Title: WRINKLED1 transcription factor: how much do we know about its regulatory mechanism?

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Abstract

Many plant species produce and build up triacylglycerol (TAG) in their seeds as a main resource to provide carbon and energy during seedling development. Plant seed oils are important not only for human diets but also as renewable feedstock of industrial uses. WRINKLED1 (WRI1), an APETALA2 (AP2) transcription factor, plays an essential role in the transcriptional regulation of TAG biosynthesis as WRI1 regulates the expression of key genes in the glycolytic and fatty acid biosynthetic pathways. Recent work has identified intrinsic structural disorder in WRI1 that may affect the stability of the protein. Furthermore, WRI1 activity is modulated by post-translational modifications and interacting partners. These progresses shed new light on regulatory functions of WRI1 at the molecular levels, paving new paths to the use of WRI1 for bioengineering of TAG in plants.

Key words

Arabidopsis, transcription factor, plant oil biosynthesis, post-translational modifications, protein stability, protein–protein interaction.

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Contents

Introduction

Plant oils (mainly triacylglycerol, or TAG) are accumulated by numerous plant species in their seeds as the storage resource (carbon and energy) to support seedling development. Plant oils are also an essential supply for human diets and for industry as renewable feed stocks. It has been predicted that global demand for plant oils will double by 2030, necessitating technological breakthroughs [1].

TAG biosynthesis requires two main steps: fatty acid biosynthesis in the plastids and TAG assembly in the endoplasmic reticulum. After export from plastids, fatty acids are converted into acyl-CoA and assembled mainly via the eukaryotic phospholipid biosynthetic pathway [2]. In the last step of oil biosynthetic process, diacylglycerol is acylated using either acyl-CoA or phospholipids to form TAG, catalyzed by diacylglycerol acyltransferase (DGAT) or phosphatidylcholine:diacylglycerol acyltransferase (PDAT) [3-5]. The regulatory mechanism of TAG biosynthesis is complicated and not completely understood, hence the identification of gene regulators and studying their regulatory mechanisms are important for both understanding the oil biosynthetic process and designing strategies to efficiently increase plant oil production [6-7].

WRI1 is a master transcriptional regulator of plant oil biosynthetic pathways

WRINKLED1 (WRI1), a member of the APETALA2 (AP2) transcription factor family, is a key regulator of plant oil biosynthesis [8-9] (also see Figure 1A). The seed oil content in *wri1-1* (the Arabidopsis loss-of-function mutant of *AtWRI1*) is reduced by 80% compared to the wild-type (WT) [10]. The transcriptomic analysis of developing seeds of WT and *wri1-1* has shown that the majority of the genes which display reduced expression in *wri1-1* are related to the glycolytic and fatty acid biosynthetic pathways [11]. Recent work has identified that many genes in glycolysis and fatty acid biosynthesis are targets of AtWRI1, such as genes encoding acetyl-CoA carboxylase, acyl carrier protein, ketoacyl-ACP synthase, a subunit of pyruvate kinase [12-14]. WRI1 is thus referred as a "master regulator" for transcriptional regulation of seed oil biosynthesis [1].

Regulators that control *AtWRI1* expression

Key regulators of seed maturation, including LEAFY COTYLEDON1 (LEC1) [15] and LEAFY COTYLEDON2 (LEC2) [12], seem to be responsible for activating *AtWRI1*. *LEC2* co-expresses with *AtWRI1*, leading to the activation of *AtWRI1* during Arabidopsis embryo development [12]. *AtWRI1* expression is increased in the gain-of-function mutant of *LEC1*, *tnp* [15], implicating a role for LEC1 in controlling *AtWRI1* [14, 16]. However, it should be noted that the gene promoter cis-elements, to which LEC1 and LEC2 recognize and bind, have yet to be identified (Figure 1B). How LEC1 and LEC2 regulate the expression of *AtWRI1* thus remain to be further elucidated [14]. In addition, the Arabidopsis transcription factor FUSCA3 (FUS3) is another positive regulator of *AtWRI1*. Comparative microarray analysis reveals that *AtWRI1* expression is reduced in the *fus3* mutant compared to WT [17]. Based on functional redundancy between LEC2 and FUS3, FUS3 has been speculated to regulate *AtWRI1* expression similar to that by LEC2 [17]. A recent ChIP-chip experiment [chromatin immunoprecipitation (“ChIP”) followed by DNA microarray (“chip”)] has confirmed that *AtWRI1* is a direct target of FUS3 [18] (Figure 1B). *AtWRI1* expression is induced by sucrose treatment [9]. AtWRI1 also activates some sugar-responsive genes, hence possibly playing a role in sugar signaling that mediates carbon flow to storage oil [9]. *FUS3* expression does not influence the expression of *LEC1* or *LEC2*, *AtWRI1* activation in response to sucrose is thus suggested to be directly regulated by FUS3 instead of indirectly through LEC1 or LEC2 [6]. It is highly possible that some plant species might have additional upstream *WRI1* regulator(s) other than those involved in seed maturation (e.g. LEC1, LEC2, and FUS3). In oil palm mesocarp, where *EgWRI1* is highly expressed, expression of key regulators for seed maturation remain low, indicating that *EgWRI1* is controlled by novel fruit-specific regulator(s) [19].

Recent work has identified that the transcription factor MYB89 represses *AtWRI1* [20]. *AtWRI1* expression is elevated in Arabidopsis loss-of-function mutant, *myb89*, and upregulation of *MYB89* leads to repression of *AtWRI1* [20]. Further ChIP assay confirmed that MYB89 directly binds the promoter of *AtWRI1*, indicating that *AtWRI1* is a direct target of MYB89 [20] (Figure1B).

AtWRI1'activity is mediated by posttranslational modifications and interacting partners

A number of mechanisms of both positive and negative transcriptional regulation of WRI1 activities have been illustrated. Recent progress also has begun to shed light on WRI1 function at the protein level. These efforts include *in silico* analysis of protein structural features, characterization of functional domains/motifs, and the identification of WRI1 interacting partners. Using CULLIN3-based E3 ligase adaptor BTB/POZMATH 1 (BPM1) protein as a prey, AtWRI1 was identified to interact with BPM1 in yeast two-hybrid (Y2H) assay [21]. AtWRI1 is able to interact with multiple other BPMs; and the degradation of AtWRI1 is mediated by the 26S proteasome through interaction with E3 adaptor BPMs [21]. This work establishes a connection between E3 ligase and plant lipid metabolism via degradation of the key transcriptional regulator, AtWRI1.

In silico analysis has revealed that AtWRI1 protein possesses intrinsic disorder features. Three intrinsically disordered regions (IDRs) are predicted using several computational programs [22]. A PEST motif, a well-characterized peptide signal sequence for protein degradation, is present in the IDR3 domain of AtWRI1 [22]. A transactivation domain (TAD) has been identified in AtWRI1 C-terminus, which does not overlap with the IDR3-PEST motif [22]. AtWRI1 has been engineered by removal of the IDR3-PEST motif or by mutation of putative phosphorylation residues in the IDR3-PEST motif. The engineered AtWRI1 proteins show enhanced stability, and overexpression of the engineered *AtWRI1* lead to increase of TAG biosynthesis in Arabidopsis compared to WT [22]. Phosphorylation is hence suggested as a possible mechanism that mediates AtWRI1 stability via modulation of the IDR3-PEST motif [22].

AtWRI1 has been recently identified as a new client of 14-3-3 proteins (a family of conserved regulatory proteins with ability to bind to phosphorylated proteins) [23]. AtWRI1 physically interacts with 14-3-3s in both yeast and plant cells [23]. Overexpression of a *14-3-3* enhances AtWRI1-mediated TAG biosynthesis, as well as transcriptional activity and stability of AtWRI1 [23]. The 14-3-3 binding motif in AtWRI1 overlaps with the binding motif for BPMs [23]. It is proposed that the interaction between AtWRI1 and 14-3-3 either prevents E3 ligase-BPMs from interacting with AtWRI1 or separates AtWRI1 from the bound E3 ligase-BPMs [23].

KIN10 (an essential SNF1-related protein kinase in the sugar signal transduction cascade) phosphorylates AtWRI1 through physical interaction[24]. The KIN10-mediated AtWRI1 phosphorylation is important for the AtWRI1 degradation. KIN10 phosphorylates AtWRI1 at residues T70 and S166 [24]. The phosphorylation deficient mutant AtWRI1$^{T70A/S166A}$ displays increased protein stability compared to WT AtWRI1 in the presence of KIN10 [24]. Interestingly, the 14-3-3 binding motif [23] and KIN10 phosphorylated residues [24] are close, suggesting that these two modulation processes might be overlapping probably via the proteasomal degradation pathway (Figure 1A).

In silico analysis also predicts that AtWRI1 is one of potential substrates of the kinase OST1 (SnRK2.6) [25]. SnRK2 kinases are essential regulators in ABA signal transduction [26-28]. The proposed OST1 phosphorylation motif in AtWRI1 protein is positioned in between the two AP2 domains [25]. SnRK2.6 mediates the stability of ABA-responsive-element Binding Factor 3 (ABF3) through phosphorylation [25]. Therefore, it is possible that SnRK2s are involved in phosphorylation of AtWRI1, which in turn affects the activity of AtWRI1 and AtWRI1-mediated oil biosynthesis.

The Mediator 15 (MED15), a subunit of the Arabidopsis mediator complex [29-30], physically interacts with AtWRI1 in both *in vitro* and *in vivo* assays [31]. *MED15* overexpression has resulted in the activation of a number of AtWRI1 target genes. ChIP assays suggest that MED15 binds the promoters of these AtWRI1 target genes [31]. However, expression of the AtWRI1 target genes remain elevated in transgenic *wri1* plants overexpressing *MED15*, indicating the presence of alternative transcription factors that interact with MED15 to regulate those genes [31].

Targets of AtWRI1 in pathways other than oil biosynthesis

While binding to the promoters of *PINs* at AW-boxes, AtWRI1 binds to the promoter of *GH3.3* (a gene involved in auxin degradation) at a novel binding motif other than AW-box, implicating a role in mediating root auxin homeostasis [32]. AtWRI1 is also able to activate the expression of *Citrus sinensis Terpene Synthase* 1 (*CsTPS1*) [33]. AtWRI1 is a homolog of the *C. sinensis* AP2 transcription factor, CitAP2.10, which stimulates the biosynthesis of (+)-valencene in orange [33]. Thus far, we do not fully understand how regulation of the alternative targets of AtWRI1 is connected to growth and development (e.g. root growth [32]). However, realizing the fact that WRI1 targets genes which might alter other physiological processes (e.g. hormone homeostasis [32]) is critical for the application of WRI1 in translational research to increase oil content.

WRI1 orthologs in other plant species

WRI1 orthologs have been identified from both monocot and dicot species, including *Brassica napus*, *Zea mays*, *Elaeis guineensis*, *Gossypium spp*, *Ricinus communis*, *Brachypodium distachyon*, *Camelina sativa*, and *Cocos nucifera* [34-39]. *AtWRI1* and *WRI1* orthologs are able to complement the phenotypes of *wri1* mutants [8, 35-36, 40]. Similar to *AtWRI1*, numerous *WRI1* orthologs are found to express highly in developing seeds. However, oil palm *WRI1* expresses highly in non-seed tissue, the oil accumulating mesocarp, and the transcript of *EgWRI1* increases as fruit ripening [19]. In addition, *WRI1* orthologs from avocado [41] and nutsedge [42] were found to express highly in mesocarp and underground stem tuber, respectively. Structural features and functional motifs, including “VYL” [35], IDR, and PEST motif [22], are conserved features across the characterized WRI1 proteins [22, 35, 38, 40].

WRI1 is important for translational research

Overexpression of *AtWRI1* or other *WRI1* orthologs have resulted in increased seed oil content in transgenic plants [8, 34, 37-38, 43-44]. Ectopic expression of *AtWRI1* and *BnWRI1* elevate oil content in leaves of transgenic Arabidopsis and Brachypodium plants [38, 45]. *ZmWRI1* overexpression, driven by the embryo-preferred *OLEOSIN* (*OLE*) promoter, has significantly increased seed oil accumulation in transgenic maize plants; however, little or no increase of oil content is resulted when the expression is driven by the *19 KD ZEIN* promoter (aimed to target the expression in starch endosperm) [37]. Using the *FUS3* promoter to drive *AtWRI1* expression, in order to facilitate the extension of oil biosynthesis in the mid-phase of seed development, has efficiently increased seed oil content in transgenic Arabidopsis plants [46].

In tobacco leaves, transient overexpression of *AtWRI1* and *WRI1* orthologs significantly increase TAG accumulation [22, 42, 47]. Transient co-expression of *WRI1* and *DGAT1* has resulted in significantly higher oil content in tobacco leaves compared to expressing *WRI1* alone, suggesting a synergistic effect between the two genes [47]. In transient expression assays using tobacco leaves, ectopic expression of stabilized forms of WRI1, resulted from mutation at the phosphorylation sites in the IDR3-PEST motif, enhances oil production [22]. Transient co-expression of *AtWRI1* and a *14-3-3* in tobacco leaves also enhances protein stability of AtWRI1, leading to increased oil production [23].

In addition, overexpression of selected transcription factors that upregulate *WRI1*, identified from plant species other than Arabidopsis, have proven to be useful for the increase of seed oil content. For instance, overexpression of soybean transcription factors, *GmZF351* and *GmDREBL*, enhance seed oil in transgenic Arabidopsis [48-49]. The two soybean transcription factors are able to activate *AtWRI1* by binding to its promoter [48-49]. Overexpression in maize of *ZmLEC1*, an activator of *ZmWRI1*, has increased seed oil up to 48% [37].

It should not be overlooked that overexpression of *WRI1* might cause abnormal growth or cell death in certain plant species [8, 14, 38]. Therefore, selection of suitable promoters of target plant species is important for the improvement of oil crops or development of new oil crops.

Future perspectives

To date, how *WRI1* expression is regulated still remains largely unknown. Also unclear is how LEC1, LEC2, and FUS3 regulate the expression of *WRI1*. It is likely that additional regulators also control the expression of *WRI1* during the developmental process or in response to environmental cues (e.g. sucrose). Addressing these questions may require detailed analyses of the *WRI1* promoters and the layered regulatory network.

Efforts have been made recently to bridge the structural features to functions of WRI1. Identification and characterization of the IDR [22], PEST motif [22], TAD [22], 14-3-3 and E3 ligase adaptor binding motif [23], and KIN10 kinase phosphorylation residues [24] in AtWRI1 protein have shed new lights onto the structure/function relationship of AtWRI1. Future work will focus on understanding the functions of individual domains and motifs, as well as how protein-protein interactions affect the dynamics of AtWRI1. Phosphorylation at T70/S166 of the IDR3-PEST motif has been shown to be important for AtWRI1 function [22, 24]. Given that AtWRI1 protein is enriched of putative phosphorylation sites [22], the fine characterization of AtWRI1 phosphorylation sites and identification of the new kinase(s) that phosphorylate AtWRI1 will be of great significance. Moreover, phosphorylation is proposed to play a dual role in mediating the stability and activity of AtWRI1 [23]. It is proposed that, in the early stage of embryo development, phosphorylation of AtWRI1 creates a binding site for 14-3-3, resulting in stabilization of AtWRI1 protein; however, during the late stage of embryo development, phosphorylation of IDR3-PEST motif in AtWRI1 facilitates the AtWRI1 degradation [23] (Figure 1C). Thus, how AtWRI1 activity is dynamically modulated by the upstream kinases (e.g. SnRK2.6 [25]), in response to diverse environmental and developmental signals, requires attention.

Recent progress also indicates that AtWRI1 has novel targets other than genes in glycolysis and fatty acid biosynthesis via binding to AW-box or novel binding sites [32]. Understanding the effects of WRI1 on these genes unrelated to oil biosynthesis might be helpful to overcome the side effects of *WRI1* overexpression aimed for the improvement of oil crops [8, 14, 38]. Genome editing and protein engineering approaches can be applied to increase WRI1 binding specificity to the oil biosynthetic genes.

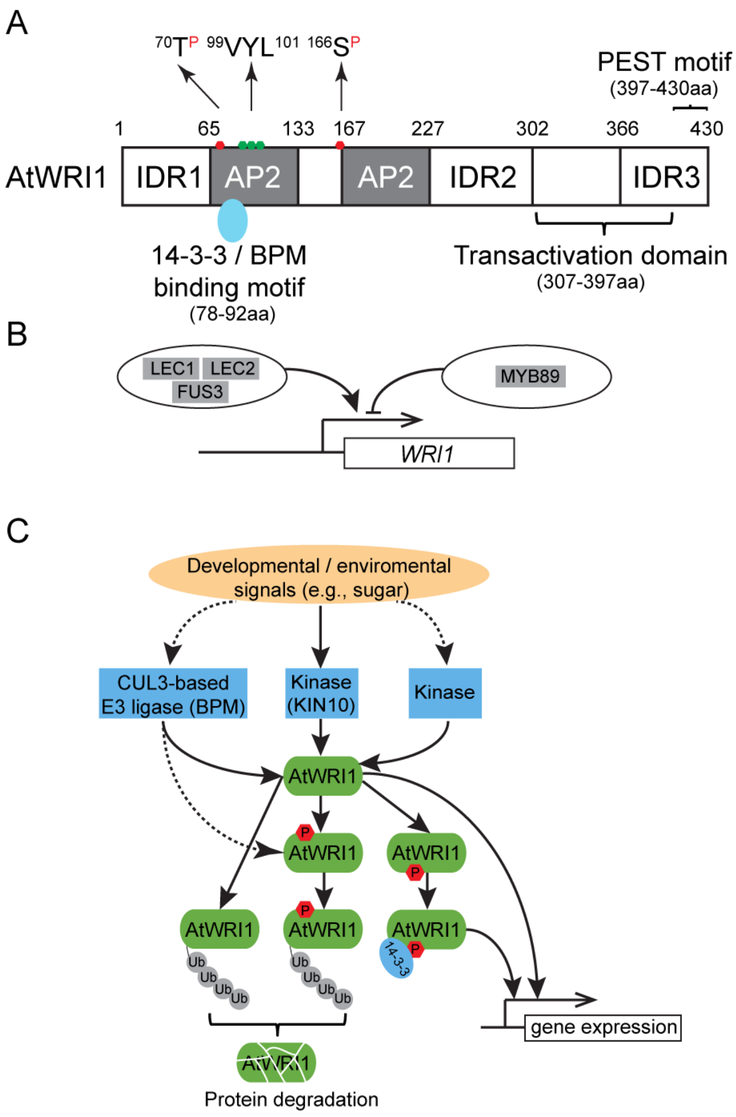

Figure Legend

Figure 1. Structural characteristics of AtWRI1 and a simplified model for the regulation of AtWRI1-mediated oil gene expression. A) Schematic representation of AtWRI1 showing the locations of the conserved functional motif of "VYL”, three intrinsically disordered regions, the PEST motif, the transactivation domain (TAD), the 14-3-3 and BPM binding motifs, and the KIN10 kinase phosphorylation residues (T70 and S166). B) Regulation of *WRI1* expression. LEC1 and LEC2 have been suggested as key regulators mediating the expression of *WRI1*,

despite the lacking of evidence for LEC1/LEC2 direct binding to the *WRI1* promoter. FUS3 and MYB89 have been shown to directly bind to the *WRI1* promoter, acting as positive and negative regulators, respectively. C) A proposed model of possible regulatory mechanisms for AtWRI1. Post-translational modifications (such as phosphorylation and ubiquitination) of AtWRI1 occur in response to different environmental cues and developmental states, leading to altered protein stability and transcriptional activity, which in turn affects the expression of AtWRI1 target genes.

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