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Possible crosstalk between the Arabidopsis TSPO-related protein and the transcription factor WRINKLED1

Kaushal Kumar Bhati [a], Ai My Luong [a], Franziska Dittrich-Domergue [b], Sabine D'Andrea [c], Patrick Moreau [b], Henri Batoko [a, *]

[a] Louvain Institute of Biomolecular Science and Technology (LIBST), University of Louvain, Croix du Sud 4-5, L7.07.14, 1348, Louvain-la-Neuve, Belgium
[b] CNRS, University of Bordeaux, Laboratoire de Biogenèse Membranaire, UMR 5200, 33140, Villenave d'Ornon, France
[c] Institut Jean-Pierre Bourgin, INRAE, AgroParisTech, Université Paris-Saclay, 78000, Versailles, France

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ABSTRACT

This study uncovers a regulatory interplay between WRINKLED1 (WRI1), a master transcription factor for glycolysis and lipid biosynthesis, and Translocator Protein (TSPO) expression in *Arabidopsis thaliana* seeds. We identified potential WRI1-responsive elements upstream of AtTSPO through bioinformatics, suggesting WRI1's involvement in regulating TSPO expression. Our analyses showed a significant reduction in AtTSPO levels in *wri1* mutant seeds compared to wild type, establishing a functional link between WRI1 and TSPO. This connection extends to the coordination of seed development and lipid metabolism, with both WRI1 and AtTSPO levels decreasing post-imbibition, indicating their roles in seed physiology. Further investigations into TSPO's impact on fatty acid synthesis revealed that TSPO misexpression alters WRI1's post-translational modifications and significantly enhances seed oil content. Additionally, we noted a decrease in key reserve proteins, including 12 S globulin and oleosin 1, in seeds with TSPO misexpression, suggesting a novel energy storage strategy in these lines. Our findings reveal a sophisticated network involving WRI1 and AtTSPO, highlighting their crucial contributions to seed development, lipid metabolism, and the modulation of energy storage mechanisms in Arabidopsis.
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1. Introduction

The Translocator protein (TSPO) is a membrane-bound protein found in various cell types, including plants [1,2]. While its exact functions are still not completely understood, several studies suggest that TSPO has diverse roles in different cell types, particularly in response to stressful conditions. TSPO-related proteins are induced in plant vegetative tissues in response to various abiotic stresses and the stress hormone abscisic acid (ABA) [3,4]. In *Arabidopsis thaliana* vegetative tissues, the transiently expressed TSPO (AtTSPO) becomes undetectable approximately 48 h after induction, even with continuous exposure to ABA, indicating potential harm to plant cells if TSPO remains consistently present [3–6]. Notably, Golgi and endoplasmic reticulum-localized TSPO are crucial in detoxifying free heme molecules in the cytoplasm [5]. During periods of stress, increased heme metabolism is harnessed by enzymes responsible for detoxifying reactive oxygen species (ROS). These enzymes get their heme prosthetic group from the cytosol. The Arabidopsis AtTSPO forms a non-covalent complex with heme, which is later degraded through selective autophagy in the vacuole [5]. This process effectively shields the cell from the cytotoxicity associated with unbound heme molecules. Additionally, under osmotic stress conditions, AtTSPO regulates water loss by modulating the expression of aquaporins in the plasma membrane. Notably, AtTSPO physically interacts with aquaporin PIP2;7 at the Golgi apparatus, and this interaction necessitates the presence of the plant-specific N-terminal extension of the AtTSPO protein [6]. Ultimately, this interaction results in complex degradation within the vacuole through the process of autophagy. However, in dehydrated Arabidopsis seed tissues, AtTSPO is readily detectable, likely due to the enrichment of ABA in these desiccated tissues [3,7]. This complexity is further compounded by the biphasic accumulation of ABA during seed development [8]. In addition, AtTSPO undergoes rapid degradation during seed imbibition (water uptake) and the subsequent utilization of reserves by the developing seedling [3,7]. Remarkably, AtTSPO becomes undetectable within just 48 h of Arabidopsis seed imbibition. However, the regulatory mechanisms governing plant TSPO expression

* Corresponding author.
E-mail address: henri.batoko@uclouvain.be (H. Batoko).

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in developing seeds, particularly those rich in energy reserves like fatty acids and proteins, remain unclear (see Table 1).

Recent research showed that constitutive expression of AtTSPO in transgenic Arabidopsis seeds leads to an increase in triacylglycerol content, implying that AtTSPO presence during seed filling might influence oil reserve accumulation [7]. WRINKLED1 (WRI1), a pivotal transcription factor in *Arabidopsis thaliana*, orchestrates the expression of numerous genes crucial for fatty acid biosynthesis and glycolysis throughout seed development [9–12]. WRI1 is instrumental in rerouting carbon from carbohydrate metabolism to fatty acid synthesis, a process vital for efficient energy and carbon allocation for oil storage during seed development [9,13]. Mutations in the WRI1 gene result in a wrinkled seed phenotype characterized by reduced oil content and altered seed composition, highlighting its essential role in lipid metabolism [9,13]. WRI1, a member of the AP2/EREBP family of transcription factors, predominantly binds to the so-called AW-box within the promoter regions of its target genes, featuring the consensus sequence [C/T]NNG[A/T]N[C/T][C/T], where N represents any nucleotide [10,14]. This motif is key to the regulation of crucial lipid metabolism genes, though the regulatory impact of WRI1 on these genes is subject to a range of factors, underscoring the importance of experimental validation to confirm these regulatory relationships. WRI1 expression is tightly controlled during seed development, peaking during the phases of active oil accumulation [10,15]. Early to mid-seed development stages see a surge in WRI1 expression, facilitating the upregulation of target genes. However, as seed maturation advances, the expression levels of WRI1 and its target genes decline, marking a decrease in lipid biosynthesis as the seed undergoes desiccation [13]. The regulation of WRI1 is multifaceted, encompassing transcriptional and post-translational mechanisms [12,16–18]. Post-translational modifications (PTMs) of WRI1, including phosphorylation, ubiquitination, and potentially proteolytic processing, play critical roles in modulating its stability [12,17]. These PTMs ensure that WRI1 activity is precisely adjusted in response to the plant's metabolic demands, environmental stressors, and developmental cues.

A bioinformatic examination of the approximately 500 bp intergenic region encompassing the *AtTSPO* promoter identified among other known *cis*-regulatory elements a putative AW-box, hinting at potential regulation of AtTSPO expression by WRI1. In this report, our findings reveal that AtTSPO expression is downregulated in the seeds of the *wri1-4* mutant and that WRI1 expression is differentially regulated in an *AtTSPO* knockout context. This points to a possible bidirectional regulatory relationship between AtTSPO and WRI1, highlighting the possible involvement of AtTSPO in the intricate network of gene regulation underpinning seed oil accumulation.

2. Materials and methods

2.1. Plant material and cultivation

We used standard practices to grow *Arabidopsis thaliana* plants, and the KO (*tspo-1*) and transgenic lines *35S:AtTSPO* (OE3 and OE8) lines were described previously [3,7]. Seedlings were propagated on half-strength Murashige and Skoog (MS) medium, containing 0.8 % agar and 1 % sucrose. Arabidopsis seeds, including the *wri1-4* mutant (Salk_008559C obtained from the Arabidopsis Biological Resource Center [ABRC]), underwent sterilization with 70 % ethanol and 0.01 % Silwet, followed by rinsing in sterile distilled water before being sown on the prepared MS medium.

2.2. Promoter analysis for AW-Box detection

The promoter sequences (~400 bp upstream of the ATG start codon) and amino acid sequences of TSPO genes from six oil crop species were retrieved from the Phytozome database (https://phytozome.jgi.doe.gov/) and Ensembl Genomes platform. The following loci were used in the analyses: AT2G47770 (AtTSPO - *Arabidopsis thaliana*); AL4G47340 (AlTSPO- *Arabidopsis lyrate*); Cagra.0551s0032 (*Capsella grandiflora*); Lus10010281, Lus10000500 and Lus10037513 (*Linum usitatissimum*). The sequences of the following loci: Csa06g054300, Csa05g001400 and Csa04g067860 from Camelina sativa; BnaA04g29550D, BnaC04g51120D, BnaA05g00220D and BnaC04g00110D from Brassica napus were retrieved from Ensembl Genomes platform. The consensus sequence 5′-CNTNG(N)7CG-3' [10] was used to screen for putative AW-box motifs via the Find Individual Motif Occurrences (FIMO) tool [19], examining both strands of DNA and allowing for overlapping motif hits. The significance of motif occurrences was determined by p-value and q-value analyses. Motif representation was created using the MEME suite [20].

2.3. Phylogenetic relationship assessment

Using MEGA X [21], we constructed protein alignments and phylogenetic trees to explore the relationships between TSPO orthologs across the specified crop species. Unrooted neighbor-joining (NJ) trees were produced with default settings, including p-distance correction and bootstrap testing (1000 replicates).

2.4. Construction of genetic vectors and transformation

The nucleotide sequence for the *AtFAE1* promoter (Rossak et al., 2001) was retrieved from TAIR database. The corresponding genetic constructs *pFAE1:AtTSPO* and *pFAE1:CsTSPO* were synthesized by GenScript (GenScript, Piscataway NJ, http://www.genscript.com/) and subcloned into the plant transformation vector expression pPZP-RSC2-hpt1 using I-SceI enzyme [22,23]. The resulting plasmids were checked by restriction and further sequencing and then introduced into Agrobacterium tumefaciens (GV3101pMP 90) for the transformation of *Arabidopsis thaliana* Col-0 using standard protocol. Selection of T1 seeds was conducted on MS media supplemented with hygromycin, and genomic DNA from resistant plants was PCR-screened using the set of specific primers (Hyg-F: CAAGACCTGCCTGAAACCGAACTG and Hyg-R: CTCCAGTCAATGACCGCTGTTATGC) for hygromycin resistance. The Arabidopsis transgenic lines were self-fertilized up to the T3 generation.

2.5. Fatty acid content analysis

Seeds of Arabidopsis (100) were weighed on a precision balance, transferred to a 7 ml screw-capped glass tube, and mixed with 2 ml methanol and 100 μl H2SO4 (% final, v/v). In addition, 50 mg of heptadecanoic acid (C17:0) was added as an internal standard. Fatty Acid transmethylation was performed by heating to 95 °C for 1h30 in a dry bath. After the reaction, the samples were cooled and 1 ml of hexane (99 %) and 2 ml of NaCl (0.9 %) solution were added to extract fatty acid methyl esters (FAMEs). The samples were centrifuged at 500 g for 5 min at RT and 500 μl of the hexane phase was collected and transferred directly to GC tubes. Analyses of FAMEs were performed as described previously [24] using an Agilent gas chromatograph (Hewlett-Packard 5890 series II; Agilent) on a 15-m 3 0.53-mm 3 1.2-mm Carbowax column (Alltech) with flame ionization detection. The oven temperature was programmed to 150 °C for 1 min, followed by a ramp of 5 °C min to 230 °C, and held at this temperature (total run time, 30 min). FAMEs

Table 1
Analysis of the AW-box presence in the promoter regions of TSPO genes across six crop species. This table elucidates the detection of the putative AW-box within the 400 bp region upstream of TSPO genes in six crop species, employing a statistical framework. The P-value associated with each motif occurrence signifies the likelihood that a sequence of equivalent length, if randomly generated, would match the specific sequence position with a score equal to or surpass the observed. This score is derived by aggregating relevant values from the position-dependent scoring matrix tailored to the motif. Furthermore, the q-value represents the estimated false discovery rate, assuming the motif's occurrence is deemed significant. Data are methodically organized in ascending order of P-value, as Grant et al. (2011) detailed, facilitating a clear understanding of motif significance and distribution.

Species	Motif number	Motif alt id	sequence name	start	stop	strand	score	p-value	q-value	Matched sequence
A. lyrata	1	CNTNGNNNNNNNCG	AL4G47340	−173	−160	−	4.86667	0.00305	1	CGTGGCCAGTGATG
	2	CNTNGNNNNNNNCG	AL4G47340	−173	−160	+	4.86667	0.00305	1	CATCACTGGCCACG
	3	CNTNGNNNNNNNCG	AL4G47340	−161	−148	−	4.61212	0.00562	1	GGTGGAGAGACACG
	4	CNTNGNNNNNNNCG	AL4G47340	−140	−127	+	4.61212	0.00562	1	CCTTGGCCTCTTTG
	5	CNTNGNNNNNNNCG	AL4G47340	−87	−74	−	4.35758	0.00855	1	CTTGGTCAGAAGAG
	6	CNTNGNNNNNNNCG	AL4G47340	−301	−288	−	4.10303	0.0108	1	TTTGGACGATTACG
	7	CNTNGNNNNNNNCG	AL4G47340	−304	−291	+	4.10303	0.0108	1	CATCGTAATCGTCC
	8	CNTNGNNNNNNNCG	AL4G47340	−343	−330	+	3.84848	0.012	1	TATTGTCTTAGTCG
A. thaliana	1	CNTNGNNNNNNNCG	AT2G47770	−366	−353	+	4.35758	0.00855	1	CTTAGTCGGTGTAG
	2	CNTNGNNNNNNNCG	AT2G47770	−293	−280	+	4.35758	0.00855	1	CATCGCAATCGTCC
	3	CNTNGNNNNNNNCG	AT2G47770	−144	−131	+	4.35758	0.00855	1	CCTTGGCCTTTTTG
	4	CNTNGNNNNNNNCG	AT2G47770	−89	−76	−	4.10303	0.0108	1	CTTGGTCAAAAGAG
	5	CNTNGNNNNNNNCG	AT2G47770	−372	−359	+	4.10303	0.0108	1	TCTTGTCTTAGTCG
	6	CNTNGNNNNNNNCG	AT2G47770	−188	−175	+	4.10303	0.0108	1	ATTAGCATTCGACG
B. napus	1	CNTNGNNNNNNNCG	BnaA04g29550D	−185	−172	−	9.86061	0.000454	1	CCTTGACAGAGACG
	2	CNTNGNNNNNNNCG	BnaA04g29550D	−197	−184	+	4.63636	0.00354	1	CCACGTGTATCACG
	3	CNTNGNNNNNNNCG	BnaA04g29550D	−339	−326	−	4.61212	0.00562	1	CCTCGCATCTCACA
	4	CNTNGNNNNNNNCG	BnaA04g29550D	−305	−292	−	4.61212	0.00562	1	CATGGCATACCCAG
	5	CNTNGNNNNNNNCG	BnaA04g29550D	−361	−348	+	4.61212	0.00562	1	CCTCAAGTGTCTCG
	6	CNTNGNNNNNNNCG	BnaA04g29550D	−206	−193	+	4.35758	0.00855	1	ATTGGCATTCCACG
	7	CNTNGNNNNNNNCG	BnaA04g29550D	−342	−329	+	4.10303	0.0108	1	TTTTGTGAGATGCG
	8	CNTNGNNNNNNNCG	BnaA04g29550D	−251	−238	+	4.10303	0.0108	1	CTTTCAGAGAGTCG
	9	CNTNGNNNNNNNCG	BnaA04g29550D	−33	−20	+	4.10303	0.0108	1	CTTAGTATCCTCCT
	1	CNTNGNNNNNNNCG	BnaA05g00220D	−337	−324	−	5.4	0.000811	1	CGACGACTGGGGCG
	2	CNTNGNNNNNNNCG	BnaA05g00220D	−307	−294	+	5.14545	0.00111	1	CTGCGTACGCCGCG
	3	CNTNGNNNNNNNCG	BnaA05g00220D	−304	−291	−	4.86667	0.00305	1	TTTCGCGGCGTACG
	4	CNTNGNNNNNNNCG	BnaA05g00220D	−340	−327	+	4.86667	0.00305	1	AATCGCCCCAGTCG
	5	CNTNGNNNNNNNCG	BnaA05g00220D	−325	−312	−	4.35758	0.00855	1	CTTTACGACAGACG
	6	CNTNGNNNNNNNCG	BnaA05g00220D	−131	−118	−	4.35758	0.00855	1	AGTTGACACAGACG
	7	CNTNGNNNNNNNCG	BnaA05g00220D	−109	−96	−	4.35758	0.00855	1	GTTGGAGAGACACG
	8	CNTNGNNNNNNNCG	BnaA05g00220D	−295	−282	−	4.10303	0.0108	1	AGTTGTCTGTTTCG
	9	CNTNGNNNNNNNCG	BnaA05g00220D	−249	−236	+	4.10303	0.0108	1	CATAGTAAAGCCCA
	1	CNTNGNNNNNNNCG	BnaC04g00110D	−268	−255	+	5.14545	0.00111	1	CTGCGTACGCCGCG
	2	CNTNGNNNNNNNCG	BnaC04g00110D	−265	−252	−	4.86667	0.00305	1	TTTCGCGGCGTACG
	3	CNTNGNNNNNNNCG	BnaC04g00110D	−141	−128	+	4.63636	0.00354	1	CCACGTGTACAACG
	4	CNTNGNNNNNNNCG	BnaC04g00110D	−298	−285	−	4.61212	0.00562	1	AGTAGACTGGTGCG
	5	CNTNGNNNNNNNCG	BnaC04g00110D	−73	−60	+	4.61212	0.00562	1	ACTTGAGCTCCTCG
	6	CNTNGNNNNNNNCG	BnaC04g00110D	−141	−128	−	4.35758	0.00855	1	CGTTGTACACGTGG
	7	CNTNGNNNNNNNCG	BnaC04g00110D	−129	−116	−	4.35758	0.00855	1	AGTTGACACAGACG
	8	CNTNGNNNNNNNCG	BnaC04g00110D	−256	−243	−	4.10303	0.0108	1	AGTTGTCTGTTTCG
	9	CNTNGNNNNNNNCG	BnaC04g00110D	−106	−93	−	4.10303	0.0108	1	TTTGGATAGACACG
	10	CNTNGNNNNNNNCG	BnaC04g00110D	−64	−51	+	4.10303	0.0108	1	CCTCGTTATTCTCT
	11	CNTNGNNNNNNNCG	BnaC04g00110D	−61	−48	−	3.87273	0.0109	1	CAAAGAGAATAACG
	12	CNTNGNNNNNNNCG	BnaC04g00110D	−238	−225	+	3.84848	0.012	1	CTTAGTTTTACCCC
	1	CNTNGNNNNNNNCG	BnaC04g51120D	−319	−306	+	9.60606	0.000604	1	CATCGTAATCGTCG
	2	CNTNGNNNNNNNCG	BnaC04g51120D	−380	−367	+	5.12121	0.00151	1	CTTCGCCCGCTCCC
	3	CNTNGNNNNNNNCG	BnaC04g51120D	−179	−166	−	4.35758	0.00855	1	CCTTGACAGAGATG
	4	CNTNGNNNNNNNCG	BnaC04g51120D	−200	−187	+	4.35758	0.00855	1	ATTCGCATTCCACG
	5	CNTNGNNNNNNNCG	BnaC04g51120D	−316	−303	−	4.10303	0.0108	1	TTTCGACGATTACG
	6	CNTNGNNNNNNNCG	BnaC04g51120D	−299	−286	−	4.10303	0.0108	1	CATGGCATAGAAAG
	7	CNTNGNNNNNNNCG	BnaC04g51120D	−245	−232	+	4.10303	0.0108	1	CTTTCAGAGAGTCG
	8	CNTNGNNNNNNNCG	BnaC04g51120D	−29	−16	+	4.10303	0.0108	1	CTTAGTATCCTCCT
	9	CNTNGNNNNNNNCG	BnaC04g51120D	−377	−364	−	10.8788	6.53e-06	0.0658	CGTGGGAGCGGGCG
C. grandiflora	1	CNTNGNNNNNNNCG	Cagra.0551s0032	−172	−159	−	4.35758	0.00855	1	CATGGAGAGAGACA
	2	CNTNGNNNNNNNCG	Cagra.0551s0032	−345	−332	+	4.35758	0.00855	1	CGTCGTCGTAATCA
	3	CNTNGNNNNNNNCG	Cagra.0551s0032	−190	−177	+	4.35758	0.00855	1	CCTTGTCTTCTGCC
	4	CNTNGNNNNNNNCG	Cagra.0551s0032	−342	−329	+	4.10303	0.0108	1	CGTCGTAATCATCA
	5	CNTNGNNNNNNNCG	Cagra.0551s0032	−229	−216	+	4.10303	0.0108	1	ATTAGCATTCCACG
	6	CNTNGNNNNNNNCG	Cagra.0551s0032	−85	−72	+	4.10303	0.0108	1	CCTTGTCATCATCA
	7	CNTNGNNNNNNNCG	Cagra.0551s0032	−38	−25	+	3.84848	0.012	1	TTTGGTTCTTTTCG
C. sativa	1	CNTNGNNNNNNNCG	Csa04g067860	−225	−212	+	4.61212	0.00562	1	ATTGGCGTTCCACG
	2	CNTNGNNNNNNNCG	Csa04g067860	−167	−154	−	4.10303	0.0108	1	CATGGAGATAGACA
	1	CNTNGNNNNNNNCG	Csa05g001400	−351	−338	+	4.89091	0.00191	1	CGAAGAGCCAGTCG
	2	CNTNGNNNNNNNCG	Csa05g001400	−396	−383	−	4.61212	0.00562	1	CCTCAGATCTGACG
	3	CNTNGNNNNNNNCG	Csa05g001400	−381	−368	−	4.35758	0.00855	1	CTTGGCTGGTTTCT
	4	CNTNGNNNNNNNCG	Csa05g001400	−149	−136	−	4.35758	0.00855	1	CATGGAGAGAGACA
	5	CNTNGNNNNNNNCG	Csa05g001400	−41	−28	+	3.84848	0.012	1	TCTTGTTTCTTTCG
	1	CNTNGNNNNNNNCG	Csa06g054300	−225	−212	+	4.61212	0.00562	1	ATTGGCGTTCCACG

(*continued on next page*)

Table 1 (*continued*)

Species	Motif number	Motif alt id	sequence name	start	stop	strand	score	p-value	q-value	Matched sequence
	2	CNTNGNNNNNNNCG	Csa06g054300	−168	−155	−	4.10303	0.0108	1	CATGGAGATAGACA
	3	CNTNGNNNNNNNCG	Csa06g054300	−251	−238	−	3.84848	0.012	1	CTTTTTCTTTTGCG
L. usitatissimum	1	CNTNGNNNNNNNCG	Lus10000500	−283	−270	−	4.63636	0.00354	1	CCATGTTTCCACCG
	2	CNTNGNNNNNNNCG	Lus10000500	−205	−192	+	4.38182	0.00602	1	CCACGATATTCACG
	3	CNTNGNNNNNNNCG	Lus10000500	−110	−97	−	4.10303	0.0108	1	CTTTGGGATTTCCT
	4	CNTNGNNNNNNNCG	Lus10000500	−113	−100	+	4.10303	0.0108	1	CATAGGAAATCCCA
	5	CNTNGNNNNNNNCG	Lus10000500	−30	−17	+	4.10303	0.0108	1	CTTACCAATCTCCG
	6	CNTNGNNNNNNNCG	Lus10000500	−66	−53	+	3.84848	0.012	1	CTTCGTCAATTTAG
	1	CNTNGNNNNNNNCG	Lus10010281	−58	−45	−	4.61212	0.00562	1	GATCGCGATGCTCG
	2	CNTNGNNNNNNNCG	Lus10010281	−31	−18	−	4.61212	0.00562	1	TGTTGTTGCCCTCG
	3	CNTNGNNNNNNNCG	Lus10010281	−63	−50	+	4.35758	0.00855	1	CATTCCGAGCATCG
	4	CNTNGNNNNNNNCG	Lus10010281	−54	−41	+	4.35758	0.00855	1	CATCGCGATCTACA
	1	CNTNGNNNNNNNCG	Lus10037513	−214	−201	−	4.63636	0.00354	1	CCATGTTTCCACCG
	2	CNTNGNNNNNNNCG	Lus10037513	−134	−121	+	4.38182	0.00602	1	CCACGATATTCACG
	3	CNTNGNNNNNNNCG	Lus10037513	−236	−223	+	4.35758	0.00855	1	TATGGTGAACTCCG
	4	CNTNGNNNNNNNCG	Lus10037513	−314	−301	−	4.10303	0.0108	1	CTTTGTTGTCGTTG
	5	CNTNGNNNNNNNCG	Lus10037513	−30	−17	+	4.10303	0.0108	1	CTTAGCATTCTCCA
	6	CNTNGNNNNNNNCG	Lus10037513	−115	−102	+	3.84848	0.012	1	CTTTCCTTCATTCG

were identified by comparison with retention times of the internal standard (C17:0) and expressed as a percentage of total FAMEs.

2.6. Protein extraction and immunoblotting

Total seed proteins were extracted for analysis via SDS-PAGE and subsequent immunoblotting, utilizing antibodies targeting AtTSPO [7], AtWRI1 (courtesy of Dr. J. Shanklin, Brookhaven National Laboratory, Upton, New York), OLE1, and 12 S [25]. In brief, protein extracts were prepared from 10 mg of seeds from each mutant/transgenic line and the wild-type control (Col-0). Seeds were homogenized in 2× NuPAGE LDS sample buffer (ThermoFisher Scientific) using glass beads in 2 ml screw-cap tubes, employing a plant tissue homogenizer for thorough disruption. The homogenized samples were incubated at 37 °C for 30 min, followed by centrifugation at 14,000 rpm for 10 min at room temperature using a benchtop centrifuge to pellet insoluble material. The clear supernatant was collected and subjected to SDS-PAGE, and proteins were then transferred to membranes for immunoblotting with the specified antibodies. The immunoblotting protocols were adapted from established procedures detailed in previous studies [3,25], which include antibody-specific dilutions and sample processing specifications.

3. Results

3.1. AtTSPO expression is significantly diminished in the wri1-4 mutant dry-seed

WRINKLED1 (WRI1), a master transcription factor, is known to enhance glycolysis and lipid biosynthesis pathways by binding to a conserved sequence known as the ASML1/WRI1 box (AW-box) located approximately 500 bp upstream of the transcription start sites of its target genes [14]. The promoter region of the Arabidopsis *TSPO* gene (At2g47770) is defined within a genomic span of fewer than 500 base pairs (bp) in the intergenic region between At2g47770 and its adjacent gene, At2g47760, which encodes an α-1,3-mannosyltransferase. While conducting a bioinformatics survey to identify putative *cis*-element regulating *AtTSPO* expression, we found two AW-box-related consensus sequences within the intergenic region proximal to the AtTSPO promoter (approximately 400 bp upstream of the ATG start codon) (Fig. 1). Considering the proposed indirect role of AtTSPO in seed lipid metabolism [7], we wondered whether such AW-box regulatory elements could be conserved in the promoter regions of TSPO genes across other Brassicaceae oilseed plants. We only depicted motifs that met a stringent P-value cutoff of <0.01 for consensus AW-box identification in Fig. 1A, with further bioinformatics analysis details provided in Supplemental Table 1. Fig. 1A–C reveal the presence of perfect consensus or closely related AW-box motifs (differing by a single nucleotide from the consensus defined by Ref. [10] within 400 bp upstream of the ATG of genes encoding *TSPO* orthologs in the analyzed plant species. This suggests a potential regulatory role of the WRI1 transcription factor over the expression of at least Brassicaceae *TSPO* genes.

To investigate our hypothesis regarding the potential relationship between WRI1 activity and TSPO expression in Arabidopsis, we employed western blot analysis using antibodies specific to the Arabidopsis WRI1 protein. We assessed TSPO protein levels in dry seeds from wild-type (WT) plants and those harboring the *wri1-4* mutant allele. This allele represents a T-DNA insertional mutation in At3g54320 (Salk_008559C, *wri1-4*), located within a large intron common to all predicted splice variants of the Arabidopsis *WRI1* gene. The mutation is associated with delayed embryo elongation, reduced seed dry weight, and a 45–55 % decrease in fatty acid content, as reported by the Arabidopsis Biological Resource Center (ABRC) and The Arabidopsis Information Resource (TAIR) (Germplasm:4515120366).

Our results revealed a significant reduction in AtTSPO levels in the mutant seeds compared to the wild type, as evidenced by decreased AtWRI1 levels in *wri1-4* seeds (Fig. 2A). This suggests a regulatory link where AtTSPO expression in dry seeds is positively influenced by functional WRI1. Further, we explored the expression patterns of WRI1 and AtTSPO during seed development via western blotting (Fig. 2B). WRI1 was detectable in extracts from immature seeds starting at 12 days post-fertilization, whereas AtTSPO was only present in mature seed extracts (Fig. 2B, lane M). This indicates that WRI1 expression during the development of Arabidopsis seeds does not directly correlate with AtTSPO expression.

Interestingly, although AtTSPO is prevalent in dry Arabidopsis seeds, it undergoes rapid degradation upon seed imbibition [7]. Concurrent monitoring of WRI1 and AtTSPO levels during Arabidopsis seed imbibition revealed that both proteins became nearly undetectable after 48 h of imbibition (Fig. 2C). To determine if AtTSPO presence affects WRI1 degradation during seed imbibition, we performed a comparative analysis using Arabidopsis knockout (KO) seeds for *AtTSPO*. In these KO seeds, WRI1 levels were markedly different from those observed in wild-type seeds (Fig. 2C and D). Following imbibition, WRI1 levels dramatically decreased within the first 3 h and then remained stable for up to 48 h (Fig. 2D),

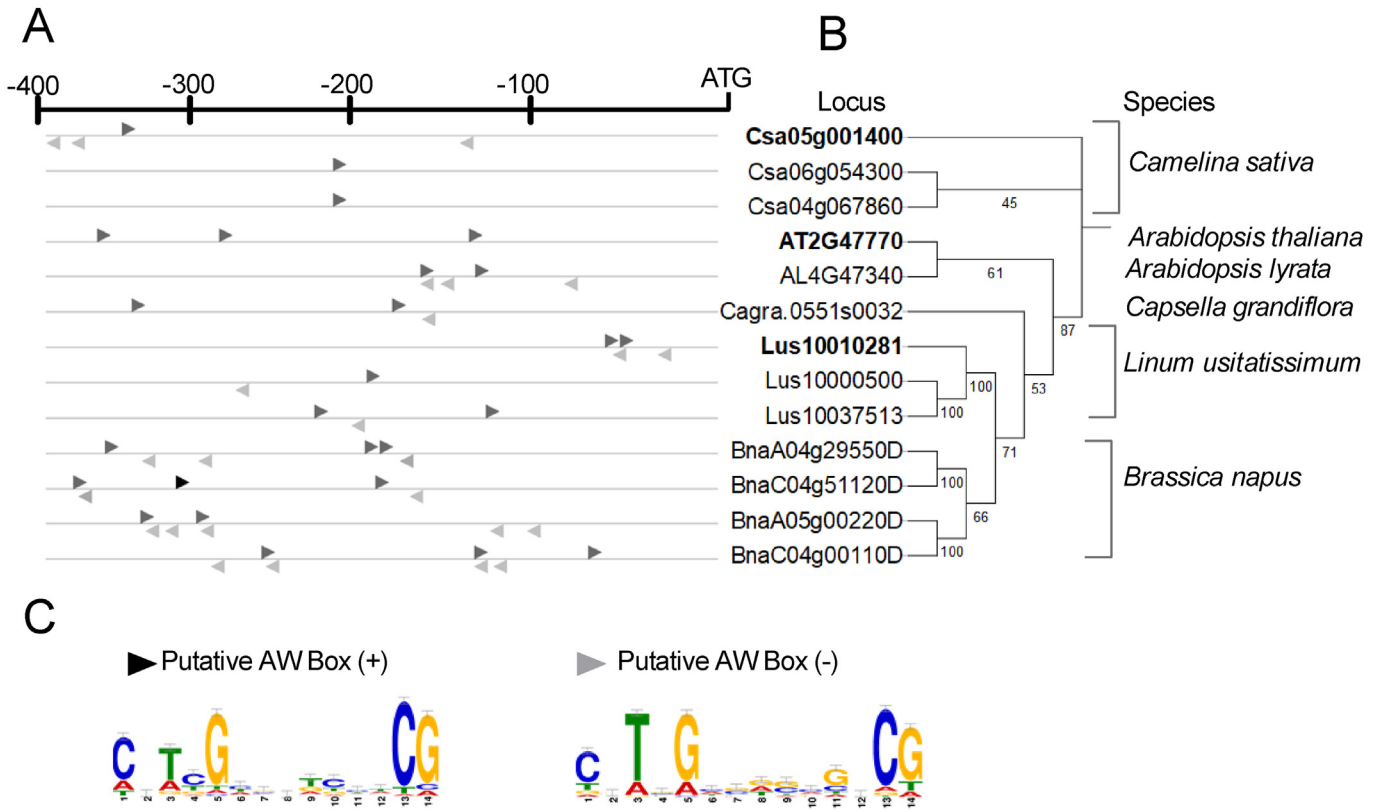


Fig. 1. Analysis of AW-Box presence in TSPO gene promoters across Brassicaceae species. (A) Examination of TSPO gene promoter regions (400 bp upstream of ATG) from various Brassicaceae species, highlighting only AW-boxes with a P-value below 0.01. Dark grey arrowheads mark AW-Box locations on the positive (+) DNA strand; light grey arrowheads on the negative (−) strand. (B) Phylogenetic comparison of TSPO proteins from selected Brassicaceae species, detailed on the right. (C) Sequence logos illustrating the conservation frequency of nucleotides with AW-Box motifs from the analyzed promoters, shown for both positive (+) and negative (−) strands.

indicating altered dynamics of WRI1 stability between imbibing wild-type and KO Arabidopsis seeds.

We concluded that this series of experiments underscores a complex interplay between WRI1 and AtTSPO in regulating seed development and response to imbibition in Arabidopsis, highlighting the potential regulatory mechanisms that influence seed physiology and lipid metabolism.

In our previous research, we demonstrated that overexpressing AtTSPO in transgenic Arabidopsis plants led to an upsurge in both triacylglycerol (TAG) and total fatty acids in the harvested dry seeds [7]. This finding prompted us to investigate if altering AtTSPO expression could influence the expression and/or functionality of WRI1, a key regulator of fatty acid biosynthesis. To explore this, we engineered transgenic Arabidopsis plants to express AtTSPO or Camelina TSPO under the control of the promoter for the Arabidopsis fatty acid elongase gene (*pFAE*). WRI1 transcriptionally regulates fatty acid elongase expression and is crucial for synthesizing long-chain (LCFAs) and very long-chain fatty acids (VLCFAs), vital components of plant membrane lipids, waxes, and storage lipids [10,13,15], highlighting the significance of WRI1 in coordinating metabolic pathways for TAG biosynthesis in oilseeds.

In our study, we systematically evaluated AtTSPO protein levels in dry seeds across several independent homozygous transgenic lines, specifically targeting lines misexpressing an additional copy of AtTSPO (At#2–5 and At#3–4) and lines misexpressing Camelina TSPO (Cs#1–2 and Cs#4–1). These were compared to wild-type (WT) and knockout (KO) Arabidopsis seeds as controls, as illustrated in Fig. 3A. Our analyses uncovered a pronounced reduction in AtTSPO levels in the transgenic lines, with decreases ranging from 40- to over 100-fold relative to WT seeds, irrespective of the TSPO variant misexpressed. Further investigation into WRI1 protein levels in dry seed extracts from these transgenic lines was conducted. Specifically, we examined seeds where AtTSPO expression was upregulated under the constitutive 35 S promoter or misexpressed under the *pFAE* promoter, with WT and KO seeds used for reference (Fig. 3B). For this analysis, two transgenic lines were selected based on their misexpression of either AtTSPO or Camelina TSPO, which resulted in relatively high (Cs#1–2) or low (At#3–4) levels (10-fold difference) of detectable AtTSPO in the dry seed (Fig. 3A). Remarkably, high molecular weight (HMW) forms of WRI1, indicative of post-translational modifications (PTMs) [12], which may suggest inactivity, were detected in all extracts except for those from lines expressing TSPO under the *pFAE* promoter's control, which showed little to no HMW forms. This finding suggests that TSPO misexpression, particularly when regulated by the *pFAE* promoter, not only reduces TSPO levels in dry seeds but might also interfere with or lessen the PTMs, such as ubiquitination, of WRI1 through a yet-to-be-determined mechanism.

To determine the potential impact of these alterations on seed oil content, we compared total fatty acids in WT seeds against those in transgenic lines expressing either Arabidopsis or Camelina TSPO. For this purpose, we compared 8 independent transgenic lines misexpressing either the Arabidopsis TSPO or the Camelina TSPO (including those studied in Fig. 3). As Fig. 4 illustrates, relative to WT seeds, total fatty acid content remained mostly unchanged in lines expressing Camelina TSPO but increased significantly - by more than 30 % - in 3 out of 4 transgenic lines misexpressing Arabidopsis TSPO. This finding suggests that despite TSPO misexpression potentially affecting WRI1's post-translational modification, such genetic alterations can enhance the oil content in

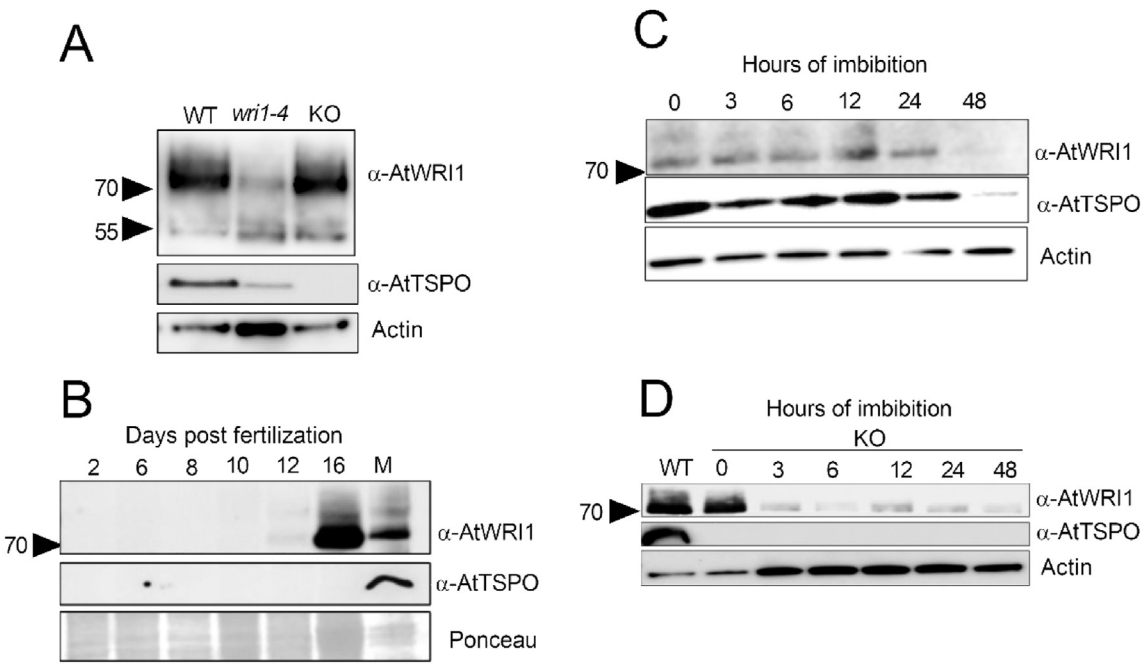


Fig. 2. WRI1-dependent regulation of AtTSPO protein levels during Arabidopsis seed development and germination. (A) Western blot analysis reveals the influence of WRI1 on AtTSPO protein abundance in Arabidopsis seeds. Protein extracts from an equal mass of dry seeds from wild type (Col-0) and the *wri1-4* mutant, with a T-DNA insertional mutation in At3g54320 were probed with specific antibodies against Arabidopsis WR1 and AtTSPO. Actin was used as a loading control. M: mature seed extract. For comparison, extracts from AtTSPO knockout (KO) seeds were included (far-right lane). (B) Temporal expression profile of WRI1 and AtTSPO during seed maturation. Protein extracts from developing pods, collected at progressive developmental stages, were subjected to western blotting. Blots were sequentially probed for WRI1 and AtTSPO. Ponceau red staining served as a loading comparison. (C, D) Dynamics of AtTSPO and WRI1 protein levels during the germination process in wild type (C) and *AtTSPO* KO (D) seeds. Proteins extracted from an equal number of seeds imbibed over a time course were analyzed. WRI1 detection is displayed in the upper blots, with corresponding AtTSPO levels in the middle blots. Actin is shown in the lower blots for normalization. Protein size markers (kDa) are indicated by the arrowheads.

harvested dry seeds.

We then expanded our analysis to include not only fatty acids but also protein reserves, by assessing the levels of 12 S globulin in seeds with altered Arabidopsis TSPO expression only (overexpression and misexpression) (Fig. 5). We used seeds from 3 independent transgenic lines, or 3 WT plants, grown and harvested simultaneously. As shown in Fig. 5A–B, we observed a notable significant decrease in 12 S, the primary reserve protein, in dry seeds from lines with TSPO misexpression (*pFAE:TSPO*), in contrast to WT or lines with constitutive overexpression of Arabidopsis TSPO (*p35S:TSPO*). A similar significant reduction was observed for oleosin 1 (OLE1), a key structural protein of lipid bodies. As a result, the 12 S/OLE1 ratio was significantly lowered in lines misexpressing TSPO (Fig. 5C). This finding indicates a distinct strategy for energy storage and mobilization in TSPO misexpressing seeds compared to their WT or TSPO overexpressing counterparts.

4. Discussion

The interplay between WRI1 and TSPO within Arabidopsis seeds unveils a fascinating regulatory mechanism pivotal to seed development and the intricacies of lipid metabolism. Our research presents compelling evidence that WRI1, a master transcription factor known for its role in enhancing glycolysis and lipid biosynthesis, also influences the expression of AtTSPO, a protein with a proposed indirect role in seed lipid metabolism. This regulatory relationship is underscored by the discovery of WRI1-responsive AW-box sequences proximal to the AtTSPO promoter, suggesting a conserved mechanism across Brassicaceae oilseed plants that may extend to broader physiological impacts.

A significant reduction in AtTSPO levels in *wri1-4* mutant seeds compared to wild type further highlights the regulatory influence of WRI1 on AtTSPO expression, impacting seed physiology and lipid content. This finding is pivotal, considering the delayed embryo elongation, reduced seed dry weight, and decreased fatty acid content associated with the *wri1-4* mutation [9,15,26]. Our exploration into the dynamics between WRI1 and AtTSPO during seed imbibition reveals a tightly regulated process where both proteins are degraded, indicating their roles are confined to the maturing seed stage, with implications for seed germination and early seedling development.

Our research revealed the nuanced role of AtTSPO in modulating the activity of WRI1, a key regulator in lipid biosynthesis pathways. Specifically, we showed that the expression of AtTSPO, under the control of the fatty acid elongase promoter (*pFAE*) but not the cauliflower mosaic virus 35 S promoter (*p35S*), notably reduced the post-translational modifications (PTMs) of WRI1, thereby possibly affecting its functionality. With transgenic Arabidopsis seeds misexpressing AtTSPO, we observed not only an alteration in WRI1's typical PTM patterns but also a notable increase in seed oil content. This phenomenon underscores a differential regulatory effect, highlighting how specific promoters driving the (mis)expression of AtTSPO can influence protein activities and metabolic outcomes. For instance, while the 35 S promoter is known to drive gene expression across various embryonic and developmental stages, its efficacy may wane in certain contexts, affecting the uniformity of gene expression, protein levels, and subsequent protein functionality [27]. In contrast, the fatty acid elongase promoter is markedly active during the initial stages of embryogenesis, a phase marked by swift cell divisions and a heightened demand for cell membrane synthesis. This demand necessitates an ample supply of very long-chain fatty acids (VLCFAs) for their incorporation into

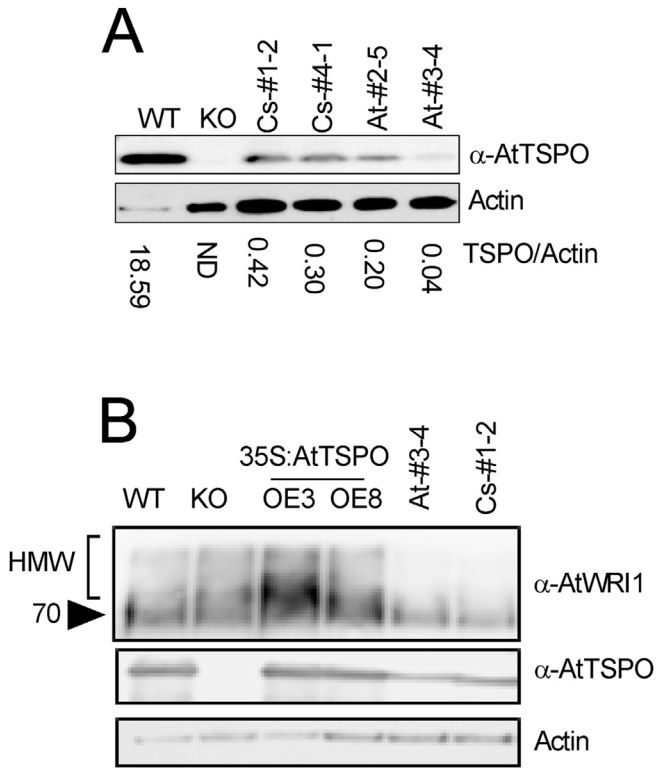


Fig. 3. Influence of AtTSPO on WRI1 protein stability. (A) Detection of AtTSPO in WT Arabidopsis expressing pFAE1:*CsTSPO* and *pFAE1:AtTSPO*. Mature seed proteins from two independent high total fatty (TFA) lines were analyzed by western blot using *anti*-AtTSPO. Quantification of AtTSPO relative to actin was performed with ImageJ. (B) WRI1 protein levels across Arabidopsis genetic variants. Total protein extracted from mature seeds of WT, *AtTSPO* KO, and transgenic lines with constitutive AtTSPO expression (OE3 and OE8 from Ref. [7]) and those expressing TSPO under the pFAE1 promoter (CsTSPO and AtTSPO) were assessed for High Molecular Weight (HMW) forms indicative of post-translational modifications of WRI1.

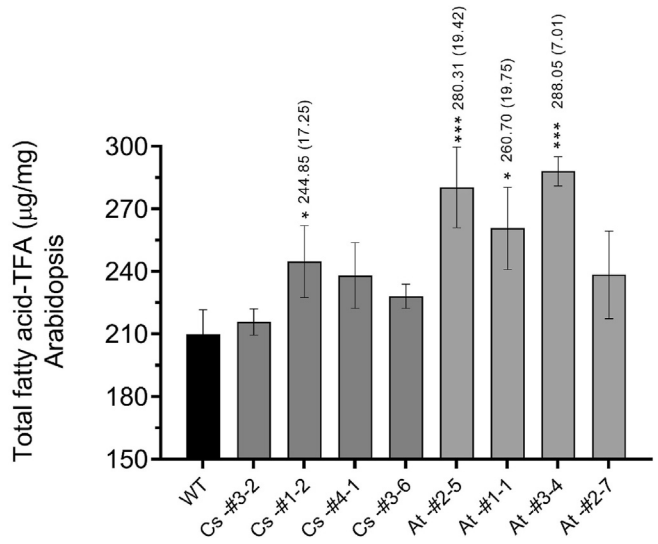


Fig. 4. Total fatty acid (TFA) content variation due to TSPO misexpression. Analysis of TFA content of T4 generation Arabidopsis seeds expressing *pFAE1-AtTSPO* (Arabidopsis TSPO expression driven by the fatty acid elongase promoter) and *pFAE1-CsTSPO* (*Camelina sativa* TSPO expression in Arabidopsis driven by fatty acid elongase promoter). Mature dry seeds from plants harboring each genetic construct underwent TFA quantification. Data were collected from four independent transgenic lines per construct (including the lines studies above), with error bars representing variance across three biological replicates. Statistical disparities were evaluated using Student's t-test, with significance differences denoted by *** ($p < 0.0001$) and * ($p < 0.05$).

triacylglycerols (TAGs), underscoring the critical role of this promoter in early seed development [28]. Elevated TSPO expression levels have been documented in cell types actively engaged in lipid storage and metabolism, highlighting its functional significance in these processes [29]. For example, in the context of high-fat diet-induced obesity in mice, lipid droplets notably accumulate in tanycytes, and the targeted inhibition of TSPO in these cells has been shown to induce lipophagy, reflecting the protein's regulatory role in lipid turnover [29]. TSPO is also implicated in regulating adipocyte differentiation and lipid storage [30].

The targeted expression of AtTSPO driven by *pFAE* during these pivotal stages of seed development points to a regulatory mechanism wherein TSPO potentially stabilizes WRI1. This stabilization likely occurs through the modulation of post-translational modifications (PTMs) that determine WRI1's degradation rate, thus ensuring the persistence of enzyme activity crucial for the lipid biosynthesis pathway [12]. Furthermore, the misexpression of AtTSPO could initiate its active degradation, possibly via the autophagy pathway, an observation previously made in vegetative tissues overexpressing AtTSPO [5]. As the seed advances toward maturation and enters dormancy with ABA levels peaking, a notable increase in protein biosynthesis is accompanied by an accumulation of TSPO (Fig. 2B). This progression highlights the intricate dynamics of TSPO expression and its regulatory implications on lipid metabolism and seed development.

However, the observed downregulation and enhanced degradation of TSPO in misexpressed transgenic lines, compared to WT or lines overexpressing AtTSPO under the *p35S* promoter, may lead to reduced TSPO levels in mature seed (Fig. 3B). This differential accumulation of TSPO, influenced by promoter selection and developmental stage-specific expression, underscores the complexity of regulatory mechanisms at play during seed development and maturation. Furthermore, the analysis of protein reserves, specifically the levels of the 12 S globulin and oleosin 1 (OLE1), in seeds with altered AtTSPO expression, offers a new perspective on the metabolic adjustments occurring in these transgenic lines. The observed reduction in levels of both 12 S globulin and oleosin 1 (OLE1) in lines misexpressing AtTSPO, culminating in a decreased 12 S/OLE1 ratio, indicates a deliberate alteration in the strategy for energy storage and mobilization within the seed. This alteration is further substantiated by our previous findings, which demonstrated that seeds overexpressing AtTSPO deplete their lipid energy reserves more rapidly compared to those deficient in TSPO or wild-type (WT) seeds during germination [7]. This nuanced balance underscores the critical role of AtTSPO in modulating metabolic processes, suggesting that TSPO's influence extends beyond simple energy storage to encompass the orchestration of energy utilization. By affecting the dynamics of lipid and protein reserve utilization, AtTSPO facilitates strategic reserve management that enhances the seed's adaptability and preparedness for germination under varying environmental conditions. This complex interplay between lipid and protein reserves, mediated by AtTSPO, highlights its significance in the broader context of seed biology, offering insights into the mechanisms that govern seed maturation, viability, and the initiation of germination.

Our findings underscore the complexity of seed physiology, highlighting the multifaceted roles of WRI1 and AtTSPO in orchestrating the metabolic pathways that determine seed composition, development, and response to environmental cues. This intricate regulatory network, involving transcriptional and post-translational mechanisms, offers a deeper understanding of plant metabolism and presents novel avenues for enhancing seed quality and oil content through genetic engineering. By manipulating the expression of key proteins like AtTSPO, we can potentially tailor seed composition to meet agricultural and industrial

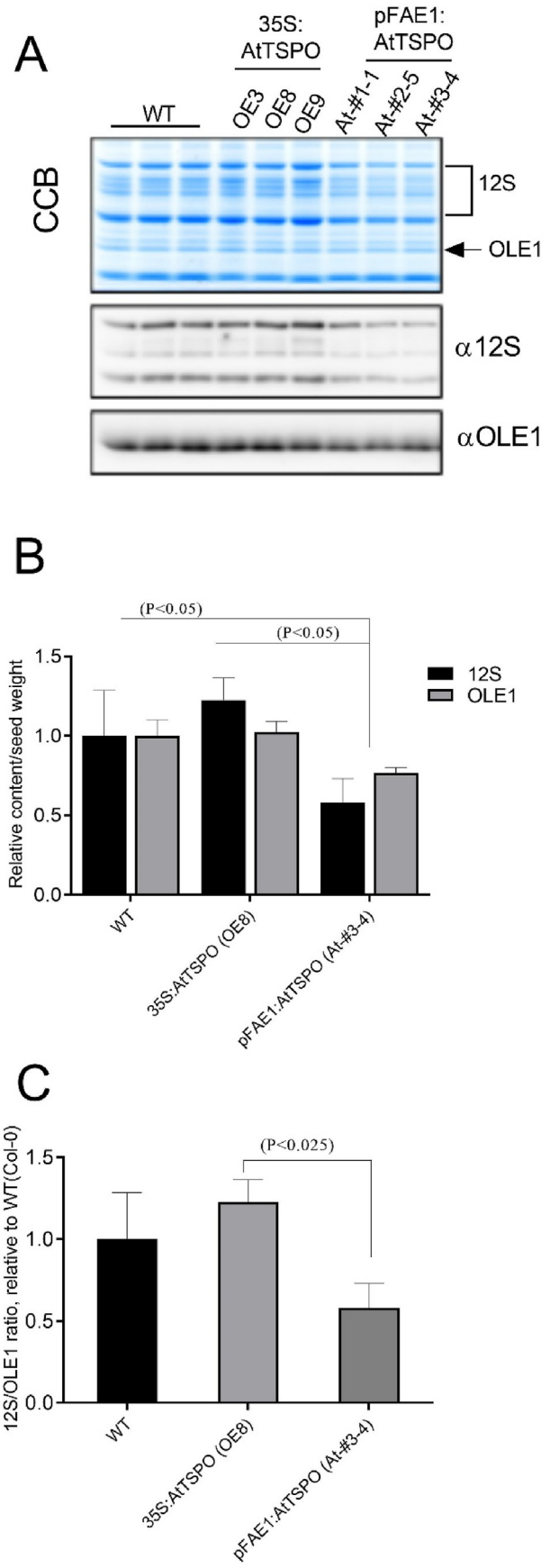


Fig. 5. Modulation of mature seed reserve protein by TSPO. We compared mature seed protein profiles between wild-type (WT) seeds and seeds from three independent transgenic Arabidopsis lines expressing (*p35S:AtTSPO*) or misexpressing (*pFAE1:AtTSPO*) the Arabidopsis TSPO. (A) Represents the protein profiles of mature seeds, with the upper panel depicting a Coomassie blue-stained SDS-PAGE gel and the lower panels showing western blot detection of 12 S globulin and oleosin 1 (OLE1). Total protein extracts from an equal mass of seeds were loaded in each lane. (B) Illustrates the relative content of 12 S and OLE1, based on quantification from the western blots in (A), averaged across three lines with standard deviation (Mean ± SD). (C) Shows the calculated ratio of 12 S to OLE1, derived from the quantified levels in (B). Relative content in (B) and (C) was normalized against Col-0 values. Statistical significance was assessed using the Kruskal-Wallis Test.

demands, paving the way for innovations in crop improvement and sustainable biofuel production.

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Authors declaration

Authors declare no conflict of interest.

CRediT authorship contribution statement

Kaushal Kumar Bhati: Formal analysis, Investigation, Writing – original draft. **Ai My Luong:** Formal analysis, Investigation, Visualization. **Franziska Dittrich-Domergue:** Formal analysis, Methodology, Visualization. **Sabine D'Andrea:** Formal analysis, Methodology, Visualization, Writing – original draft. **Patrick Moreau:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. **Henri Batoko:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.05.011.

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