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

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

3
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5 Running head
6 **The *AtGRDP1* gene deregulation alters the fruits**

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21
22

23 **Abstract**

24

25 Glycine Rich Proteins (GRPs) are induced at different developmental stages and in
26 specific plant tissues. Recently, we described a novel Arabidopsis gene encoding a short
27 glycine-rich domain protein (AtGRDP1). This gene is involved in abiotic stress
28 responsiveness; the *Atgrdp1*-null mutant seeds were more sensitive to stress, while the
29 opposite phenotype was achieved by *AtGRDP1* overexpression. In this study, we
30 analyzed the phenotype of the fruits produced by Arabidopsis *Atgrdp1* mutants and
31 *35S::AtGRDP1* overexpression lines. Our analyses revealed important changes in
32 silique length, seed number, seed weight and morphology in the analyzed lines. In
33 particular, *Atgrdp1* mutant lines exhibited several defects including short siliques, a
34 diminished number of seeds per silique, and reduction in seed size and weight as
35 compared to Col-0. The overexpression of the *AtGRDP1* gene also generated
36 phenotypes with alterations in size of silique, number of seeds per silique, and size and
37 weight of seed. In addition, the expression analysis of *AtGRDP1* gene showed that it
38 was expressed in floral and fruit organs, with the highest expression level in mature
39 siliques. The alterations in the siliques and seeds traits in the *Atgrdp1* mutant line, as
40 well as the phenotypes observed in *AtGRDP1* overexpression lines, suggest a role of the
41 *AtGRDP1* gene in the Arabidopsis fruit development.

42

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

44

45 **Introduction**

46

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

53

54 In Arabidopsis, we recently reported a novel gene family “DUF1399-GRDP” composed of
55 four members, two of which (*AtGRDP1* and *AtGRDP2*) were partially characterized by our
56 group. The main feature of the both novel proteins is the presence of short glycine-rich
57 domain, a domain of unknown function (DUF1399), and a potential RNA-binding domain
58 [5,6]. Glycine-rich domain proteins (GRDPs) have been reported in other plants, such as
59 Eucalyptus and common bean [5,6,7,8,9]. The initial characterization of *AtGRDP1* gene
60 revealed an important role during abiotic stress tolerance [5]. Moreover, the overexpression
61 of the *AtGRDP2* paralogue in Arabidopsis, lettuce and common bean resulted in salt
62 tolerant phenotypes. *AtGRDP2* gene is mainly expressed in Arabidopsis floral organs; and
63 its involvement in Arabidopsis development was evidenced through the analysis of *Atgrdp2*
64 mutants and 35S::*AtGRDP2* overexpression lines [6].

65

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

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

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72 **Materials and Methods**

73

74 **Plant material and growth conditions**

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

84

85 **Stereoscopic and environmental scanning electron microscopy (eSEM) analysis**

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

92 For eSEM analysis, dried seeds for each line were glued onto pure carbon containing
93 polymer films, and fixed onto sample holders. The seed coat morphology of Col-0,
94 *Atgrdp1-null* mutant and 35S::*AtGRDPI-3*, -5 and -6 overexpression lines were observed,
95 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.

118

119

120 Quantification of *AtGRDP1* transcript in different floral organs and fruits

121 Buds, flowers, and siliques of 0.3-05 cm, 0.7-0.9 cm, 1-1.5 cm, and > 2 cm, and also
122 mature siliques of 30-day-old plants were frozen in liquid nitrogen and processed to
123 obtain a total RNA using the Concert Plant RNA Kit (Invitrogen, Carlsbad, USA). All
124 possible remaining genomic DNA was removed by DNase Turbo (Ambion, Austin, TX,
125 USA). Quantitative RT-PCR (qRT-PCR) analysis were performed with 30 ng of total
126 RNA by the one-step assay using the iScript™ One-Step RT-PCR kit with SYBR
127 Green (Applied Biosystems, USA). All reactions were performed in 10 µl reaction
128 mixture containing, 5 µl of Power SYBR Green RT-PCR Mix (2X), 200 nM of each
129 oligonucleotide, and 0.08 µl of RT Enzyme Mix (125X), using the StepOne Real-Time
130 PCR Detection System (Applied Biosystems). The thermal cycling conditions used
131 were as reported by [5]. For each RNA sample, three biological replicates ($n = 3$) were
132 analyzed with their respective technical replicates. The *ubiquitin 5 (UBQ5)* gene from
133 *A. thaliana* was used as expression control. The relative gene expression levels of
134 *AtGRDP1* gene was presented as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{AtGRDP1} - Ct_{UBQ5}$ [11]. The
135 primers used for the *AtGRDP1* gene were: UTR3-F
136 5'AAATGGAGGCGGTTGCGGT3' and UTR3-R
137 5'CAGATCCTCACAGTCTTTGGC3'; For the *UBQ5* gene loading the primers
138 employed were UBQ5-F 5'TCGACGCTTCATCTCGTCCT3' and UBQ5-R
139 5'CGCTGAACCTTTCCAGATCC3'. Experiments were repeated at least twice with
140 similar results.

141

142

143

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 \pm standard error
149 of the mean (SEM). Significant differences are represented with letters at ($P < 0.05$).

150

151

152

153 **Results**154 **Evaluation of silique traits in *Atgrdp1*-null mutant and 35S::*AtGRDP1* overexpression**
155 **lines**

156

157 Different silique traits such as length and seeds per fruit were examined in 35 day-old
158 *Atgrdp1* mutant and 35S::*AtGRDP1* overexpression lines. Our analyses revealed silique
159 size alterations in the *Atgrdp1* loss-of-function mutant, displaying a 38.5% reduction in the
160 silique length when compared to parental Col-0 siliques (Fig 1A, B). The 35S::*AtGRDP1*
161 transgenic lines also showed differences in siliques. All overexpressing lines had smaller
162 siliques than parental Col-0, observing a reduction of 29.4% in the 35S::*AtGRDP1*-3 line,
163 22.4% in the 35S::*AtGRDP1*-5 line, and 35.7% in the 35S::*AtGRDP1*-6 line (Fig 1A, B).

164

165 Regarding the number of seeds per silique (Fig. 1C), the 35S::*AtGRDP1*-5 line produced
166 the highest number of seeds per silique (58.2 seeds); whereas, the *Atgrdp1* mutant (with
167 42.2 seeds), 35S::*AtGRDP1*-3 line (with 45.7 seeds) and 35S::*AtGRDP1*-6 (with 32.7
168 seeds) generated the lowest number of seeds per silique when compared with the parental
169 Col-0 with 54.3 seeds/silique (Fig. 1C). In the Figure 1D, we showed stereomicroscope
170 images of immature green siliques of all lines, where *Atgrdp1* mutant and 35S::*AtGRDP1*
171 lines had reduced space among seeds probably due to shorter siliques. Moreover, *Atgrdp1*
172 mutant and 35S::*AtGRDP1*-3 and 35S::*AtGRDP1*-6 lines displayed aborted ovules (Fig.
173 1D).

174

175

176

177 **Seed weight and morphology of *Atgrdp1*-null mutant and 35S::*AtGRDPI***
178 **overexpression lines**

179 We evaluated the seed weight of the *Atgrdp1*-null mutant and 35S::*AtGRDPI*
180 overexpression lines. Seeds of *Atgrdp1*-null mutant plants were lighter than those of the
181 parental Col-0 plants; these mutant seeds were 16% lighter than Col-0 seeds. Conversely,
182 we found that the 35S::*AtGRDPI-3* and 35S::*AtGRDPI-6* overexpression lines generated
183 seeds with higher weight than Col-0, achieving 11.5 and 22%, respectively (Fig. 2A). In the
184 case of 35S::*AtGRDPI-5* line produced seeds whose mean weight values were similar to
185 Col-0 (Fig. 2A). Additionally, an environmental scanning electron microscopy (eSEM)
186 analysis was carried out to observe changes among the seeds from Col-0, *Atgrdp1*-null
187 mutant and 35S::*AtGRDPI* overexpression lines. Parameters evaluated were seed shape and
188 size (Fig. 2B, C). Consistent with seed weight data, *Atgrdp1*-null mutant seeds were smaller
189 and disclosed a round-shape. These small seeds displayed 20% reduction in width and 15%
190 in length versus Col-0. Interestingly, seeds of 35S::*AtGRDPI* overexpression lines
191 exhibited altered seed width and length; the biggest differences were observed in
192 35S::*AtGRDPI-6* seeds (Fig. 2B). In addition, we observed that the surface of the seed testa
193 in the *Atgrdp1*-null mutant and 35S::*AtGRDPI* overexpression line displayed variations in
194 the columella shape compared to Col-0 seeds (Fig. 2C).

195

196 **Expression profile of *AtGRDPI* gene in Arabidopsis floral organs and fruits**

197 *AtGRDPI* organs-specific expression pattern was evaluated by qRT-PCR in different floral
198 and siliques stages of 30 days-old Arabidopsis ecotype Col-0 plants. The floral organs
199 analyzed were buds, and flowers; the fruits were immature developing siliques in four sizes
200 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

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

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206 Discussion

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

218

219 Variations on seeds and siliques were also observed when the overexpression lines
220 (*35S::AtGRDPI-3*, *-5*, and *-6*) were analyzed. All overexpression lines of *AtGRDPI* gene
221 had smaller siliques than the parental Col-0. The *35S::AtGRDPI-3* and *-6* showed a lower
222 number of seed per silique, since they possessed aborted ovules. On the other hand,
223 *35S::AtGRDPI-5* was the line that presented highest number of seeds per silique, even
224 more than Col-0, showing a reduced space among seeds. Interestingly, the *35S::AtGRDPI-*
225 *6* generated the biggest seeds; this transgenic line also had the highest expression rate of the
226 *AtGRDPI* gene of the three overexpression lines [5].

227

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

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

242

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249 **Legend to figures**

250

251 **Figure 1. Silique morphology of *Atgrdp1*-null mutant, and 35S::*AtGRDPI***252 **overexpressing lines.** (A) Silique phenotype of 35-days-old Col-0, *Atgrdp1* and253 35S::*AtGRDPI* overexpression lines grown under long day conditions. The scale bar

254 corresponds to 0.5 cm. (B) Measure of length of mature seeds were represented graphically

255 (C). The seed number per mature silique was represented graphically. The measures were

256 carried out using three siliques per plant, and twelve plants per line were analyzed ($n = 36$).257 Error bars denote \pm SE and different letters are used to indicate significant differences

258 among lines according to the One-way ANOVA analysis and Tukey's multiple comparison

259 tests ($P < 0.05$). (D) Representative images of a cut section in green silique from 35-day-260 old Col-0, *Atgrdp1*-null mutant, and 35S::*AtGRDPI* overexpression plants grown under

261 long day conditions. The yellow arrows indicate the abortive events in silique. The scale

262 bar corresponds to 600 μm .

263

264 **Figure 2. Seed morphology of *Atgrdp1* mutant line and 35S::*AtGRDPI* overexpressing**265 **lines.** (A) Weight of seeds (mg) from the Col-0, *Atgrdp1*-null mutant, and 35S::*AtGRDPI*266 over-expression lines. Error bars represent the means \pm SE ($n = 500$) with three replicates.267 (B) Width and length of seeds (μm) from the Col-0, *Atgrdp1*-null mutant, and268 35S::*AtGRDPI* overexpression lines. Error bars represent the means \pm SE ($n = 15$) with

269 three replicates. Different letters indicate significant differences between the Col-0,

270 *Atgrdp1*-null mutant, and 35S::*AtGRDPI* overexpression lines. One-way ANOVA thought271 Tukey's multiple comparisons test was used to analyze the data ($P < 0.05$). (C) Scanning272 electron micrographs showing: whole seed, scale bar corresponds to 200 μm ; seed coat,

273 scale bar corresponds to 50 μm ; seed coat detail, zoom 30x from micrograph of seed coat;
274 and embryos, scale bar corresponds to 300 μm of Col-0, *Atgrdp1* mutant line, and
275 35S::*AtGRDPI* overexpression lines.

276

277 **Figure 3. Expression profile of *AtGRDPI* gene in floral and fruits organs. (A)**

278 Estimation of *AtGRDPI* transcript in flowers and fruits of 30 day-old Arabidopsis Col-0
279 plants by qRT-PCR. Buds, flowers, immature developing siliques in four sizes 0.3-0.5 (1),
280 0.7-0.9 (2), 1-1.5 (3) and 2 cm (4) and mature siliques (5) were analyzed. The *A. thaliana*
281 *ubiquitin5* (*UBQ5*) gene was used as loading control, and the relative gene expression
282 levels of *AtGRDPI* gene were presented as $2^{-\Delta\text{Ct}}$, where $\Delta\text{Ct} = \text{Ct}_{\text{AtGRDPI}} - \text{Ct}_{\text{UBQ5}}$. Bars
283 represent the means \pm SE ($n = 3$). Different letters indicate significant differences at ($P <$
284 0.05) analyzed with One-way ANOVA thought to Tukey's multiple comparison test.

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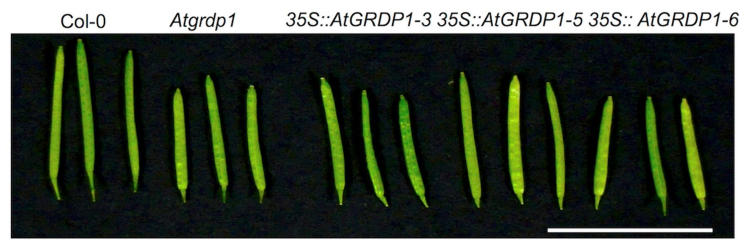
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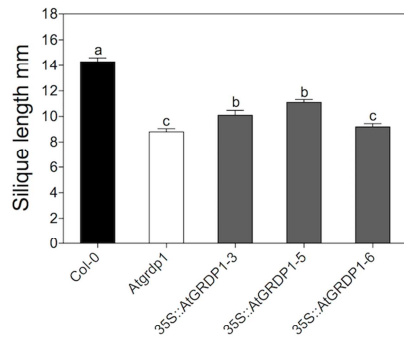
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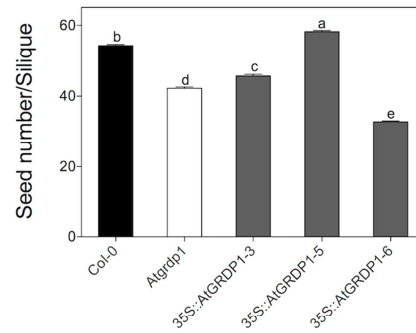
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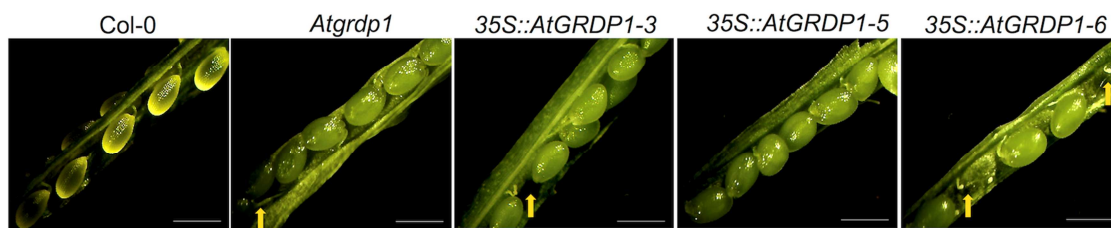
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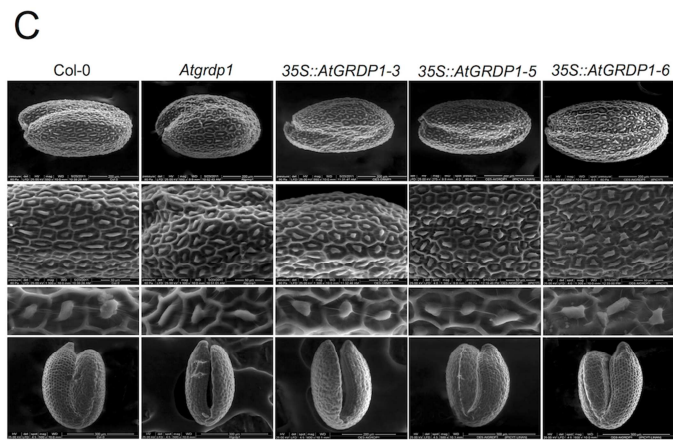
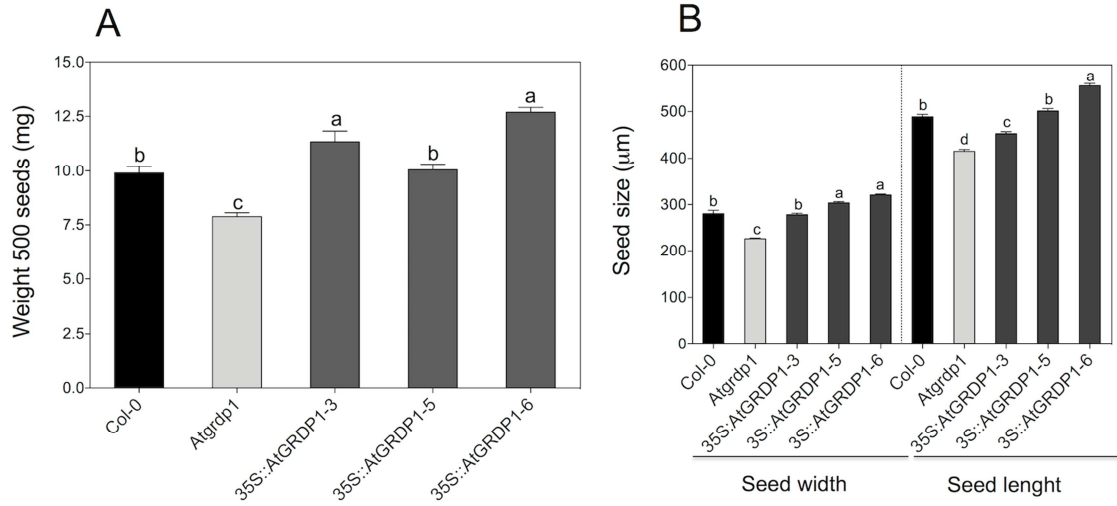
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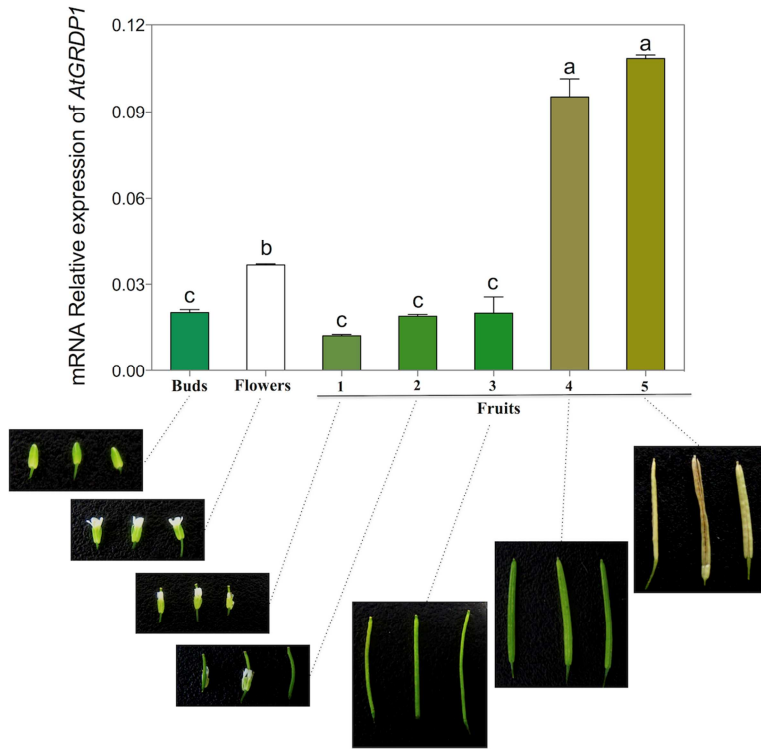
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Highlights

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

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ACCEPTED MANUSCRIPT