

Wrinkled 1 (WRI1) Homologs, AP2-Type Transcription Factors Involving Master Regulation of Seed Storage Oil Synthesis in Castor Bean (*Ricinus communis* L.)

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ABSTRACT

Among APETALA2 (AP2)-type plant specific transcription factor family, *WRINKLED1* (WRI1), has appeared to be a master gene transcriptionally regulating a set of carbon metabolism- and fatty acid synthesis (FAS)-related genes responsible for seed specific triacylglycerols (TAGs) storage in oil plants. B3 type transcription factors, such as ABI3 and FUS3, are known to be involved in seed development, such as seed storage protein synthesis and maturation. Based on the recent whole genome sequence data of castor bean (*Ricinus communis* L.), putative WRI1 homologs (*RcWRI1*, *RcWRI2*) specifically expressed in castor bean seed have been identified by comparing organ specific expression profiles among seed development-related transcription factors, seed storage specific genes (*Ricin*, *RcOleosin*) and a set of FAS genes including genes for sucrose synthase (*RcSUS2*), biotin carboxyl carrier protein (a subunit of acetyl-CoA carboxylase, *RcBCCP2*) and ketoacyl-acyl carrier protein synthase (*RcKAS1*). Immunoreactive signals with WRI1, FUS3 and ABI5-related polypeptides were also detected in seed specifically, consistent with the expression profiles of seed development-related genes. The WRI1 binding consensus sites, [CnTnG](n)(7)[CG], designated as the AW-box, were found at the promoter region of *RcBCCP2* and *RcKAS1*. Thus, *RcWRI1* possibly play a pivotal role in seed specific TAGs storage during seed development by directly activating FAS -related genes.

Keywords: AP2; Castor Bean; Fatty Acid; Oil Seed; *Ricinus communis* L.; Transcription Factor; WRI1

1. Introduction

The castor bean plant is a tropical shrub that is one of the most important and valued oilseed crops. Castor bean was originated in Africa, but now is broadly cultivated in many tropical and subtropical regions [1]. Triacylglycerols (TAGs) are the major storage compound ranging from 40% to 60% (weight per dry weight) in the castor bean seed. About 90% of the seed TAGs is composed of ricinoleic acid (12-hydroxy-oleate), which is unusual hydroxylated fatty acid highly valued for chemical and pharmacological application [2]. It has been proposed that castor bean has a potential to produce biodiesel, because of its unique properties that the seed has high oil content, toxic storage protein (ricin) confers oil source without competition to food resource and the plant can tolerate to severe environments where food crops are unfavorable [3].

According to several lines of evidence of genetic

regulation mechanisms involving seed development and seed storage accumulation biosynthesis [5-7]. Genetic and molecular biological studies using *Arabidopsis thaliana* have currently unveiled that a set of specific transcription factors are temporarily and spatially expressed during the seed development and its maturation and that those seed specific transcription factors collaborate in regulation of seed specific genes for storage proteins and TAGs synthesis [8,9]. Several genes responsible for *A. thaliana* mutants involved in the seed development and the early embryonic morphology have been identified, namely *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *FUSCA 3* (*FUS3*) and *LEAFY COTYLDON 1* and *2* (*LEC1*, *LEC2*), etc. [10]. *ABI3*, *FUS3* and *LEC2* appeared to be key regulators belonging to B3-type transcription factor family, whereas *LEC1* is a NFY-B, a HAP3 subunit of the CCAAT-binding transcription factors [7]. It is known that, at the downstream of *LEC1*, *LEC2*, *ABI3* and *FUS3* form a complicated network interacting to each other to

promote embryogenesis, seed storage accumulation and establishment of dormancy [11]. Recently, *WRINKLED* 1 (WRI1) transcription factor has been identified to be an important regulator for a set of carbohydrate metabolism and FAS in maturing *Arabidopsis* seed [8,12]. TAGs contents in the WRI1 mutant seeds decreases by 80% compared with that in wild type *Arabidopsis* seeds, while WRI1 mutant seeds accumulate high sucrose [13]. It has been reported that WRI1 accelerates the expression of genes encoding enzymes of glycolysis, fatty acid elongation and the biotin and lipoic acid synthesis reactions [14]. Among LEC2, ABI3 and FUS3, WRI1 appeared to be regulated at the downstream of LEC2 [15]. It has been reported that WRI1 specifically binds to a conserved *cis*-element motif (AW-box) which are identified in upstream promoter regions of seed specific FAS related gene *KASI*, *BCCP2* and *SUS2* in *Arabidopsis* [9]. Over expression of the WRI1 homologs in *Arabidopsis*, *Brassica* and maize resulted in significant increase of seed TAGs contents [16-18]. Recently, whole genome analysis projects with the next-generation DNA sequencer has unveiled a set of whole genome data in various oil crops (maize, soybean, castor bean, jatropha and palm tree), indicating that the genetic manipulation of oil crops has significant potential for improvement of vegetable oil production in an aspect of breeding [19-23].

Castor bean (*Ricinus communis* L.) is one of the most profitable oil crops and suitable for research of industrial oil and drug such as castor oil and ricinoleic acid, etc. In order to address the key regulating mechanism of TAG biosynthesis in castor bean, we searched for transcriptional factors involved in seed storage synthesis by inspecting the available Castor Bean Genome Database (<http://castorbean.jcvi.org>) [21,24]. We focused on the expression profiles of transcription factors (3 of WRI1s, 1/2 of B3-type and 1 of bZIP-type) and FAS-related genes in developing seeds. We profiled the expression levels of WRI1 homologs in castor bean organs and identified *Rc* WRI1 as a putative master transcription factor regulating seed specific FAS. Immunoblot also indicated that seed specific transcription factors are coexpressed in developing seed with the WRI1-related polypeptides, suggesting that a seed specific transcription factor network composing B3-type transcription factors and WRI1 homolog is conserved in the seed storage oil synthesis mechanism between castor bean and *Arabidopsis*.

2. Materials and Methods

2.1. Plant Materials

Seeds of castor bean (*Ricinus communis* L.) were supplied from Matsuura Kampo Co. (Nagoya, Japan). Three to five seeds of castor bean were sown in the pots (16 cm × 20 cm) filled with soil in the Kaizuka field,

Kyushu University on 10 April 2011, and the seedlings were thinned to one plant per pot upon germination in order to get uniform plant stand. Chemical compound fertilizer, mamekase (N:P₂O₅:K₂O-3:10:10) (5 g pot⁻¹) and lime (5 g pot⁻¹) were well mixed with the soil before sowing.

2.2. RNA Extraction and Semi-Quantitative RT-PCR of Castor Bean WRI1 Homologs and Fatty Acid Synthesis(FAS)-Related Genes

BLAST search was conducted using WRI1 homologs, AINTEGUMENTA (AtANT) and other AP2-type transcription factors, DREB1A, ERF1, of *Arabidopsis thaliana* in the castor bean gene index at phytozome v.9.0 in JGI (<http://www.phytozome.net/>) identified castor bean orthologs. Gene index numbers and/or accession numbers and specific primer sequences of the castor bean genes were shown in **Table 1**.

RNA preparation by the SDS/phenol/LiCl method, cDNA synthesis and PCR were carried out as described previously [25,26] using Rev Tra Ace reverse transcriptase (Invitrogen) and GoTaq kit (Promega) according to the manufacturer's manuals and the thermal cycler PC-816 (ASTEC, Fukuoka, Japan). Thermal cycle conditions in PCR were as follows; initial denaturing at 94°C for 2 min, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 61°C for 30 s and extending at 72°C for 40 s, and then final extending at 72°C for 5 min. The PCR products were separated in 1.5% agarose gel and visualized with FluorChem (AlphaInnotech, San Leandro, CA, USA) after ethidium bromide staining.

2.3. Preparation of Extracts from Castor Bean Tissues

Protein samples were prepared from various organs with minor modification as described previously [27]. Frozen tissues (2 g) were homogenized on an ice-cooled motor in 6 mL of lysis buffer containing 25 mM Tris-HCl [pH 7.4], 10 mM EDTA-Na, 10% glycerol, 0.1% β-mercaptoethanol, 1 mM PMSF, 5 mM *n*-aminocaproic acid, 1 mM benzamidine, 1 μg·mL⁻¹ leupeptin and 1% Triton X-100. The resultant homogenates were centrifuged at 10,000 × g for 20 min at 4°C. Protein concentrations of samples were measured by a Bio Rad protein assay kit (BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

2.4. Raising Anti-WRI1, ABI3 and ABI5-Peptide Specific Antibodies and Immunoblot

To purpose to detect endogenous transcription factors in-

Table 1. Oligo DNA Primers used for RT-PCR analysis.

Gene	Accession number/ Gene Index	Primer sequence
RcWRI1-5	AB774159/ #30069	CCTTGGATCCATGGACTCTTGCA- CAATGAT
RcWRI1-3		CCTCGACCAAAGCTTAAACCAGA- ACC
RcWRI2-5	AB774160/ #30114	CCGTGGATCCAATGCCGTACCA- ATTTCGA
RcWRI2-3		TTGACCATGGATAATCCGAAATC- GCACAGGAAC
RcWRI3-5	AB774161/ #30131	CCTTGGATCCTACGCCACTCAAG- AGGA
RcWRI3-3		GCAAGGATATAAGCTTATTTGCG- CGAG
RcABI3-5	AB774162/ #30204	GCTTGGATCCGATAATGGTGTGC- CAGCAAC
RcABI3-3		TGTAGTCGACTATGAATCTTCT- TGGAGT
RcBCCP2-5	AB774163/ #29630	AATGGCATCGACATCGGTTCCCTTG
RcBCCP2-3		TTCACAAATGGGGGTTACCAG- GT
RcKAS1-5	AB774164/ #29693	GTGTGTCACCTGAGGAGGTTAACT
RcKAS1-3		GGAAGTGATGTCGATGACTCTGTC
RcSUS2-5	AB774165/ #29986	TATCGCTACATAGCCGACAGGAA- G
RcSUS2-3		ACATGCCAGACACTGACTGGAG- TT
Ricin-5	X03179/ #60629	TTAGCAGCGACATCAGGGAACA- GT
Ricin-3		GCAGGACATACACTGCAAGA- GA
RcOLE1-5	AY360218/ #29917	AGCTACTGACAATGGCTGATCGTC
RcOLE1-3		CCGAAGCAGACCCCA- CAAACAA
RcActin-5	AY360221/ #30206	TACATGTTACCACCCTGCGGAA
RcActin-3		GAAGCACTTCTGTGGACAATGG- A

Numbers of Gene indexes were referred for Pytozome ver.9.

involved in seed development, specific peptide epitope sequences were designed as follows; H₂N-CVSKYRQVARRHHNGRWEAR-COOH for WRI1, H₂N-YHMLSAHSPHSLFNVASC-COOH for ABI3 and H₂N-ENGQLKLALADLERRRQQC-COOH for ABI5, which appeared to be highly conserved amino acid sequences present in orthologs of WRI1, ABI3 and ABI5 among various higher plants, respectively. The anti peptide specific polyclonal antibodies were raised in rabbits with those synthetic oligopeptide antigens (Sigma-Aldrich Co., St. Louis, USA) [25]. After SDS-polyacrylamide electrophoresis (SDS-PAGE), separated poly-

peptides were electrotransferred onto a PVDF membrane at 10 V·cm⁻¹ for 120 min in 25 mM Tris-base containing 0.05% SDS and 20% methanol) and then the PVDF membrane (Millipore) was blocked by incubating in 1 × TBS (25 mM Tris-HCl [pH 7.4], 150 mM NaCl) containing 3% skim milk (TBS-milk) for 60 min. The PVDF membrane was incubated with an appropriate antibody (anti-WRI1 antibody, anti-ABI3 antibody or ABI5 antibody) (one to 3000 dilution [v/v]) in TBS-milk containing 0.05% Tween 20 at room temperature for 2 hours. Subsequently, the PVDF membrane was incubated at room temperature for 60 min in TBS containing horseradish peroxidase (HRP)-conjugated protein G (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) (one to 10,000 dilution [v/v]), and 0.05% Tween 20. The immunodecorated protein bands were visualized by an ECL Plus kit (GE Healthcare Bio-Sciences) and FluorChem Imaging Analyzer.

3. Results

3.1. Phylogenetic Analysis of WRI1-Related Transcription Factor Genes

A phylogenetic tree of WRI1 homologs in dicots and monocots, showed that WRI1-related genes can be classified into two subfamilies (**Figure 1(a)**). The phylogenetic tree of WRI1 homologs and AP2 homologs indicates that the castor bean WRI1-related genes formed a distant group from other AP2-related genes including *AtERF1*, *AtDREB1A* and *RAV1* (**Figure 1(a)**). The *RcWRI1,2,3* and other WRI1 orthologs share a very similar domain structure, composing of two AP2-type domains, as expected. Closely associated to the WRI1 homolog clade, *AtANT* also contains two AP2 type domains, but appeared to be involved in floral development rather than seed storage regulation [28]. *RcWRI1* encodes have a 49.3 kDa peptide relatively larger than *RcWRI2* (37.3 kDa, 327 aa) and *RcWRI3* (36.6 kDa, 330 aa). The predicted amino acid sequences of *RcWRI1*, *RcWRI2* and *RcWRI3* have similarities of 49%, 43% and 45%, respectively, to that of *AtWRI1*. Comparison among AP2/ERF type transcription families indicates that the WRI1 orthologs forms an unique subfamily in the amino acid sequence similarity and the domain structural similarity.

3.2. The Expression Profiles of Seed Development-Related Transcription Factor Genes

A significant signal of *RcWRI1* was detected specifically in filling seeds (2, 3.5 cm diameters) and weakly in matured seed, while the expression levels of *RcWRI1* were not or marginal in leaf and pod tissues at 32 cycles (**Figure 1(b)**). *RcWRI2* was detected at 35 cycles in seed

specifically as like *RcWRI1*, but the expression levels were weaker than those of *RcWRI1*, accordingly to the cycle numbers. In contrast, *RcWRI3* were detected at the highest level in leaf, and weakly in filling-seeds, but not in pod at 35 cycles (**Figure 1(b)**). The relative signals of *RcABI3* was expressed in filling seed-specific manner as same as *RcWRI1* (**Figure 1(b)**).

3.3. The Expression Profiles of FAS-Related Genes

A metabolic cascade of FAS in oil storage tissue in *Arabidopsis* seed indicates that sucrose synthase (SUS) in cytosol plays an important role in cleavage of sucrose into UDP-glucose and fructose cytosol for subsequent FAS in plastids [14]. Among a set of plastid enzymes involved in FAS, a set of genes including biotin carboxyl carrier protein (BCCP) and ketoacyl-acyl carrier protein synthase (KAS) appeared to be regulated transcriptionally by *WRI1* [9]. We focused on castor bean orthologs of *SUS2*, *BCCP2* and *KAS1* to examine whether those FAS-related genes and *RcWRI1* are coexpressed in developing seed specific manner. Among castor

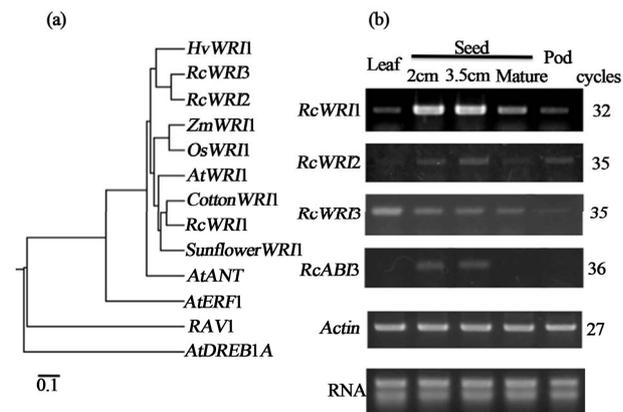


Figure 1. Wrinkled 1 homologs and a B3-type transcription factor in various organs of castor bean plant. (a) A phylogenetic tree of *WRI1* homologs and AP2/ERF transcription factors from castor bean and other higher plants was aligned by ClustalW (UPGMA). The genes used were follows: *RcWRI1*, 2, 3 (Table 1); *AtWRI1*, At3g54320; *AtDREB1A*, At4g25480; *AtERF1*; At3g23240; *AtANT*(AINTGUMENTA), At4g37750; *RAV1*, At1g13260; Cotton-*WRI1*, Gorai.011G225700; *HvWRI1*, TC250449; *OsWRI1*, Os12g0126300; *SunflowerWRI1*, TC52295; *ZnWRI1*, GRMZM2G124524. Gene index numbers and tentative consensus (TC) numbers were referred to Phytozome ver 9.0 (<http://www.phytozome.net/>) and TGI database (<http://compbio.dfci.harvard.edu/tgi/plant.html>), respectively. (b) The expression levels of mRNAs of castor bean *WRI1* homologs and an *ABI3* homolog in various organs are shown. Semi-quantitative RT-PCR was carried out at indicated numbers of thermal cycles as described in Materials and Methods. *RcActin* was used as a standard.

bean homolog genes, *RcSUS2*, *RcBCCP2* and *RcKAS1* were identified to have the highest similarities of 75%, 49% and 85% to *AtSUS2* (At5g49190), *AtBCCP2* (At5g15530) and *AtKAS1* (At5g46290), respectively.

Significant signal of *RcSUS2* were detected broadly in leaf, filling seeds (2, 3.5 cm diameters), matured seed and pod tissues at 27 cycles (**Figure 2**). *RcBCCP2* was expressed in developing seed at relatively high levels, while the expression was weak in leaf and pod but marginal in matured seed. *RcKAS1* was also expressed in developing seeds (2, 3.5 cm diameters), while the expression was weak or marginal in leaf, matured seed and pod. In contrast to *RcBCCP2* and *RcKAS1*, *Oleosin* involved in oil body formation were detected at high level both in developing seeds and matured seed at 24 cycles (**Figure 2**). Significant signals of *Ricin* was detected at 27 cycles in developing seed-specific manner as same as *RcBCCP2* and *RcKAS1* (**Figure 2**).

3.4. Immunochemical Detection of Seed-Specific Transcription Factors in Various Organs of Castor Bean

Conserved amino acid sequence motives of *WRI1* is shown with alignments of AP2/ERF-related proteins (**Figure 3(a)**). Alignments of the second AP2-type domains among *WRI1* homologs revealed a highly conserved motif of 19 amino acids (VSKYRGVVARHHHNGRWEAR) that is specific to *WRI1* orthologs but not to other AP2/ERF with single AP2-domain (**Figure 3(a)**). *AtANT* has different 6 amino acids in the region of the 19 amino acid motif for *WRI1* specific epitope. Specificities of the antibodies were con-

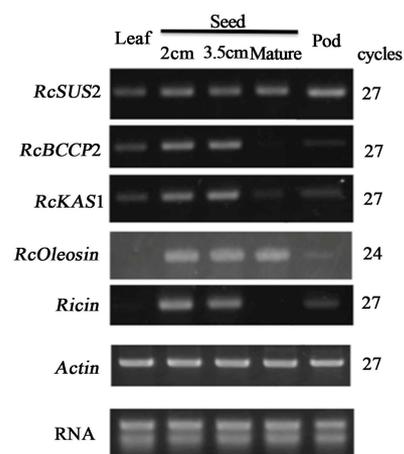


Figure 2. The expression profiles of FAS-related and seed storage protein genes. The expression levels of *SUS2*, *BCCP*, *KAS* *Oleosin* and *Ricin* in various organs were analyzed by semi-quantitative RT-PCR at indicated numbers of thermal cycles with the specific primer sets (Table 1). *RcActin* was used as a standard.

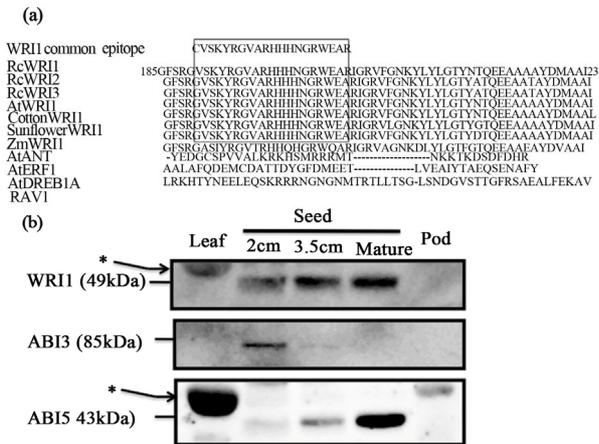


Figure 3. Immunoblot of WRI1-related polypeptides and seed specific transcription factor-related polypeptides in various organs of castor bean. (a) Alignments of deduced amino acid sequences of WRI1 homologs and other AP2/ERF homologs. A highly conserved amino acid motif (VSKYRGVARHHHNGRWEAR) present in the second AP2-type domain was used as an epitope for raising anti-WRI1 specific antibody. (b) Immunoblot of tissue extracts prepared from leaf, developing seed, mature seed and pod of castor bean plant was carried out by anti-WRI1 antibody, anti-ABI3 antibody and anti-ABI5 antibody. Each 50 µg protein per lane was subjected to SDS-PAGE (12.5% acrylamide gels) and immunoblot with specific antibodies as indicated. (*) indicates nonspecific signal of RuBisCO protein.

firmly by immunoblot using recombinant proteins of castor bean WRI1, ABI3 and ABI5 homologs (data not shown).

Immunoblot with the anti-WRI1 antibody showed a significant signal in filling seeds and the matured seed with a relative molecular mass of about 50 kDa (Figure 3(b)). Weak signals with the antibody were also detected in leaf at about 55 kDa, indicating a nonspecific signal possibly derived from RuBisCO large subunit. Immunoreactive signals with anti-ABI3 antibody were detected significantly in 2 cm seed, but not or marginally in leaf, 3.5 cm seed, matured seed and pod. In contrast to the anti-ABI3 antibody, immunoreactive signals with anti-ABI5 antibody were detected significantly in matured seed, weakly in 3.5 cm seed but not or marginally in leaf, 2 cm seed and pod.

4. Discussion

The castor bean WRI1 homologs formed a monophyletic cluster separated from other clusters of AP2/ERF group, *AtERF1*, *RAV1* and *AtDREB1A*. The phylogenetic tree or WRI1 homologs indicates that WRI1 cluster have three clade. *RcWRI1* and *AtWRI1* belong to one clade of the WRI1 cluster and has the highest similarity (62%) in amino acid sequence to that of cotton WRI1 homolog, and

relatively high similarity (66%) in amino acid sequences to those of *AtWRI1*. Other two castor bean WRI1 homologs, *RcWRI2* and *RcWRI3*, belong to another clade of WRI1 cluster, and have similarity (%) in amino acid sequences to those of *HvWRI1*. It is possible that *RcWRI1* belonging to the same clade of *AtWRI1* and *ZmWRI1* plays an important role in seed specific expression of FAS genes, because *AtWRI1* and *ZmWRI1* appeared to promote the master regulation of a set of FAS-related genes [8,18]. It was reported that *AtWRI1* binds to a specific sequence motif, AW-box, in the promoter regions of *BCCP2* and *KAS1* and other FAS genes [9]. According to castor bean genome sequence data base, the 5'-promoter regions within 300 bp from starting Met of *RcBCCP2* and *RcKAS1* genes possess 2 and 3 boxes similar to the AW-box consensus motif, respectively. It is reasonable that *RcWRI1* is expressed specifically in seed and that *RcWRI1* plays pivotal roles in master regulation of seed specific FAS genes via binding to AW-box as same as *AtWRI1* does. In contrast to *RcWRI1* and *RcWRI2*, *RcWRI3* is expressed mainly in leaf but not reproductive organs, seed and pods, suggesting that *RcWRI3* regulates FAS genes involved in membrane lipid synthesis in vegetative organs such as, glycerolipids (DGDG, MGDG and SQDG) in thylakoid apparatus indispensable for photosynthesis.

RT-PCR and immunoblot indicates that mRNA and protein of *RcABI3* are expressed specifically in developing seed, suggesting that a network of B3 type transcription factors play pivotal roles in regulation of seed development and seed storage proteins in castor bean as same as in *Arabidopsis*. Furthermore, the observation that a significant signal of ABI5-related polypeptide is detected specifically in matured seed is consistent with previous studies that ABI5 is required for establishing and maintaining seed dormancy [11].

The present study revealed a line of evidence about seed specific transcription factors and FAS-related genes in castor bean: 1) Among WRI1 homologs in castor bean, *RcWRI1* mRNA is expressed specifically in developing seed.; 2) The *RcABI3* and ABI5-related proteins function in different stages of seed development; 3) The expression of seed specific FAS-related genes, *RcBCCP2* and *RcKAS1* possessing AW-box motives in the promoter regions, are tightly associated with those of *RcWRI1* and *RcKAS1*. These observations suggest that *RcWRI1* is one of master transcription factors possibly regulating seed specific FAS-related genes. It is necessary for clarifying direct interaction of *RcWRI1* with AW-box and transcriptional activation of *RcBCCP2* and *RcKAS1* by *RcWRI1* recombinant protein and/or chromatin-immunoprecipitation assay with anti-WRI1 antibody. While both WRI1 and ABI3-related polypeptides were

detected in developing seeds by immunoblot, it is possible that other B3-type transcription factors (including LEC2 orthologs) are required for direct regulation of *ReWRI1* expression rather than *ABI3*. It remains to be clarified whether other novel master regulation factors are involved in regulation of key steps of seed oil synthesis in addition of *WRI1-FAS* genes cascade.

Increasing evidence suggests that the network of various transcription factors, such as AP2-, B3- and bZIP-type transcription factors regulate seed development and storage compound synthesis. Therefore, identifications and molecular application technique of the signaling molecules regulating seed development and oil synthesis are important for and improvement of vegetative oil crops.

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