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Original Paper

Genetic disruption of *CRC* 12S globulin increases seed oil content and seed yield in *Arabidopsis thaliana*

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Abstract Oils and proteins represent two major carbon reserves within oil seeds. Attempts to increase seed oil production through the genetic suppression of seed protein, however, have generally been unsuccessful. In those experiments, the total protein content remains stable because of compensation by storage proteins from different gene families. *Arabidopsis thaliana* may offer a solution to this problem, as only a small number of 12S-globulin and 2S-albumin proteins, which are major seed storage proteins, are found in these seeds. We obtained T-DNA-tagged mutants for the major 12S-globulin genes, *CRA1*, *CRB*, and *CRC*, and found elevated levels of oil in *crc* seeds. This was associated with the transcriptional upregulation of genes encoding the LEC2 and WRI1 transcription factors, diacylglycerol acyltransferase (DGAT1), and plastidial pyruvate kinase β subunit 1 (PKp-β1), all of which are important for oil production. Furthermore, *cra1*, *crb*, and *crc* single-mutant plants developed substantially more branches, thereby producing more flowers and fruits than did wild-type plants. Thus, *cra1*, *crb* and *crc* mutations resulted in 19%, 22% and 41% increases in seeds and 24%, 25% and 62% increases in oil content per plant, respectively, as compared with wild-type plants. Our results suggest that the perturbation of storage-protein synthesis in developing seeds of Arabidopsis influences the capacity of whole plants for producing sink organs such as shoot branches, flowers and seeds.

Key words: Arabidopsis, 12S globulin, seed yield, shoot branching, triacylglycerol.

Proteins, starches, and oils (triacylglycerol; TAG) are three major carbon reserves within seeds. In oil seeds, starches accumulate during the earliest stage of seed development, whereas proteins and oils accumulate during middle and late stages (Ruuska et al. 2002). Pathways involved in protein or oil synthesis may compete for translocated carbons, as protein levels negatively correlate with oil content in oil seeds (Grami et al. 1977; Kennedy et al. 2011). One strategy to increase seed oil content, therefore, is to genetically suppress protein levels. Kohno-Murase et al. (1994, 1995) demonstrated that levels of cruciferin (a 12S globulin) or napin (a 2S albumin) can be suppressed in oilseed rape (*Brassica nupus* L.) using antisense RNA technology. When cruciferin levels are reduced, however, napin levels are elevated, and vice versa. Similarly, knock down of a major seed storage protein in soybean (*Glycine max*) or rice (*Oryza sativa*) promotes the synthesis of other storage proteins (Kawakatsu et al. 2010; Schmidt et al. 2011). Because of these compensatory responses, a plant expressing a small number of seed storage proteins is required to effectively evaluate negative correlations between protein and oil content in seeds.

In Arabidopsis (the Columbia strain), major seed storage proteins are composed of 12S globulin and 2S albumin, which constitute ~87% and 10% of total seed protein, respectively (Higashi et al. 2006). The 12S globulins are encoded by *Cruciferin A1* (*CRA1*, At5g44120), *Cruciferin B* (*CRB*, At1g03880), *Cruciferin C* (*CRC*, At4g28520), and *Cruciferin 2* (*CRU2*, At1g03890), whereas the 2S albumins are encoded by five genes named *At2S1* to *At2S5* (Fujiwara et al. 2002; Gruis et al. 2002). CRA1 and CRC each account for >35% of total seed protein, whereas CRB and At2S3 account for 12% and 8% of total seed protein, respectively. In contrast, CRU2 and the other 2S-albumin isoforms are expressed at very low levels under normal growth conditions (Higashi et al. 2006). To evaluate the correlation between protein and oil content in Arabidopsis seeds, therefore, the major 12S-globulin genes *CRA1*, *CRB* and *CRC* represent good targets for disruption.

We isolated T-DNA-tagged lines for *CRA1*, *CRB*,

Abbreviations: DAF, days after flowering; DGAT1, diacylglycerol acyltransferase 1; RT-PCR, reverse transcription-polymerase chain reaction; TAG, triacylglycerol; PKp-β1, plastidial pyruvate kinase β subunit 1

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and *CRC* in Arabidopsis and measured protein and oil content within mutant and wild-type seeds. The disruption of a single 12S-globulin gene was compensated for by the upregulation of other major 12S-globulin genes and *At2S1*. However, we found significantly elevated levels of TAG in *crc* seeds with concomitant upregulation of key genes involved in TAG production. Interestingly, branching was elevated in each of the 12S-globulin mutants, thereby increasing the number of both flowers and fruits. Thus, disruption of a 12S-globulin gene results in increased seed numbers and seed oil yields per plant as compared with wild type. We discuss how the suppression of 12S-globulin synthesis within developing seeds may affect the capacity of whole plants for producing sink organs such as shoot branches, flowers and seeds.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (Columbia strain) seeds were sown on 100 g of peat moss (Golden Peatban; Sakata Seed Corporation, Yokohama, Japan), which had been cracked and placed into a plastic pot (8 cm long, 8 cm wide, and 6.5 cm deep). Plants were grown at 22°C under a 16-h day/8-h night photoperiod. The photon flux density was ~150 μmol m^{-2} s^{-1}. T-DNA-tagged lines for *cra1* (Salk_002668), *crb* (Salk_045987), and *crc* (GK-283D09) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). Homozygous mutants were identified by PCR using gene-specific (Supplemental Table S1) and T-DNA-specific (5′-TGGTTCACGTAGTGGGCCATCG-3′ and 5′-ATATTGACCATCATACTCATTGC-3′ for SALK LBa1 and GABI o8409, respectively) primers.

Seed productivity measurements

Seed productivity was measured using 5- and 6-week-old plants. These measurements included the number of stems that developed from a rosette base, the number of axillary branches that developed from the stems, and the number of opened flowers and fruits. Under growth conditions described above, flowering was essentially finished after 6 weeks. All seeds were harvested using a handmade plastic apparatus, which resembles the Arasystem (Lehle Seeds, Texas, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Developing seeds were collected from fruits 8–10 or 12–14 d after flowering (DAF). Total RNA was extracted from each sample using the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan), and cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Tokyo, Japan). RT-PCR was performed on a PTC-100 thermal cycler (Bio-Rad, Tokyo, Japan) using Ex-taq polymerase (Takara Bio Inc., Shiga, Japan), gene-specific primers (Supplemental Table S1) and the following number of reaction cycles: 14 for *CRA1*, *CRB*, *CRC*, and *2S1*; 24 for *FUS3*; 30 for *LEC2*; 24 for *WRI1*; 23 for *DGAT1*; 21 for *Pkp-β1*; and 26 for *Ubiquitin10* (*UBI10*). Each cycle consisted of 94°C for 30 s, 56°C for 30 s and 72°C for 45 s. PCR bands were stained with 1 mg l^{-1} ethidium bromide, and the intensity of each band was quantified using ImageJ v. 1.46 (http://rsbweb.nih.gov/ij/) and normalized to *UBI10*.

SDS-PAGE analysis

Fifty dry seeds were frozen in liquid nitrogen in a 1.5-ml plastic tube and ground into powder using a Kontes pellet pestle motor (VWR International, Illinois, USA). Total protein was extracted using 100 μl SDS buffer containing 100 mM Tris-HCl, pH 8.0; 0.5% SDS; 10% glycerol; and 2% 2-mercaptoethanol. After boiling for 3 min, the sample was centrifuged at 15,000 rpm for 3 min (MRX-150, TOMY, Tokyo, Japan), and the supernatant was collected. Protein content was determined using a Bio-Rad protein assay reagent (Bio-Rad, Tokyo, Japan) with γ-globulin as the standard.

The equivalent of a two-seed aliquot was loaded into each lane and separated by SDS-PAGE using a 12.5% gel. Protein bands were stained using Coomassie Brilliant Blue R-250, and the intensity of each polypeptide band was quantified using ImageJ. The identity of each band was confirmed by MALDI-TOF-MS (Autoflex III, Bruker Daltonics, Yokohama, Japan).

Determining seed-oil content

Total lipids were extracted from seeds using the method from Folch et al. (1957), with modifications. Briefly, 500 seeds were immersed in 1 ml of boiling 2-propanol for 5 min. After the seeds were chilled on ice, chloroform was added (2 ml) and seeds were homogenized using an NS-51 homogenizer equipped with an NS 7 blade (Microtec Co., Ltd., Chiba, Japan). After centrifugation, the supernatant was recovered and the pellet was re-extracted using 3 ml of a mixture of chloroform and methanol (2 : 1, v/v). After a second centrifugation, the supernatant was recovered and combined with the original supernatant. Then, 1.2 ml of 0.9% KCl was added, and the sample was mixed vigorously. After centrifugation, the lower layer was recovered and evaporated using a rotary evaporator (Eyela, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Lipid residues were weighed and dissolved in chloroform to a concentration of 10 mg ml^{-1}. Lipid extracts (1 mg) were separated by silica gel thin-layer chromatography using a silica gel 60 glass plate that was 20×20 cm and 0.25 mm thick (Merck, Darmstadt, Germany) and a mixed solvent that contained hexane, diethyl ether, and acetic acid (80 : 30 : 1, v/v/v). TAG was identified under UV_{365} illumination after spraying with 0.01% primuline in 80% acetone. TAG content was quantified using gas chromatography (GC-18A, Shimadzu, Kyoto, Japan) as described (Inatsugi et al. 2002).

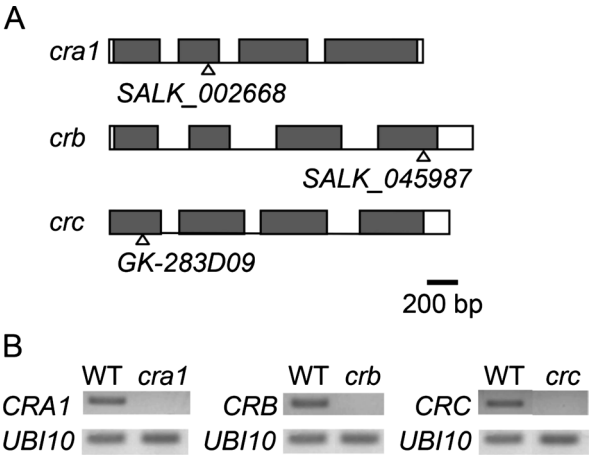

Figure 1. Isolation of T-DNA-tagged lines for 12S-globulin genes in Arabidopsis. (A) The structures of Arabidopsis *CRA1*, *CRB*, and *CRC* genes are shown. Triangles indicate the T-DNA insertion sites for the *cra1* (*SALK_002668*), *crb* (*SALK_045987*), and *crc* (*GK-283D09*) mutants. Gray boxes represent exons, white boxes represent untranslated regions, and lines represent introns. (B) RT-PCR analysis of 12S-globulin genes in developing seeds at 12–14 DAF.

Results

Isolation of Arabidopsis 12S-globulin mutants

To suppress 12S-globulin levels, we obtained and purified Arabidopsis T-DNA-tagged mutants for *CRA1*, *CRB*, and *CRC* (Figure 1A). RT-PCR analysis showed that each mutant accumulated no intact mRNA for the corresponding gene in developing seeds (Figure 1B). When mutant seeds were harvested after 6 weeks of plant growth, they were not different from wild type with respect to size, shape, and weight (data not shown).

Polypeptide profiles in wild-type and mutant 12S-globulin seeds

In wild-type seeds, the CRA1, CRB, and CRC polypeptides are processed into α (29.2, 27.4, and 34.7 kDa, respectively) and β (20.9, 20.8, and 21.2 kDa, respectively) subunits (Higashi et al. 2006). When subjected to SDS-PAGE analysis, α subunits were detected at their expected relative masses, whereas β subunits were detected as a single band (Figure 2A). CRU2 polypeptides were not detected in the wild-type protein extracts by our MALDI-TOF-MS analysis. The α-subunit band for CRA1, CRB, and CRC was not detected in protein extracts from *cra1*, *crb*, and *crc* seeds, respectively. As such, each mutant was null with respect to the gene product. Figure 2B shows the relative polypeptide abundance for each lane of Figure 2A. Compared with wild-type seeds, the amount of CRC α subunit was unchanged in *cra1* seeds; the amount of CRA1 α subunit was elevated in *crc* seeds; and the amount of 2S albumin (large subunit) was elevated in *crb* and *crc* seeds. This demonstrates that the deletion of

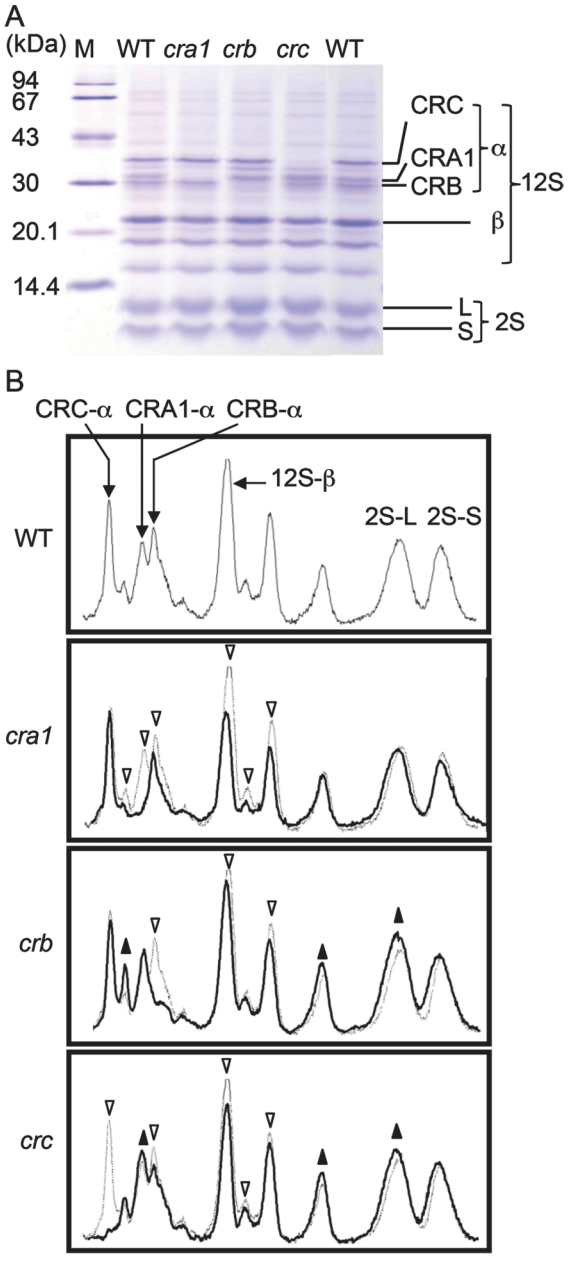

Figure 2. SDS-PAGE analysis of total seed protein in 12S-globulin mutants. (A) Each lane contains SDS-soluble seed protein that is equivalent to two dry seeds. The α-subunit bands for CRA1, CRB, and CRC are indicated with arrows. The gel was stained with Coomassie Brilliant Blue. (B) Relative levels of SDS-soluble polypeptides in mutant 12S-globulin seeds. Each trace was made from a single lane shown in (A) using ImageJ software. Each mutant-seed trace is overlaid with the wild-type trace (gray line). Closed and open triangles indicate protein bands that are increased and decreased, respectively, in mutant extracts.

some 12S-globulin genes was partially compensated for by the upregulation of other 12S-globulin proteins or 2S albumins. The total amount of SDS-soluble protein was, however, also reduced in both *cra1* and *crc* seeds as compared with wild type (Figure 3).

TAG content is elevated in crc seeds

TAG was extracted and measured for both wild-type

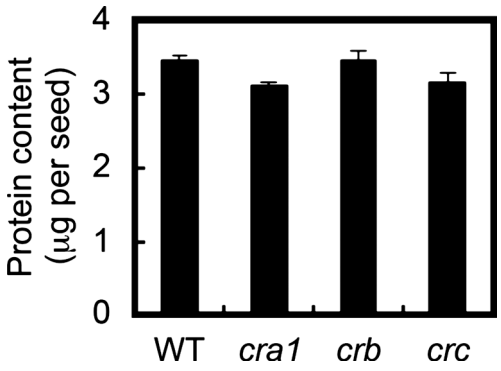

Figure 3. Protein content in wild-type and mutant 12S-globulin seeds. SDS-soluble proteins were extracted from 50 seeds (mean±S.E., n=3 plants).

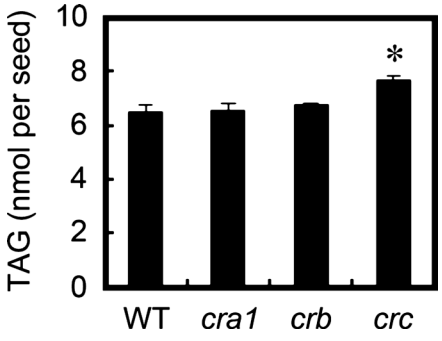

Figure 4. Oil content in wild-type and mutant 12S-globulin seeds (mean±S.E., n=3 plants). An asterisks indicates a significant difference from wild type using Tukey's multiple comparison test (p<0.05).

and mutant seeds. TAG levels were significantly higher in *crc* seeds than in wild-type seeds, whereas TAG levels were not affected in *cra1* seeds and tended to be higher (although not significantly) in *crb* seeds (Figure 4). The composition of major fatty acids was similar between wild-type and mutant seeds (data not shown), demonstrating that TAG synthesis, but not the synthesis/desaturation of specific fatty acids, was enhanced in *crc* seeds.

TAG and seed yield are increased in 12S-globulin mutants

Seed germination was not affected in 12S-globulin mutants. In addition, wild-type and mutant seedlings were indistinguishable in terms of growth, bolting, and flower production (data not shown). After 5 weeks of growth, however, each line of 12S-globulin mutants had notably more stems and shoot branches than wild type (Figures 5A, B), and *crb* and *crc* single-mutants produced 45.7% and 47.0% more flowers/fruits than wild type, respectively (Figure 5C). By 6 weeks of growth, *cra1*, *crb*, and *crc* mutants produced 26.8%, 14.3%, and 21.4% more stems than wild type, respectively (Figure 5D). In addition, all 12S-globulin mutants developed more shoot branches, in particular the *crc* mutants (28%; Figure 5E).

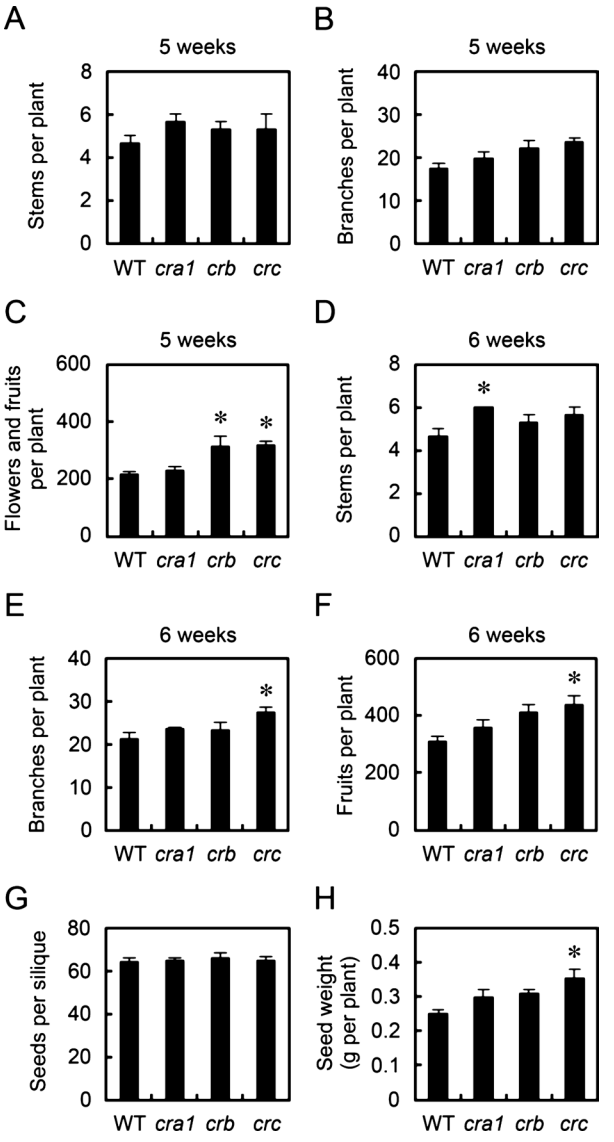

Figure 5. Whole-plant morphological features and seed yield in both wild-type and mutant 12S-globulin plants (mean±S.E., n=3 plants). (A–C) The number of stems (A), shoot branches (B), and flowers and fruits (C) for 5-week-old plants are indicated. (D–F) The number of stems (D), shoot branches (E), and fruits (F) for 6-week-old plants are indicated. (G) The number of seeds per fruit (mean±S.E., n=10 fruits) (H), and the total seed yield (in grams) per plant are indicated. Asterisks indicate a significant difference from wild type using Tukey's multiple comparison test (p<0.05).

Eventually each 12S-globulin mutant produced more flowers and fruits than wild-type plants (Figure 5F), but the average number of seeds per fruit was not affected (Figure 5G). Thus, 12S-globulin mutants produced more seeds than wild type; in particular, this was true for the *crc* mutants (40.5% more; Figure 5H). TAG yield per plant also increased by 24.1%, 24.5%, and 62.3% in *cra1*, *crb*, and *crc* mutants, respectively, as compared with wild type (Figure 6).

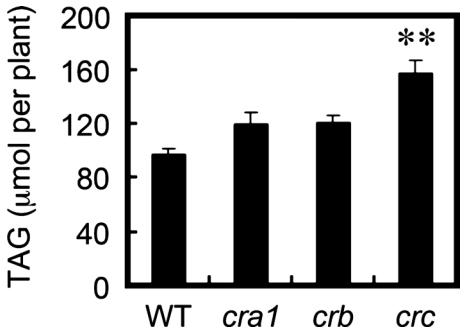

Figure 6. TAG yield for wild-type and mutant 12S-globulin plants. The total TAG yield (mean±S.E., $n=3$ plants) represents the average TAG content per seed (Figure 4) multiplied by the seed yield per plant (Figure 5H) divided by the seed weight (data not shown). Double asterisks indicate a significant difference from wild type using Tukey's multiple comparison test ($p<0.01$).

Disruption of 12S-globulin genes affects the transcript levels of genes related to seed metabolism

We performed RT-PCR analysis of genes related to seed metabolism in wild-type and mutant 12S-globulin seeds. Disruption of *CRA1* upregulated the levels of *CRC* transcripts in developing seeds, and vice versa (Figure 7A). In contrast, *CRB* disruption did not affect levels of *CRA1* or *CRC* transcripts. Finally, transcript levels for *At2S1* were markedly elevated in all mutant 12S-globulin seeds as compared with wild type. These results demonstrate that under these growth conditions the elimination of some 12S-globulin genes is compensated for by the upregulation of other 12S-globulin or 2S-albumin genes.

FUS3 encodes a transcription factor that regulates the expression of 12S-globulin and 2S-albumin genes (Kagaya et al. 2005; Kroj et al. 2003). At early stages of seed development (8–10 DAF), *FUS3* transcripts were upregulated in all mutant 12S-globulin seeds as compared with wild type. At later stages of development (12–14 DAF), however, only *crc* seeds maintained substantially higher levels of *FUS3* transcripts than wild-type seeds. At 12–14 DAF the levels of *CRA1*, *CRC*, and *At2S1* transcripts were highest in *crc*, *cra1*, and *crc* seeds, respectively.

LEC2 and WRI1 are transcription factors that regulate fatty-acid and TAG synthesis (Kroj et al. 2003; Maeo et al. 2009). At 8–10 DAF, levels of *LEC2* and *WRI1* transcripts were upregulated in all mutant 12S-globulin seeds as compared with wild type (Figure 7B). At 12–14 DAF, however, elevated levels of *WRI1* transcripts were maintained only in *crc* seeds.

Transcripts for *DGAT1* and *PKp-β1* were elevated in all mutant 12S-globulin seeds as compared with wild type (8–10 DAF). At 12–14 DAF, however, elevated levels of *DGAT1* transcripts were maintained only in *cra1* and *crc* seeds. Levels of *PKp-β1* transcripts had decreased in all mutants by 12–14 DAF and were lower than those in wild type in *cra1* and *crb* mutants. These results are consistent with data concerning *LEC2* and *WRI1* that were presented above.

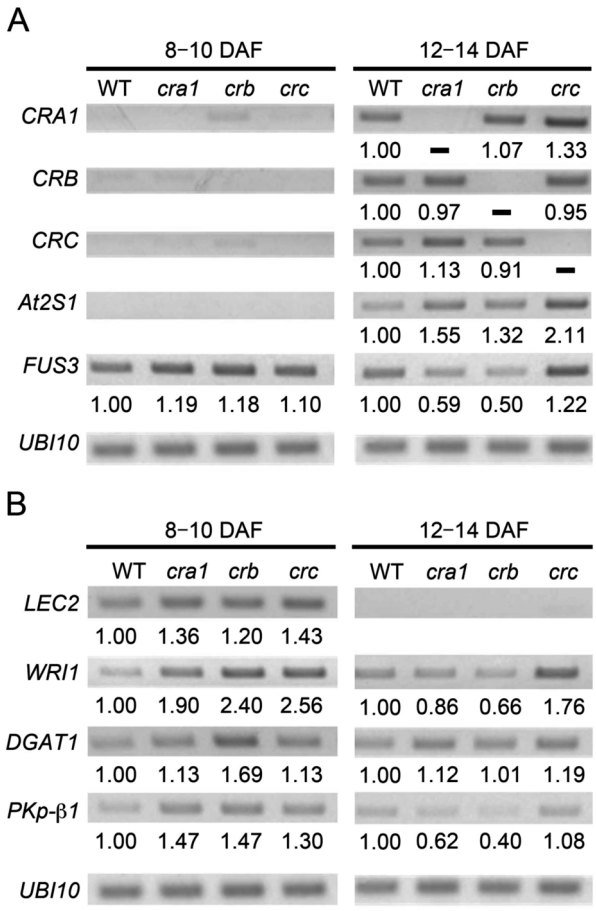

Figure 7. Transcript levels for genes related to seed metabolism in wild-type and mutant 12S-globulin seeds. Transcript levels for genes related to the synthesis of seed-storage proteins (A) and TAG (B) were examined by RT-PCR. RNA was extracted from developing seeds at 8–10 and 12–14 DAF.

Discussion

We have analyzed Arabidopsis mutants for the 12S-globulin genes *cra1*, *crb*, and *crc*. Disruption of *crb* increased levels of 2S albumins, whereas *crc* disruption increased levels of CRA1 and 2S albumins. These protein-level results were supported by RT-PCR analysis, which showed that *CRA1* transcripts were elevated in *crc* seeds and that *At2S1* transcripts were elevated in *cra1*, *crb*, and *crc* seeds. In *cra1* seeds, *CRC* transcripts were substantially elevated, but CRC protein levels were not increased. There is a clear difference, therefore, between *cra1* and *crc* mutants.

Correlations between total protein and TAG levels were generally inconsistent between mutant lines. TAG levels were elevated most dramatically in *crc* seeds, which

is consistent with our finding that *crc* seeds contained the highest levels of both *LEC2* and *WRI1* transcripts at 8–10 and 12–14 DAF. TAG levels were not statistically elevated in *cra1* or *crb* seeds, however, despite the upregulation of *LEC2* and *WRI1* transcript levels at 8–10 DAF. Our data demonstrate that the disruption of some 12S-globulin genes affects the transcription of a wide range of genes involved in seed metabolism, including genes that encode transcription factors (FUS3, LEC2 and WRI1), seed storage proteins (2S albumins), and enzymes involved in fatty-acid and TAG biosynthesis (PKp-β1 and DGAT1). Li et al. (2007) reported that the levels of *CRC* transcripts increase earlier than those of *CRA1* and *CRB* transcripts during seed development. Thus, disruption of *CRC* could have profound effects on *LEC2* and *WRI1* transcription during seed development compared to that of *CRA1* or *CRB*. To further understand the effects of 12S-globulin gene disruption on TAG levels in Arabidopsis seeds, we are constructing *cra1 crb crc* triple mutants.

We found that 12S-globulin mutants produce more flowers than wild type because of enhanced shoot branching. As the growth of both seedlings and plants before flowering was indistinguishable among all tested plants, we speculate that parental plants may monitor in some way the metabolism of developing seeds, and that metabolic alterations within seeds affect the development of sink organs such as shoot branches, flowers and seeds. In Arabidopsis, T-DNA-tagged lines for amino acid permease 2 (AAP2), which is involved in the translocation of amino acids from source to sink tissues and the phloem loading of amino acids (Zhang et al. 2010), exhibit reduced levels of seed storage proteins and increased levels of TAG. In *aap2* mutants, the total seed yield is dramatically increased because of the concomitant increases in the numbers of shoot branches and fruits (Zhang et al. 2010). These phenotypes are similar to those observed for all 12S-globulin mutants. Thus, the inhibition of 12S-globulin synthesis in *cra1*, *crb*, and *crc* mutants may affect the efficiency of amino-acid translocation, thereby upregulating the rate of shoot branching, as is seen with *aap2* mutants. There are a number of factors, however, that can promote shoot branching, including cytokinin (Ongaro and Leyser 2008). Future studies must determine whether cytokinins and other branch-inducing hormones underlie the enhanced shoot branching that characterized 12S-globulin mutants.

In summary, the 12S-globulin mutant *crc* exhibited increased levels of seed oil. The disruption of 12S-globulin genes also increased the capacity of parental plants for producing sink organs such as shoot branches, flowers and seeds. Further characterization of Arabidopsis 12S-globulin mutants including *cra1 crb crc* triple mutants is necessary to uncover the molecular link between the disruption of 12S-globulin genes in seeds and the enhanced shoot branching in whole plants. Determining this link may facilitate increased seed oil production. Disruption of 12S-globulin genes might improve oil production in nonfood oil crops such as *Camelina sativa*, which is a close relative of Arabidopsis. Disruption of 12S-globulin genes in *C. sativa* is going on in our laboratory.

Acknowledgements

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Supplemental Table S1. Primer sequences

Primer	Sequence
CRA1-F	5'-CCACAAAAAGTAAGAAATATAAG-3'
CRA1-R	5'-TTCTGAAGTTGCTGTGCTGT-3'
CRB-F	5'-GCTCTTCGTGGCTCCATC-3'
CRB-R	5'-CAAGTCGTACCAAACCGAAC-3'
CRC-F	5'-GTTCTCTTCATCCATCTCTCAC-3'
CRC-R	5'-ATGTCACGGAACCCTTGTTGTC-3'
2S1-F	5'-ATGGCAAACAAGTTGTTCCTCG-3'
2S1-R	5'-CCTTCATTCCCTTCTTTCTACTAA-3'
FUS3-F	5'-CACTGAAACCCAAAGAGATCC-3'
FUS3-R	5'-GGAATCCCTTCCTTGCATTC-3'
LEC2-F	5'-GTATGTCCTCGAGAACACAG-3'
LEC2-R	5'-CTTGAGAACTTCCACCAC-3'
WRI1-F	5'-CTGCTTCCTCTTCCTCTGAG-3'
WRI1-R	5'-CAGTACTTGAGAGCAGCCAG-3'
DGAT1-F	5'-ATGGCGATTTTGGATTCTG-3'
DGAT1-R	5'-ACCGACGGTCGATACGTAAACG-3'
PKp-β1-F	5'-CTCAGACATTGCTATCGCTG-3'
PKp-β1-R	5'-GCCAGATTGGCTGTGTAC-3'
UBI10-F	5'-GCCAAGATCCAAGACAAAGA-3'
UBI10-R	5'-TTACGAGCAAGCATCATCAA-3'