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ORIGINAL PAPER

Improving seed size, seed weight and seedling emergence in *Camelina sativa* by overexpressing the *Atsob3-6* gene variant

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Abstract Seedling stand establishment is a critical factor affecting crop yield in low-precipitation agricultural regions. This is especially true for small seeded crops, such as *Camelina* (*Camelina sativa*) and canola (*Brassica napus*), that need to be planted shallow. Deeper planting would be desirable so that seeds can access soil moisture and bigger seeds could improve emergence and stand establishment by providing the energy necessary for seedling elongation. *AHL* (*AT-Hook Containing, Nuclear Localized*) genes play an important role in seedling growth and development. AHL proteins contain two structural units, the DNA-binding AT-hook motif and the Plant and Prokaryote Conserved (PPC) domain, required for protein–protein interactions. Our previous studies demonstrate that *AtAHL29/SOB3* (*Suppressor of phytochrome B-4 #3*) regulates seedling development in *Arabidopsis* (*Arabidopsis thaliana*). Activation-tagged overexpression of *AtSOB3* (*Atsob3-D*) represses the long-hypocotyl phenotype of an *Arabidopsis* phytochrome B mutant. In contrast, overexpression of the *Atsob3-6* variant (*Atsob3-6-OX*), with a non-functional AT-hook, confers a long-hypocotyl phenotype. In this study, we demonstrate the role of *Atsob3-D* and *Atsob3-6-OX* in modulating seed size and hypocotyl length in the brassicas *Arabidopsis* and *Camelina*. In *Arabidopsis*, *Atsob3-D* reduces seed weight whereas *Atsob3-6-OX* increases seed weight and size when compared to the wild type. Similarly, *Atsob3-6-OX* transgenic *Camelina* seedlings are taller than the wild type, and produce larger and heavier seeds. These larger *Atsob3-6-OX Camelina* seeds also confer better emergence in deep-soil planting when compared to the wild type. Taken together, *Atsob3-6-OX* increases seed size, seed weight, seedling hypocotyl length and stand establishment in the oilseed crop *Camelina*.

Keywords *Arabidopsis* · *Camelina* · Seed · Seedling · Emergence · *Atsob3-6*

Introduction

Most breeding programs have been targeting seed number for crop yield improvement (Harper et al. 1970; for review see Sadras 2007; Sadras and Egli 2008; Venable 1992). For some crops, however, seed size may be a better target for yield than seed number due to high heritability (Gnan et al. 2014; Sadras and

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Slafer 2012). In small-seeded crops such as brassicas, seed size is among the most important agronomic traits impacting seedling stand establishment (Harker et al. 2015). In cultivars of hybrid broccoli (*Brassica oleracea*), seedlings from large seeds had better establishment, higher seed weights and crop yield (Ahmed and Zuberi 1973; Elliott et al. 2008; Heather and Sieczka 1991). With increased seed size, enhanced germination and emergence was also reported in the monocot triticale (Kaydan and Yagmur 2008).

Seed size can also affect other developmental characteristics of plants. In barley and tomato, a positive correlation between seed size and seminal root, total root weight and lateral root number has been reported (Bertholdsson and Kolodinska Brantestam 2009; Khan et al. 2012). Seedlings from bigger seeds displayed improved tolerance to root diseases such as *Rhizoctonia solani* (Sturrock et al. 2015). In contrast, seed size had no effect on plant density and seed yield of spring and winter types of Argentine canola (*B. napus*). However, Argentine canola plants from large seeds had larger leaves, more leaves and higher biomass than plants from small seeds (Major 1977; Mendham et al. 1981). Increasing seed size of *B. napus, B. rapa* and *Sinapsis alba* has shown a positive linear association with early biomass, which may increases crop competition with weeds and pest pressure (Bodnaryk and Lamb 1991; Harker et al. 2015). Vigorous canola growth from larger seeds shortened time to flowering, flowering period and reduced the risk of high temperature exposure during pod development (Harker et al. 2015).

In dryland cropping systems, seeds size and the length/strength of the seedling hypocotyl can have a major impact on stand establishment. For example, in the inland Pacific Northwest (PNW), wheat is often rotated with legumes such as pea, garbanzo and lentils. In high rainfall ($>$ 38 cm) zones, a crop is grown every year with two- to three-year rotation cycles. In lower rainfall ($\leq$ 38 cm) zones, crops are often grown once every two to three years with the land remaining fallow in between crops to recharge soil moisture (Schillinger et al. 2010). In recent years, oilseed crops such as canola (*B. napus*) and *Camelina* (*Camelina sativa*) have also been included in these rotations. However, the relatively small seed size and subsequent shallow planting depth of canola and *Camelina* can have an impact on stand establishment in low-rainfall dryland cropping systems.

Camelina, with its relatively short growing season (85–100 days) and low moisture requirement, is a relatively new crop in the US that is being incorporated into some of these low-rainfall areas of the inland PNW (Shonnard et al. 2010; Schillinger et al. 2010). *Camelina* is also a potential biodiesel feedstock crop due to its high oil content (30–40%) (Pavlista et al. 2007, 2011). Because of *Camelina'*s small seed size, planting depth must remain relatively shallow. This shallow planting depth can have an impact on seedling emergence and stand establishment if seeds cannot be planted deep enough to access soil moisture while having the energy to emerge above the surface. *Camelina* seedling emergence may be facilitated by developing varieties with longer hypocotyls and larger seeds.

In the model brassica, *Arabidopsis* (*Arabidopsis thaliana*), the *AHL* (*AT-Hook Containing, Nuclear Localized*) gene family has been implicated in regulating hypocotyl elongation. For example, *SUPPRESSOR OF PHYTOCHROME B-4 # 3* (*SOB3/AtAHL29*) was identified in an activation-tagging gene-overexpression mutant screen for suppression of the long-hypocotyl phenotype conferred by a mutation in the photoreceptor phytochrome B. In this screen, *Atsob3-D* was shown to repress hypocotyl elongation whereas the *Atsob3-4* null-mutant, when combined with the *Atesc-8* null mutation in *AtSOB3*'s closest family member *ESCAROLA* (*AtESC/AtAHL27*), confers a long-hypocotyl phenotype (Street et al. 2008). AHL proteins contain two conserved regions, the DNA-binding AT-hook motif (Fujimoto et al. 2004) and the Plant and Prokaryote Conserved (PPC) domain which is involved in protein–protein interactions (Zhao et al. 2013).

The AT-hook motif binds AT-rich DNA and has a conserved R-G-R region which is necessary for protein-DNA interactions (Aravind 1998; Raymond and Nissen 1990; Zhao et al. 2013). Street et al. (2008) identified a missense mutation in the R-G-R motif, *Atsob3-6,* which confers a long-hypocotyl phenotype when overexpressed (*Atsob3-6-OX*). According to a molecular model proposed by Zhao et al. (2013), AHLs interact with each other and themselves, as well as with other nuclear proteins including transcription factors (TFs), via the PPC domain, to form homo- or hetero-complexes which modulate plant growth and

development. In order to coordinate this process, a functional AT-hook with a conserved R-G-R is required. The *Atsob3-6* mutation (R-G-H), in the R-G-R core of the AT-hook motif, blocks DNA binding and abolishes the normal repressive function of these TF complexes on hypocotyl growth, resulting in a long-hypocotyl phenotype (Zhao et al. 2013). Additionally, a connection between AHLs and the growth-promoting brassinosteroid hormones is suggested by the observation that AHL12 and ESC interact physically with a plant-specific NAC (NAM, ATAF1,2, CUC2) transcription factor called ATAF2 (Zhao et al. 2013). ATAF2 promotes hypocotyl elongation by repressing the expression of two genes, *PHYTOCHROME B ACTIVITION TAGGED SUPPRESSOR 1* (*BAS1*) and *SUPPRESSOR OF PHYTOCHROME B#4-7* (*SOB7*), which encode brassinosteroid-inactivation enzymes (Peng et al. 2015).

In this study, we test the hypothesis that *AtSOB3* can regulate seed size together with hypocotyl elongation. We demonstrate that seed weight and hypocotyl length of *Atsob3-D* was significantly smaller than the wild type, whereas the dominant-negative mutation, *Atsob3-6-OX*, conferred heavier and larger seeds with a longer hypocotyl in *Arabidopsis*. We further demonstrate that the increase in hypocotyl length is due to cell elongation rather than cell proliferation. Our results demonstrate that the *Atsob3-6-OX* allele increased seed size, weight and hypocotyl elongation when overexpressed in *Camelina*. We also demonstrate that transgenic *Camelina* seeds overexpressing *Atsob3-6* have improved seedling emergence in deep-planting assays when compared to non-transgenic siblings.

Materials and Methods

Plant materials and growth conditions- All of the *Arabidopsis* lines used in this study were in the Columbia ecotype (Col-0) background. *Atsob3-D* and *Atsob3-6-OX* lines have been described previously (Street et al. 2008; Zhao et al. 2013). All *Camelina* plant material used in this study was in the variety Calena. Transgenic seeds from homozygous single-locus insertion lines were used in this study. *Arabidopsis* seeds were sterilized with 70% ethanol plus 0.05% Tween20 for 15 min, 95% ethanol plus 0.05% Tween20 for 10 min followed by washing with 95% ethanol for 5 min and air drying. After sterilization, seeds were sown onto 0.5X Linsmaier & Skoog (LS) Modified Basal Medium containing 15 g/L sucrose and 6 mg/L Gellan Gum Powder (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) then incubated at 4 °C in darkness for 4 days, followed by 8 h of red light treatment before being transferred to continuous white light (22 µmol m^{-2} s^{-1}, 25 °C) in a Percival E 30 B (Percival scientific, Perry, IA, USA) chamber for 6 days. *Camelina* seedlings were grown under long-day conditions (16 h of light and 8 h of dark) with a light intensity of 300 µmol m^{-2} - s^{-1} at 25 °C.

Hypocotyl measurement- Six-day-old *Arabidopsis* seedlings were harvested and scanned into TIFF format images at 1200 dpi using an Epson Perfection V600 flatbed scanner (Epson America Inc., Long Beach, CA, USA). NIH ImageJ software version 1.48 was used to measure hypocotyl lengths. Seven-day-old *Camelina* seedlings were harvested and hypocotyls were measured using a ruler.

Seed size, seed number and weight analysis- 100 seeds of each line were scanned into TIFF format images at 4800 dpi using an Epson Perfection V600 flatbed scanner. SmartGrain7 software from NIAS (Tanabata 2012- National Institute of Aerobiological Sciences, Japan) was used for size analysis. For weight analysis, three technical replicates of 100 seeds from each line were counted and weighed using a Mettler Toledo analytical balance (Mettler Toledo LLC, Columbus, OH, USA). To determine the number of *Camelina* seeds per pod, 10 different pods of each plant from 10 biological replicate plants were counted and seed numbers were recorded.

Semi quantitative RT-PCR- *Camelina* total RNA was extracted from ten-day-old seedlings using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, and USA). During extraction, genomic DNA contamination was eliminated via the On-Column DNase I Digestion Set (Sigma-Aldrich, St. Louis, MO, USA). First strand cDNA was synthesized using the iScript Reverse Transcription Supermix (BioRad, Hercules, CA, USA). *CsActin* was used to normalize mRNA levels in cDNAs. No-RT controls were used to detect DNA contamination in RNAs. Primers used for PCR are listed in Table S1.

Plasmid construction and plant transformation- The *Atsob3-6* allele was amplified by PCR using

AtSOB3 full length primers with *Eco*RI/*Xho*I adapters. This fragment was used to generate pUSH5 via insertion into the pBinGlyRed3 plasmid using enzymes *Eco*RI plus *Xho*I where the gene of interest was driven by the soybean glycinin promoter with *Ds*Red as a selectable marker. The resulting construct was transformed into *Agrobacterium tumefaciens* strain *GV3101*. This construct was then transform into *Camelina* plants using the floral dip method as described by Lu and Kang (2008). Single-locus homozygous T_3 seeds were used for further analysis. Primers are included in Table S1.

Hypocotyl images- Seedlings were fixed with 4% paraformaldehyde in 0.05 M Pipes buffer (pH 7.2) at 4 °C for 24 h. The seedlings were then rinsed for 10 min, three times with 0.05 M Pipes before being stained with 0.1% safranin-O in Pipes buffer overnight on a rotator. The seedlings were rinsed for 10 min in water, three times, then images were taken on a Leica SP8 Confocal Microscope (Leica, Germany) using a white light laser excitation set at 561 nm with emission collected between 582 and 640 nm. Magnification was 20X with an individual image size of 1024px. Tile scan Z-stacks were employed to image an entire longitudinal section of the hypocotyl and the images were reconstructed using LASX software from Leica. The number of cells in individual columns in the epidermis of four-day-old-hypocotyls were recorded. Three different cell-columns of three biological replicate seedlings were counted and cell lengths were measured using ImageJ.

Seedlings emergence assay- For seedling emergence in compacted commercial sunshine mix #1 (Sun Gro Horticulture, USA), the potting mix was air dried for a week in a greenhouse after which, a 500 ml beaker full of soil was moistened with 200 ml of water. A 2 cm layer of moist soil was placed at the bottom of a transparent beaker and then split in half with a label. In one half, 15 transgenic seeds were placed around the edges of the beaker so that the seedlings could be visible from outside. In the other half, 15 wild-type seeds were arranged in the same manner. All seeds were then covered with the moist potting mix and the soil was compacted to the final depth of 6 cm. The beakers were then covered with black plastic pots to provide darkness. The numbers of emerged seedlings on the soil surface were recorded after 7 days of growth at 25 °C. All the germinated seedlings for both the transgenic and the wild type were carefully extracted from soil. Seedling hypocotyls were measured using a ruler. For seedling emergence in Palouse silt loam soil, seeds were sown as described above with seeds place on moist soil and then covered with dry soil without any compaction.

Statistical analysis- MS Excel software was used for statistical analysis and graph generation. Data analyses were conducted by comparing the raw data of all individuals of each transgenic line with those of the wild-type line using Student's *t* test with unequal variances. Differences between the transgenic lines and wild-type lines were then compared and significance levels were determined at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$ unless otherwise stated. Error bar denotes SEM.

Results

AtSOB3 regulates hypocotyl elongation and seed size in *Arabidopsis*

Previous studies have shown that *AtSOB3* controls hypocotyl elongation and that the *Atsob3-6* dominant-negative allele confers a dramatic long-hypocotyl phenotype (Street et al. 2008; Zhao et al. 2013) (Fig. 1). To examine the role of *AtSOB3* in seed development, we analyzed the size and weight of *Atsob3-D,* Col-0 and *Atsob3-6-OX* seeds. Our results

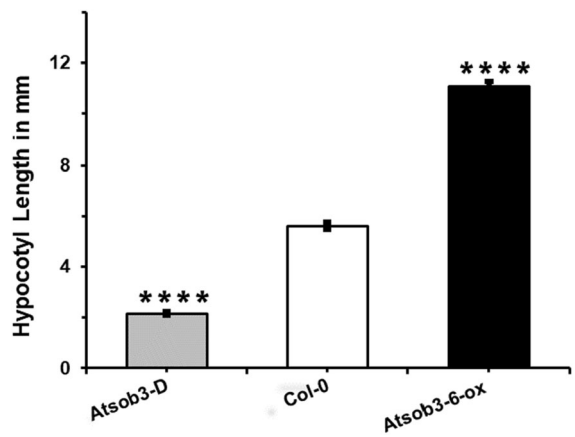

Fig. 1 *AtSOB3* regulates hypocotyl elongation in *Arabidopsis*. *Atsob3-D* suppressed hypocotyl elongation and *Atsob3-6-OX* promoted hypocotyl elongation when compared to the wild type (Col-0). Six-day-old seedlings for all three genotypes were grown under continuous white light (22 µmol m^{-2} s^{-1}) at 25 °C. $n = 60$, $****p < 0.0001$

have demonstrated that *Atsob3-D* repressed seed weight but did not change seed area compared to the wild type (Fig. 2). In contrast, *Atsob3-6-OX* confered larger (Fig. 2a and Fig S1) and heavier (Fig. 2b) seeds than the wild type. The results also demonstrated that the *Atsob3-D* seeds were more circular with a lower length-to-width ratio when compared to the wild type and *Atsob3-6-OX* (Table S2).

The *Atsob3-6-OX* controls hypocotyl cell elongation in *Arabidopsis*

In order to investigate whether the long-hypocotyl phenotype of *Atsob3-6-OX* is due to cell division or cell elongation, we performed microscopic analysis of epidermal cells of six-day-old Col-0 and *Atsob3-6-OX* seedlings. In six-day-old *Atsob3-6-OX* seedlings, the individual cell count and measurement became impractical because of hypocotyl twisting during growth (Fig S2). Therefore, we examined four-day-old seedlings. In four-day-old seedlings there were no significant differences in cell number in the longitudinal section of hypocotyl between both genotypes. However, epidermal cells of *Atsob3-6-OX* were longer than those of the wild type (Fig. 3).

The *Atsob3-6* allele controls hypocotyl elongation, seed size and seed weight when overexpressed in *Camelina*

To test the hypothesis that *Atsob3-6-OX* can increase seed size in *Camelina,* a brassica crop which is closely related to *Arabidopsis* (Kagale et al. 2014), we transformed *Atsob3-6* into *Camelina* and compared stable homozygous transgenic seeds and seedlings of *Atsob3-6-OX* with those of the wild type. The results demonstrated that the transgenic lines *Atsob3-6-OX-1* and *Atsob3-6-OX-2* possessed longer hypocotyls when planted on the surface of the soil (Fig. 4a) and larger (Fig. 4b and Fig S3) and heavier seeds (Fig. 4c) than the wild type. The results also showed that the number of seeds per pod was also increased in *Atsob3-6-OX* transgenic *Camelina* (Table S3). Semi-quantitative RT-PCR results demonstrated transcript accumulation of *Atsob3-6* in transgenic *Camelina*, but not in the wild type (Fig S4).

Larger *Camelina* seeds of *Atsob3-6-OX* conferred better emergence than those of the wild type

In order to evaluate whether the larger *Camelina Atsob3-6-OX* seeds improve seedling emergence from subsurface planting, we first planted *Atsob3-6-OX-1*, *Atsob3-6-OX-2* and wild-type seeds in moist, compacted sunshine mix #1, a standard greenhouse soil blend. We observed that 24.33% of the *Atsob3-6-OX-2* and 22.33% of *Atsob3-6-OX-1* transgenic seedlings and 2.22% of the wild type emerged through 6 cm of lightly compacted moist potting mix (Fig. 5a and Table 1). In these experiments, all seeds from all genotypes germinated. Though we do not know if germination rates were altered, hypocotyl measurements of all germinated seedlings (emerged as well as not-emerged) demonstrated that *Atsob3-6-OX*

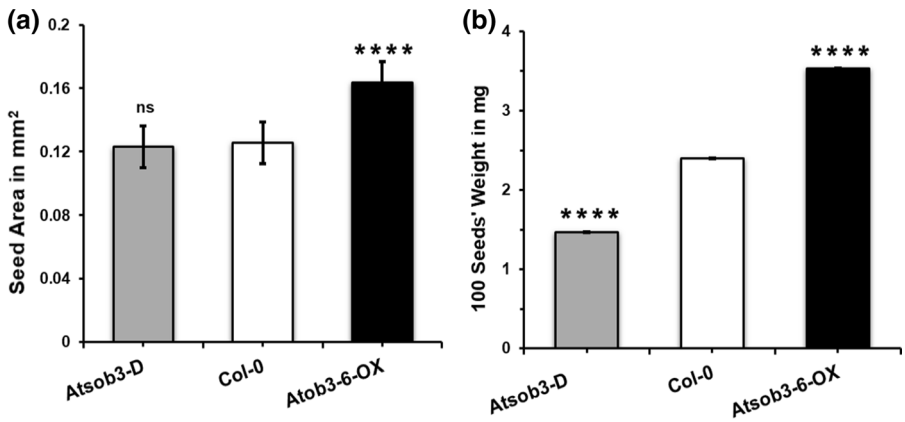

Fig. 2 *AtSOB3* regulates seed size and seed weight in *Arabidopsis*. Seed size of Col-0, *Atsob3-D*, and *Atsob3-6-OX*, was analyzed using Smart Grain software. *Atsob3-D* reduced seed weight but did not change seed area (a), whereas *Atsob3-6-OX* increased seed weight and seed area (b). $n = 110$ for area and 300 for weight, ****$p < 0.0001$

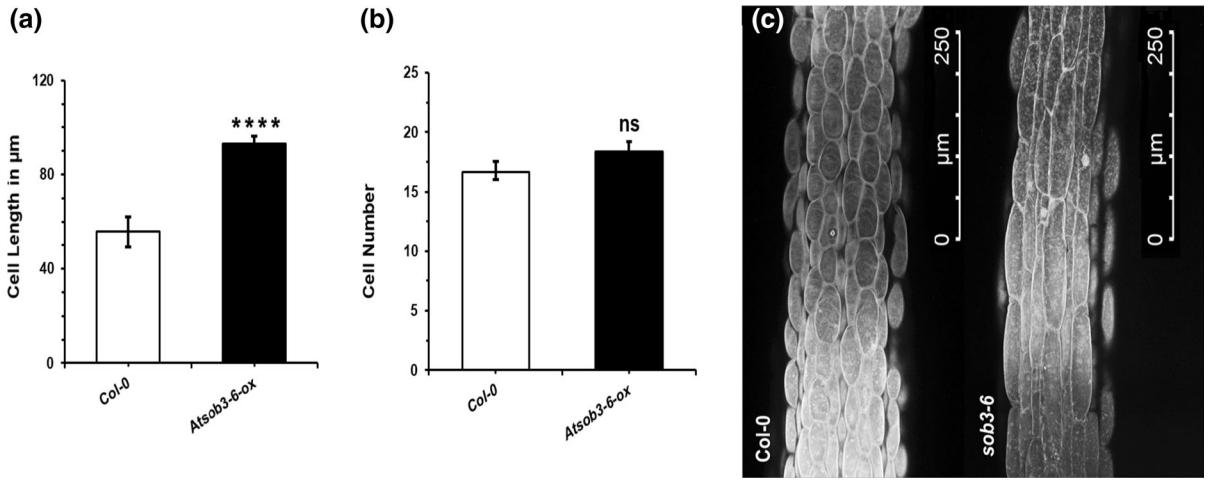

Fig. 3 *Atsob3-6-OX* controls cell elongation in *Arabidopsis* hypocotyls. *Atsob3-6-OX* increased cell length (**a**) but did not increase cell number length (**b**) in epidermal cells of four-day-old hypocotyls. Microscopic images show short and long cells of Col-0 and *Atsob3-6-OX* respectively (c). $^{ns}p > 05$, $^{****}p < 0.0001$

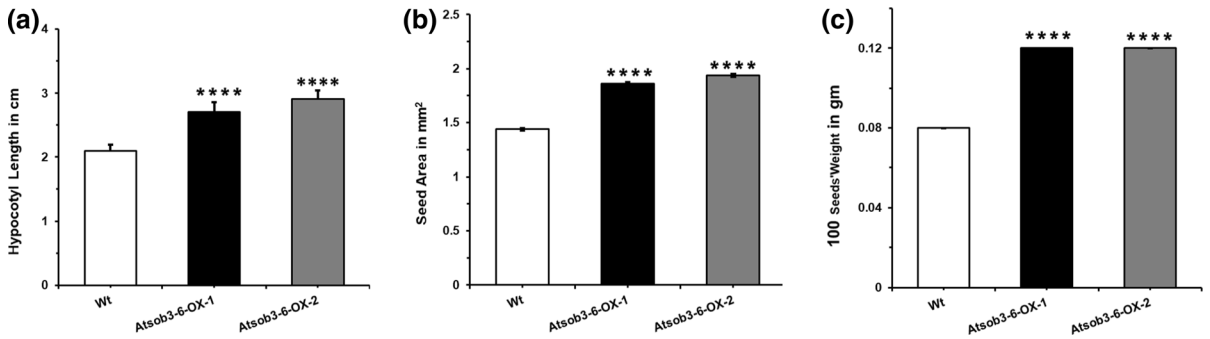

Fig. 4 The *Atsob3-6* allele regulates hypocotyl length, seed size and seed weight when overexpressed in *Camelina*. Two independent *Atsob3-6-OX* transgenic *Camelina* lines displayed increased hypocotyl length (**a**), seed size (**b**) and seed weight (**c**) when compared to the wild type (Wt). $n = 60$ for hypocotyl length. $n = 100$ for seed area. $n = 300$ for seed weight, $^{****}p < 0.0001$

conferred longer hypocotyls in subsurface planting (Fig. 5b). We also tested *Camelina* seedling emergence from subsurface planting in dry Palouse silt loam with 30% of *Atsob3-6-OX* transgenic seedlings and 0% of wild-type seedlings emerging (Fig. 5c and Table S4).

Discussion

AtSOB3 regulates hypocotyl elongation in *Arabidopsis*

Our results confirm that overexpression of the dominant-negative allele, *Atsob3-6-OX*, doubles the hypocotyl length in *Arabidopsis* when compared to the wild type whereas overexpression of *Atsob3-D* confers a short hypocotyl. These results are consistent with hypocotyl analyses previously published in Street et al. (2008) and Zhao et al. (2013a, b proposed that AHLs interact with other family members, as well as other non-AHL DNA-binding proteins, to control hypocotyl growth. This model is based on the observations that the mutant protein encoded by *Atsob3-6* can no longer bind DNA but is still capable of interacting with other proteins suggesting that AtSOB3-6 titrates away the activity of negative regulators of hypocotyl elongation (Zhao et al. 2013). In this study, we demonstrate that the long-hypocotyl phenotype of *Atsob3-6* is due to cell

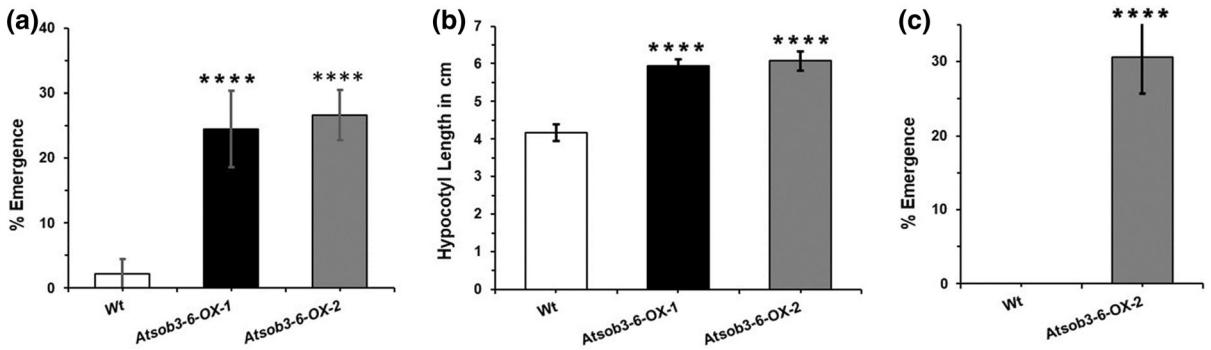

Fig. 5 *Atsob3-6-OX* confers better seedling emergence in *Camelina*. Seeds of two independent *Atsob3-6-OX* lines and the wild type were germinated beneath 6 cm of lightly compacted potting mix at 25 °C for 7 days before measuring percent emergence (**a**) and total hypocotyl length within and above the soil (**b**). Seedling emergence of *Atsob3-6-OX-2* and wild-type seedlings was also measured 7 days after planting beneath 6 cm of dry Palouse silt loam (c). $n = 36$, ****$p < 0.0001$

Table 1 Transgenic *Camelina* seedling emergence assay in compacted potting mix. Seeds of two independent *Atsob3-6-OX* lines and wild type were planted beneath 6 cm of lightly compacted potting mix at 25 °C for seven days before scoring percent seedling emergence. ***$p < 0.001$

Genotype	Wt.	*Atsob3-6-OX-1*	*Atsob3-6-OX-2*
No of seed planted	45	45	45
No of emerged seedlings	1	10	11
% emergence	2.2	***22.2	***24.4

elongation rather than the cell division. This is consistent with the previously reported result of Gendreau et al. (1997), which demonstrates that hypocotyl elongation in *Arabidopsis* is mainly due to cell elongation rather than cell division.

AtSOB3 regulates seed size in *Arabidopsis*

Favero et al. (2016) demonstrated that the *Atsob3-6-OX* confers tall seedling hypocotyls without any undesirable adult phenotypes. In addition, the regulatory role of *AtSOB3* on overall plant development in *Arabidopsis* involves both auxin and brassinosteroid hormone signaling pathways (Favero et al. 2017, 2016; Peng et al. 2015). Auxin and brassinosteroid signaling pathways have been known to play important roles in seed and seedling development (Jiang et al. 2013; Li and Li 2016; Sun et al. 2017). Based on these results, we anticipated that *AtSOB3* may play a role in seed development. To test this hypothesis, we compared the seed size of wild-type *Arabidopsis*, *Atsob3-D*, and *Atsob3-6-OX*. *Atsob3-D* seeds confer a similar surface area with seeds that weigh less than the wild type. In contrast, *Atsob3-6-OX* seeds confer relatively larger surface area with heavier seeds than the wild type. *Atsob3-D* also changes the length-to-width ratio of seeds making them more circular. Together, our data demonstrate that *AtSOB3* controls seed shape, seed size, and seed weight.

Transgenic *Atsob3-6-OX Camelina* displays increased hypocotyl elongation and seed size

Earlier studies have shown that *AHL* genes are present in almost all land plant species ranging from mosses (*Physcomitrella patens*) to higher plants (Rensing et al. 2008; Zhao et al. 2014). Previous studies have also shown that *AHL* genes are highly conserved between *Arabidopsis* and brassica crops (Lagercrantz 1998; Lagercrantz and Lydiate 1996; Osborn et al. 1997; Zou et al. 2014). *Camelina* and *Arabidopsis* are closely related brassica species (Kagale et al. 2014). Thus, we hypothesized that *Atsob3-6-OX* increases hypocotyl elongation and seed size in *Camelina*. Our

data demonstrate that transgenic *Camelina* seeds expressing *Atsob3-6-OX* are 28% bigger and 50% heavier with 50% longer hypocotyls than the wild type. These results suggest that *SOB3-like AHLs* may behave similarly in both *Arabidopsis* and *Camelina* with respect to seed and seedling development.

Overexpression of *Atsob3-6* (*Atsob3-6-OX*) in *Camelina* improves seedling emergence

In this study we demonstrate that the *AHL* gene family regulates seed size and seedling emergence in *Arabidopsis* and *Camelina*. Overexpression of *Atsob3-6* variants increases seed size, seed weight and hypocotyl elongation in both species. Furthermore, *Atsob3-6-OX* in *Camelina* improves seedling emergence in deep planting assays when compared to the wild type. Based on these data, transgenic *Camelina* overexpressing *Atsob3-6* could be particularly helpful for improving yield and stand establishments in dryland cropping areas, by allowing seeds planted deeper in the soil greater access to available moisture while providing the energy for hypocotyl elongation to access the sunlight necessary for photosynthesis, growth and development.

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