Seed-specific down-regulation of Arabidopsis *CELLULOSE SYNTHASE 1* or *9* reduces seed cellulose content and differentially affects carbon partitioning

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Abstract

Key message

Seed-specific down-regulation of *AtCESA1* and *AtCESA9,* which encode cellulose synthase subunits, differentially affects seed storage compound accumulation in Arabidopsis.

High amounts of cellulose can negatively affect crop seed quality, and therefore diverting carbon partitioning from cellulose to oil, protein and/or starch via molecular breeding may improve seed quality. In order to determine the effect of seed cellulose content reduction on levels of storage compounds, *Arabidopsis thaliana CELLULOSE SYNTHASE1* (*AtCESA1*) and *AtCESA9* genes, which both encode cellulose synthase subunits, were individually down-regulated using seed-specific intron-spliced hairpin RNA (hpRNAi) constructs. The selected seed-specific *AtCESA1* and *AtCESA9* Arabidopsis RNAi lines displayed reduced cellulose contents in seeds, and exhibited no obvious visual phenotypic growth defects with the exception of a minor effect on early root development in *AtCESA1*RNAi seedlings and early hypocotyl elongation in the dark in both types of RNAi line. The seed-specific down-regulation of *AtCESA9* resulted in a reduction in seed weight compared to empty vector controls, which was not observed in *AtCESA1* RNAi lines. In terms of effects on carbon partitioning, *AtCESA1* and *AtCESA9* RNAi lines exhibited distinct effects. The down-regulation of *AtCESA1* led to a ~3% relative increase in seed protein content ($P = 0.04$) and a ~3% relative decrease in oil content ($P = 0.02$), but caused no alteration in soluble glucose levels. On the contrary, *AtCESA9* RNAi lines did not display a significant reduction in seed oil, protein or soluble glucose content. Taken together, our results indicate that the seed-specific down-regulation of *AtCESA1* causes alterations in seed storage compound

accumulation, while the effect of *AtCESA9* on carbon partitioning is absent or minor in Arabidopsis.

Key words: *Arabidopsis thaliana,* cellulose, cellulose synthase, hpRNAi, seed quality

Introduction

Seeds provide important sources of food for human consumption, feed for livestock and raw materials for industry (Shewry and Casey 1999). During seed development, sucrose derived from photosynthesis mainly from the leaves is transported to developing seeds where it serves as a carbon source for the synthesis of different storage compounds that can have substantial economic value and include lipids, starch and protein (Weber *et al.*, 1997; Shewry, 2007; Ufaz and Galili, 2008; Weselake *et al.*, 2009). Most seed crops retain their storage reserves within their embryos or endosperm, and their proportion varies immensely among different plant species (Weber et al. 1997).

The amount of arable land and water available for crop production is becoming more limited, and both biotic and abiotic stress factors, as well as their associated yield losses, are escalating due to climate change. As such, improving the quality and quantity of important seed storage compounds for food end-use is going to be essential to meet the rapidly increasing global food demand. Over the past few decades, various approaches have been assessed to achieve this goal, and some success has been made through conventional breeding and genetic manipulation in terms of boosting the seed protein, oil or starch content of different crop species (Knowles 1983; Slominski et al. 2004; Weselake et al. 2009; An and Suh 2015; Gacek et al. 2018). Studies on the use of genetic engineering to increase seed storage protein content in the Brassicaceae family, however, are limited. Furthermore, it has been shown that seed oil and protein content are inversely correlated in oilseeds, which is likely linked to the limited success in obtaining high oil and protein seeds in early studies (Knowles 1983).

Thus, diverting the flow of carbon into desired storage compound biosynthesis from the biosynthesis of lower-value seed compounds such as cellulose (the major component of seed

66 crude fibre), which shares its carbon source with oil, protein and starch in seeds, represents a
67 potentially useful alternative strategy for this purpose (Tomlinson et al. 2004; Ekman et al. 2008;
68 Iyer et al. 2008; Shi et al. 2012). As a strong carbon sink in plants, the biosynthesis of cellulose
69 consumes a considerable amount of the carbon produced by photosynthesis (Somerville, 2006;
70 Weber et al., 1997). Cellulose is a major component of plant cell walls and plays an important
71 role in plant morphogenesis and mechanical strength in vegetative tissues (Haigler et al. 2001);
72 however, high cellulose content in seeds is generally not desirable from an economic standpoint
73 as the complex nature of cellulose renders seed meal largely indigestible for monogastric
74 animals, leading to the ineffective utilization of plant biomass (Weber et al. 1997; Matthäus
75 1998; Annison and Choct 2005; Jiang and Deyholos 2010; Li et al. 2017). In addition, mutations
76 in certain cellulose synthase genes (*AtCESA*; i.e. *AtCESA4, AtCESA7* and *AtCESA8)* in
77 *Arabidopsis thaliana* (hereafter Arabidopsis) have been shown to lead to increased disease
78 resistance owing to the specific activation of novel defense pathways through the alteration of
79 secondary cell wall integrity (Hernández-Blanco et al. 2007). As such, reducing seed cellulose
80 content might not only increase available carbon for the biosynthesis of high-value seed storage
81 compounds, but could also increase the digestibility and value of the meal obtained from seeds
82 and may also enhance disease resistance.

83 Cellulose is synthesized by a large multimeric complex that contains cellulose synthase
84 subunits (CESAs) as their catalytic domains (Somerville, 2006). The model species Arabidopsis
85 possesses 10 CESA-encoding genes, among which *AtCESA1, AtCESA2, AtCESA3* and *AtCESA9*
86 are expressed at relatively high levels in developing seeds (Beeckman et al. 2002). The
87 expression levels of these genes in different embryo developmental stages seems to vary
88 (Beeckman et al. 2002). *AtCESA1* and *AtCESA2* have higher levels of expression in late-heart

and torpedo stages of the whole embryo, whereas *AtCESA3* and *AtCESA9* show stronger expression in cotyledons than in other parts of the embryo (Beeckman et al. 2002). AtCESA1 and AtCESA3 are essential components of the primary cell wall synthesizing protein complex, together with some combination of AtCESA2, AtCESA5, AtCESA6 or AtCESA9 (Persson et al., 2007; Desprez et al., 2007), and are involved in cell expansion in the Arabidopsis embryo, and thus affect the shape of the developing embryo (Beeckman et al. 2002). However, *AtCESA1* down-regulation cannot be compensated for by any of the other genes even when overexpressed with strong promoters (Burn et al. 2002). Although *AtCESA2* and *AtCESA9* share high sequence similarity, it is AtCESA9 that is reported to have a direct involvement in secondary cell wall cellulose deposition in the developing Arabidopsis seed coat (Beeckman et al. 2002; Somerville 2006; Stork et al. 2010; Mendu et al. 2011). Thus, *AtCESA1* and *AtCESA9* have specific functions in the primary and secondary cell wall cellulose synthesis in Arabidopsis developing seeds, respectively, and have been identified as essential genes in the biosynthesis of embryo and seed coat cellulose (Beeckman et al. 2002; Burn et al. 2002; Stork et al. 2010).

Although a number of *AtCESA* knock-out /knock-down Arabidopsis mutants are available, almost all of them exhibit defective phenotypes in terms of plant growth and development (Li et al. 2017). For instance, the knock-out of *AtCESA1* has been found to cause severe morphological alterations in various plant organs including shoots, roots and embryos (Arioli et al. 1998; Beeckman et al. 2002; Gillmor et al. 2002; Persson et al. 2007). Similarly, although an *AtCESA9* T-DNA mutation resulted in a 25% reduction in seed cellulose content without affecting cellulose content in other tissues, these T-DNA mutants displayed a decrease in seed size and weight compared to wild-type controls (Stork et al. 2010). The effect of *CESA* down-regulation on seed storage compound biosynthesis, however, is not characterized.

In an attempt to mitigate the reported undesirable phenotypic effects of mutating or constitutively down-regulating *AtCESA1* or *AtCESA9*, we endeavored to assess the effects of seed-specific down-regulation of these genes in Arabidopsis with respect to morphology and carbon partitioning among seed storage compounds. While the down-regulation of either *AtCESA* gene resulted in reduced cellulose content in seeds, only minor morphological alterations were noted that did not appear to affect plant development. Furthermore, *AtCESA1* and *AtCESA9* RNAi lines displayed distinct effects on the levels of seed protein, oil and carbohydrates, indicating differential roles of the products of these genes in carbon partitioning.

Materials and Methods

Plant growth conditions

Arabidopsis ecotype Col-0 seeds were placed at 4ºC in the dark for 3 days prior to sowing on Sunshine #4 potting mix (Sun Gro Horticulture, Vancouver, Canada). T_1 and T_2 transgenic plants were grown in plastic tray inserts [36 wells (2.35" x 2.15" x 2.33" per each well)/ insert], and were supplemented with N:P:K 20:20:20 fertilizer once a week (Chissa-Asahi Fertilizer Co., Ltd, Tokyo, Japan). All Arabidopsis plants were grown from sowing onwards in a growth chamber at 22 ºC with a photoperiod of 18 h day/6 h night and 250 or $\mu molm^{-2}s^{-1}$ light intensity as described previously (Liu et al. 2019; Bhuiyan et al. 2016; Singer et al. 2016).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from flash frozen developing siliques of wild-type Col-0 Arabidopsis plants using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). DNA digestion was carried out to remove traces of DNA using the On-Column DNase I Digest Set (Sigma-

Aldrich). First-strand cDNA synthesis was achieved using the Superscript IV First-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Invitrogen, Life Technologies Inc.) using 350 ng total RNA as template and an oligodT primer. The resulting cDNA samples were stored at -20 ºC until further use.

Vector construction for down-regulation of *AtCESA* genes with RNAi technology

Target gene down-regulation was achieved in this study using RNAi technology, whereby the expression of inverted repeats of *AtCESA1* and *AtCESA9* regions, respectively, was driven with a seed-specific promoter to elicit a reduction of cellulose in seeds without compromising cellulose biosynthesis in other plant organs. Two seed-specific plant transformation RNAi constructs (Fig. S1) targeting *AtCESA1* (CESA1ss) and *AtCESA9* (CESA9ss) were developed using standard protocols (Singer et al. 2016). First, a 320-bp *CESA1*-specific sequence and a 371-bp *CESA9*-specific sequence were selected from available cDNA sequences using the Geneious Blast search function (Geneious Basic 5.6.5; https://www.geneious.com) and a lack of potential off-target effects was confirmed in each case using dsCheck software (http://dscheck.rnai.jp/). Sense and antisense fragments were then amplified in both cases from silique-derived cDNA using primers shown in Table S1, which possessed appropriate restriction sites at their 5' termini to facilitate cloning. Fragments were amplified using Pfx50 DNA Polymerase (Invitogen) and the following thermal parameters: 94ºC for 2 min, 40 cycles of 94ºC for 15s, 60ºC for 30s, and 68ºC for 1.0 min, followed by a final extension at 68ºC for 5 min. All resulting fragments were cloned into the pGEM®-T Easy vector (Promega, Madison, USA) and verified by sequencing.

Subsequently, the RNAi fragments were introduced in opposite orientations on either side

158 of an intronic spacer derived from pHannibal (Wesley et al., 2001) within the auxiliary
159 pAUX3131 vector (Goderis et al., 2002) that had been previously modified to contain the seed-
160 specific *Phaseolus vulgaris β-phaseolin* promoter (Frisch et al. 1995) and transcriptional
161 terminator (Singer et al. 2016). The *β-phaseolin* promoter exhibits high levels of activity during
162 microsporogenesis and embryogenesis, with little to no activity in vegetative tissues
163 (Chandrasekharan et al. 2003). The resulting *AtCESA1* and *AtCESA9* RNAi cassettes were then
164 introduced into a modified plant transformation vector derived from pPZP-RCS1 (Goderis et al.
165 2002), which contained the kanamycin-resistance gene *neomycin phosphotransferase II* (*NPTII*)
166 under the control of the *nopaline synthase* (*nos*) promoter.
167
168 **Generation of transgenic Arabidopsis lines**
169 The resulting binary RNAi vectors, as well as an empty vector negative control, were
170 introduced individually into *Agrobacterium tumefaciens* strain GV3101 via electroporation. The
171 recombinant bacteria were then used for the transformation of Arabidopsis ecotype Col-0 using
172 the floral dip method (Clough and Bent, 1998). Kanamycin-resistant seedlings were selected on
173 selection medium (Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (0.21%
174 [w/v] MS basal salts, 1% [w/v] sucrose, 0.03% [w/v] MES, 0.8% [w/v] phytagar, 20 mg/ml
175 kanamycin, 99.6 µg/ml timentin, pH 5.7-5.8). Homozygous lines with a single copy of the
176 transgenic cassette were identified using segregation analysis of T_2 and T_3 seeds respectively.
177 For every experiment, transgenic experimental lines, as well as empty vector controls, were
178 grown in the same growth chamber at the same time.
179
180

Quantitative real-time RT-PCR

Total RNA was extracted from both T_2 and T_3 homozygous developing siliques of transgenic and empty vector control lines (14 days after flowering [DAF]) , and cDNA was synthesized as described in a previous section. Quantitative RT-PCR assays were performed with 3 biological replicates and 3 technical replicates using 1 µl of 1/5 diluted cDNA as template along with SYBR Green PCR Master Mix in a total reaction volume of 10 µl according to the manufacturer's instructions (Applied Biosystems, Life Technologies Inc.). *AtCESA1* expression was analyzed in seed-specific *AtCESA1* RNAi and control lines using the *AtCESA1* gene-specific primers AtCESA1qRTF and AtCESA1qRTR (Table S1). In the case of *AtCESA9* expression analysis, *AtCESA9* gene-specific primers AtCESA9qRTF and AtCESA9qRTR (Table S1) were used. Constitutively expressed *PROTEIN PHOSPHATASE 2A SUBUNIT 3* (*PP2AA3*) transcript was amplified as the internal control using primers PP2AA3F and PP2AA3R (Table S1; Singer et al., 2016). Assays were conducted using a Step OnePlus Real-Time PCR System (Life Technologies) using the following thermal parameters: 95ºC for 2 min, followed by 40 cycles of 95ºC for 15s and 60ºC for 1 min. Dissociation curves were generated in each reaction to confirm amplification specificity. Levels of gene expression relative to the internal control were obtained using the comparative Ct method (Schmittgen and Livak 2008).

Morphological and histochemical analysis

All morphological and histochemical analyses were carried out using three independent homozygous lines bearing each construct, respectively. Seed weight analysis was performed by counting approximately 150-250 T_3 seeds from each line in triplicate for each construct using the particle counter function of the FuorChem SP Imager and AlphaEase software (Alpha Innotech

Corp., San Leandro, CA), followed by weighing using an analytical balance (OHAUS Corporation, Florham Park, NJ). The ability of seeds to uptake tetrazolium salt was determined by imbibing 3 batches of seeds from 3 independent homozygous RNAi lines and control lines in an aqueous solution of 1% (w/v) tetrazolium violet (2,3,5-triphenyl- tetrazolium) at 30 ºC for 24 h-72 h (Stork et al. 2010).

Root growth assessments were performed as described previously with slight modifications (Corrales et al. 2014). Briefly, surface sterilized homozygous seeds were grown in half-strength MS agar square plates; (245 mm-245 mm) without antibiotics, and were placed vertically in a plant growth chamber under previously mentioned growth parameters following cold stratification for 3 days at 4 ^{0}C. Root lengths were measured every other day following germination for 12 days. Root length measurements were taken by analyzing pictures using AxioVision4.8 software. The experiment was conducted 3 times with 3 batches of seeds in each case. For measuring dark grown hypocotyl length, seeds were prepared as described above and following cold stratification were exposed to fluorescent white light (120 μM $m^{-2}S^{-1}$) for 2h to synchronize germination. Subsequently, plates were wrapped in three layers of aluminum foil and placed vertically in a growth chamber with previously described growth conditions, with the exception of light levels (no lights were applied in this case). Hypocotyl lengths were measured 6 days following germination. Weights of 12-day old seedlings (approximately 10 plants of each line) were also measured for *AtCESA1* and *AtCESA9* RNAi lines, as well as control lines, using an analytical balance (OHAUS Corporation, Florham Park, NJ) as described previously (Corrales et al. 2014). Seed germination was assessed by placing seeds on half-strength MS agar plates and counting the number of germinated seeds daily for 7 days. Germination assays were replicated in triplicate, with 30 seeds per replication.

Crystalline cellulose content analysis

The crystalline cellulose content of seed samples from 3 independent T_3 homozygous lines bearing each construct (2-3 biological replicates of each), respectively, was estimated by determining the acid-insoluble glucose content using a microscale method based on that of Updegraff (1969) with slight modifications (Griffiths et al. 2014). Briefly, 10 to 20 mg of seed were ground in liquid nitrogen using a mortar and pestle, followed by incubation at 50 ºC for 16 h after washing with 70% aqueous ethanol. The dry weights of the homogenized seeds were recorded, and samples were then treated with 2 ml of Updegraff reagent (acetic acid: nitric acid: water, 8:1:2 (v/v/v). Samples were vortexed and incubated at 100 ºC for 1 h, followed by centrifugation at 13000 rpm for 5 min. The resulting pellets were washed with distilled water and twice with acetone, and then incubated at room temperature overnight until the samples were completely dry. Samples were then treated with 1 ml 72% (w/v) H_2SO_4: water, vortexed, incubated at room temperature for 90 min and centrifuged at 10,000 rpm for 5 min. The acid insoluble glucose present in the resulting supernatant was measured using a colorimetric method in microtiter plates after diluting 10X with distilled water (Foster et al. 2010). Briefly, 75 µl of diluted sample was treated with 150 µl of freshly prepared cold (4 °C) anthrone reagent (2mg/ml (w/v) anthrone [Sigma-Aldrich] in concentrated H_2SO_4) in microtiter plates. The anthrone mixtures were then incubated for 30 min at 80 °C, and absorbance was recorded at a wavelength of 620 nm in a spectrophotometer (Synergy H4 Hybrid reader, Biotek Instruments, Winooski, USA). A standard curve was prepared from a standard serial dilution of glucose, and total amounts of cellulose-derived glucose were calculated per weight of dry seed mass.

Total protein content analysis

Total protein content was determined in the seeds of 3 independent T_3 homozygous lines bearing each construct, respectively (3-4 biological replicates of each), using a modified "Dumas" method (AOAC 992.23). Briefly, the nitrogen content was estimated in duplicate T_3 homozygous dry seed samples (80-105 mg), using a nitrogen analyzer (model FP-428, Leco Instruments Ltd., Mississauga, ON, Canada). The total protein content in the seed samples was then determined by multiplying the nitrogen content by a conversion factor of 6.25 (AOAC 992.23). The total caffeine (157 mg) and EDTA (ethylenediaminetetraacetic acid; 102 mg) contents were used as standards for calibration.

Determination of total lipid content and fatty acid composition in seeds

Total lipid content and fatty acid composition of mature T_3 seeds from three independent homozygous lines bearing each construct, respectively (3-4 biological replicates of each), were determined using GC/MS as described previously (Pan et al., 2013; Singer et al., 2016). In brief, approximately 10 mg of seeds were weighed and placed in Teflon-lined screw-capped glass tubes along with C17:0 TAG (100 µl) as an internal standard. Transmethylation was carried out using 3N methanolic HCl (Supelco, Sigma-Aldrich, Oakville, Ontario) at 80 ºC for 16 h. Subsequently, tubes were cooled on ice and 0.9% aqueous NaCl was added to stop the reaction. The resulting fatty acid methyl esters (FAMEs) were extracted twice with hexane, followed by drying under nitrogen gas at 37 ºC.

The resulting FAMEs were then redissolved in 1 ml of iso-octane containing C21:0 methyl ester (0.1 mg/ml) and analyzed by gas chromatography (GC)-mass spectrometry (MS). For GC analysis (Agilent Technologies 7890A GC system), a split/splitless inlet was used and

the injection volume was 1 µl in a ten-to-one split mode; FAME separation was performed in a DB-23 capillary column (Agilent Technologies: 30 m × 250 µm × 0.25 µm) with helium as the carrier gas (1.2 ml/min). The temperature program was as follows: 165 ºC for 4 min, ramping from165 to 180 ºC in 5 min, then 180 to 230 ºC in 5 min. Compounds were detected by mass spectrometry (Agilent Technology 5977A Mass Selective Detector) and peaks were identified with the software NIST MS Search 2.0.

Determination of seed glucose and seedling starch content

Seed soluble glucose content was determined in three independent homozygous lines bearing each construct, respectively (2-3 biological replicates of each), as described previously with slight modifications (Lock et al. 2009). Briefly, approximately 50 mg of seeds were homogenized in 1.5 ml of 80% (v/v) aqueous ethanol and incubated at 70 ºC for 1 hour. The samples were centrifuged at 15000 rpm for 5 min, and the supernatant was collected in a new glass vial. The pellet was subjected to 3 sequential washings with 0.5 ml of 80% aqueous ethanol and the supernatants were combined and evaporated to dryness at room temperature under a stream of nitrogen gas. The residue, which represents the soluble carbohydrate fraction, was dissolved in 0.1 ml of water and used for glucose quantification. Glucose was determined using the glucose oxidase/peroxidase method (600 units/L glucose oxidase, 325 units/L peroxidase; Megazyme International, Ireland) following the manufacturer's guidelines.

Total starch content was determined using the amyloglucosidase/α-amylase method according to the manufacturer's instructions (AOAC Official Method 996.11; Megazyme International, Ireland). Briefly, ~100 - 130 mg of 6-day-old seedlings were frozen in liquid nitrogen and ground to a fine powder. Ground samples were extracted with 5 mL of 80% ethanol

(v/v) and incubated for 5 min at 85ºC to remove soluble glucose. Subsequently, another 5 mL of 80% ethanol (v/v) was added to the samples, which were then vortexed and centrifuged for 10 min at ~3000 rpm. The ethanol was removed from the pellet, which was re-extracted with 10 mL of 80% aqueous ethanol (v/v) and processed as above, without incubating at 85 ºC. The pellet was then completely dried under a stream of N_2 gas and the starch in the pellet was converted to glucose using the amyloglucosidase/α-amylase method, and glucose content was then determined using the glucose oxidase/peroxidase method as described above.

Statistical analysis

Probability values (P-values) were calculated using two-tailed student's t-tests (assuming unequal variance) for comparisons of means derived from empty vector control lines and *AtCESA1* or *AtCESA9* RNAi lines for cellulose content, seed weight, protein content and oil content. Statistical significance was declared at $P \leq 0.05$ for all comparisons.

Results

Seed-specific *AtCESA* RNAi Arabidopsis lines exhibit reduced *CESA* expression

Seed-specific *AtCESA1* and *AtCESA9* RNAi constructs (Fig. S1) were developed in order to down-regulate the expression of these genes in seeds, and determine their effects on seed cellulose and storage compound content. Initial expression analyses of *AtCESA1* or *AtCESA9* in these lines (~ 3 individual lines for each case) was performed using cDNA derived from developing T_1 siliques containing T_2 seeds to confirm *AtCESA* down-regulation (Fig. 1a and 1C). The relative expression level of *AtCESA1* was reduced by up to 79% in *AtCESA1* RNAi lines compared to control lines (Fig. 1a), whereas a high variability in the degree of down-regulation

of the *AtCESA9* gene was observed among the *AtCESA9* RNAi lines tested, with the greatest reduction being approximately 70% in two lines compared to control lines (Fig.1c). Furthermore, developing T_2 siliques containing T_3 seeds were also harvested from 3 independent *AtCESA1* and 3 independent *AtCESA9* RNAi lines (utilized throughout this study), respectively, followed by the identification of homozygous lines in each case. Appropriate alterations in *AtCESA* expression were also confirmed in this generation (Fig. 1b and 1d), with up to 68% (CESA1ss4-13) and 55% (CESA9ss6-4) reductions in expression compared to empty vector controls, respectively.

AtCESA down-regulation resulted in reduced crystalline cellulose content of T_3 seeds

Cellulose content (estimated using acid-insoluble glucose content) of T_3 seeds was measured to determine whether the reduced expression of *AtCESA1* or *AtCESA9* in developing seeds inhibited cellulose biosynthesis. As shown in Fig. 2, the average level of crystalline cellulose was reduced by approximately 25% for *AtCESA1* and 17% for *AtCESA9* seed-specific RNAi lines compared to the empty vector controls. Cellulose content fluctuated among different RNAi lines, with most of the *AtCESA1* RNAi lines containing between ~ 30 to 40 µg/mg acid-insoluble glucose, *AtCESA9* RNAi lines containing between ~40 to50 µg/mg, and control lines containing ~50 to 60 µg/ mg. All independent transgenic *AtCESA1* and *AtCESA9* RNAi lines tested were found to have lower cellulose content than control lines (Fig. S2).

The effects of _AtCESA_ down-regulation on growth and morphology

There were no obvious visual phenotypic growth defects in soil-grown plants (Fig. S3a) of any transgenic lines studied here. The fresh weight of 12 day old *AtCESA1* and *AtCESA9*

RNAi plants (6.73 mg/per seedling [±1.4], 7.57 mg/per seedling [±2.0] respectively) was similar to the control lines (6.96 mg/per seedling [±1.58]) (Fig. S3b). Similarly, there was no difference in seed germination between transgenic lines and the controls (Table S2), whereas a minor effect on root development was noted during the early stages of development in *AtCESA1* RNAi seedlings (Fig. 3a). In this case, *AtCESA1* RNAi lines displayed significantly shorter roots and a slower rate of root growth, 2 and 4 days after germination (DAG), with *AtCESA1* lines exhibiting root growth rates mean value of 0.12 mm/hour (±0.01) and 0.13 mm/hour (±0.03) compared to 0.24 mm/hour (±0.04) and 0.19 mm/hour (±0.03) in control plants (Table 1). However, by 10 DAG, root growth rates were similar in *AtCESA1* RNAi lines compared to control lines. Conversely, *AtCESA9* RNAi seedlings showed no discrepancies in root growth compared to control plants (Table 1; Fig. 3b). However, dark grown hypocotyl length was also reduced significantly in both *AtCESA1* and *AtCESA9* RNAi lines, with relative hypocotyl length reductions of 37% and 12% compared to empty vector control lines, respectively (Fig 4; Table 2).

In contrast, while seed weight was not affected in *AtCESA1* RNAi lines, the seed-specific down-regulation of *AtCESA9* resulted in an overall 7% reduction in the average 100 seed weight compared to the control lines (Fig. 5; ranging from 1.93 mg – 2.48 mg in empty vector lines and 1.76 mg – 2.23 mg in *AtCESA9* RNAi lines). In addition, when the 3 independent transgenic lines were analyzed separately, seeds from 2 *AtCESA9* RNAi lines demonstrated a slight but significant weight reduction, whereas no independent *AtCESA1* RNAi transgenic line exhibited any significant change in seed weight (Fig S4).

Compositional changes in the seed coat that alter its permeability can be detected by the rate and levels of violet coloration of the embryo when imbibed in tetrazolium violet

(Vishwanath et al. 2013) (Fig. 6). When transgenic seeds from this study were incubated in a solution of tetrazolium violet, *AtCESA1* and *AtCESA9* RNAi seeds displayed a somewhat increased capacity for tetrazolium violet uptake compared to control seeds (Fig. 6). A small amount of violet coloration was observed at the hilum region of the mature control seeds, whereas violet coloration was observed throughout the embryo in the mature *AtCESA1* and *AtCESA9* RNAi seeds after 50-60 hours.

The effect of *AtCESA1* and *AtCESA9* down-regulation on seed protein, oil, and soluble glucose contents

As shown in Fig. 7, T_3 homozygous seeds from *AtCESA1* RNAi lines exhibited a significant overall 3% increase ($P < 0.05$) in protein content (w/w) compared to control seeds. On average, the protein content in *AtCESA1* seed-specific RNAi lines was 28.4% (samples ranged from 28.4% - 29.3%), compared to 27.6% for the control lines (samples ranged from 26.1% - 28.4%) (Fig. 7; Fig S5). While no significant differences were observed between empty vector controls and the 3 independent *AtCESA1* RNAi transgenic lines on an individual basis, we observed a trend of increased protein content in each case (Fig. S5). Conversely, the seed-specific down-regulation of *AtCESA9* did not have a consistent effect seed protein content overall (Fig. 7; Fig. S5).

With regards to seed oil content, there was again a general trend for individual *AtCESA1* seed-specific down-regulation lines to exhibit reductions in seed oil content; however, this was only significant in 1 of the 3 independent lines tested (Fig. S6). Overall, this translated into a small but significant reduction (3% overall) in seed oil content in *AtCESA1* RNAi lines compared to empty vector controls (Fig. 8). As was the case with protein content, *AtCESA9*

RNAi down-regulation did not cause significant variation in total oil content (Fig. 8, Fig. S6). In empty vector control seeds, oil content ranged from 27.25% to 30.33%, while that of *AtCESA1* RNAi lines ranged from 26.86 to 29.36% and *AtCESA9* RNAi lines from 27.08% to 30.32% (Fig S6). To determine whether *AtCESA1* or *AtCESA9* down-regulation affected fatty acid composition, we also carried out compositional analysis of the oil extracted from T_3 homozygous seeds and we observed that the effect is minor in both cases (Table 3; Fig S7). *AtCESA1* RNAi lines exhibited a small but significant increase in C16:$1^{\Delta 9 cis}$ and C22:$1^{\Delta 11 cis}$ while *AtCESA9* RNAi lines showed a significant increase in C16:0, C16:$1^{\Delta 9 cis}$ and a significant reduction in C18:$1^{\Delta 9 cis}$ and C20:$1^{\Delta 11 cis}$ fatty acids.

Since the changes in seed protein and oil content in *AtCESA* RNAi lines were small or absent, we also assessed whether additional carbon available through the reduction of cellulose was redirected to soluble glucose biosynthesis. Despite a trend for individual independent *AtCESA1* and *AtCESA9* RNAi lines to exhibit increased seed glucose content (Fig. S8), this did not translate into significant overall increases in seed glucose content in either case (Fig. 9). In addition, we also performed starch content analysis of seedlings derived from *AtCESA* RNAi and control lines to determine whether the down-regulation of *AtCESA1* or *AtCESA9* down-regulation affected starch accumulation at this developmental stage. We observed a small, but significant increase in the starch content of *AtCESA9* RNAi seedlings compared to controls, whereas *AtCESA1* RNAi seedlings did not show any significant alteration (Fig. 10; Fig. S9).

Discussion

Enhancing the quality and quantity of economically important seed storage compounds in crops is critical to the seed crop industry and different approaches have been undertaken for

411 achieving this task (Shewry 2007; Ufaz and Galili 2008; Weselake et al. 2009; Weichert et al.
412 2010). Diverting carbon from other sources to oil and protein biosynthesis has been attempted
413 previously; however, this strategy has been met with only limited success since the biosynthetic
414 processes driving the production of different storage compounds are inversely correlated
415 (Knowles 1983; Ekman et al. 2008). In oilseeds, a large portion of carbon generated through
416 photosynthesis is partitioned into cell wall fibre, which negatively affects seed value (Somerville
417 2006; Weselake et al. 2009). High levels of fibre content in seed meal lower the available energy
418 content in the meal, thus limiting the feed value of the meal (Campbell et al. 2016; Le et al.
419 2017). Specifically, cell wall materials enriched in cellulose are non-digestible by monogastric
420 animals, thus limiting the effective utilization of meal and its economic value.
421 Sucrose entering sink tissues is hydrolyzed by the catalytic action of either SUCROSE
422 SYNTHASE or INVERTASE (Barratt et al. 2009), and the resulting hexoses are either used to
423 produce pyruvate through glycolysis for the downstream production of storage compounds
424 (mainly oil and protein in oilseed species such as Arabidopsis) or to synthesize cell wall
425 components including cellulose through the action of CELLULOSE SYNTHASE (Haigler et al.
426 2001; Baud et al. 2008). When the cellulose biosynthetic pathway in the developing seed is
427 attenuated, the biosynthesis of storage reserves, therefore, could theoretically be affected by
428 increasing the flow of carbon into oil, protein or starch in particular crop species, although the
429 biochemical interactions between pathways are yet to be fully understood. As such, this could
430 represent a useful strategy to increase seed value (Tomlinson et al. 2004; Ekman et al. 2008; Iyer
431 et al. 2008; Shi et al. 2012).
432 Many previous studies in which cellulose biosynthesis has been disrupted either through
433 mutation or constitutive down-regulation have led to plants with severe defects in morphogenesis

with characteristic symptoms such as tissue swelling, cell wall gaps, altered vascular morphology and aberrant cell wall thickenings with ectopic deposition of callose and lignin (Arioli et al. 1998; Gillmor et al. 2002; Caño-Delgado et al. 2003; Schrick et al. 2004; Somerville 2006). These characteristics affect plant growth, leading to abnormal root growth, shorter internode length, small leaf size, abnormal vascular tissue swellings and dwarf phenotypes (Arioli et al. 1998; Burton et al. 2000; Gillmor et al. 2002; Caño-Delgado et al. 2003). In an attempt to minimize abnormal morphological and growth phenotypes while still reducing cellulose content in seeds, we down-regulated the expression of two *AtCESA* genes (*AtCESA1* and *AtCESA9*), which are preferentially expressed in Arabidopsis seeds, in a seed-specific manner using RNAi (Fig. 1). The resulting Arabidopsis lines produced seeds with reduced cellulose contents and no obvious morphological defects, which is likely due to the seed-specific and only partial down-regulation observed in these lines. Although previous reports suggest that the involvement of AtCESA9 in cellulose biosynthesis in the embryo may be minor (Beeckman et al. 2002; Stork et al. 2010), we observed reduced total seed cellulose content in our seed-specific *AtCESA9* RNAi Arabidopsis lines. Beeckman *et al*. (2002) have observed varying *AtCESA9* expression levels in different cell types at different developmental stages of the embryo. Thus, *AtCESA9* may have some function in cellulose biosynthesis at specific developmental stages within the embryo in addition to its function in seed coat cellulose biosynthesis (Stork et al. 2010; Mendu et al. 2011).

Although no gross morphological differences were noted in any of our transgenic lines, we did observe a slight retardation in root growth in the *AtCESA1* RNAi lines early on development (between 2 and 4 DAG), which was not the case in *AtCESA9* RNAi lines, as is consistent with previous reports (Stork et al. 2010) (Fig. 3; Table 1). Root growth rates of the *AtCESA1* RNAi lines in the current study appeared to normalize in the later stages of root

development (between 10-12 DAG) (Table 1). While severe root growth defects have been previously reported in *AtCESA1* mutants, constitutive antisense *AtCESA1* Arabidopsis lines have been found to display a much weaker phenotype in this regard (Burn et al. 2002). Furthermore, we also observed reduced dark grown hypocotyl length for both RNAi lines compared to the empty vector controls, suggesting that both of these cellulose synthase genes may contribute to cell expansion during germination in the dark (Beeckman et al. 2002). This phenotype was more apparent in *AtCESA1* RNAi lines than in *AtCESA9* RNAi lines, although the effect observed in the current study was much weaker than in previously reported *AtCESA1* mutants (Beeckman et al. 2002). The smaller reduction in hypocotyl elongation in *AtCESA9* RNAi lines compared to *AtCESA1* RNAi lines suggests that its effect on cell expansion during early germination may be less than that of *AtCESA1*. These findings contrast with previous studies in which *AtCESA9* mutants were not found to display any difference in dark grown hypocotyl elongation compared to wild-type in seedlings (Burn et al. 2002; Stork et al. 2010). Therefore, in the current study, it is likely that effects on vegetative growth were very minor or absent due to the seed-specific nature of our RNAi vectors. The minor changes in early seedling development that were observed here likely derive from the fact that early seedling growth is reliant upon storage compound reserves within the embryo (Sanjaya et al. 2011).

In addition to effects on hypocotyl growth observed in the seed-specific *AtCESA9* RNAi lines, we also observed a reduction in seed weight in *AtCESA9* RNAi lines (but not *AtCESA1* RNAi lines). This did not appear to affect germination or vegetative growth of the plants, which corresponds with results obtained previously with *AtCESA9* T-DNA mutants (Stork et al. 2010). The seed weight reduction of the *AtCESA9* mutant reported by Stork et al. (2010) was more substantial (up to 24% reduction/100 seeds) than the 7% seed weight reduction observed in the

current study. Stork et al. (2010) also reported the presence of altered seed coat cell size, cell shape, and internal angle uniformity in the seeds of the *AtCESA9* T-DNA mutants, which were suggested to have arisen due to reduced cellulose content in their seed coats. While we did not carry out an in-depth analysis of embryos or seed coats in our transgenic lines, we did observe somewhat enhanced sensitivity to tetrazolium salt uptake for seeds from both *AtCESA1* and *AtCESA9* seed-specific RNAi lines compared to empty vector controls (Fig. 8), which suggests that seed coat permeability may have been affected by *AtCESA* down-regulation. This reduction in seed coat permeability was not severe as we only observed a difference in salt uptake after 48 hours of treatment (for most samples by 60 hours after treatment), whereas severe seed coat disruptions reported previously typically yield this same effect within 24-48 hours (DeBolt et al. 2009; Stork et al. 2010; Vishwanath et al. 2013). Thus, the effect of seed-specific partial down-regulation of *AtCESA1* or *AtCESA9* for seed coat cellulose biosynthesis may be less than in previously reported knock-out mutants. Indeed, the seed-specific nature of the RNAi construct may result in a smaller effect in the maternal seed coat in terms of reducing gene expression than in the seed embryo/endosperm (Zakharov et al. 2004).

Brassicaceae oilseed meals with increased storage protein content have been observed previously for varieties with lower amounts of seed coat fibre, such as yellow seeded canola-type *Brassica napus* varieties (Simbaya et al. 1995; Jia et al. 2012). In this case, the dietary fibre content (i.e. lignin) in seed meal obtained from yellow seeded canola was negatively correlated with meal protein content, while the meal protein content was higher than in brown-seeded canola (Simbaya et al. 1995). As such, we examined the effect of seed-specific down-regulation of *AtCESA1* or *AtCESA9* on the major storage components in Arabidopsis seeds by reducing the major seed insoluble fibre component, cellulose. In the current study, in addition to reduced

503 cellulose content, we also observed a small but significant increase in seed protein content in
504 *AtCESA1*, but not *AtCESA9*, RNAi lines (Fig 7). To the best of our knowledge, this is the first
505 instance in which seed protein has been assessed in lines in which *AtCESA*s have been down-
506 regulated/mutated, and a direct relationship between *AtCESA*s and protein biosynthesis has not
507 been previously reported.

508 Links between cellulose and oil biosynthesis may also exist, as evidenced by the negative
509 effect of lipid or fatty acid synthesizing mutants on cell wall synthesis (Schrick et al. 2004, 2012;
510 Li et al. 2015). While the precise mechanisms driving such an interaction remain to be
511 elucidated, diverting carbon flow from cell wall components to oil biosynthesis has been
512 reported previously. For example, increased oil content was observed in Arabidopsis seeds as a
513 result of down-regulating the expression of a transcription factor (GLABRA2) involved in
514 mucilage production (Lin et al., 2012). In the current study, however, we did not observe
515 increased seed oil content in seed-specific *AtCESA1* or *AtCESA9* RNAi lines, but instead saw a
516 modest reduction in total seed oil content in *AtCESA1* RNAi lines (Fig. 8) and only minor effects
517 on fatty acid composition.

518 Due to the lack of change in seed protein or oil contents in *AtCESA9* RNAi lines in this
519 study, we also assessed whether additional carbon resulting from *AtCESA* down-regulation in
520 seeds might instead be directed towards soluble glucose or starch production since reduced
521 cellulose biosynthesis itself may lead to an accumulation of soluble sugars as they are not being
522 used for cellulose or other seed storage compound biosynthesis. In addition, sucrose synthase,
523 which is involved in UDP-glucose production though the hydrolysis of sucrose, has been
524 previously identified as an integral component of the cellulose biosynthetic machinery (Fujii et
525 al. 2010). However, increases in seed glucose levels were not significant in either *AtCESA1* or

526 *AtCESA9* RNAi lines in this study (Fig. 9). Seedling starch levels followed a trend that was very
527 similar to that observed for seed glucose content, with *AtCESA9* RNAi seedlings, but not
528 *AtCESA1* RNAi seedlings, exhibiting a small but significant increase in starch content (Fig. 10).
529 Taken together, these results suggest that the effect of *AtCESA9* down-regulation on seed
530 storage compound biosynthesis appears to be minor. Since *AtCESA9* is mainly involved in seed
531 coat cellulose synthesis (Stork et al. 2010) and the seed coat is the main protecting layer of the
532 embryo, it is possible that a relatively high proportion of the carbon pool may be redirected
533 towards the synthesis of non-cellulosic cell wall components, such as hemicellulose or pectin, to
534 overcome the structural weakness of the seed coat in this case (Stork et al. 2010). As such, it
535 would be beneficial to evaluate other cell wall components in addition to cellulose, and to also
536 examine cell wall ultrastructure of our *AtCESA9* RNAi lines in the future.
537 Increasing protein content while reducing the complex fibre content in seeds has been
538 gaining attention over the past few years, especially within the oilseed industry (Tan et al. 2011;
539 Yoshie-Stark et al. 2008). Enhancing total protein content through transgenic engineering
540 approaches involving the over-expression of particular genes has been reported previously for
541 different plant species including Arabidopsis, with some success (Mcallister et al. 2012; Lam et
542 al. 2003). Our results suggest that it may also be possible to obtain seeds with reduced cellulose
543 and increased protein content simultaneously by down-regulating *AtCESA1* expression within the
544 seeds, which is a novel finding in this area, even though the change is incremental. Since the
545 seed-specific *AtCESA1* RNAi lines did not exhibit negative phenotypes regarding plant growth
546 and development, future experiments comprising the co-expression of genes involved in nitrogen
547 utilization in combination with seed-specific *AtCESA1* down-regulation may provide higher
548 amounts of protein in seeds. However, the reduced oil content seen in *AtCESA1* RNAi lines may

549 hinder the direct use of this strategy, although a multigene approach involving the co-expression
550 of genes involved in seed oil accumulation could potentially overcome this issue (van Erp et al.,
551 2014; Vanhercke et al., 2014). Indeed, while the down-regulation of starch synthesis on its own
552 did not lead to increased oil content in Arabidopsis leaf tissues, the co-expression *WRINKLED1*
553 (*WRI1*), which encodes a transcription factor that up-regulates genes encoding enzymes in
554 glycolysis and fatty acid biosynthesis, led to increased lipid content (Sanjaya et al. 2011). In
555 addition, the overexpression of enzymes involved in lipid biosynthetic pathways such as
556 diacylglycerol acyltransferase (DGAT) has been proven to be effective in terms of increasing oil
557 content is seeds (reviewed by Yang et al., 2018), which may also be a useful strategy to recover
558 oil content in the *AtCESA1* RNAi lines. Therefore, the lines developed in this study could
559 potentially be used as a starting point for the future development of crop varieties with enhanced
560 seed quality in terms of low fibre and high protein.
561
562 **Abbreviations used**
563 CESA, *CELLULOSE SYNTHASE*; *AtCESA*, Arabidopsis *CELLULOSE SYNTHASE*; hpRNAi,
564 intron-spliced hairpin RNA; *PP2AA3*, *PROTEIN PHOSPHATASE 2A SUBUNIT 3*; FAME, fatty
565 acid methyl esters; DAF, days after flowering; TAG, triacylglycerol
566
567
568 **Author contribution statement**
569 SDS, RJW and GC conceived and designed the experiment. KNJ carried out most experiments
570 and drafted the manuscript. RJW, SDS, GC, and JAO supervised the studies and all authors
571 revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Table

Table 1 Average root growth rate with standard deviation (±SD) of the seedlings of *AtCESA* RNAi lines and control lines

Data comprise the mean root growth rate (mm/hour) ±SD from a minimum of 10 *AtCESA* RNAi and control homozygous seedlings (from 3 independent transgenic events), respectively. CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines; DAG, Days after germination. Data are representative of 3 experiments conducted with similar results.

Plant line	**Root growth rate (mm/ hour)**				
	2 DAG	4 DAG	6 DAG	10 DAG	12 DAG
CESA1ss	0.12 ±0.01 *	0.13 ±0.03 *	0.24 ±0.04	0.37 ±0.02	0.48 ±0.08
CESA9ss	0.20 ±0.05	0.24 ±0.03	0.27 ±0.03	0.36 ±0.07	0.52 ±0.05
Con	0.24 ±0.04	0.19 ±0.03	0.30 ±0.06	0.38 ±0.04	0.49 ±0.08

Data were analyzed using two-tailed student's T-tests. (*) indicates values that are significantly lower than empty vector controls at $P \leq 0.05$.

Table 2 Average hypocotyl length with standard deviation (±SD) of the seedlings of *AtCESA* RNAi lines and control lines

Data comprise the mean hypocotyl length (mm) ±SD from a minimum of 18 *AtCESA* RNAi and control homozygous seedlings (from 3 independent transgenic events), respectively, 6 days post-germination. CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines; Data are representative of 3 experiments conducted with similar results.

Plant line	Hypocotyl length (mm)
CESA1ss	8.27 ±1.07*
CESA9ss	11.67 ±2.51*
Con	13.29 ±1.38

Data were analyzed using two-tailed student's T-tests. (*) indicates values that are significantly lower than empty vector controls at $P \leq 0.05$.

Table 3. Average fatty acid compositions of the seed oil from *AtCESA1* and *AtCESA9* RNAi lines (T_3 seeds)

Data are means ±SD of seed samples from 3 independent homozygous *AtCESA1* RNAi, *AtCESA9* RNAi and control lines (at least 3 biological replicates were used in each case). Two technical replicates were utilized in each case. Fatty acid composition is presented as the percentage of each fatty acid with respect to total fatty acids on a mol% basis. CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines.

Fatty acid	**Fatty acid composition (mol %)**		
	Con	**CESA1ss**	**CESA9ss**
C16:0	8.6 ±0.14	8.69 ±0.13	8.85 ±0.1 ▲
C16:1	0.36 ±0.02	0.39 ±0.02 ▲	0.39 ±0.04▲
C18:0	4.18 ±0.07	4.14 ±0.1	4.17 ±0.08
C18:1	16.37 ±0.6	16.46 ±0.76	16.31 ±0.55 ▼
C18:1c11	1.65 ±0.07	1.61 ±0.03	1.67 ±0.03
C18:2	24.46 ±0.07	24.3 ±0.3	24.6 ±0.12
C18:3	16.05 ±0.29	16 ±0.3	15.97 ±0.39
C20:0	2.43 ±0.05	2.43 ±0.06	2.43 ±0.06
C20:1	18.79 ±0.31	18.86 ±0.22	18.47±0.18 ▼
C20:2	2.52 ±0.07	2.52 ±0.09	2.54 ±0.06
C20:3	0.42 ±0.07	0.42 ±0.02	0.42 ±0.02
C22:1	1.93 ±0.06	1.98 ±0.06▲	1.93 ±0.05

Data were analyzed using two-tailed student's T-tests. (▲/▼) indicates values that are significantly greater/lower than empty vector controls at $P \leq 0.05$.

819

Figure captions

Fig. 1 Relative expression of Arabidopsis *AtCESA1* and *AtCESA9* genes in RNAi and empty vector control lines. Total RNA was obtained from *AtCESA1* T_1 siliques containing T_2 seeds (a), *AtCESA1* T_2 siliques containing T_3 seeds (b), *AtCESA9* T1 siliques containing T_2 seeds (c) and *AtCESA9* T_2 siliques containing T_3 seeds (d) 14 days after flowering. Equal amounts of total RNA were used for cDNA synthesis and 1/5 dilutions of the resulting reactions were used for quantitative RT-PCR. The Arabidopsis *PROTEIN PHOSPHATASE 2A SUBUNIT 3* (*PP2AA3*) gene was used as the internal reference. Control blocks represents the mean from 3 individual empty vector lines. CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines.

Fig. 2 Contents of acid-insoluble glucose in T_3 homozygous Arabidopsis seeds from *AtCESA1* and *AtCESA9* seed-specific RNAi lines, as well as empty vector controls. The mean values represent the average of measurements from 3 independent transgenic lines, with 2-3 biological replicates of each, while bars denote standard error of the mean. Two technical replicates were carried out in each case. Significant differences in transgenic seeds compared to control seeds are indicated by an asterisk ($P \leq 0.05$). CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines.

Fig. 3 Growth rates of *AtCESA1* and *AtCESA9* seed-specific RNAi seedlings, as well as empty vector controls. Seedlings grown vertically on solid medium were photographed 8 days post-germination. *AtCESA1* seed-specific RNAi (from 3 independent homozygous transgenic events) **(a)** and *AtCESA9* seed-specific RNAi (from 3 independent homozygous transgenic events) **(b)**

seedlings are shown to the right of the vertical black lines, while empty vector control seedlings are displayed to the left in both instances. The picture shown here is representative of 3 experiments conducted with similar results, with at least 10 seedlings assessed per construct in each experiment. Scale bars represent 1 cm.

Fig.4 Phenotype of *AtCESA1* and *AtCESA9* seed-specific RNAi seedlings, as well as empty vector controls 6 days post germination. *AtCESA1* seed-specific RNAi **(a)** and *AtCESA9* seed-specific RNAi **(b)** seedlings are shown to the left of the vertical black lines, while empty vector control seedlings are displayed to the right in both instances. The picture shown here is representative of 3 experiments conducted with similar results, with at least 10 seedlings assessed per construct in each experiment. Scale bars represent 1 cm.

Fig. 5 One hundred-seed weight of T_3 homozygous Arabidopsis seeds from *AtCESA1* and *AtCESA9* seed-specific RNAi lines, as well as empty vector controls. The mean values represent the average of 3 independent homozygous transgenic lines, with at least 3 biological replicates of each, while bars denote standard error of the mean. Three technical replicates were carried out in each case. Significant differences compared to the control are indicated by an asterisk ($P \leq 0.05$). CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines

Fig. 6 Permeability to tetrazolium salt of T_3 seeds from *AtCESA1* and *AtCESA9* seed-specific RNAi lines, as well as empty vector controls after 60 hours. *AtCESA1* seed-specific RNAi **(a),** *AtCESA9* seed-specific RNAi **(b)** and empty vector control seeds **(c)** were imbibed in 1% aqueous tetrazolium violet solution and subsequently observed under a dissecting light

867 microscope. Scale bar represents 200 μm.

868

869 **Fig. 7** Total protein content of T_3 homozygous Arabidopsis seeds from *AtCESA1* and *AtCESA9*
870 seed-specific RNAi lines, as well as empty vector controls. Mean values are percentages of dry
871 seed weight and include the average of T_3 seed samples from 3 independent homozygous
872 transgenic lines, with at least 3 biological replicates of each, while bars denote standard error of
873 the mean. Two technical replicates were carried out in each case. Significant differences
874 compared to the control are indicated by an asterisk ($P \leq 0.05$). CESA1ss, Arabidopsis *AtCESA1*
875 seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector
876 control lines

877

878 **Fig. 8** Seed oil content of T_3 homozygous Arabidopsis seeds from *AtCESA1* and *AtCESA9* seed-
879 specific RNAi lines, as well as empty vector controls. The mean values are percentages of dry
880 seed weight and include the average of T_3 seed samples from 3 independent transgenic lines,
881 with at least 3 biological replicates of each, while bars denote standard error of the mean. Two
882 technical replicates were carried out in each case. Significant differences compared to the control
883 are indicated by an asterisk ($P \leq 0.05$). CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi
884 lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines

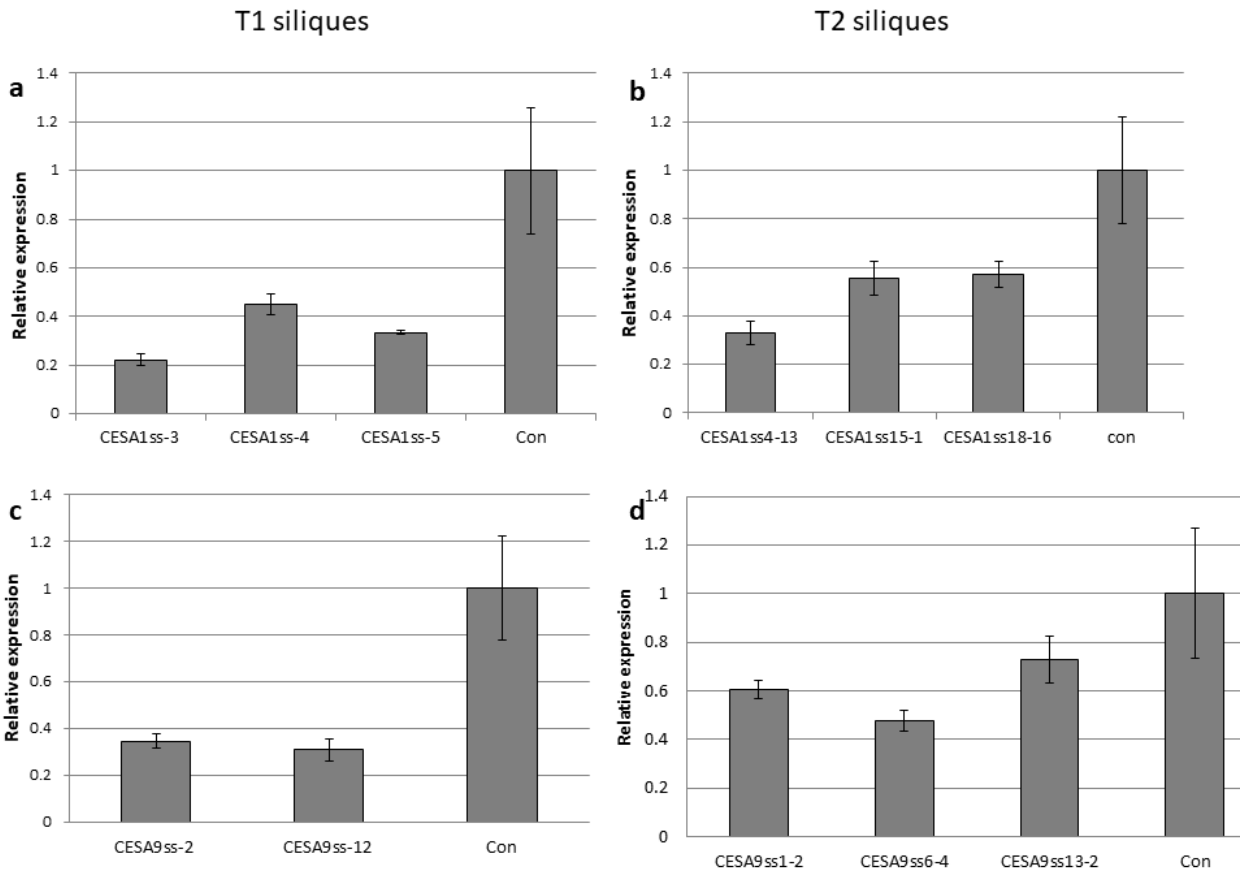
885

886 **Fig. 9** Seed glucose concentration in T_3 homozygous Arabidopsis seeds from *AtCESA1* and
887 *AtCESA9* seed-specific RNAi lines, as well as empty vector controls. The mean values are the
888 average μmol of glucose in g of tissue of T_3 seed samples from 3 independent transgenic lines,
889 with at least 2 biological replicates of each, while bars denote standard error of the mean. Three
890 technical replicates were carried out in each case. Significant differences compared to controls

are indicated by an asterisk ($P \leq 0.05$). CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines

Fig. 10 Seedling starch content in T_3 homozygous Arabidopsis seedlings from *AtCESA1* and *AtCESA9* seed-specific RNAi lines, as well as empty vector controls. Depicted are the mean values of % starch per mg T_3 seedling tissue (fresh weight) from 3 independent transgenic lines, with at least 2 biological replicates of each, while bars denote standard error of the mean. Two technical replicates were carried out in each case. Significant differences compared to controls are indicated by an asterisk ($P \leq 0.05$). CESA1ss, Arabidopsis *AtCESA1* seed-specific RNAi lines; CESA9ss, *AtCESA9* seed-specific RNAi lines; Con, empty vector control lines

1


T1 siliques
T2 siliques
a
b
c
d
Relative expression
CESA1ss-3
CESA1ss-4
CESA1ss-5
Con
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CESA1ss15-1
CESA1ss18-16
con
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CESA9ss6-4
CESA9ss13-2

2

3 **Fig. 1**

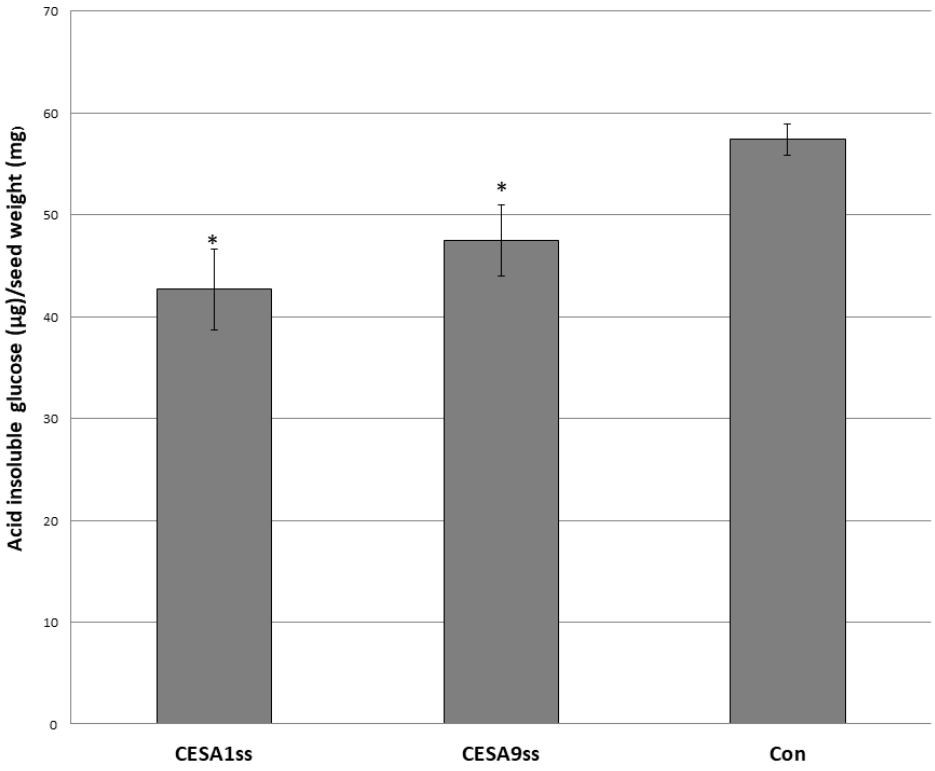

70
60
50
40
30
20
10
0
Acid insoluble glucose (µg)/seed weight (mg)
*
*
CESA1ss
CESA9ss
Con

Fig. 2

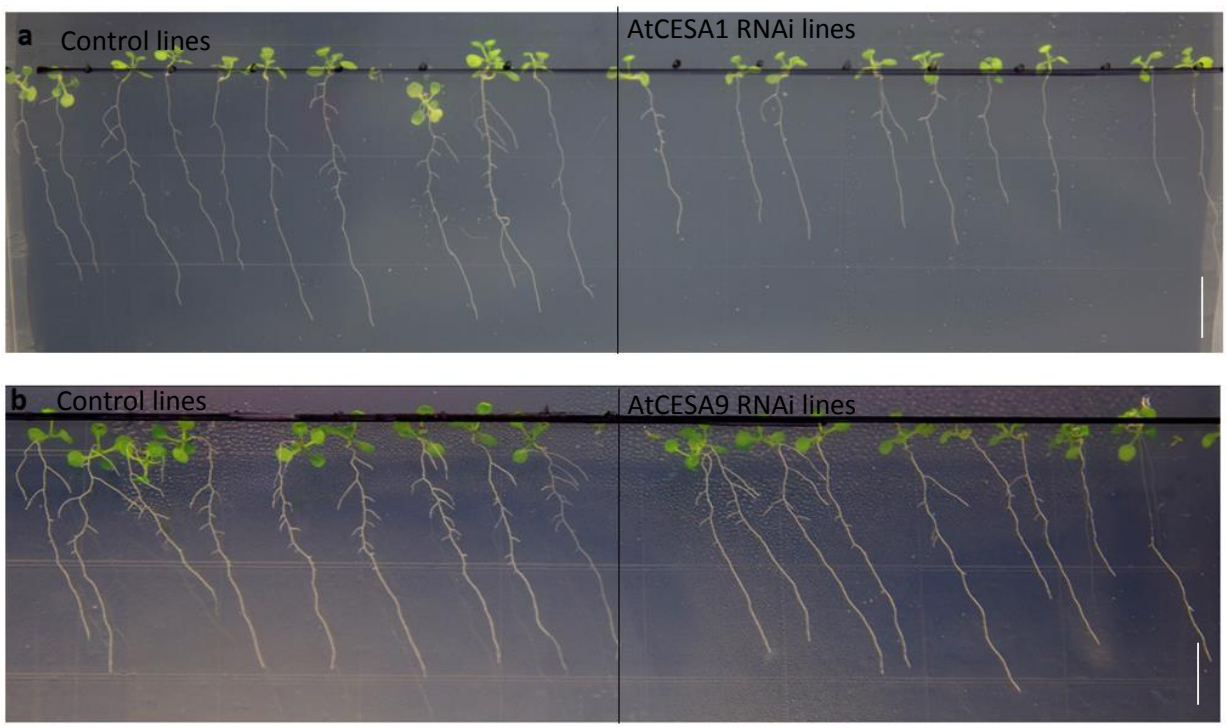

a
Control lines
AtCESA1 RNAi lines
b
Control lines
AtCESA9 RNAi lines

Fig. 3

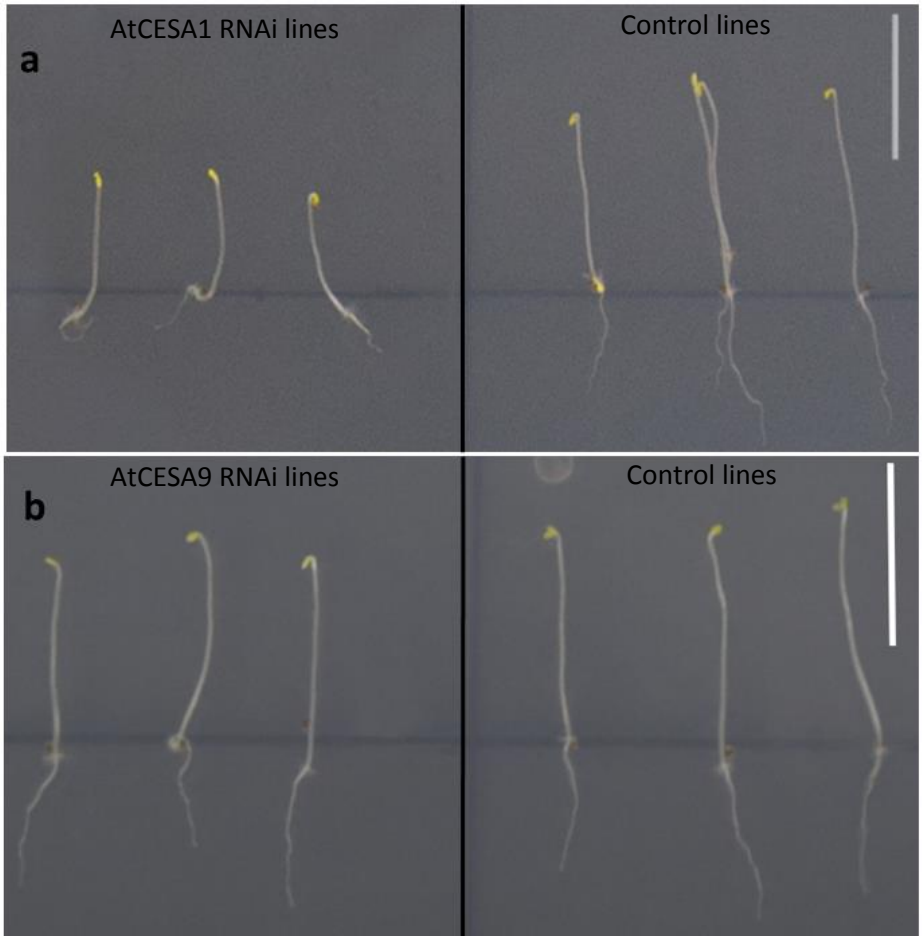
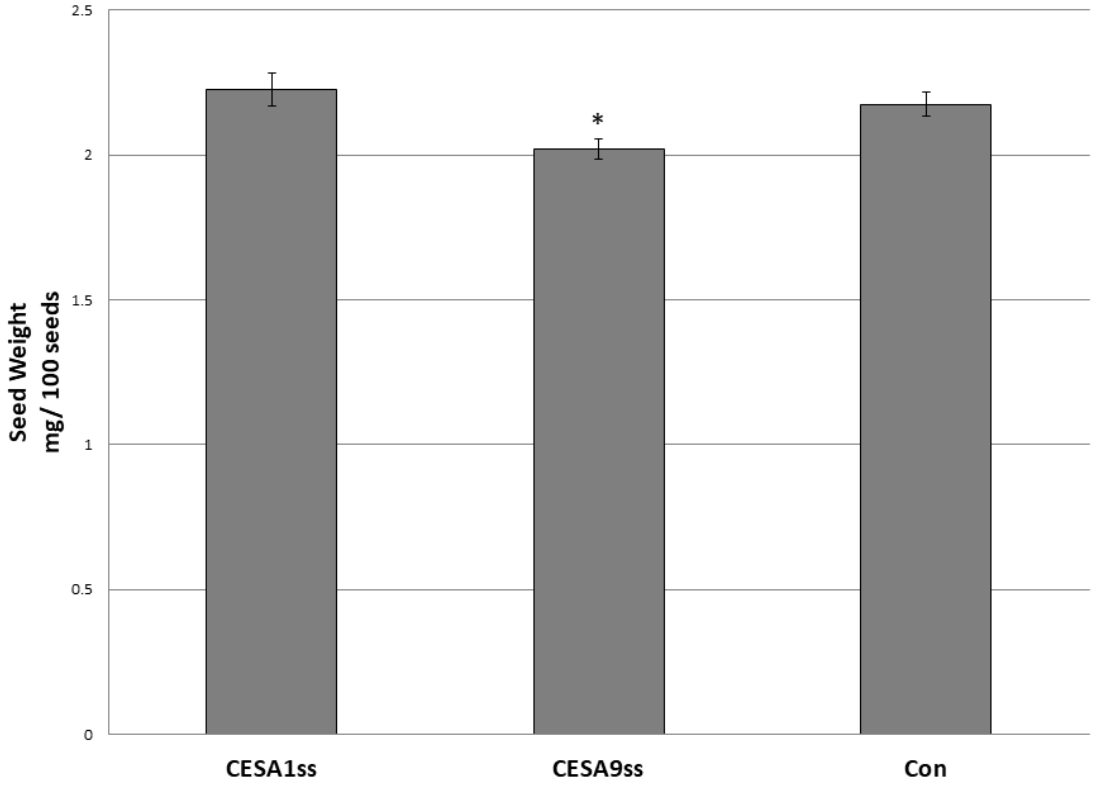

a
AtCESA1 RNAi lines
Control lines
b
AtCESA9 RNAi lines
Control lines

Fig. 4



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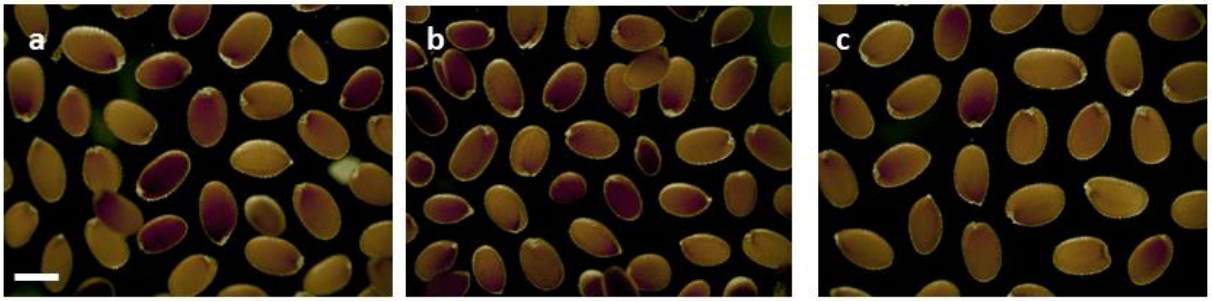

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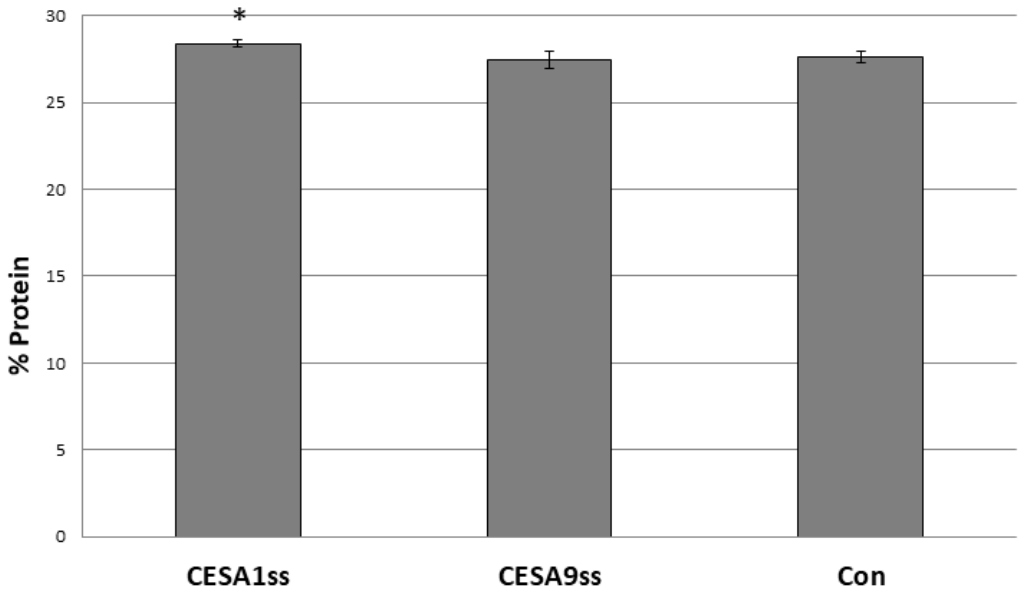

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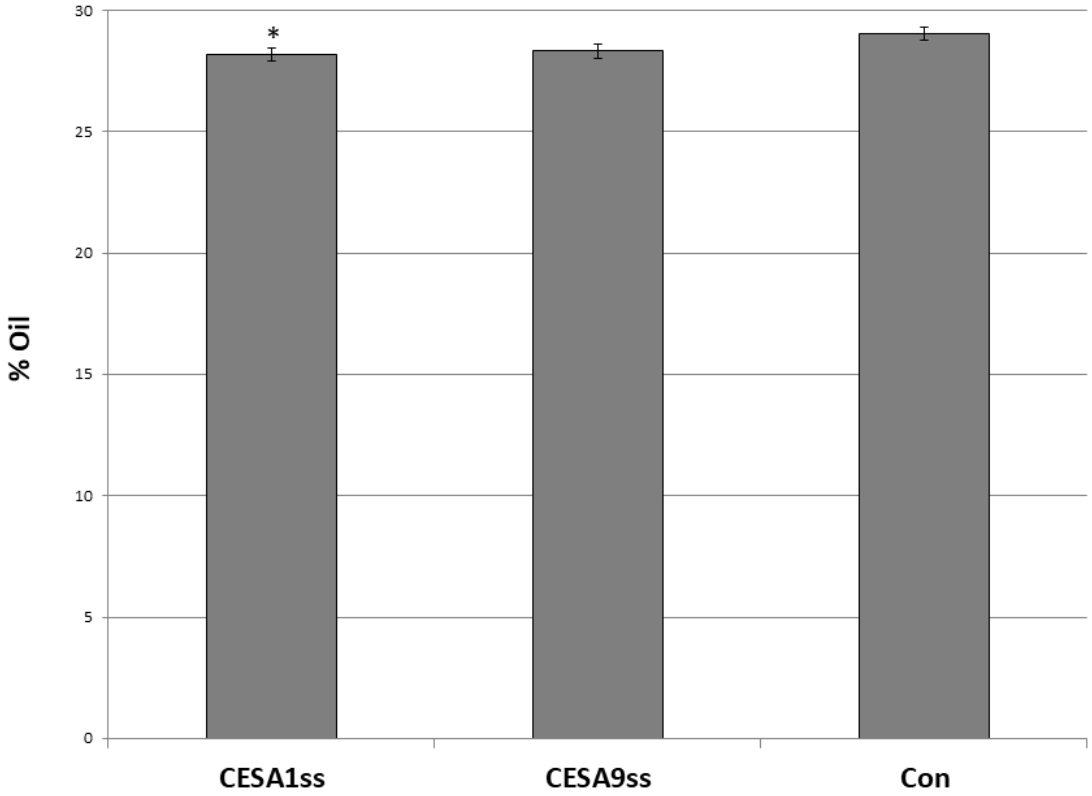

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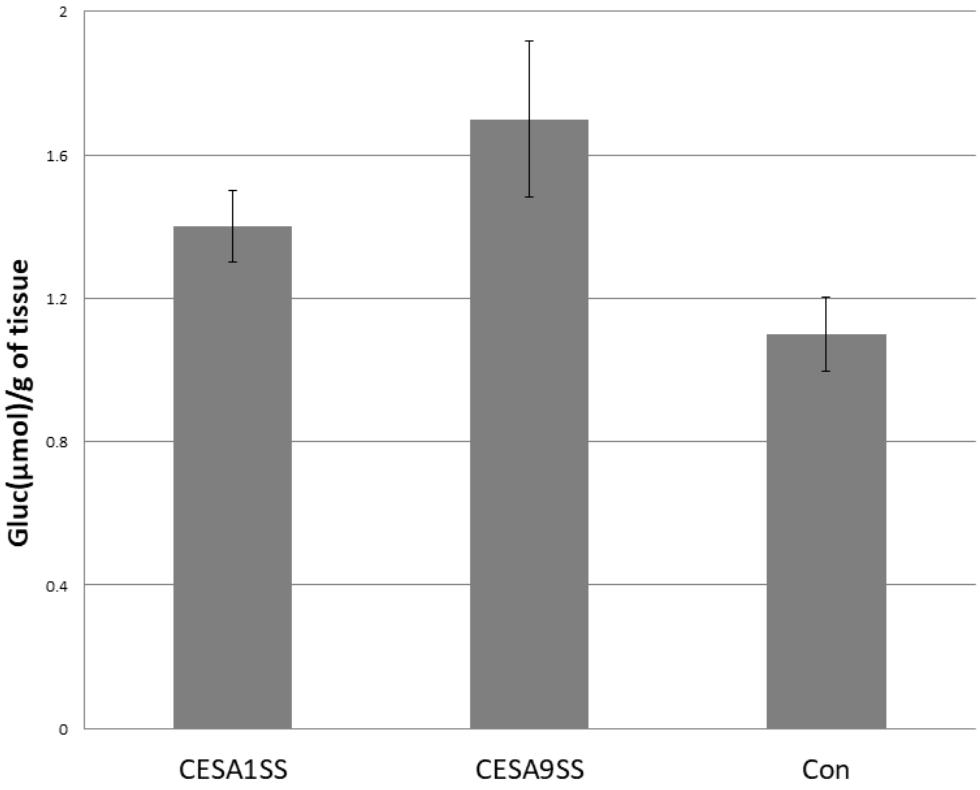
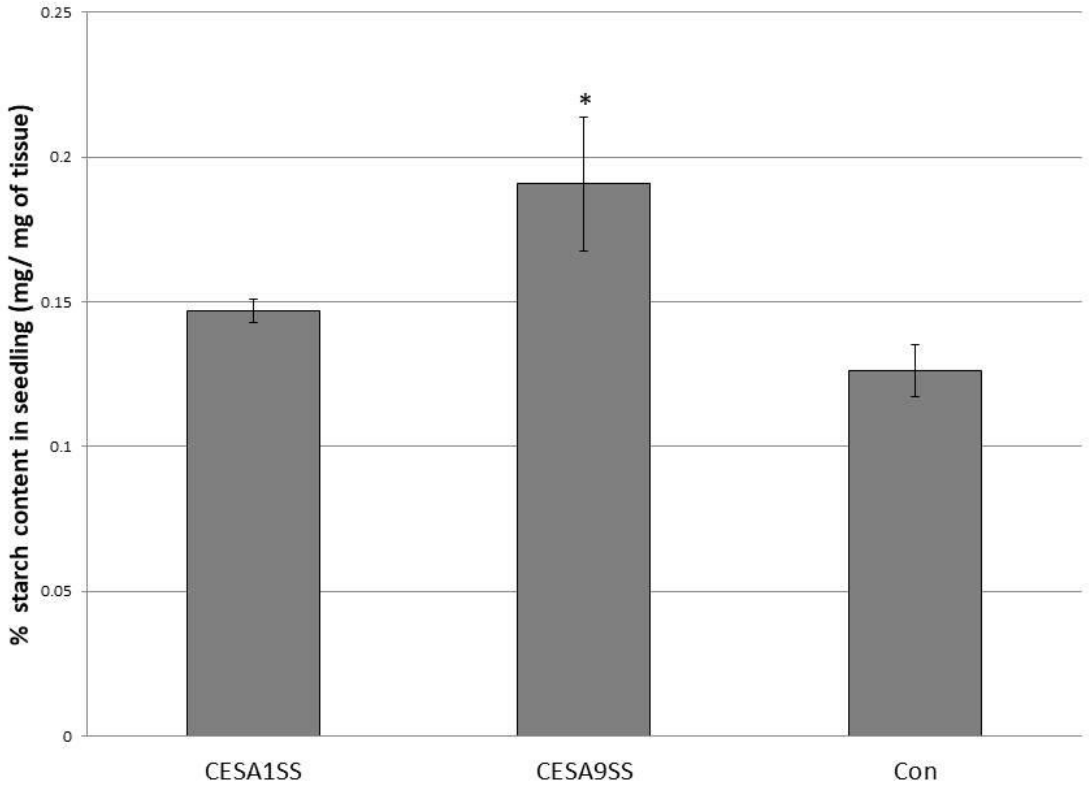


Fig. 9



38

39 **Fig. 10**

40