Expression of the genes coding for plastidic acetyl-CoA carboxylase subunits is regulated by a location-sensitive transcription factor binding site

Expression of the genes coding for plastidic acetyl-CoA carboxylase subunits is regulated by a location-sensitive transcription factor binding site

Natsumi Fukuda, Yuki Ikawa, Toshihiro Aoyagi and Akiko Kozaki*#

Department of Biology, Shizuoka University, 836 Ohya Suruga-ku Shizuoka, Japan, 422-8529

#to whom correspondence should be addressed:

phone: +81-54-238-4957

FAX: +81-54-238-4957

E-mail: sakozak@ipc.shzuoka.ac.jp

Abstract

Plastidic acetyl-CoA carboxylase (ACCase) regulates the rate of fatty acid synthesis. This enzyme is composed of biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyltransferase (CT), which consists of α and β subunits. Among these components, CTβ is encoded by the plastidic genome. In *Arabidopsis*, *BC* and *CTα* are each encoded by a single gene, and there are two genes for BCCP, *BCCP1* and *BCCP2*. Promoter analysis revealed that the 5'-UTR containing the AW box is necessary for the expression of these genes in seeds and seedlings. The results indicated that there are other transcription factors besides WRI1 that bind to the AW box and regulate these genes in organs other than seeds.

Although the AW boxes at 748 and 532 bp upstream from the transcription start sites (TSSs) of the *BC* and *CTα* genes, respectively, were not functional in seeds, the latter was functional in seedlings. In addition, when these AW boxes were moved to approximately 200 bp upstream from the TSS, they became active in seeds but not in seedlings. These results suggest that the distance from the TSS affects the function of the AW box, and the AW box alone is not sufficient for expression in seedlings.

A comparison of the protein levels of BC, BCCP1, BCCP2 and CTβ between a *wri1* mutant, a WRI1-overexpressing line and control plants showed that protein levels of BCCP2 and BC but not BCCP1 and CTβ are affected by WRI1. The results suggest that ACCase subunits are differentially regulated by WRI1.

Keywords

Acetyl-CoA carboxylase (ACCase), WRINKLED1 (WRI1), fatty acid synthesis, Arabidopsis, seed, promoter

Introduction

Acetyl-CoA carboxylase (ACCase) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the first committed step of fatty acid synthesis. De novo fatty acid synthesis is an essential process required for the biogenesis of membrane lipids and storage oils. Plant fatty acids are synthesized mainly in plastids, and the production of malonyl-CoA catalyzed by ACCase is the rate-limiting step for fatty acid biosynthesis (Ohlrogge et al. 1995).

Plants contain ACCase in the cytosol in addition to the plastids, and malonyl-CoA produced by cytosolic ACCase is used for the elongation of fatty acids in the synthesis of very-long-chain fatty acids and the biosynthesis of flavonoids. In most plants, plastidic ACCase and cytosolic ACCase are different forms. Plastid ACCase is a heteromeric enzyme that is composed of three different domains: biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and carboxyltransferase (CT), which is composed of non-identical α and β subunits. On the other hand, cytosolic ACCase is composed of a large multifunctional polypeptide (Sasaki et al. 1993; Sasaki et al. 1995).

Among the subunits of ACCase, only the β-subunit of CT is plastid encoded, and the other subunits are nuclear-encoded polypeptides. In *Arabidopsis*, BC and *CTα* are each encoded by a single gene and BCCP is encoded by two genes, *BCCP1* and *BCCP2.* To meet the requirements of fatty acid synthesis, each subunit of ACCase should be coordinately regulated, and these genes have been demonstrated to be coordinately expressed in the developing embryo in *Arabidopsis* (Ke et al. 2000; Ruuska et al. 2002).

Recently, a transcription factor with an AP2 domain, WRINKLED1 (WRI1), was identified as the master regulator of genes involved in fatty acid synthesis and the glycolytic pathway in *Arabidopsis* seeds. Seeds from a *wri1* mutant contained reduced amounts of storage oils (Focks and Benning 1998; Cernac and Benning 2004). The expression of *BCCP1* and *BCCP2* was reported to be regulated by WRI1 in seeds (Maeo et al. 2009; Baud et al. 2010), but fatty acid synthesis occurs in many young tissues in addition to seeds. WRI1 is expressed exclusively in developing seeds, indicating that the expression of genes for fatty acid synthesis in other tissues than seeds is regulated by other transcription factors.

In this study, we investigated the regulatory region of promoters of genes for ACCase subunits in seeds and seedlings and found that the WRI1 binding sequence is essential for expression in both seeds and seedlings, and that the WRI1 binding site should be close to the transcription start site (TSS) in order to have functional activity. Furthermore, we analyzed the effect of WRI1 on the protein levels of ACCase subunits.

Materials and methods

Plant materials and growth conditions

The seeds of sGsL s of Col-0, 35S:WRI1 and a *wri1*-10 mutant with an insertion of a T-DNA fragment were kind gifts from Dr. Kenzo Nakamura (Maeo et al. 2009).

Seeds of transgenic plants were surface sterilized in 20% bleach for 15 min, rinsed five times with sterile water and then plated on half-strength Murashige and Skoog medium, pH 5.7, 0.8% agar, and 1 % sucrose with kanamycin (30 μg mL^{-1}). Plates were first placed at 4°C for 2 days and then transferred to a growth chamber at 23°C with a light period of 16 h (65 μmol m^{-2} sec^{-1}) and a dark period of 8 h. Seedlings were transferred to soil at 7-10 days after germination if needed.

To collect developmental stage siliques, colored threads were used to tag flowers on the day of flowering (day 0) when petals just appeared. The siliques that developed from the tagged flowers were collected at specific time points.

Construction of binary vector and transformation

Primers used in PCR reactions are described in Table 1.

Promoter deletion series: DNA fragments containing upstream sequences of the *BCCP1*, *BCCP2*, *BC* and *CTα* genes were amplified by PCR using the primer sets described in Table 1. Amplified PCR fragments were sequenced and cloned upstream of the GUS gene in pBI101.

5'-UTR -980 and 5-'UTR -297: The 5'-UTR of *BCCP2* was amplified by PCR using primers BCCP2-UTRF and R. Fragments of 980 or 297 bp from upstream of the TSS were amplified using primers BCCP2p-980F/BCCP2p-TSSR. The 5'-UTR was digested with SalI and XhoI, and the 980 and 297 bp fragments were digested with SalI and BamHI. Both the 5'-UTR and upstream fragments were ligated into the SalI /BamHI sites of pBI101.

CTα229ΔAW532: DNA fragments, from 956 to 545 bp and from 532 to 1 bp upstream of the TSS of CTα gene, were amplified using primers CTα-956F/CTα-545HR and CTα-532F/CTα-TSSR, respectively. Amplified PCR fragments were digested with SalI/HindIII and HindIII/BamHI, respectively, and were ligated into the SalI /BamHI sites of pBI101.

AW-BC203 and AW-CTα229: Primers BC-AW203F and CTα-AW229F containing the AW box were synthesized. A 203 bp of BC fragment was amplified using primers BC-AW203F/BC-TSSR, and a 229 bp of fragment was amplified using primers CTα-AW229F/CTα-TSSR. Amplified fragments were digested as described previously

and ligated into the SalI/BamHI sites of pBI101.

BCCP1+2UTR and *BCCP2*+1UTR: The 5'-UTR of *BCCP2* was amplified by PCR using primers BCCP2-UTRBF/BCCP2-UTRXbR. A 991 bp fragment upstream of the TSS of the *BCCP1* gene was amplified using primers BCCP1p-991F/BCCP1p-TSSR. The 5'-UTR and *BCCP1* fragments were digested with BamHI/XbaI and SalI/BamHI, respectively. Fragments were ligated into the SalI/XbaI sites of pBI101. The 5'-UTR of *BCCP1* was amplified by PCR using primers BCCP1p-UTREF/BCCP1p-TISR. A 980 bp fragment upstream of the TSS of the *BCCP2* gene was amplified using BCCP2p-980F/BCCP2p-TSSER. The 5'-UTR and *BCCP2* fragments were digested with BamHI/EcoRI and SalI/EcoRI, respectively. Fragments were ligated into the SalI/BamHI sites of pBI101.

Transformation: *Arabidopsis thaliana* (ecotype Col-0) was transformed with *Agrobacterium tumefaciens* (strains GV3101) using the floral dip method (Clough and Bent 1998).

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from seeds at 10 days after flowering (DAF) and 10-day-old seedlings from wild-type *Arabidopsis* using TriPure (Roche) and Fruitmate (TAKARA). cDNA was synthesized using 1 μg of RNA as a template and ReverTra Ace (TOYOBO). *S*

emiquantitative RT-PCR was performed (25 cycles) using the primers described in Table 2.

GUS staining assay and GUS fluorometric assay

Histochemical staining of GUS activity was conducted on transgenic plants (a minimum of ten plants was assayed for each independently transformant line). Fresh tissue was incubated in reaction solution (1 mM X-Gluc, 50 mM sodium phosphate buffer pH 7.0, 1 mM K^+ferricyanide/ferrocyanide mixture, 0.01% Triton X-100, 10 mM 2-mercaptoethanol, 20% methanol, 1 mM EDTA) at 37°C for 12 h. After staining, the tissue was fixed in a mixture of ethanol:acetic acid at a 6:1 ratio and rinsed with 70% ethanol. Observations and photographs were made using a dissecting microscope.

GUS activity in protein extracts was measured using the fluorogenic substrate 4-methylumbelliferyl b-D-glucuronide (MUG). Proteins were extracted from seeds at 10 DAF and seedlings from transgenic plants (five to ten independent plants). Total protein extracts were prepared by grinding the tissues in extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol) containing 0.1% (w/v) SDS and 1% Triton X-100, followed by centrifugation at 13,000 g for 10 min. GUS

activity in supernatants was measured in extraction buffer containing 1 mM MUG and 20% methanol. Reactions were stopped with 0.2 M Na_2CO_3 and the amount of 4-methylumbelliferone was calculated by relating relative fluorescence units with those of a standard of known concentration. The protein concentration of extracts was determined using Protein Assay Kits (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)

The recombinant GST-WRI1 fusion protein was expressed in *E. coli* BL21 CodonPlus cells (Agilent Technologies) using a pGEX-4T vector (GE Healthcare) and purified from cells according to the manufacturer's instructions. The purified GST-WRI1 was used for EMSA.

EMSAs were performed as described previously (Kozaki et al. 2004) with slight modifications. For the EMSA, 5'-end biotin-labeled probes, biotin-F and biotin-R were synthesized (Table 2). Oligonucleotides containing the AW box with sequences for primers annealing (biotin-F and -R) at both ends, BC-probe, CTα-probe and BCCP2-probe (Table 2) were synthesized, and dsDNAs were synthesized. Synthesized dsDNAs were amplified by PCR using primers (biotin-F and -R). In the reaction, 100 ng of purified protein GST-WRI1 was incubated for 20 min on ice with 30-50 fmol of the labeled probes in binding buffer (10 mM Tris-Cl, pH 7.5, 75 mM NaCl, 1 mM DTT, 6% glycerol, 0.1% BSA, 0.05% NP40, 50 ng/μL poly (dI-dC)). After incubation, the mixture was loaded on a 5% polyacrylamide gel and run in 0.25× Tris-Borate-EDTA buffer at 4°C. DNA-protein complexes were transferred to Hybond-N+ membrane (GE Healthcare) and detected with Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

Protein extraction and immunological detection

To analyze seed proteins, seeds were harvested at 8-10 DAF and homogenized in liquid nitrogen using a mortar and pestle. The powdered seeds were transferred into PBS buffer and protein was extracted. The protein concentration was determined using a protein assay kit (Bio-Rad). Proteins separated by SDS-PAGE were transferred to a PVDF membrane (Pall). BCCP1 and 2 were detected using horseradish peroxidase (HRP)-conjugated avidin (Sigma-Aldrich). BC and *CTα* subunits were detected using antisera as described in Madoka et al. (2002). These antisera were a gift from Dr. Yukiko Sasaki. The antigen-antibody complexes were detected using a chemiluminescent detection system (Nacalai Tesque) with horseradish peroxidase-linked anti-rabbit IgG (KPL).The signal was detected using LAS-4000 MINI

(Fuji Film).

Results

5'-UTR is essential for seed-specific expression of genes for plastidic ACCase subunits from Arabidopsis

In *Arabidopsis*, *CTα* and BC are each encoded by single genes (At2g38040 and At5g35360, respectively) and BCCP is encoded by two genes, *BCCP1* (At5g16390) and *BCCP2* (At5g15530).

To investigate the regulatory region of these genes, their 5'-upstream sequences were introduced into vector pBI101 ahead of the GUS gene, and these constructs were introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. First, we used approximately 1 kb fragments upstream of the TSS or translation initiation site (TIS) of these genes to construct reporter vectors (Fig. 1A). Histochemical GUS staining of seeds at 4, 10, and 15 DAF from transgenic plants (at least 10 independent plants for each construct) were analyzed. Strong GUS staining was detected in seeds at 10 DAF from plants carrying 5'-upstream sequence with the 5'-UTR of these genes, whereas no GUS staining was detected in seeds from plants carrying 5'-upstream sequence without the 5'-UTR (Fig. 1B, C, D and E). The expression of genes for ACCase subunits increased throughout the early developmental stage of seeds and peaked between 8 and 11 DAF and decreased to undetectable levels around 12-15 DAF in *Arabidopsis* (Ruuska et al. 2002; Ke et al. 2000). The GUS staining patterns of plants carrying the 5'-upstream sequence with the 5'-UTR agreed with previous observations. A similar GUS staining pattern was observed when the upstream sequences of *BCCP1*, *BCCP2*, *BC* and *CTα* genes were deleted to 296 bp, 297 bp, 203 bp and 229 bp from the TSS, respectively (Fig. 1). We also determined the GUS activity in seeds at 10 DAF from plants containing each construct (Fig. 2A). Although the activities varied dependent on the transgenic lines, the results agreed well with the results of GUS staining. The observations suggested that 5'-UTRs are necessary for the expression of these genes in seeds and approximately 300 bp upstream of TSS is enough in addition to the 5'-UTR.

Among the genes for ACCase subunits, expression of the *BCCP2* gene was strongly induced by WRINKLED 1 (WRI1) and it was found to contain WRI1 binding sequences (AW boxes) in the 5'-UTR (Maeo et al. 2009). We searched for AW boxes in the promoter regions of genes for other ACCase subunits, and we found that all four genes contained AW-boxes in the 5'-UTRs. In addition, we found consensus sequences of the AW box at 748 bp and 532 bp upstream from the TSS of the *BC* and *CTα* genes, respectively

(Fig. 3).

In addition to the AW box, another conserved sequence (cAAAAGtAggggttT) found in genes for plastidial pyruvate kinase PKp-β1 and BCCP2 is reported to be important for regulation by WRI1 (Baud et al. 2009). We found this sequence in the 5'-upstream sequence of *BC* and *CTα* but not in those of the *BCCP1* genes (Fig. 3A and C). Together with the GUS staining results, the conserved sequence (cAAAAGtAggggttT) seemed not to be necessary for the expression of *BCCP1, BC* and *CTα* genes in seeds.

Distance between the AW box and TSS affects gene expression

Next, we stained 10-day-old seedlings of transgenic plants to investigate the regulatory region for expression in organs other than seeds. Seedlings from transgenic plants containing sequences upstream from the TIS except for plants with the *BCCP2* promoter showed GUS staining as observed in seeds (Fig. 1). To examine the expression of these genes in 10-day-old seedlings, we performed RT-PCR (Fig. 4). As observed in GUS staining experiments, the expression of BCCP2 was very low in seedlings although other genes were expressed. Among plants carrying a 5'-upstream sequence without the 5'-UTR, only seedlings carrying the *CTα* promoter showed GUS staining. The results of a spectrophotometrical assay of GUS activity (Fig. 2B) supported the GUS staining results. These results indicated that the 5'-UTRs of these genes except for *CTα* are important for expression in seedlings as well as in seeds. Because the *CTα* gene contains its AW box 532 bp upstream from the TSS, the upstream sequence was deleted to -523 bp from the TSS. When the AW box upstream of the TSS was deleted, no GUS staining was observed in the seedlings (Fig. 5). To examine the importance of the AW box for expression in seedlings, we constructed the reporter vector with a CT*α* promoter region from the TSS to -956 bp that lacked the AW box at -532 bp (CTαΔAW532 in Fig. 5A). The seedlings with CTαΔAW532 showed no GUS staining (Fig. 5B). These results suggested that the AW box is important for the expression of ACCase subunit genes in seedlings.

Although the AW box located upstream of the TSS of the *CTα* gene was functional in seedlings, that of the *BC* gene (746 bp upstream of the TSS) was not functional. The sequences of the AW box located upstream of the TSS and the 5'-UTR of the BC gene differ by only one nucleotide (Fig. 3B) and appeared to be bound by WRI1. To examine whether WRI1 binds to AW boxes located upstream of the TSS of the *BC* and *CTα* genes, we performed an EMSA. WRI1 was expressed in *E. coli* as a fusion protein with GST. The 20 bp sequences containing the AW box at 746 bp and 532 bp upstream of the TSS of the *BC* and *CTα* genes, respectively, were used as probes. As a positive control, a

20 bp sequence containing the AW box from 47 bp downstream of the TSS of the *BCCP2* gene was used as a probe. All of the probes used for the experiments were shifted by the addition of WRI1, and the shifted bands were eliminated by the inclusion of unlabeled competitor DNA fragments (Fig. 6). The results showed that the AW boxes upstream of the TSS of both the *BC* and *CTα* genes were actually bound by WRI1.

Although WRI1 bound to both AW boxes upstream of the TSS of the *BC* and *CTα* genes, only the AW box upstream of the *CTα* gene was functional. We speculated that the distance between the AW box and the TSS affected promoter activity. To examine this idea, we constructed reporter vectors, in which the AW boxes upstream of the TSS of the *BC* and *CTα* genes were located approximately 200 bp upstream of the sequences of these genes (Fig. 7A). We obtained *Arabidopsis* lines containing these reporter genes and stained both seeds and seedlings.

Contrary to our expectation, seedlings from both of transgenic plants showed no GUS staining. On the other hand, seeds from both plants were stained. (Fig. 7B) These results indicated that the AW boxes upstream of the TSS are functional in seeds when they are located close to the TSS, whereas the AW box was not sufficient for expression in seedlings.

The 5'-UTR of *BCCP2* contains two AW boxes, and expression of the *BCCP2* gene is much more strongly induced by WRI1 than genes with a single AW box (Maeo et al. 2009). We examined whether the 5'-UTR of *BCCP2* gene is functional when it is moved upstream of the TSS. The 5'-UTR of *BCCP2* was moved to 980 bp or 297 bp upstream of TSS of *BCCP2* gene and fused to GUS genes to construct vectors (Fig. 8A). The vector was introduced into *Arabidopsis*, and GUS activity was examined by staining. When the 5'-UTR was moved to 980 bp or 297 bp upstream of the TSS, GUS staining was not detected in either in seeds or seedlings (Fig. 8B), although when the sequence of the 5'-UTR was located downstream of the TSS, 980 bp or 297 bp upstream sequences of the TSS of *BCCP2* gene was sufficient for expression in seeds (Fig. 1). This result supported the idea that the AW box is functional in seeds only when it is located close to the TSS.

When the 5'-UTR of *BCCP2* was moved to 297 bp upstream of the TSS, the closest AW box was located 419 bp upstream of the TSS, indicating that when the AW box was further than approximately 400 bp from the TSS was not functional in seeds.

Seed-specific expression of the BCCP2 gene

Our GUS staining results (Fig. 1), and previous expression data (Thelen and Ohlrogge 2002), showed that the *BCCP2* gene is specifically expressed in developing seeds. Other ACCase genes, including *BCCP1*, are strongly expressed in developing

seeds and young tissue. As shown in Fig. 1, a distance of approximately 300 bp from the TSS in addition to the 5'-UTR was sufficient for the expression of these genes in seedlings. In order to clarify which part of the promoter, the 5'-UTR or 5'-upstream sequence of the TSS, determined the seed-specific expression of the *BCCP2* gene, we made chimeric promoters composed of the 5'-upstream sequence of the TSS of *BCCP1* and the 5'-UTR of *BCCP2* (*BCCP1*+2UTR in Fig. 9A) and of the 5'-upstream sequence of the TSS of *BCCP2* and the 5'-UTR of *BCCP1* (*BCCP2*+1UTR in Fig. 9A) and the chimeric promoters were placed upstream of the GUS gene.

GUS expression was examined by staining of seeds and seedlings from the transgenic plants (Fig. 9B). The GUS staining pattern of transgenic plants carrying *BCCP1*+2UTR was similar to that of plants carrying the *BCCP2* promoter; seeds showed strong GUS staining but no GUS staining was observed in seedlings. On the other hand, both seeds and seedlings from the *BCCP2*+1UTR plants showed GUS staining, although the GUS staining in seedlings was weaker than that of plants with 991 bp of *BCCP1* 5'-upstream sequence with 5-'UTR (Fig. 1). These results suggested that the different expression patterns of *BCCP1* and *BCCP2* genes were not caused by the difference in the sequence of the 5'-upstream regions of these genes.

The effect of WRI1 on protein levels of ACCase subunits

In order to investigate whether WRI1 affects the protein levels of ACCase subunits in seeds, we examined the protein levels of BC, BCCP1, BCCP2 and CTβ in seeds from a *wri1* mutant and transgenic plants overexpressing WRI1 (35S:WRI1). Among these proteins, only CTβ is encoded by the plastid genome. Because ACCase subunits are expressed in pods where WRI1 is not expressed, we extracted proteins from seeds at 10 DAF without pods. To detect CTβ and BC, we used antibodies against pea CTβ and tobacco BC, respectively, and BCCPs were detected by avidin conjugated with horseradish peroxidase (HRP) (Madoka et al. 2002).

Among these proteins, the BCCP2 protein showed the most significant differences in the *wri1* mutant or 35S:WRI1 plants compared with control plants (sGsL) (Fig. 10). BCCP2 levels in the *wri1* mutant were much lower than in control plants, and that in 35S:WRI1 plants was higher than in the controls. In contrast, there was no significant difference in BCCP1 levels. BC levels in the *wri1* mutant were slightly lower than in the controls and CTβ levels were not affected by WRI1.

Discussion

In this report, we analyzed the regulation of genes for heteromeric ACCase in

Arabidopsis. We show that the AW box is essential for the expression of these genes in both seeds and seedlings. Although WRI1 is exclusively expressed in developing seeds, there are other AP2 domain transcription factors that may have similar or the same binding properties to WRI1 and are expressed in other organs (Masaki et al. 2005), and these transcription factors may regulate ACCase expression in other organs.

Baud et al. (2009) reported that a conserved sequence (cAAAGtAggggttT) is found in the promoters of some WRI1 regulated genes, such as genes for BCCP2, plastidial pyruvate kinases β subunit (PKp-β1), pyruvate dehydrogenase E1 β subunit (PDH-E1β) and so on, and these sequences are necessary for the expression of these genes. Although our results show that these sequences are not required for the expression of genes for BCCP1, BC and CTα subunits, the conserved sequences are found close to the TSS of the *BC* and *CTα* genes in addition to the *BCCP2* gene. The function of the conserved sequences remained to be resolved.

The AW boxes at 748 bp and 532 bp upstream of the TSS of the *BC* and *CTα* genes, respectively, were not functional in seeds (Fig. 1 and 3). In addition, when the 5'-UTR of *BCCP2* was moved to 297 bp upstream of the TSS (the AW boxes were located 419 bp and 527 bp upstream of the TSS), the expression of *BCCP2* in seeds was not detected (Fig. 8). However, when the AW boxes upstream of the TSS of the *BC* and *CTα* genes were moved to approximately 200 bp upstream of the TSS, they became functional in seeds (Fig. 7). Taken together, the AW box is location sensitive and should be located less than 400 bp from the TSS.

The transcriptional efficiency from some regulatory motifs, such as CArG box (binding motif of myocardin) and NF-KB motif (binding motif of NF-kB), are reported to be dependent on the distance from the TSS (Tabach et al. 2007). Our finding explains why AW boxes in genes involved in fatty acid synthesis and glycolysis are close to the TSS.

Previous reports (Thelen and Ohlrogge 2002) and our present results show that *BCCP2* gene expression is seed specific (Fig. 1). Fig. 9 shows that the region upstream of the TSS of the *BCCP1* gene does not contain the region necessary for expression in seedlings. The minimum regulatory region of the *BCCP1* gene required for expression in seedlings probably exists in the 5'-UTR. However, the GUS staining in seedlings of *BCCP2*+1UTR plants was very weak (Fig. 9) and the GUS activity in seedlings of plants carrying a fragment comprising 298 bp upstream from the TSS of *BCCP1* gene was significantly lower than that of plants with a 991 bp upstream sequence (Fig. 2B). These results indicate that there are other sequences more than 298 bp upstream from the TSS of *BCCP1*, which enhance the promoter activity in seedlings in the presence of the

AW box. In addition, moving the AW box from 532 bp to 229 bp upstream of the TSS of *CTα* resulted in the loss of function in seedlings although it was functional when it was located in the original position of the CTα promoter (532 bp upstream of the TSS) (Fig. 1 and 7). On the other hand, the 229 bp sequence from the TSS of the *CTα* gene showed strong promoter activity in seedlings in the presence of the 5'-UTR (Fig. 1). These results also support the hypothesis that there are other regulatory sequences for the expression in seedlings in addition to the AW box in the 5'-UTR and/or 5'-upstream sequence of the TSS. Further analysis will clarify the regulatory sequence for expression in seedlings.

The protein levels of BCCP1 in seeds from *wri1* mutants and 35S:WRI1 plants were similar to those of control plants (Fig. 10). This was an unexpected result because the *BCCP1* genes contains an AW box in the 5'-UTR, and the expression levels of the *BCCP1* gene was reduced in seeds of the *wri1* mutant (Baud et al. 2010). On the other hand, the protein levels of BCCP2 were dramatically affected by WRI1 (Fig. 10). This result agrees with the previous observation that the *BCCP2* gene is drastically activated by WRI1 (Maeo et al. 2009; Baud et al. 2009). The genes for PKp-β1, BCCP2 and KAS1, which are strongly activated by WRI1, contain two AW boxes around their TSSs. Maeo et al. (2009) showed that a mutation in one of AW boxes in PKp-β1 reduced the transcriptional activity, indicating that transcriptional activity is affected by the number of AW boxes. The strong activation of the *BCCP2* gene by WRI1 may be caused by the existence of two AW boxes in the 5'-UTR.

The level of CTβ protein encoded by *accD*, a plastid-encoded gene, was not affected by WRI1 (Fig. 10). The *accD* gene is a housekeeping gene regulated by nuclear-encoded plastid RNA polymerase (NEP) (Hajdukiewicz et al. 1997). The expression of the *accD* gene is reported to cooperatively increase with other genes for ACCase subunits in developing *Arabidopsis* seeds (Ke et al. 2000), but the regulation of *accD* gene expression in developing seeds is still unclear.

The *wri1* mutant was shown to be associated with an 80% reduction in seed oil content (Focks and Benning 1998). Among the ACCase subunits, BCCP2 was most significantly affected in its protein level (Fig. 10). Both antisense and mutant of *BCCP2* genes showed only slight effects on fatty acid accumulation in seeds (Thelen and Ohlrogge 2002; Li et al. 2011), suggesting that the reduction in BCCP2 level observed in the *wri* mutant (Fig. 10) may not be the cause of the drastic reduction in oil content in *wri1* seeds. On the other hand, a mutation of the PKp-β1 gene, which is also strongly regulated by WRI1, caused a 60% reduction in oil content (Andre et al. 2007). It is still unclear how much impact WRI1 has on ACCase activity.

If the level of BCCP2 does not affect the fatty acid content in seeds (Thelen and Ohlrogge 2002; Li et al. 2011), why is BCCP2 strongly induced by WRI1? WRI1 induces the *AtGLB1* gene encoding PII in developing seeds in a similar profile to *BCCP2*. PII protein has been shown to interact with BCCP in the presence of ATP and low concentrations of 2-oxoglutarate (2-OG), causing the inhibition of ACCase activity (Baud et al. 2010; Feria Bourrellier et al. 2010). In plastids, the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle assimilates ammonium using 2-OG in the presence of ATP. Under nitrogen-rich condition, the GS-GOGAT cycle is activated to consume 2-OG and it might cause the low 2-OG level conditions to inhibit ACCase activity. BCCP2 might function to trap PII to maintain ACCase activity under such conditions.

Further analysis of transcriptional and post-translational regulation of plastidic ACCase will elucidate the regulation of lipid metabolism in seeds and other organs in relation to other metabolic processes, especially in response to the nutrition status of cells.

Acknowledgements

We thank K Nakamura for providing plant materials and Y Sasaki for providing antisera.

References

Andre C, Froehlich JE, Moll MR, Benning C (2007) A heteromeric plastidic pyruvate kinase complex involved in seed oil biosynthesis in Arabidopsis. Plant Cell 19 (6):2006-2022.

Baud S, Feria Bourrellier AB, Azzopardi M, Berger A, Dechorgnat J, Daniel-Vedele F, Lepiniec L, Miquel M, Rochat C, Hodges M, Ferrario-Mery S (2010) PII is induced by WRINKLED1 and fine-tunes fatty acid composition in seeds of Arabidopsis thaliana. Plant J 64 (2):291-303.

Baud S, Wuilleme S, To A, Rochat C, Lepiniec L (2009) Role of WRINKLED1 in the transcriptional regulation of glycolytic and fatty acid biosynthetic genes in Arabidopsis. Plant J 60 (6):933-947.

Cernac A, Benning C (2004) WRINKLED1 encodes an AP2/EREB domain protein involved in the control of storage compound biosynthesis in Arabidopsis. Plant J 40 (4):575-585.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16 (6):735-743

Feria Bourrellier AB, Valot B, Guillot A, Ambard-Bretteville F, Vidal J, Hodges M (2010) Chloroplast acetyl-CoA carboxylase activity is 2-oxoglutarate-regulated by interaction of PII with the biotin carboxyl carrier subunit. Proc Natl Acad Sci U S A 107 (1):502-507.

Focks N, Benning C (1998) wrinkled1: A novel, low-seed-oil mutant of Arabidopsis with a deficiency in the seed-specific regulation of carbohydrate metabolism. Plant Physiol 118 (1):91-101

Hajdukiewicz PT, Allison LA, Maliga P (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. EMBO J 16 (13):4041-4048.

Ke J, Wen TN, Nikolau BJ, Wurtele ES (2000) Coordinate regulation of the nuclear and plastidic genes coding for the subunits of the heteromeric acetyl-coenzyme A carboxylase. Plant Physiol 122 (4):1057-1071

Kozaki A, Hake S, Colasanti J (2004) The maize ID1 flowering time regulator is a zinc finger protein with novel DNA binding properties. Nucleic Acids Res 32 (5):1710-1720.

Li ZG, Yin WB, Song LY, Chen YH, Guan RZ, Wang JQ, Wang RR, Hu ZM (2011) Genes encoding the biotin carboxylase subunit of acetyl-CoA carboxylase from Brassica napus and parental species: cloning, expression patterns, and evolution. Genome 54 (3):202-211.

Madoka Y, Tomizawa K, Mizoi J, Nishida I, Nagano Y, Sasaki Y (2002) Chloroplast transformation with modified accD operon increases acetyl-CoA carboxylase and causes extension of leaf longevity and increase in seed yield in tobacco. Plant Cell Physiol 43 (12):1518-1525

Maeo K, Tokuda T, Ayame A, Mitsui N, Kawai T, Tsukagoshi H, Ishiguro S, Nakamura K (2009) An AP2-type transcription factor, WRINKLED1, of Arabidopsis thaliana binds to the AW-box sequence conserved among proximal upstream regions of genes involved in fatty acid synthesis. Plant J 60 (3):476-487.

Masaki T, Mitsui N, Tsukagoshi H, Nishii T, Morikami A, Nakamura K (2005) ACTIVATOR of Spomin::LUC1/WRINKLED1 of Arabidopsis thaliana transactivates sugar-inducible promoters. Plant Cell Physiol 46 (4):547-556.

Ohlrogge J, Savage L, Jaworski J, Voelker T, Post-Beittenmiller D (1995) Alteration of acyl-acyl carrier protein pools and acetyl-CoA carboxylase expression in Escherichia coli by a plant medium chain acyl-acyl carrier protein thioesterase. Arch Biochem Biophys 317 (1):185-190.

Ruuska SA, Girke T, Benning C, Ohlrogge JB (2002) Contrapuntal networks of gene expression during Arabidopsis seed filling. Plant Cell 14 (6):1191-1206

Sasaki Y, Hakamada K, Suama Y, Nagano Y, Furusawa I, Matsuno R (1993) Chloroplast-encoded protein as a subunit of acetyl-CoA carboxylase in pea plant. J Biol Chem 268 (33):25118-25123

Sasaki Y, Konishi T, Nagano Y (1995) The Compartmentation of Acetyl-Coenzyme A Carboxylase in Plants. Plant Physiol 108 (2):445-449.

Tabach Y, Brosh R, Buganim Y, Reiner A, Zuk O, Yitzhaky A, Koudritsky M, Rotter V, Domany E (2007) Wide-scale analysis of human functional transcription factor binding reveals a strong bias towards the transcription start site. PLoS One 2 (8):e807.

Thelen JJ, Ohlrogge JB (2002) Both antisense and sense expression of biotin carboxyl carrier protein isoform 2 inactivates the plastid acetyl-coenzyme A carboxylase in Arabidopsis thaliana. Plant J 32 (4):419-431.

Table 1. Primers used for promoter analysis

	Name	Sequence
promoter		
BCCP1	BCCP1p-991F	GCGTCGACTTTGTTCTTCTTTTTCCG
	BCCP1p-296F	GCGTCGACAATTGGAGACACCGACAG
	BCCP1p-TSSR	GCGGATCCGGATTCGGAACGCGTTTC
	BCCP1p-TISR	GCGGATCCTCGTCTTCTTATTGTTATTG
BCCP2	BCCP2p-980F	GCGTCGACATCAGACAAAAGAGAGACAAC
	BCCP2p-297F	GCGTCGACTATCTGCATTTACTGAAG
	BCCP2p-TSSR	GCGGATCCGTCATAACATAGTGCCTCGG
	BCCP2p-TISR	GCGGATCCTGTTGAGACAGTGGACGATG
BC	BCp-1009F	GCGTCGACTAATATATCTTCTTATGAC
	BCp-203F	GCGTCGACCCTTGTAAGCTTAATGGGC
	BCp-TSSR	GCGGATCCCGCCCGACTGGTTTGATTTG
	BCp-TISR	GCGGATCCTTTCCAAATGCAGGAGGC
CTα	CTαp-956F	GCGTCGACTAATACCCTTATTAAAGGCC
	CTαp-523F	GCGTCGAATTTCTCAGCTGCTCTAGAATG
	CTαp-229F	GCGTCGACGCTCTCACCCGAGAGACCC
	CTαp-TSSR	GCGAATCCTTCCTTGCCAATTGCCAGAAC
	CTαp-TISR	GCGGATCCTTTTTGCGTTGAGTTCTT
CTαΔAW532	CTα-545HR	GAAGCTTTTGTTTCTTCTCCTACTGC
	CTαΔAW532	GAAGCTTCCATTTCTCAGCTGCTCTAG
AW-BC203	BC-AW203F	GTCGACTTCGATACTCACGAGGATTCCTTGTAA GCTTAATGGGC
AW-CTα229	CTα-AW229F	GTCGACGCTCGAATCTAGCGAAGCCGCTCTCAC CCGAGAGACCCG
BCCP1+2UTR	BCCP2-UTRBF	GCGGATCCATGGCACGATTGAACTGATAG
	BCCP2-UTRXbR	GTCTAGATGTTGAGACAGTGGACGATG
BCCP2+1UTR	BCCP1p-UTREF	GGAATCCGACATCTTCTCTCTCTCTTTC
	BCCP2p-TSSER	GGGATTCGTCATAACATAGTGCCTCG
5'UTR-980	BCCP2-UTRF	GTCGACATGGCACGATTGAACTGATAG
5'UTR-297	BCCP2-UTRR	GCGCTCTGACGCCATTGTTGAGAC

Table 2. Primers used for RT-PCR and EMSA

	Name	Sequence
RT-PCR		
BCCP1	BCCP1F	GCTACAGAAGAGTCTATTTC
	BCCP1R	CCATTTCTCATGGTGCCG
BCCP2	BCCP2F	CTCTCTGAATTTATGGCT
	BCCP2R	GCTAAAAGGATCCTTCTTC
BC	BCF	CAGGTGGAGCATCCTGTG
	BCR	CTAAACCGTTGCGTTTGTC
CTα	CTαF	GCTCTAGAATGCCCCTTATTCATCGG
	CTαR	GCGAGCTCTCACAGCTTCAGGTCAATAC
EMSA		
biotin-labeled	biotin F	TAGTAACGGCCTCCAGTGTG
biotin-labeled	biotin R	CATGCTCGAGCGGCCGCCAG
BC	BC-probe	TAGTAACGGCCTCCAGTGTGTTCGATACTCACG AGGATTCTGGCGGCCGCTCGAGCATG
CTα	CTα-probe	TAGTAACGGCCGCCAGTGTGGCTCGAATCTAGC GAAGCCACTGGCGGCCGCTCGAGCATG
BCCP2	BCCP2-probe	TAGTAACGGCCGCCAGTGTGCTTCCTCGGTTTC ATCGTCCCTGGCGGCCGCTCGAGCATG

Figure legends

Fig. 1. GUS expression analysis of transgenic plants containing promoters of ACCase subunit genes. **(A)** Schematic diagram of the promoter: GUS constructs analyzed. The numbers under the bars indicate the endpoints of the promoter sequences. The right ends of dark boxes indicate the translational initiation sites (TIS). Transcriptional start sites are indicated by TSS. The GUS staining results are summarized on the right side of each bar. B-E. Histochemical staining of seeds at 4, 10 and 15 days after flowering (DAF) and seedling from plants containing promoters of **(B)** *BCCP1*, **(C)** *BCCP2*, **(D)** *BC* and **(E)** *CTα* genes.

Fig. 2. Quantitative analysis of GUS activities of transgenic plants containing promoters of ACCase subunit genes. **(A)** GUS activities in seeds at 10 days after flowering (DAF) from transgenic plants. **(B)** GUS activities in seedlings from transgenic plants. Values represent means of 5-10 independent transgenic lines. Error bars represent SD.

Fig. 3. The AW box and cAAAAGtAggggttT consensus in the promoters of ACCase subunit genes. **(A)** Location of the AW box and cAAAAGtAggggttT consensus in the promoters of ACCase subunits genes. Transcriptional start sites are indicated by TSS. **(B)** Sequences of the AW box identified in each promoter. **(C)** Sequence of the cAAAAGtAggggttT consensus identified in each promoter. The numbers indicate the position from the transcriptional start site. Asterisks indicate that complementary sequences are present.

Fig. 4. RT-PCR analysis of genes for ACCase subunits. Total RNA was isolated from seedlings and seeds at 10 days after flowering (DAF) from wild-type *Arabidopsis*. Semi-quantitative RT-PCR analyses (25 cycles) were performed with primers described in Table 2.

Fig. 5. GUS expression analysis of transgenic plants containing CTα promoter lacking the AW box at upstream of the transcriptional start site (TSS). **(A)** Schematic diagram of the 523 bp upstream from the transcriptional start site of CTα (CTα-523 bp) and 956 bp upstream from TSS where the AW box was deleted (CTαΔAWbox532). These promoters were ligated upstream of GUS gene. **(B)** Histochemical staining of seeds at 10 days after flowering (DAF) and in seedlings.

Fig. 6. Binding of WRI1 to the AW box at upstream of the transcriptional start site (TSS) of the *BC* and *CTα* genes. Electrophoretic mobility shift assay using the AW box sequences at 748 and 532 bp upstream of the TSS of the *BC* and *CTα* genes, respectively, as probes. As a positive control, the AW box sequence 47 bp downstream of the TSS of the *BCCP2* gene was used as a probe. The black arrowhead indicates the shifted band and the white arrowhead indicates free probes.

Fig. 7. Activity of the manipulated *BC* and *CTα* upstream sequences where the AW boxes were move to approximately 200 bp from the transcriptional start site (TSS). **(A)** Schematic diagram of the upstream sequences of the *BC* and *CTα* genes with the AW box at the 5'-end. The AW boxes upstream of the TSS of the *BC* and *CTα* genes were placed at 203 and 229 bp upstream of the TSS of *BC* (AW-BC203) and *CTα* (AW-CTα229), respectively. These promoters were ligated upstream of GUS gene. **(B)** Histochemical staining of seeds at 10 days after flowering (DAF) and in seedlings.

Fig. 8. Activity of the manipulated *BCCP2* promoter where the 5'-UTR was moved to upstream of the transcriptional start site (TSS). **(A)** Schematic diagram of the manipulated *BCCP2* promoter where the 5'-UTR was moved to 980 bp (5'UTR-980) or 297 bp (5'UTR-297) upstream of TSS. **(B)** Histochemical staining of seeds at 10 days after flowering (DAF) and in seedlings.

Fig. 9. Activity of a chimeric promoters with the 5'-UTR of the *BCCP2* gene and the 5'-upstream sequence of *BCCP1* and with the 5'-UTR of the *BCCP1* gene and the 5'-upstream sequence of *BCCP2* . **(A)** Schematic diagram of the chimeric promoters with the 5'-UTR of the *BCCP2* gene and the 5'-upstream sequence of *BCCP1*(*BCCP1*+2UTR) and with the 5'-UTR of the *BCCP1* gene and the 5'-upstream sequence of *BCCP2* (*BCCP2*+1UTR). **(B)** Histochemical staining of seeds at 10 days after flowering (DAF) and in seedlings.

Fig. 10. Protein level of ACCase subunits in *wri1* mutant and WRI1 overexpressing plants. Proteins extracted from 10 days after flowering (DAF) seeds of sGsL (control), *wri1* mutant and 35S:WRI1 plant were separated on SDS-PAGE and subjected to western blotting analysis. The blots were incubated with horseradish peroxidase-conjugated avidin to detect BCCP1 and 2, or antisera against each subunit

to detect BC and CTβ.

Fig.1

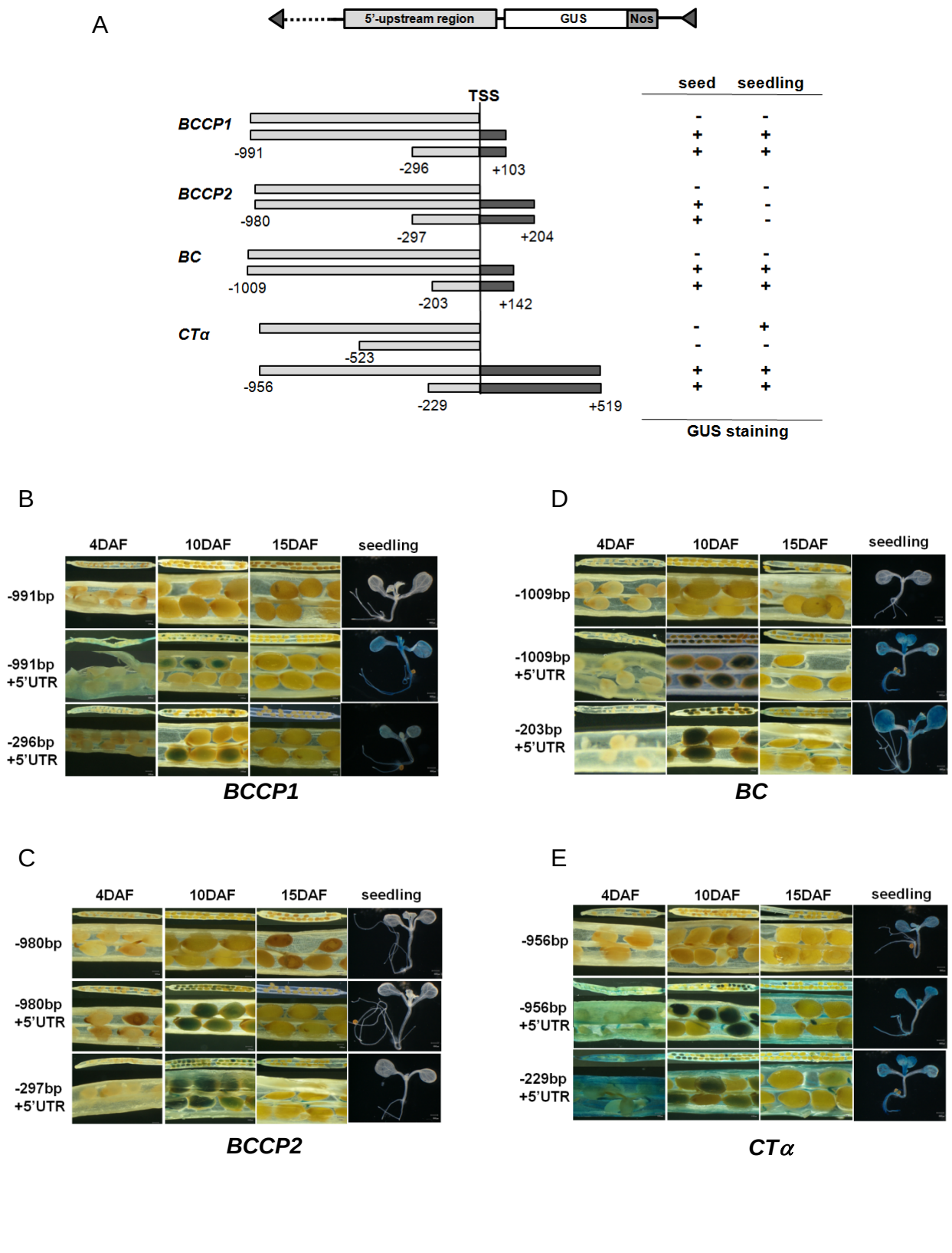
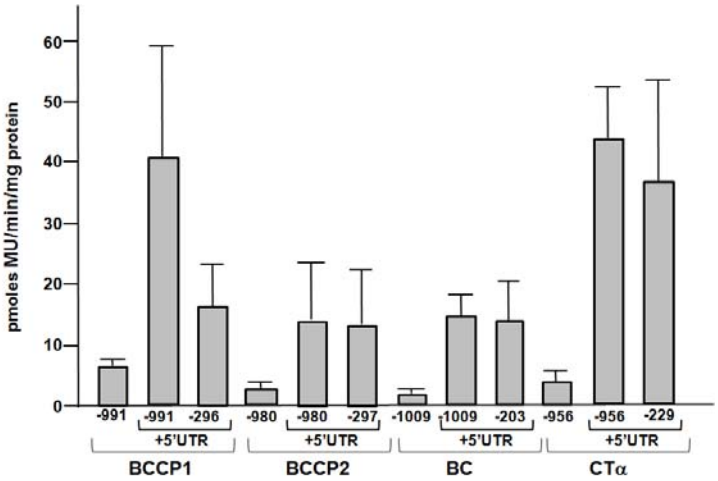

A
5'-upstream region
GUS
Nos
TSS
seed
seedling
BCCP1
-991
-296
+103
BCCP2
-980
-297
+204
BC
-1009
-203
+142
CTα
-523
-956
-229
+519
GUS staining
B
4DAF
10DAF
15DAF
seedling
-991bp
-991bp +5'UTR
-296bp +5'UTR
BCCP1
D
-1009bp
-1009bp +5'UTR
-203bp +5'UTR
BC
C
-980bp
-980bp +5'UTR
-297bp +5'UTR
BCCP2
E
-956bp
-956bp +5'UTR
-229bp +5'UTR
CTα

Fig.2

A


pmoles MU/min/mg protein
60
50
40
30
20
10
0
-991
-991
-296
-980
-980
-297
-1009
1009
-203
-956
-956
-229
+5'UTR
+5'UTR
+5'UTR
+5'UTR
BCCP1
BCCP2
BC
CTα

B

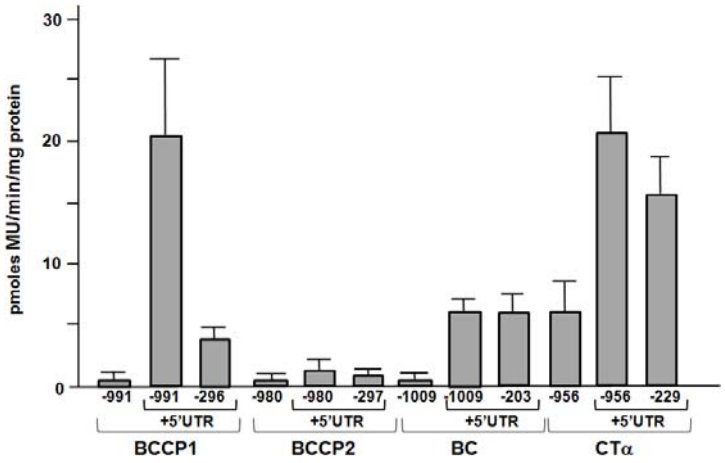
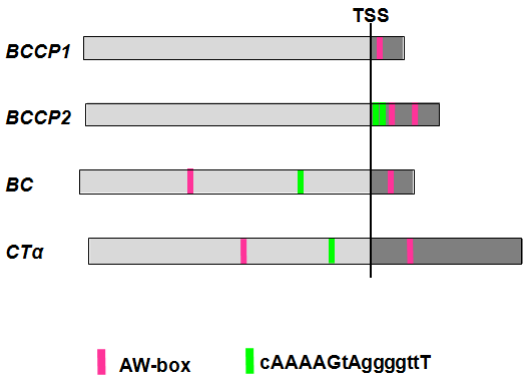

pmoles MU/min/mg protein
30
20
10
0
-991
-991
-296
-980
-980
-297
-1009
1009
-203
-956
-956
-229
+5'UTR
+5'UTR
+5'UTR
+5'UTR
BCCP1
BCCP2
BC
CTα

Fig. 3

A



B

BCCP1	CTTTGGTTTCATCG	47/60
BCCP2	CTTCGTAAGCATCG	69/82
BCCP2	CCTCGGTTTCATCG	176/189
BC	CCTCGTGAGTATCG*	-761/-748
BC	CCTCGTGATTATCG*	75/88
Ctα	CTTCGCTAGATTCG*	-545/-532
Ctα	CATCGAAGAGATGC*	153/166
		position

C

BCCP2	CAAAAGGAGCGGTTT	26/40
BCCP2	TAAAAGAGTTGGGTT	59/73
BC	TAAAAGCAAAGCAAT	-336/-350
Ctα	AAAAAGAAAAACTAT*	-181/-195
		position

Fig.4

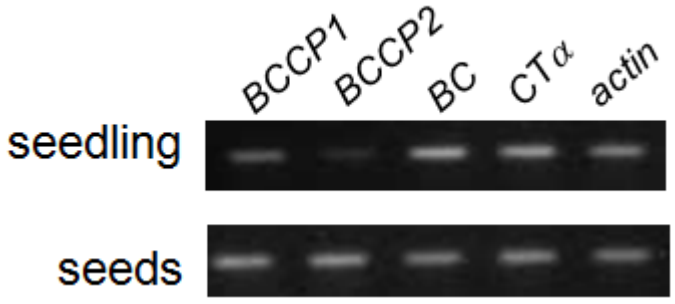
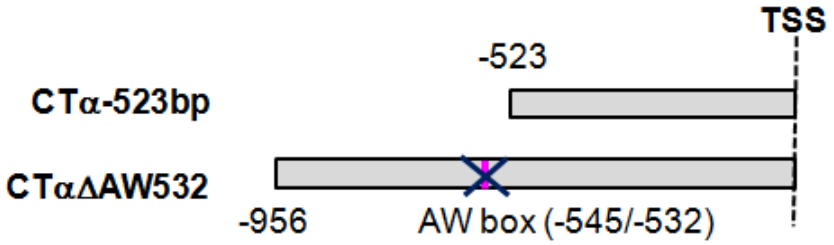


Fig.5

A


TSS
-523
CTα-523bp
CTαΔAW532
-956
AW box (-545/-532)

B

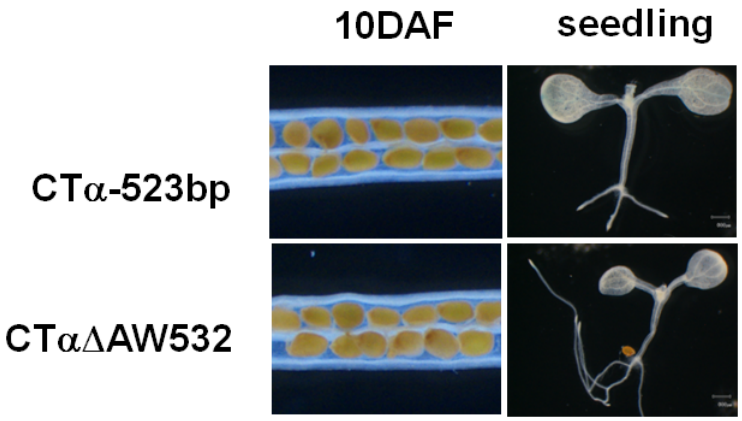

10DAF
seedling
CTα-523bp
CTαΔAW532

Fig.6

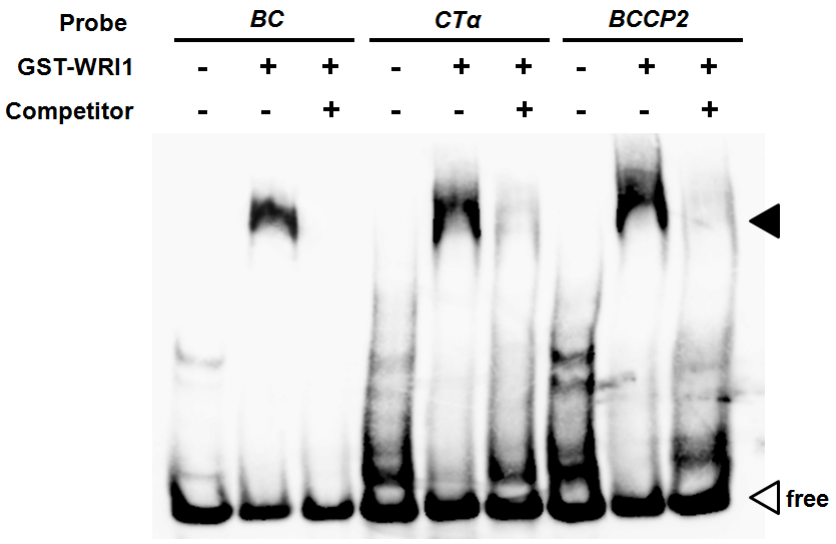
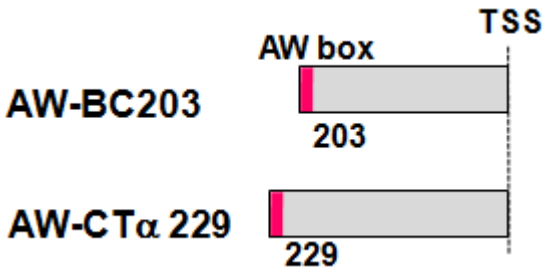


Fig. 7

A


TSS
AW box
AW-BC203
203
AW-CTα 229
229

B

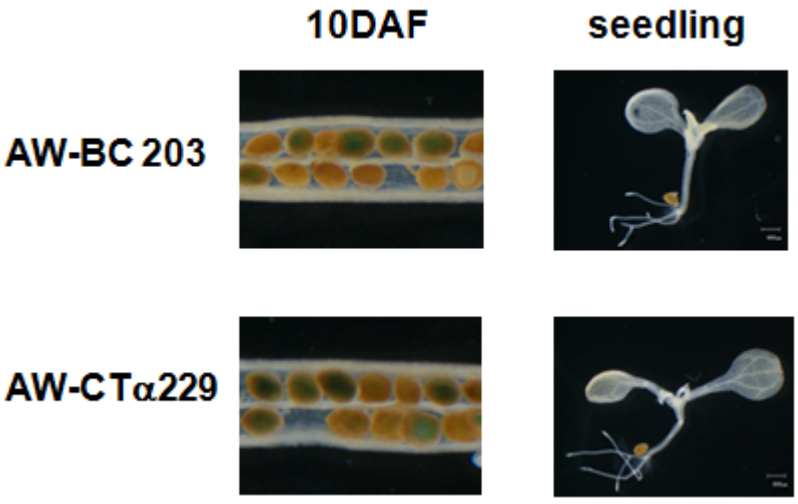
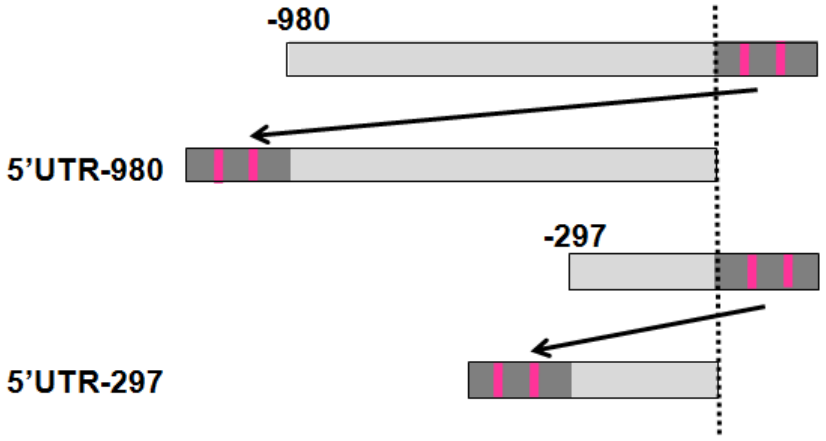

10DAF
seedling
AW-BC 203
AW-CTα229

Fig.8

A



B

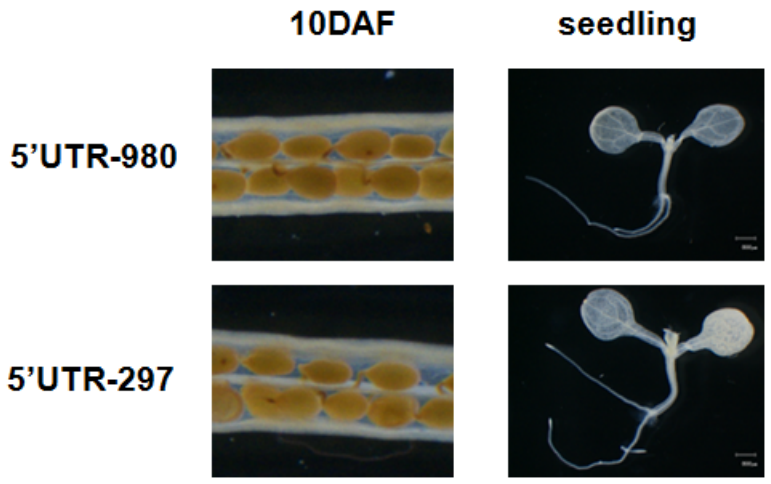
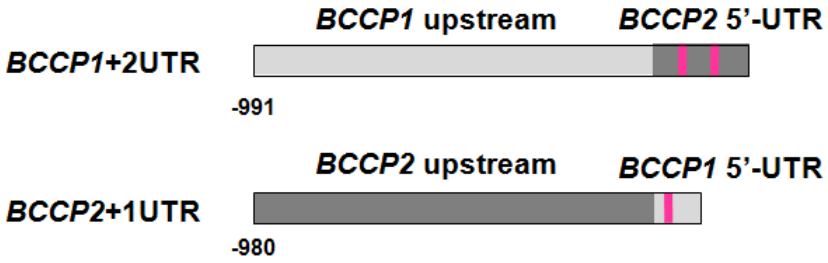


Fig.9

A


BCCP1 upstream
BCCP2 5'-UTR
BCCP1+2UTR
-991
BCCP2 upstream
BCCP1 5'-UTR
BCCP2+1UTR
-980

B

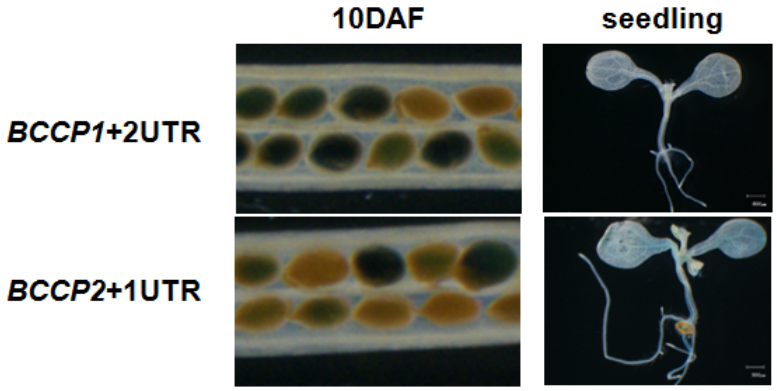

10DAF
seedling
BCCP1+2UTR
BCCP2+1UTR

Fig.10