Low-glutathione mutants are impaired in growth but do not show an increased sensitivity to moderate water deficit

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Abstract

Glutathione is considered a key metabolite for stress defense and elevated levels have frequently been proposed to positively influence stress tolerance. To investigate whether glutathione affects plant performance and the drought tolerance of plants, wild-type Arabidopsis plants and an allelic series of five mutants (*rax1*, *pad2*, *cad2*, *nrc1*, and *zir1*) with reduced glutathione contents between 21 and 63 % compared to wild-type glutathione content were phenotypically characterized for their shoot growth under control and water-limiting conditions using a shoot phenotyping platform. Under non-stress conditions the *zir1* mutant with only 21 % glutathione showed a pronounced dwarf phenotype. All other mutants with intermediate glutathione contents up to 62 % in contrast showed consistently slightly smaller shoots than the wild-type. Moderate drought stress imposed through water withdrawal until shoot growth ceased showed that wild-type plants and all mutants responded similarly in terms of chlorophyll fluorescence and growth retardation. These results lead to the conclusion that glutathione is important for general plant performance but that the glutathione content does not affect tolerance to moderate drought conditions typically experienced by crops in the field.

Introduction

Crop yield is severely constrained by environmental stress factors resulting in a gap between the yield potential and the actual yield. The yield gap is predicted to increase in the future due to climate change and due to increasing temperature and extended phases of moderate to severe drought in particular [1-4]. Understanding the tolerance and protection mechanisms of

plants is mandatory to breed crops that are able to ensure high yield even under intermittent phases of stress. Plant growth and stress defense are controlled by a multitude of different factors building tight regulatory networks that provide the plasticity that is required to ensure survival and yield of plants even under adverse conditions. The tripeptide glutathione (GSH) is considered a key metabolite in plant defense reactions against biotic and abiotic stress factors [5]. One important function of GSH is the detoxification of reactive oxygen species and organic peroxides that are frequently formed in access in stress situations [6,7]. H_2O_2 is in part detoxified via the glutathione-ascorbate cycle in which ascorbate peroxidases reduce H_2O_2 at the expense of ascorbate [8]. Dehydroascorbate ultimately resulting from ascorbate oxidation is subsequently reduced by GSH resulting in the formation of glutathione disulfide (GSSG), which is then again reduced by glutathione-disulfide reductases (GRs). The glutathione-ascorbate cycle and dehydroascorbate reductase in particular was recently shown to play a central role in minimizing drought-induced grain yield loss in rice [9]. Arabidopsis mutants lacking GR in the cytosol have been shown to have a slightly less negative glutathione redox potential (E_{GSH}) [10]. It has also been shown that cytosolic GR plays a crucial role in leaf responses to intracellular H_2O_2 and in regulation of gene expression through salicylic acid and jasmonic acid signaling pathways [11].

Oxidation of two GSH molecules results in one GSSG molecule, which makes E_{GSH} dependent on both the degree of oxidation and the total amount of glutathione [12]. The glutathione pool in the cytosol, plastids, mitochondria and peroxisomes is, however, in a reduced state with almost 100 % GSH (in the low mM range) and only nanomolar concentrations of GSSG [13,14]. GSH is synthesized in two enzyme-dependent steps catalyzed by glutamate-cysteine ligase (GSH1) and glutathione synthase (GSH2). The biosynthetic pathway is controlled by negative feedback control of GSH on the first enzyme GSH1 [15]. With GSH as a key metabolite in stress defense reactions it is not surprising that

82 several independent genetic screens for mutants sensitive to abiotic or biotic stresses resulted
83 in isolation of mutants with diminished GSH content caused by mutations in GSH1 [16-21].
84 Mutants with intermediate GSH levels of 20 to 40 % are frequently described with a wild-type
85 like phenotype under control conditions [18], although some reports also indicate slightly
86 retarded growth [22]. A causal link between GSH and plant growth is particularly emphasized
87 by the Arabidopsis mutants *zinc tolerance induced by iron 1* (*zir1*), which develops as a dwarf
88 and contains only 15 % of wild-type GSH, and *root meristemless 1* (*rml1*) in which
89 postembryonic development is largely abolished due to an almost complete lack of GSH
90 [20,21].

91 As a key metabolite in the glutathione-ascorbate cycle and a putative cofactor for other ROS
92 scavenging systems like glutathione S-transferases (GSTs), glutathione has frequently been
93 linked to general stress responses in plants [5]. During drought stress responses guard cells are
94 known to produce H_2O_2 that may be exploited for signaling but also needs to be detoxified to
95 avoid serious damage [23,24]. Large-scale changes of cellular redox homeostasis and
96 particularly of E_{GSH} have been considered to link primary stress responses to downstream
97 targets [12,25]. Stress-dependent changes in E_{GSH} can be visualized in live cells with redox-
98 sensitive GFP (roGFP) [13,26,27] and the question has been raised whether severe water
99 stress might trigger an oxidative response that might be involved in drought signaling. While
100 harsh hypo-osmotic treatments (1 M mannitol) did not cause any acute effects as measured by
101 the roGFP2 biosensor [28]. Jubany-Mari and colleagues reported a gradual drought-induced
102 oxidative shift of about 10 mV in the cytosol over a period of several days [29].

103 The latter finding raised the question whether changes in E_{GSH} participate in the water deficit
104 response and whether mutants with altered E_{GSH} are impaired in their response. First
105 experiments to answer this question have provided contradicting results. Overexpression of
106 the first and regulatory enzyme in GSH biosynthesis, GSH1, in tobacco has been reported to

confer tolerance to drought stress [30]. Consistent with this an increased GSH level was reported to confer tolerance to drought and salt stress while the partially GSH-deficient mutant *pad2* displays a significantly lower survival rate than wild-type plants after a two-week drought treatment [31]. In contrast, it has been reported recently that GSH deficiency in *pad2* plants does not affect the water deficit response during a 9-day drought period [32]. Similarly, it has also been reported that partial depletion of GSH in the *gsh1* mutant alleles *cad2*, *pad2*, and *rax1*, did not adversely affect the leaf area of seedlings exposed to short-term abiotic stress [22]. Surprisingly, the negative effects of long-term exposure to oxidative stress and high salt concentrations on leaf area were less marked in the GSH synthesis mutants than in wild-type plants. The apparent contradiction between these studies may result from different stress treatments and different scoring systems recording either survival [31] or biomass increase and leaf area [22,32]. Furthermore, the informative value of multiple studies on the role of GSH in stress tolerance is limited by the fact that frequently only individual mutants are compared to wild-type plants. Schnaubelt *et al.* considered this point by testing three different mutants, which, however, all contained intermediate levels of GSH with little phenotypic variation under non-stress conditions [22,33]. In addition, the severe stress regimes used in this study with high salt (75 mM NaCl) and osmotic stress (100 mM sorbitol) and the evaluation of clearly visible macroscopic markers is unlikely to provide a refined picture of stress sensitivity in Arabidopsis. Such a strategy can be particularly problematic when used to assess whether the growth of mutant or transgenic lines is impacted by changes in stress signaling pathways because difficulties with experimentation including possible physical damage of roots and uptake of osmotica during transfer and possible uptake and breakdown of osmotica [2,34]. Similarly, harsh drought treatment ultimately leading to wilting and death of soil-grown plants is not suitable to compare the performance of different genotypes with different growth characteristics, such as smaller plants [35].

To investigate the potential role of GSH in the response of Arabidopsis plants to moderate water deficit in more detail, we extended the allelic series of GSH-deficient mutants used by Schnaubelt *et al*. [22,33] by the latest additions, namely the mutants *nrc1* [19] and *zir1* [20] of which the latter is particularly interesting given its reported dwarf phenotype. Wild-type plants and all mutants were compared side-by-side for their growth and drought tolerance by using advanced non-invasive high-throughput shoot phenotyping enabling continuous recording of growth responses. We demonstrate that GSH-deficient mutants display diminished growth that is more severe in low GSH mutants, while even in mutants with pronounced growth deficits the decrease in GSH does not negatively impact on tolerance to moderate drought treatment.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana L. (Heyn.) ecotype Columbia-0 (Col-0) was used for all experiments as a wild-type control. Mutants with defects in GSH1 (At4g23100) were provided by the colleagues who reported their isolation and initial characterization. Arabidopsis plants were grown on a mixture of soil (www.floragard.de), sand and perlite in 10:1:1 ratio and kept in controlled growth chambers under long day conditions with 16 h light at 19 °C and 8 h dark at 17 °C. Light intensity was kept between 50 and 75 µE m^{-2} s^{-1} and relative humidity at 50 %. Seeds of wild-type and all mutants were harvested at the same time and used at a similar age for all further experiments. For initial phenotypic and physiological characterization seedlings were grown on vertically oriented agar plates under sterile conditions. Seeds were surface-

sterilized with 70 % (v/v) ethanol for 5 min and plated on half-strength standard MS medium (M0222.0050; Duchefa, www.duchefa-biochemie.nl), supplemented with 0.5 % (w/v) sucrose and solidified with 0.8 % (w/v) agar. Seeds were then stratified for 2 d in the dark at 4 °C and germinated under long day conditions with 16 h light at 22 °C and 8 h dark at 18 °C. Light intensity was 75 µE m^{-2} s^{-1} and relative humidity at 50 %.

Details on growth conditions applied in large-scale non-invasive phenotyping experiments are provided in the respective method descriptions.

Analysis of low-molecular-weight thiols

Approximately 20 mg plant material from 5-day-old seedlings grown on plates under sterile conditions was homogenized and extracted in a 10-fold volume of 0.1 N HCl. Samples were centrifuged for 10 min at 4 °C. 25 µL of the supernatant were mixed with 20 µL of 0.1 M NaOH and 1 µL of 100 mM freshly prepared dithiothreitol (DTT) to quantitatively reduce disulfides. Samples were vortexed, spun down and kept for 15 min at 37 °C in the dark. Afterwards, 10 µL 1 M Tris/HCl pH 8.0, 35 µL water and 5 µL 100 mM monobromobimane in acetonitrile (Thiolyte® MB, Calbiochem, www.merckmillipore.com) were mixed and added to the samples. The samples were vortexed, spun down and kept for 15 min at 37 °C in dark. 100 µL of 9 % (v/v) acetic acid were added, samples were vortexed and centrifuged at 13,000 g for 15 min at 4 °C. 180 µL of the supernatant were filled in HPLC vials. Thiol conjugates were separated by HPLC (SpherisorbTM ODS2, 250 × 4.6 mm, 5 µm, Waters, http://www.waters.com) using buffer C (10 % (v/v) methanol, 0.25 % (v/v) acetic acid, pH 3.7) and D (90 % (v/v) methanol, 0.25 % (v/v) acetic acid, pH 3.9). The elution protocol was employed with a linear gradient from 4 to 20 % D in C within 20 min, with the flow rate set to

178 1 mL/min. Bimane adducts were detected fluorimetrically (UltiMate™ 3000, Thermo Fisher,
179 http://www.thermofisher.com) with excitation at 390 nm and emission at 480 nm.

180

181 Phenotypic characterization of shoot growth and drought stress
182 response

183 Shoot growth was analyzed automatically by using the GROWSCREEN FLUORO setup
184 described earlier [36,37]. Seeds of WT and all *gsh1* mutant lines were stratified for 3 days at 4
185 °C in the dark and then placed in pots individually. Subsequently seeds were germinated and
186 on day seven after germination seedlings with similar size were transferred into larger pots (7
187 x 7 x 8 cm) and randomized on trays with 30 plants on each tray. In exceptional cases plants
188 died after this transfer and were removed from further analysis. The plants were then grown in
189 growth chambers under fully controlled conditions at 22/18 °C, 170 µmol m^{-2} s^{-1} PAR, and
190 8/16 h day/night regime. The soil water content (SWC) was recorded gravimetrically. After
191 initial soaking the SWC was allowed to decrease until a value of approx. 40 % was reached.
192 Subsequently this SWC was held through intermittent addition of water. Starting from day 15
193 after sowing all plants were initially documented for the projected leaf area (PLA) and
194 chlorophyll fluorescence every second day. During the exponential growth phase of shoots in
195 weeks 5 and 6 after sowing all growth parameters were collected on a daily basis. All readings
196 were taken around midday to ensure that the rosettes are oriented almost horizontally above
197 the soil. The PLAs A_1 and A_2 of two consecutive days were used to calculate the relative
198 shoot growth rate (RGR_{Shoot}) (% d^{-1}) according to the equation $RGR_{Shoot} = 100 \times 1/t \times$
199 $\ln(A_2/A_1)$. Chlorophyll fluorescence was recorded after dark adaptation for at least 30 min
200 with a camera-based system to calculate color coded images of F_v/F_m as a measure of the

potential quantum yield of photosystem II. For further analysis, average values for F_v/F_m for whole rosettes were calculated.

For drought stress experiments the plants were split into two subpopulations of which the first population was well-watered throughout the experiment while the second population was exposed to drought from day 24 onwards until growth ceased on day 37. Subsequently plants were again watered to a soil water content of about 40 % and allowed to recover. All plants were harvested after 44 days to determine fresh and dry weight.

Determination of rosette morphology

Leaf circumference, rosette compactness, rosette stockiness, and eccentricity were calculated from the PLA as described earlier [36].

Ratiometric roGFP2 imaging

5-day-old Arabidopsis seedlings stably expressing Grx1-roGFP2 in the cytosol were imaged by CLSM. Seedlings were mounted on a glass slide in a drop of distilled water and immediately transferred to a Zeiss LSM780 confocal microscope. Images of root tips were collected with a 40x lens (Zeiss C-Apochromat 40x/1.2 NA water immersion). RoGFP2 was excited with the 405 and 488 nm laser lines in multi-track mode with a pixel size of 0.415 µm in x and y and 1.58 µs pixel dwell time with line switching and averaging four scans per line. The pinhole was set to 73 µm (2.0 - 2.3 Airy units dependent on the wavelengths). Emission was collected from 504 to 530 nm. Autofluorescence excited at 405 nm was collected from 431 to 470 nm. Images were processed using a custom built MATLAB tool with x,y noise

filtering, fluorescence background subtraction and autofluorescence correction as described previously [38].

Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 7. Different data sets were analyzed for statistical significance with the One- and two-way Analysis of Variance (One- and two-way ANOVA) followed by Tukey's multiple comparisons test.

Results

Severe osmotic stress imposed by mannitol causes partial oxidation of cytosolic glutathione

To investigate the impact of osmotic challenge on Arabidopsis plants, we initially grew seedlings expressing the E_{GSH}-sensor Grx1-roGFP2 in the cytosol on 300 mM mannitol to mimic severe drought stress. In addition to wild-type seedlings, the effect of mannitol on E_{GSH} was also studied in *gr1* mutants deficient in cytosolic GR because impaired reduction capacity for GSSG renders the glutathione pool more sensitive to stress-induced oxidation [10]. While the 405/488-nm fluorescence ratio for Grx1-roGFP2 in wild-type root tips was close to values for full reduction measured after incubation with DTT, the readout for the cytosol of *gr1* roots was significantly higher with intermediate ratios between full reduction and full oxidation of the sensor (Fig 1A). From these measurements cytosolic redox potentials

of about -310 mV in the wild-type and close to the midpoint of the sensor at -280 mV for *gr1* can be deduced. In both cases, for wild-type and *gr1* seedlings, treatment with 300 mM mannitol did not cause significant changes in the fluorescence ratio during the first 16 h. This indicates that within this time window the seedlings were still fully capable of maintaining their thiol redox homeostasis in the cytosol. In contrast to this observation, continuous growth on 300 mM mannitol for 5 days caused a pronounced ratio shift in wild-type and *gr1* root tips. While the ratio values in wild-type root tips reached intermediate values, the ratios in *gr1* root tips approached full oxidation in the root cap while the meristematic region appeared still partially reduced (Fig 1B).

Fig 1. Long-term osmotic stress causes partial oxidation of cytosolic Grx1-roGFP2 in Arabidopsis root tips. (**A**) 5-day-old seedlings transferred to 300 mM mannitol. The dashed horizontal lines indicate the ratio values resulting from treatment with 10 mM DTT for full reduction (blue) and 25 mM H_2O_2 for full oxidation (red). (**B**) 5-day-old seedlings germinated and continuously grown on 300 mM mannitol. All values are means ± SD ($n \geq 10$). Letters indicate significant differences (One-way ANOVA with Tukey's multiple comparisons test; $p \leq 0.05$).

Based on our observation that long-term drought stress impacts on E_{GSH} and causes oxidation in the cytosol, as well as on published reports on decreased drought tolerance of GSH-deficient mutants [31], we set out to test the hypothesis that the GSH content of Arabidopsis plants correlates with drought sensitivity.

An allelic series of *gsh1* mutants

Several genetic screens have led to identification of different *gsh1* mutant alleles that have all been reported to be GSH-deficient albeit at different degrees. For the degree of GSH depletion, however, values between 20 and 40 % GSH compared to wild-type plants are frequently cited, which precludes unambiguous ranking of the mutants according to their GSH content. While GSH levels may indeed vary between growth conditions, the annotation of mutants with concentration range of GSH makes selection of the most appropriate alleles for comparative experimental work difficult. To rank all five available mutants of the allelic series that can be maintained in their homozygous state according to decreasing GSH content, seedlings were grown under controlled conditions on agar plates and analyzed for their GSH content. The HPLC analysis revealed a separation of wild-type and the different *gsh1* mutants into 3 distinct classes (Fig 2A). Wild-type seedlings contained 273 ± 39 nmol g^{-1} FW glutathione and *rax1* seedlings 142 ± 46 nmol g^{-1} FW. The third class included *pad2*, *cad2*, *nrc1* and *zir1* which all contained less than 73 nmol g^{-1} FW glutathione. Although not statistically different the measured mean content of glutathione in *zir1* was only 45 ± 20 and thus lower than in the other three mutants of this class. Depletion of GSH due to mutations in GSH1 was accompanied by a concomitant increase of cysteine as one of the GSH1 substrates (Fig 2B).

Fig 2. Glutathione content in a series of allelic mutants deficient in glutamate-cysteine ligase. (**A,B**) HPLC analysis of total glutathione presented as GSH equivalents (**A**) and cysteine (**B**) in 5-day-old seedlings. The presented values are means ± SD (n = 5). Lower case letters indicate statistically different values (One-way ANOVA with Tukey's multiple comparisons test; $p < 0.05$).

Soil-grown GSH-deficient mutants display growth phenotypes but are insensitive to moderate water deficit

To study the relationship between glutathione content and biomass gain under drought, we analyzed the growth of *gsh1* mutants and wild-type in a sub-lethal drought stress assay. A large population of soil-grown plants was separated into two sub-populations of which one was used as a well-watered control group while the second was exposed to drought stress until growth ceased (Figs 3A, 4 and 6B). At the end of the drought period plants did not yet show obvious signs of wilting (Fig 4). After the drought period, all plants were watered again reaching a SWC of about 40 - 50 % for another week before the plants were harvested (Fig 3A). Within the population of plants grown with sufficient water supply throughout the entire growth period no distinct growth phenotype could be observed for *cad2* (Figs 3B and 5C). The glutathione-deficient mutants *rax1*, *pad2* and *nrc1* were consistently smaller than wild-type plants by up to 30 % at the end of the 6-week growth period (Figs 3B and 5C). Growth retardation was apparent already on day 22 after sowing (Fig 5A). In contrast to mutants with intermediate levels of GSH, *zir1* showed a distinct dwarf phenotype with only 19 % of the wild-type biomass.

Fig 3. Drought-induced growth reduction is not affected by the GSH content of Arabidopsis plants. (A) Watering regime and soil water content during the experiment for drought stressed plants (dashed line) and control plants (solid line). (**B**,**C**) Shoot fresh weight under control and drought conditions respectively at the end of the growth period after 6 weeks. Plants were grown in soil-filled pots under short day conditions and exposed to water stress as illustrated in panel **A**. Values are means ± SD ($n \geq 10$). Letters in each graph indicate significant differences (One-way ANOVA with Tukey's multiple comparisons test; $p < 0.05$).

Fig 4. Photographs of representative plants during the drought stress experiment. DAS: days after sowing.

Fig 5. Projected leaf area (PLA) in wild-type and *gsh1* mutants grown under control and drought stress conditions in soil-filled pots. (**A**,**B**) Continuous recording of PLA during the entire growth period for well-watered control plants (**A**) and drought-stressed plants (**B**). The inset in (**A**) shows the PLA of well-watered plants 22 DAS to emphasize that different growth could be seen here already. Identifiers for the different growth curves are provided in (**B**). (**C**,**D**) PLA at the time of harvest 44 DAS for control (**C**) and drought-stressed (**D**) plants. (**E**,**F**) PLA for wild-type and all *gsh1* mutants under control (**E**) and water deficit conditions (**F**) measured at the end of the growth period. For calculation of the regression the origin of co-ordinates (point 0/0) was included as an additional virtual data point. (**G**) Relative PLA. The calculated linear regression indicates a direct correlation between PLA under drought and control conditions for all plant lines under investigation. All values are means ± SD ($n \geq 16$ randomly distributed on 8 trays).

Lack of water supply for an interim period of 13 days during the entire growth phase of 44 days caused a pronounced growth retardation in wild-type plants by about 50 % as compared to the well-watered controls recorded as a decrease in shoot fresh and dry weight as well as PLA (Figs 3B, 3C, 5A-D and S1A and S1C Figs). The shoot water content at the end of the experiment was similar in control and drought-stressed plants indicating that the applied drought stress did not cause serious damage, such as permanent lesions (S1B and S1D Figs). Direct comparison of drought-stressed *gsh1* mutants and the respective wild-type revealed that for the intermediate GSH-deficient mutants the relative retardation was less pronounced than under control conditions or could not even be detected statistically (Figs 3C, 5D and S1C Fig). The major exception with the most pronounced difference to the wild-type is *zir1* for which the retardation as clearly visible in all recorded parameters. The severe dwarf phenotype of *zir1* enabled further analysis of the PLA at the end of the experiment for all

346 mutants despite the lack of consistent phenotypic differences for the intermediate mutants.
347 Comparison of the PLA with the dry weight (DW) for well-watered control plants and
348 drought-stressed plants resulted in linear relationships indicating that the specific leaf area
349 (PLA g^{-1} DW) in each group of plants is not affected by glutathione deficiency (Figs 5E and
350 5F). To further test whether mutants with very low GSH are more seriously affected by
351 drought than mutants with intermediate levels of GSH and wild-type plants, we plotted the
352 PLA of drought-stressed plants against the PLA of control plants. The linear relationship
353 strongly indicates that even low GSH mutants that grow as dwarfs are not severely affected in
354 their ability to withstand moderate water deficit (Fig 5G).

355 Drought-induced growth retardation was also apparent from the continuous recording of the
356 PLA (Figs 5A-D) and the RGR_{Shoot} calculated from these measurements (Figs 6A and 6B).
357 Regular measurement of the PLA over the entire growth period also enabled the calculation of
358 the relative shoot growth rate (RGR_{Shoot}) on a daily basis. RGR_{Shoot} in the control population
359 gradually decreased from 28 % d^{-1} at the start of the recordings on day 16 after sowing to 12
360 % d^{-1} at the end of the growth period (Fig 6A). Over the entire growth period no obvious
361 deviations in RGR_{Shoot} were apparent between the wild-type and the different *gsh1* mutants
362 with intermediated GSH content. For *zir1* RGR_{Shoot} showed a similar decrease over time, but
363 there were a few intermittent days with significantly lower RGR_{Shoot} than in all other plants
364 (Fig 6A). In the drought-stressed population RGR_{Shoot} decreased significantly faster during the
365 growth period approaching zero after 13 days of water withdrawal (Fig 6B and 6C). The
366 drought stress period started by chance during a phase when *zir1* already showed particularly
367 low values for RGR_{Shoot} in the control population (Fig 6A and 6B). From this point onwards
368 RGR_{Shoot} was consistently lower in *zir1* compared to all other lines for almost the entire
369 drought period (Fig 6B). Immediately after re-watering on day 37 the RGR_{Shoot} increased to
370 peak values of about 20 % d^{-1} on day 41 and subsequent decline to values of about 15 % d^{-1}

371 on day 43 for all lines including *zir1*. After re-watering, the glutathione-deficient mutants
372 even showed a tendency of faster growth compared to wild-type plants (Fig 6B and 6C).

373

374 **Fig 6. Relative shoot growth rate (RGR_{Shoot}) in wild-type and *gsh1* mutants grown und**
375 **control and drought-stress conditions.** Continuous recording of RGR_{Shoot} during the entire
376 growth period for control (**A**) and drought-stressed plants (**B**). The period of water withdrawal
377 for the drought-stressed population is indicated by a grey shadow. Water withdrawal ended
378 when RGR_{Shoot} approached zero on day 37. (**C**) Comparison of relative growth rates for plants
379 in well-watered and drought stressed populations at three critical time points during the
380 experiment. Symbols for the different lines are used as described in panel **A**.

381

382 With each measurement of all individual plants the potential quantum yield of photosynthesis
383 (F_v/F_m) was recorded after dark adaptation for at least 30 min. From measurements of the
384 whole rosette single average values for the respective rosettes were calculated. In plants kept
385 under well-watered control conditions throughout the entire growth phase, F_v/F_m reached
386 values between 0.71 and 0.72 for wild-type plants and for the mutants with intermediate
387 levels of GSH (*rax1*, *pad2*, *cad2* and *nrc1*). Slightly but significantly lower values for F_v/F_m
388 of 0.70 to 0.71 were only found in the more severely GSH depleted *zir1*. While the
389 differences between control plants and drought stressed plants were very small in all lines
390 under investigation there was a tendency towards slightly increased values for F_v/F_m in
391 drought-stressed plants both at the last day of the drought period and just before harvest at the
392 end of the experiment after full recovery of drought stressed plants (Figs 7A and 7B). This
393 difference reached significance only for *pad2*, *cad2* and *nrc1* on the last day of the drought
394 phase and in *cad2* at the time of harvest.

395

Fig 7. Potential quantum yield of PSII (F_v/F_m) of Arabidopsis wild-type and *gsh1* mutants grown under control and drought stress conditions. (**A**) F_v/F_m on the last day of the drought period 37 DAS. (**B**) F_v/F_m at the end of the experiment 43 DAS. All values are means ± SD ($n \geq 16$ plants). Letters in each graph indicate significant differences as determined by Two-way ANOVA with Tukey's multiple comparisons test; $p < 0.05$). For Col-0 and *zir1* control plants and drought-treated plants were compared separately (indicated by index numbers). Values for plants grown under control conditions are shown in white and values for plants exposed to water deficit are shown in grey bars.

Recording of PLA and subsequent image analysis enabled the extraction of additional morphological characteristics of the rosette shape. These factors, included the area by circumference, the compactness of rosettes as a measure for the length of petioles, the rosette stockiness as a measure of how indented the rosette is, and the eccentricity as a parameter describing the shape of the rosette compared to a circle. Generally, all parameters reflected the measurements of PLA (S2 Fig). The only exception was *zir1* which showed an increased stockiness compared to all other lines. Due to the earlier decrease in RGR_{Shoot} *zir1* also developed a more compact rosette, a plateau in rosette stockiness and a more pronounced eccentricity after onset of drought.

Discussion

Glutathione affects growth under non-stress conditions

Side-by-side comparison of all available viable *gsh1* mutants for their glutathione content revealed different levels in the different mutants. The observation that *rax1* contains more glutathione than *cad2* and *pad2* confirms an earlier report by Parisy et al. [18] and the lowest glutathione content in *zir1* is in line with the original report of this mutant [20]. *nrc1* has very

similar glutathione levels as *cad2* which is in line with their similar Cd^{2+}-sensitivity [19]. Phenotypic comparison showed that growth of all mutants except *cad2* was impaired albeit to different degrees. *zir1* mutants were significantly smaller than all others. Comparison of our results with findings from earlier reports on individual mutants or side-by-side comparison of a subset of mutants revealed some pronounced differences. While Schnaubelt et al. [22] reported 14-day-old shoots of *rax1*, *pad2* and *cad2* to be significantly smaller than wild-type shoots, the original reports on the initial characterization of *cad2*, *rax1*, and *pad2* all found no phenotypic difference between mutant and wild-type seedlings [16-18]. While Ball et al. [17] found no effect of light on the phenotypic appearance of *rax1* at all developmental stages, a slight retardation of *pad2* seedlings observed under low light conditions got reverted under high light [22]. GSH levels may vary significantly between experiments due to some non-controlled experimental differences [18]. Thus, the use of a complete allelic series of mutants grown under exactly the same conditions in an automated phenotyping setup should avoid such limitations with lab-based phenotyping and provide more robust data. Comparison of shoot phenotypes further supports the conclusion that phenotypes are not linearly correlated with the GSH content as long as the GSH content can be kept above a certain threshold [20]. A possible explanation for the lack of a linear correlation between glutathione content and growth may be that the available mutants originate from different EMS-treated populations that were screened for sensitivities to different stress factors. This implies that, despite several rounds of backcrossing, the mutants may still contain additional cryptic mutations that are not linked to glutathione homeostasis. Such mutations may affect growth properties in a pleiotropic way similar to recent findings on ascorbate-deficient mutants [39]. In any case, this further emphasizes the added value of investigating several allelic mutants with slight deviations in GSH particularly in the low GSH range side-by-side.

The lower threshold for maintaining a wild-type-like phenotype is clearly passed in the *zir1* mutant resulting in pronounced dwarfism [20]. Glutathione is a key metabolite with essential functions in detoxification, cellular redox homeostasis and as a co-factor [5,40]. GSH is a co-factor in detoxification of methylglyoxal [41] and Fe-S cluster transfer by glutaredoxins [42]. In addition, GSH acts as a S-donor in glucosinolate biosynthesis as well as a co-substrate of ascorbate peroxidases and glutathione S-transferases (GSTs) for peroxide detoxification and of GSTs for conjugation of electrophilic xenobiotics [43-46]. Beyond this glutathione is the most abundant low molecular thiol with low millimolar concentrations in the cell [47] and as such together with glutathione reductases important for maintaining highly reducing redox potentials in the cytosol, plastids, mitochondria and peroxisomes [10,13,14]. With this multitude of functions, it is not surprising that a decrease in GSH levels eventually impair some processes, even though this may be indirectly. Although the experiments reported here were not designed to answer questions on which molecular processes are impaired, the results nevertheless indicate that a certain threshold of GSH depletion needs to be reached before growth is impaired. Below this threshold the developmental phenotype is strongly dependent on the GSH concentrations as evidenced by the *zir1* mutant but also the *rml1* mutant, which has less than 5 % GSH and arrests in growth after germination [21]. The availability of at least one viable mutant with low GSH, which shows already strong and consistent growth impairment, appears particularly useful to further test hypotheses regarding a causal link between GSH content and stress sensitivity.

Glutathione content and drought tolerance

Plants reprogram their metabolism and growth when exposed to water limitation [2] and these changes in growth and physiological status can often be monitored non-invasively at the level

of whole plants [48]. The potential quantum yield of photosynthesis F_v/F_m is a parameter for the functional status of photosystem II and it is well known that severe drought stress causes a decline in F_v/F_m [49,50]. At the same time, it is also established that F_v/F_m during the growth process increases with leaf size due to a relative increase of the lamina which typically shows high F_v/F_m values compared to leaf borders with low F_v/F_m value [36]. The slightly increased F_v/F_m in plants exposed to moderate water deficit might thus correspond to reduced growth and concomitant earlier leaf differentiation and hence a higher fraction of leaves with F_v/F_m values typical for full-grown leaves [36]. Similarly, the lower F_v/F_m values for *zir1* mutants at the end of the drought period and still after a recovery phase may thus be a consequence of delayed development and less matured leaves compared to wild-type and all other mutants. Alternatively, the lower F_v/F_m with values around 0.70-0.71 may also be indicative of partial photoinhibition in *zir1*. Photoinhibition and concomitant production of reactive oxygen species (ROS) is a common feature of stress responses. Given that GSH is involved in detoxification of ROS via the glutathione-ascorbate cycle the growth phenotype appears to be consistent with reduced growth in the ascorbate-deficient mutant *vtc2-1* [51]. This apparent link between ascorbate content and plant growth, however, has been questioned recently after identification and characterization of a true null allele *vtc2-4* that despite low ascorbate content has a wild-type-like phenotype [39].

External supply of 400 µM GSH has been reported to render Arabidopsis plants more drought tolerant [52]. Vice versa *pad2* mutants are reported to be more sensitive to drought than wild-type plants [31]. Our results, however, show no such drought sensitivity. Instead, our systematic approach rather indicates that soil-grown Arabidopsis plants exposed to moderate water deficit generally respond with a gradual decline in growth but no signs of wilting at the point of complete growth arrest. This response is not affected by the GSH content. This is even true when GSH is down to 21 % of wild-type levels, which causes severe growth

494 retardation in *zir1* under non-stress conditions. Direct comparison of wild-type plants and
495 mutants with GSH levels of 62 to 21 % compared to wild-type all have similar specific leaf
496 areas, i.e. PLA/DW ratios, irrespective of whether plants had been grown under control
497 conditions or exposed to drought stress (Fig. 5). A temporary phase of moderate water deficit
498 caused by lack of water supply for 13 days led to a pronounced decrease in growth, but even a
499 slight increase in the specific leaf area. This result indicates that plants that experienced
500 temporary water deficit subsequently grew slightly faster than well-watered control plants.
501 Re-growth after rewatering has been accounted for by cell expansion resulting in actual
502 growth and not only a reversible change due to increased turgor [53]. Interestingly, plants
503 exposed to mild drought stress have been observed to accumulate sugars and starch [54]. This
504 may contribute to a quick restart of growth after re-watering in all lines including *zir1*.
505 Important in this context is the observation that the linearity for the specific leaf area across
506 all mutants and the wild-type is maintained, which indicates that even the very low
507 glutathione mutant *zir1* did not get severely damaged during the stress treatment.

508

509 Conclusion

510 Our analysis provides a systematic, quantitative foundation to the observation that glutathione
511 deficiency causes retarded growth of both roots and shoots. Direct side-by-side comparison of
512 mutants with different degrees of glutathione depletion show no gradual decrease in growth,
513 but a minor retardation, which is similar for all mutants with 25-62 % GSH and more severe
514 only for mutants with only 21 % GSH. This suggests that under non-stress conditions partial
515 depletion of the cellular glutathione pool is tolerable while passing a threshold below about 25
516 % GSH leads to gradual impairment of plant growth.

Our systematic analysis did not show any indication that GSH content of Arabidopsis plants correlates with drought resistance. As such it contrasts earlier reports. Moderate water deficit applied through water withdrawal until shoot growth ceased showed that wild-type plants and all mutants responded similarly in terms of all morphological parameters analyzed, as well as the photosynthetic machinery as analyzed by chlorophyll fluorescence. Taken together the results indicate that glutathione is important for general plant performance, but does not affect tolerance to moderate drought conditions typically experienced by crops in the field.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (DFG) within the Research Training Group GRK 2064 (AM.; MS), grant ME1567/9-1/2 within the Priority Program SPP1710 (AM), the Emmy-Noether programme (SCHW1719/1-1; MS), and Excellence Initiative (EXC 1028; SK). The Seed Fund grant CoSens from the Bioeconomy Science Center, NRW (AM; MS) is gratefully acknowledged. The scientific activities of the Bioeconomy Science Center are financially supported by the Ministry of Innovation, Science and Research within the framework of the NRW Strategieprojekt BioSC (No. 313/323-400-002 13). SB received financial support through a fellowship from the Higher Education Commission, Pakistan. We thank Bastian Welter (University of Cologne) for technical assistance with HPLC measurements, Silvia Braun and Thorsten Brehm (FZ Jülich) for support of the shoot phenotyping and drought stress experiments, and Dr. Kerstin Nagel for discussion of phenotyping approaches and critical reading of the manuscript.

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672

673

674 Supporting information captions

675 **S1 Fig. Shoot dry weight and shoot water content for wild-type and *gsh1* mutants.** (**A**,**C**)
676 Shoot dry weight for well-watered control plants (**A**) and drought-stressed plants (**C**). (**B**,**D**)
677 shoot water content under control (**B**) and drought conditions (**D**). Values are means ± SD (n
678 ≥ 10). Letters in each graph indicate significant differences (One-way ANOVA with Tukey's
679 multiple comparisons test; $p < 0.05$).

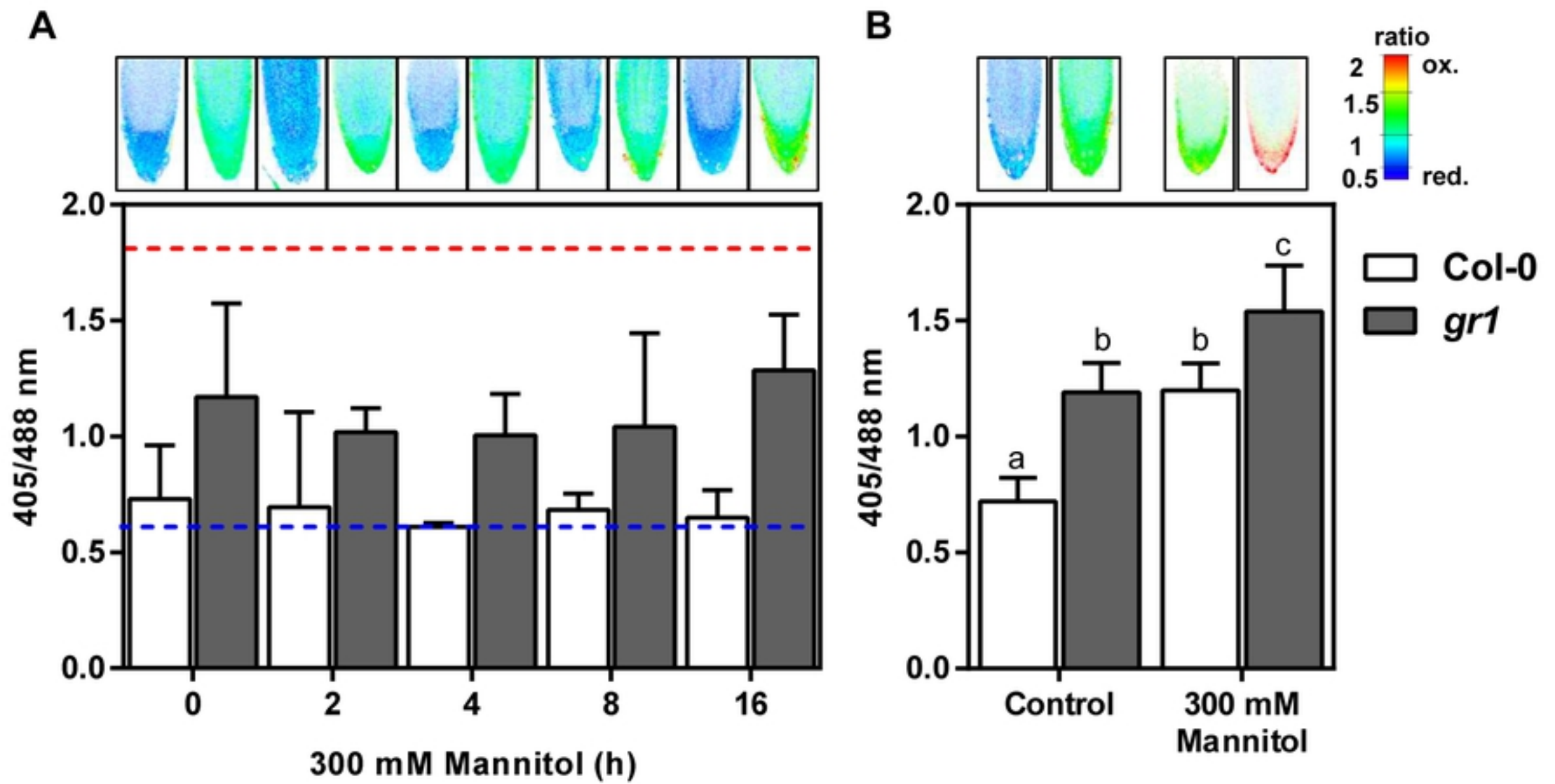
680

681 **S2 Fig. Morphological characteristics of rosettes grown under control and drought-**
682 **stress conditions.**

683

684

685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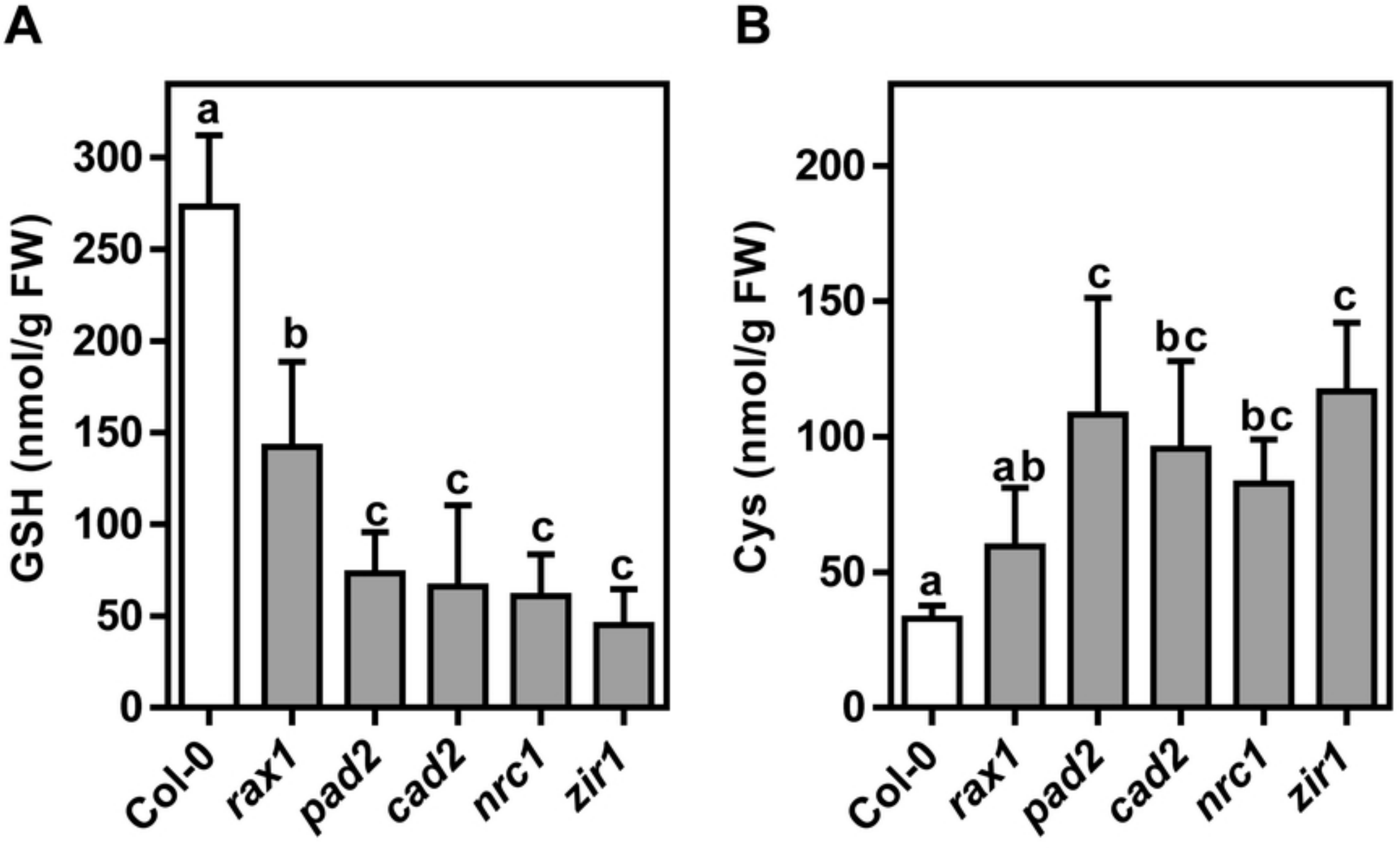
B
ratio
2
1.5
1
0.5
ox.
red.
2.0
1.5
1.0
0.5
0.0
405/488 nm
0
2
4
8
16
300 mM Mannitol (h)
a
b
b
c
Control
300 mM Mannitol
Col-0
gr1

Figure 1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GSH (nmol/g FW)
300
250
200
150
100
50
0
a
b
c
c
c
c
Col-0
rax1
pad2
cad2
nrc1
zir1
B
Cys (nmol/g FW)
200
150
100
50
0
a
ab
c
bc
bc
c
Col-0
rax1
pad2
cad2
nrc1
zir1

Figure 2

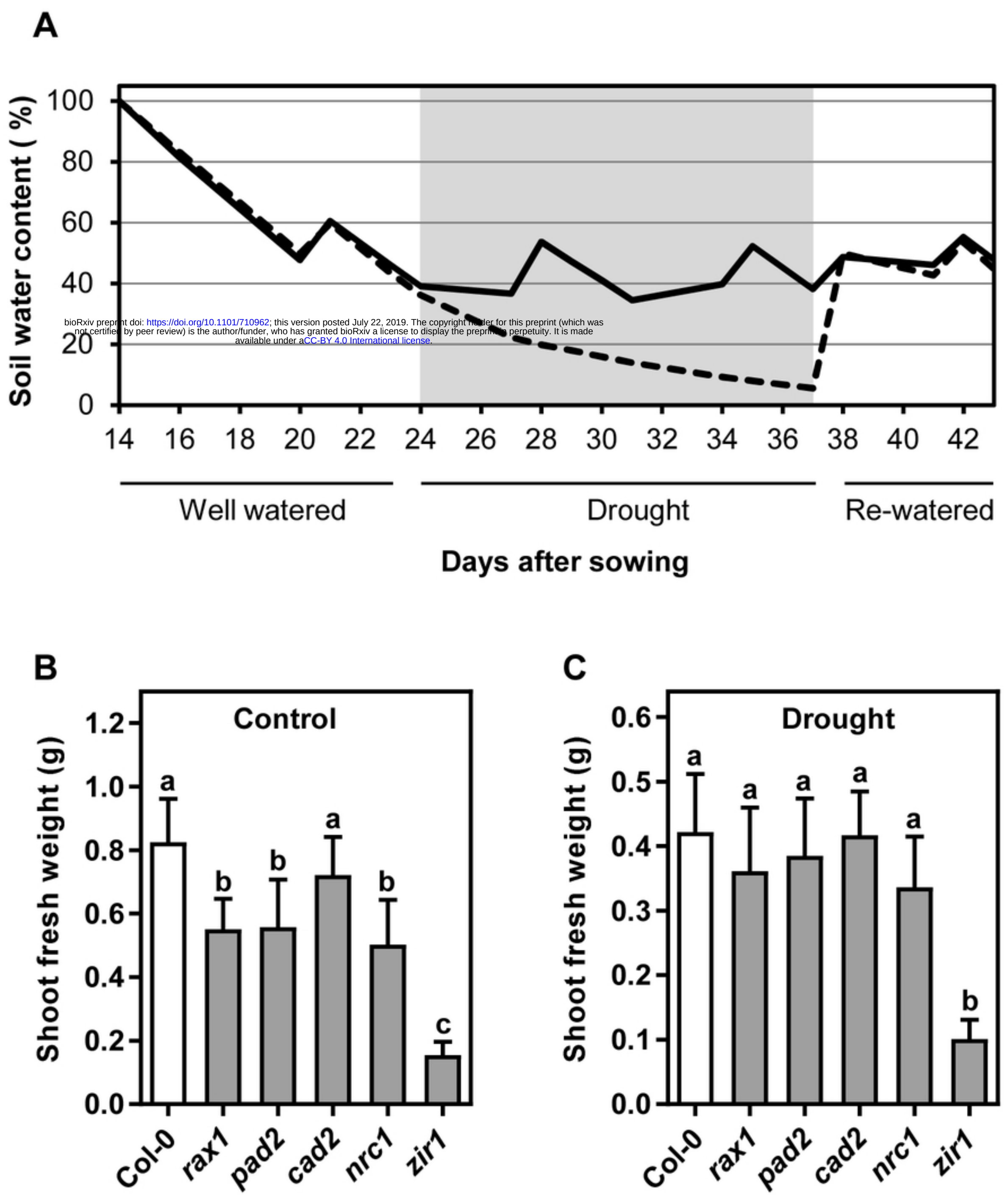
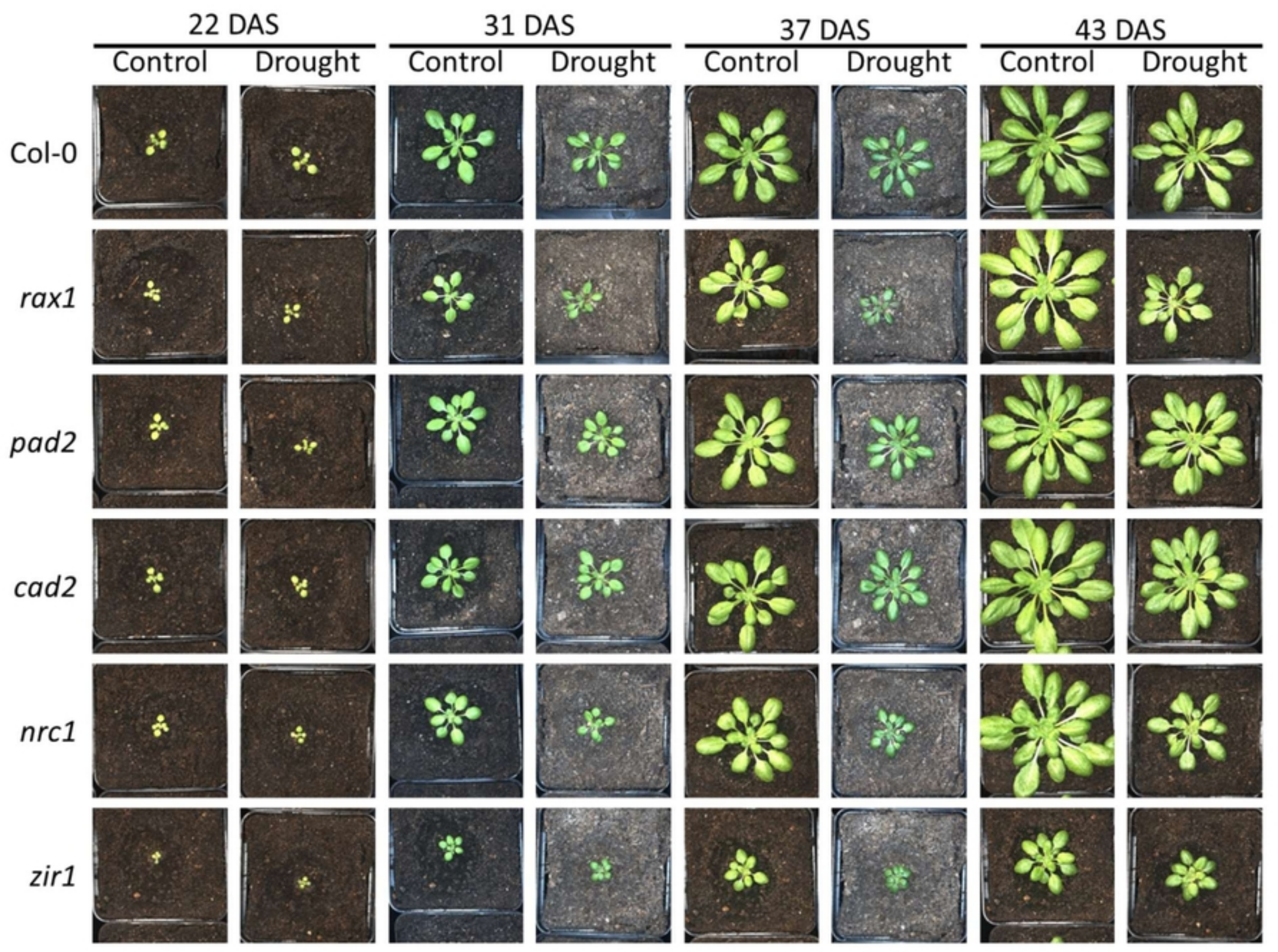


Figure 3


22 DAS
31 DAS
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Control
Drought
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Drought
Col-0
rax1
pad2
cad2
nrc1
zir1

Figure 4

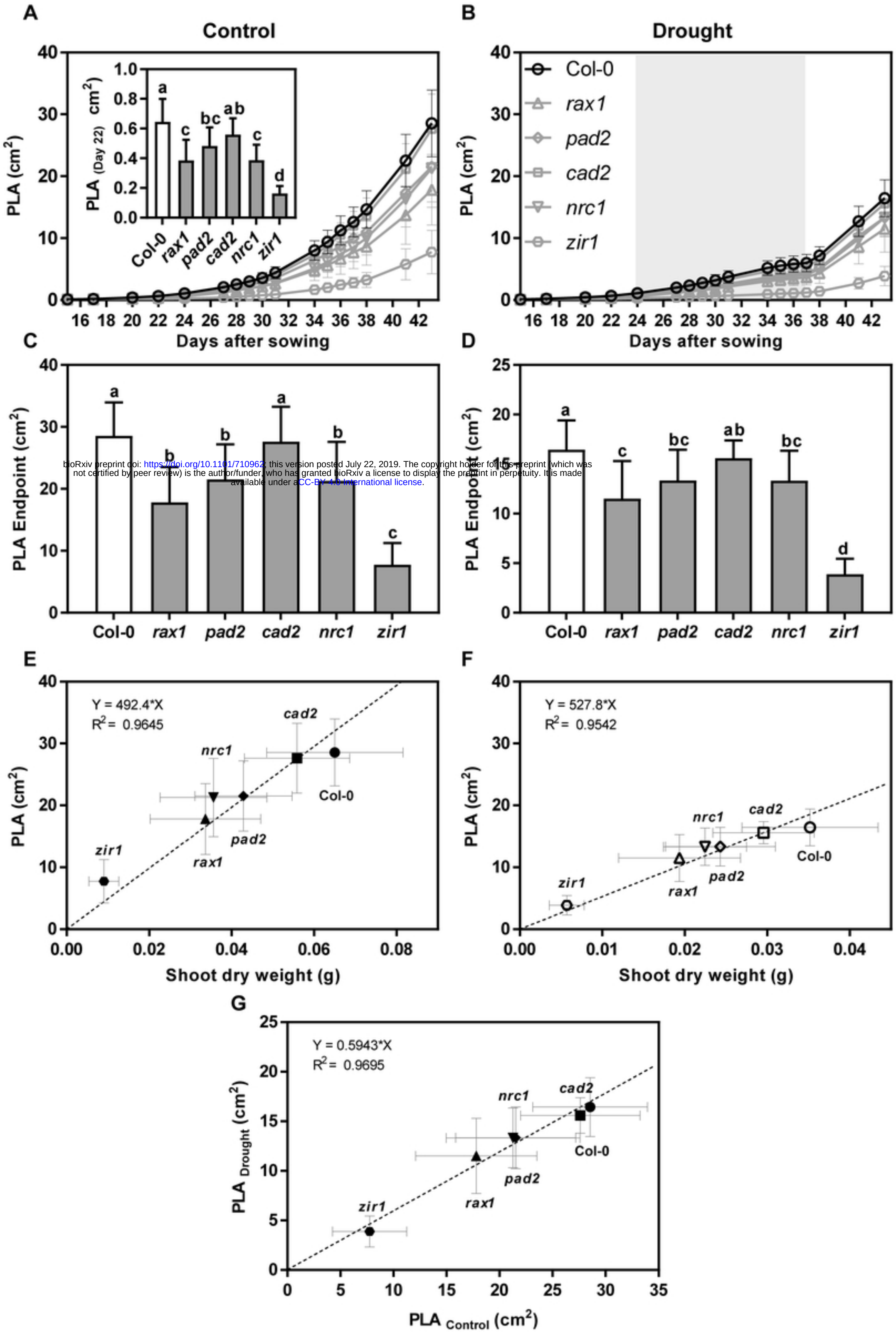

Figure 5

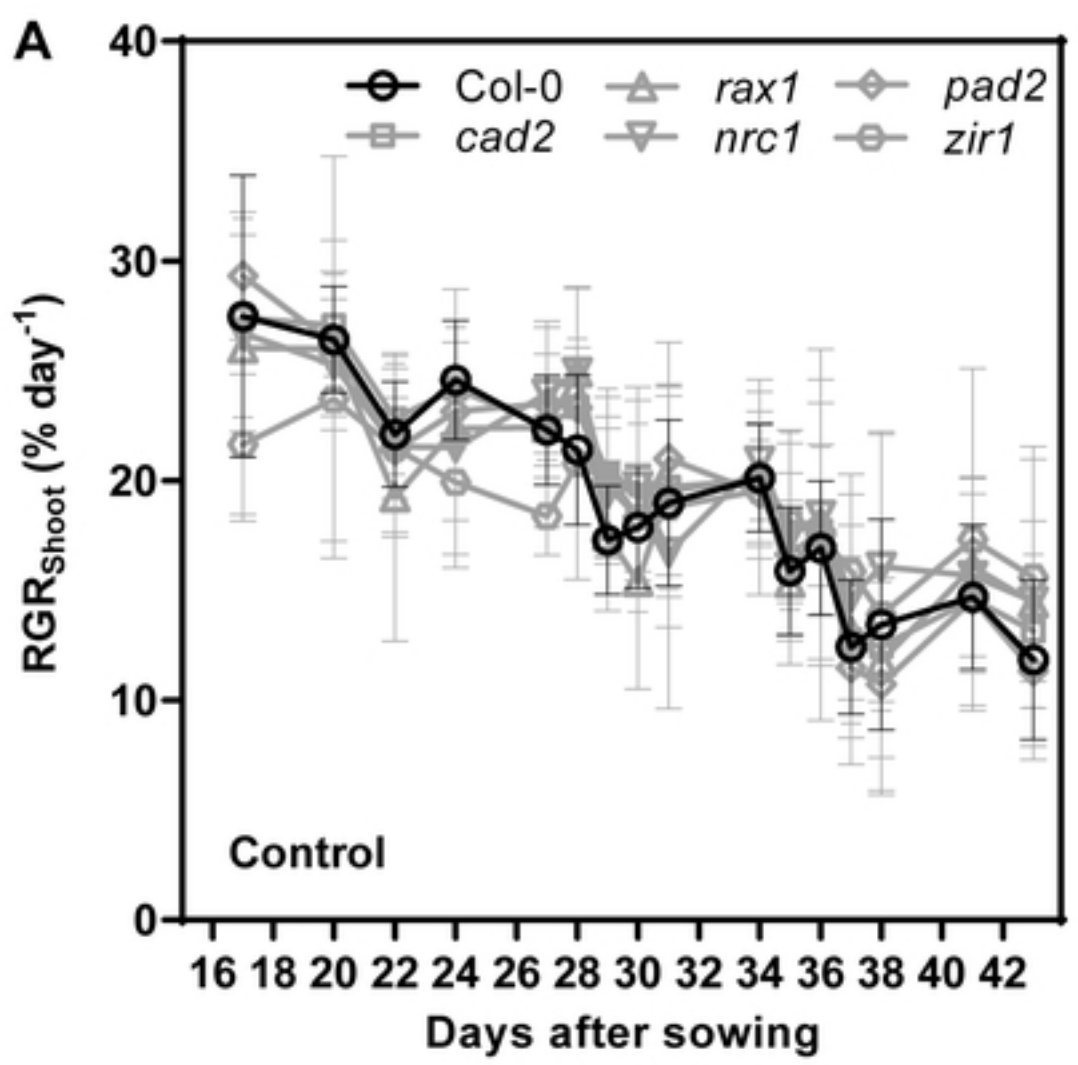

Figure 6

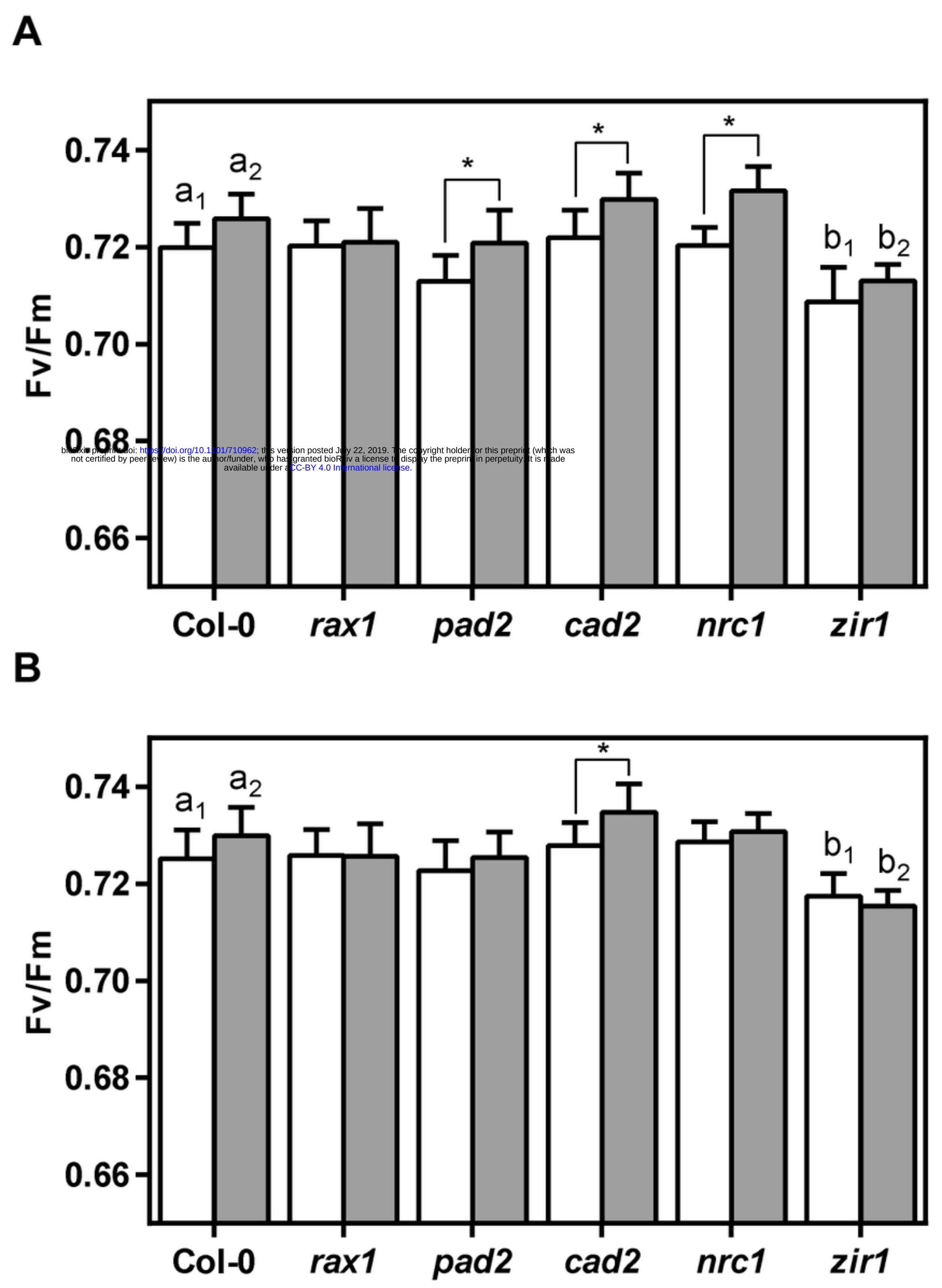

Figure 7