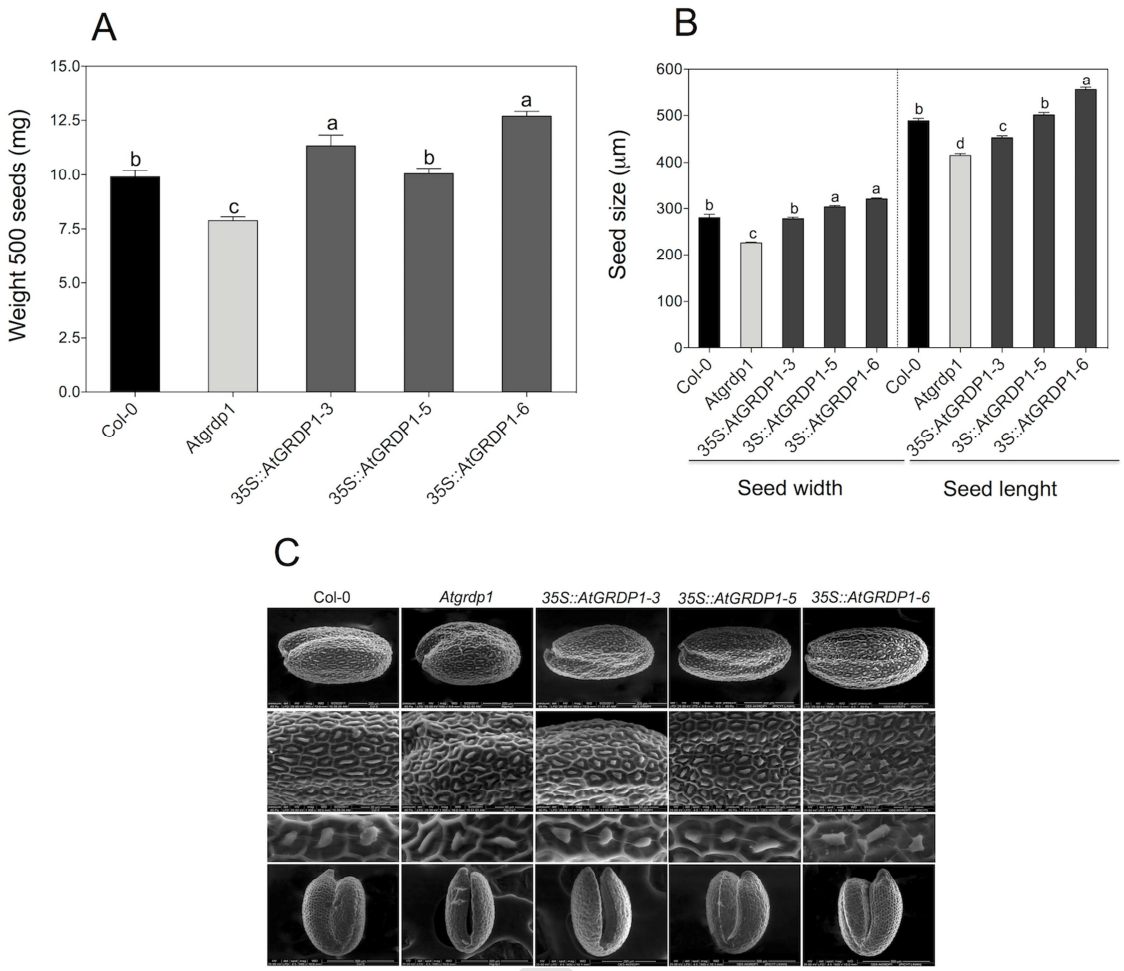
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10.0
7.5
5.0
2.5
0.0
b
c
a
b
a
Col-0
Atgrdp1
35S::AtGRDP1-3
35S::AtGRDP1-5
35S::AtGRDP1-6
B
Seed size (µm)
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500
400
300
200
100
0
b
c
b
a
a
b
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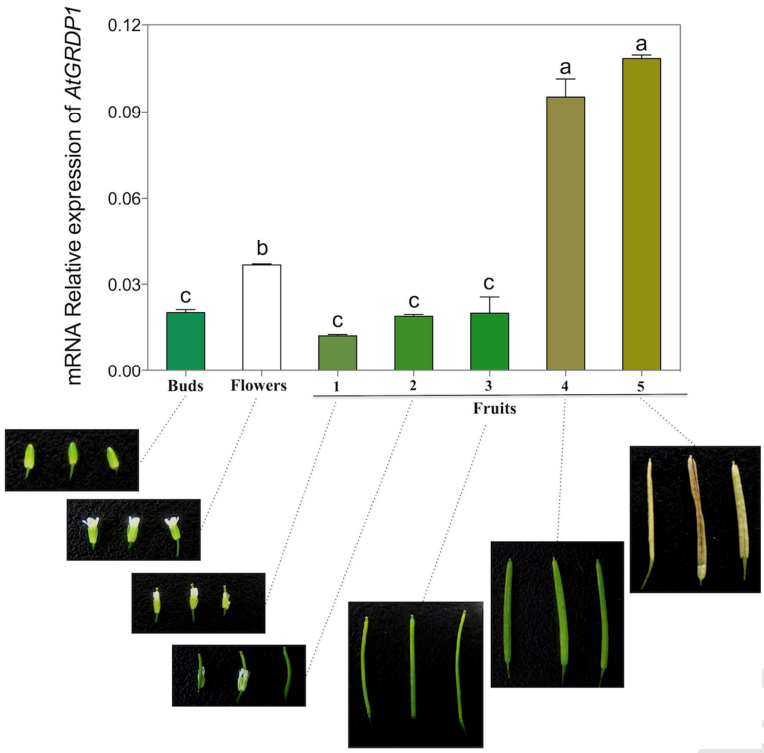

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Highlights

The highest expression of AtGRDP1 was found in mature siliques

Seed number and size was altered by deregulation of *AtGRDP1* expression

Silique size was smaller in *Atgrdp1*-null mutant and 35S::*AtGRDP1* overexpressing lines

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