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Permalink
https://escholarship.org/uc/item/576387d2

Journal
PLoS pathogens, 4(12)

ISSN
1553-7366

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Publication Date
2008-12-12

DOI
10.1371/journal.ppat.1000237

Peer reviewed
The Chromatin Remodeler SPLAYED Regulates Specific Stress Signaling Pathways

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Abstract

Organisms are continuously exposed to a myriad of environmental stresses. Central to an organism’s survival is the ability to mount a robust transcriptional response to the imposed stress. An emerging mechanism of transcriptional control involves dynamic changes in chromatin structure. Alterations in chromatin structure are brought about by a number of different mechanisms, including chromatin modifications, which covalently modify histone proteins; incorporation of histone variants; and chromatin remodeling, which utilizes ATP hydrolysis to alter histone-DNA contacts. While considerable insight into the mechanisms of chromatin remodeling has been gained, the biological role of chromatin remodeling complexes beyond their function as regulators of cellular differentiation and development has remained poorly understood. Here, we provide genetic, biochemical, and biological evidence for the critical role of chromatin remodeling in mediating plant defense against specific biotic stresses. We found that the Arabidopsis SWI/SNF class chromatin remodeling ATPase SPLAYED (SYD) is required for the expression of selected genes downstream of the jasmonate (JA) and ethylene (ET) signaling pathways. SYD is also directly recruited to the promoters of several of these genes. Furthermore, we show that SYD is required for resistance against the necrotrophic pathogen Botrytis cinerea but not the biotrophic pathogen Pseudomonas syringae. These findings demonstrate not only that chromatin remodeling is required for selective pathogen resistance, but also that chromatin remodelers such as SYD can regulate specific pathways within biotic stress signaling networks.

Introduction

In eukaryotic organisms genomic DNA is packaged into chromatin, which can repress transcription by blocking the access of regulatory proteins to DNA. Dynamic changes in chromatin structure are now recognized as a robust mechanism of transcriptional control [1–3]. Changes in chromatin structure are brought about by a number of different mechanisms including: chromatin modifications, which covalently modify histone proteins; incorporation of histone variants; and chromatin remodeling, which utilizes ATP hydrolysis to alter histone-DNA contacts [1,3–5]. ATP-dependent chromatin remodeling complexes are present in all eukaryotic organisms and can be grouped into three main classes: the SWI/SNF ATPases, the imitation switch (ISWI) ATPases, and the chromodomain and helicase-like domain (CHD) ATPases [2,3].

Significant advances have been made in understanding the mechanism of ATP-dependent chromatin remodeling complex action [1,4]. However, the biological role of chromatin remodeling complexes remains poorly understood, particularly in multicellular organisms where null mutations tend to be lethal [3,6]. Studies that have investigated the biological role of chromatin remodeling complexes in multicellular organisms have largely focused on their role as regulators of cellular differentiation and development [2,3]. In particular, Arabidopsis has served as a valuable model due to the fact that mutants in genes encoding a number of chromatin remodeling complex proteins are viable. One of the most well characterized chromatin remodeling complex proteins in Arabidopsis is the SWI/SNF class chromatin remodeling ATPase SPLAYED (SYD). Loss of SYD activity causes defects in many different developmental pathways including stem cell maintenance, patterning, developmental transitions and growth [3,7–9].

The biological role of altering chromatin structure in response to stress via chromatin modifications and incorporation of histone variants has been documented [10–14]. However, the biological role of chromatin remodeling complexes or their specificity remains poorly understood. The role of chromatin remodeling in response to stress has been best studied in yeast where it has been shown that chromatin remodeling complexes are required for stress tolerance and are recruited to specific promoters upon stress [15–19]. However, few studies performed in multicellular organisms have investigated the role of chromatin remodeling in mediating stress responses. One study conducted in the human cell culture line SW480 demonstrated that chromatin remodeling...
Author Summary

In eukaryotes, genomic DNA is organized into a complex DNA-protein structure termed chromatin. The organization of chromatin serves to compact DNA within the nucleus and plays a central role in regulating transcription by controlling the access of DNA to transcriptional machinery. Chromatin structure can be altered through several mechanisms, one of which is chromatin remodeling, a process that disrupts DNA-protein interactions resulting in altered accessibility of specific DNA regions to regulatory proteins in the transcriptional machinery. In this study, we investigated the biological role of chromatin remodeling in defense responses to biotic stresses using the model plant Arabidopsis. We found that a chromatin remodeling protein, SPLAYED, is required for gene expression within specific biotic stress signaling networks. Consistent with this observation, loss of SPLAYED chromatin-remodeling activity resulted in increased susceptibility to a fungal pathogen, Botrytis cinerea, but not to a bacterial pathogen, Pseudomonas syringae. These results demonstrate that reduced stress tolerance in a chromatin-remodeling mutant plant can be stress specific, and is not simply due to a decrease in overall fitness as a result of non-specific global mis-regulation of gene expression.

Results/Discussion

We investigated the role of chromatin remodeling in stress signaling using Arabidopsis, a multicellular organism where viable chromatin remodeling null mutants exist [9]. In a previous study we showed that the SWI/SNF class chromatin remodeling ATPase SID transcript is upregulated rapidly following mechanical wounding [21]. We also demonstrated that mechanical wounding is a response common to numerous biotic stresses that a plant may encounter [21]. The upregulation of SID in response to wounding suggests that SYD may be recruited to remodel promoters within stress signaling networks. To begin delineating the placement of SYD in stress signaling we first examined whether SYD is required for expression of other transcripts upregulated rapidly in response to wounding. This demonstrated that SYD is not required for the expression of the rapid wound response genes ETHYLENE RESPONSE FACTOR #18 (ERF#18) or CCR4 ASSOCIATED FACTOR 1-like (CAF1-like) (Figure S1A). We next investigated the role of SYD in the ethylene (ET), jasmonate (JA), and salicylic acid (SA) stress signaling pathways, which respond to abiotic and biotic stresses such as wounding and pathogen infection (Figure 1A) [22,23]. As shown in Figure 1B, basal expression of the plant defensin PDF1.2a, a marker for intact ET and JA signaling, is lost in syd-2 null mutants [9]. As basal levels of PDF1.2a are generally low, but detectable, we increased cycle number to improve our ability to detect basal differences between syd-2 and wild-type (WT) [24,25]. In contrast, basal expression of PATHOGENESIS-RELATED (PR1), a marker for intact SA signaling, is maintained in syd-2 plants (Figure 1B). These data suggest that SYD is required for ET and JA signaling but not SA signaling.

The loss of basal PDF1.2a but not PR1 expression in non-stressed syd-2 plants suggests that SYD impacts specific stress signaling pathways. To explore the role of SYD under inductive stress treatments we inoculated plants with the necrotrophic pathogen Botrytis cinerea and the virulent biotrophic pathogen Pseudomonas syringae. As resistance to B. cinerea requires ET and JA signaling, whereas resistance to P. syringae is predominantly mediated by SA signaling [22,26], use of these two pathogens allows further experimental evaluation of requirements for SYD function in defense signaling. We first monitored expression of key genes in the ET/JA pathway in response to B. cinerea treatment. The expression of the transcription factor ETHYLENE RESPONSE FACTOR1 (ERF1), which requires both ET and JA for induction [22,27–29], is similar in WT and syd-2 plants (Figure 2A). In contrast to ERF1, the expression of PDF1.2a requires SYD in response to B. cinerea (Figure 2A). In addition, we examined the expression of PR1 in plants treated with B. cinerea and determined that this gene is expressed at similar levels in WT and syd-2 plants (Figure 2A). We next assayed the expression of a suite of genes involved in SA biosynthesis and signaling in response to P. syringae and found that SYD is not required for their expression (Figure 2B and Figure S2). Additionally, expression of PR1, but not upstream genes (PAD4, ICS1, NPR1, and WRK170), is enhanced in syd-2 plants. The apparent lack of detectable enhancement in PR1 expression levels by RT-PCR (Figure 1B) is likely due to signal saturation inherent to ETBr staining. However, this pattern of transcriptional alteration is similar to what is observed in myc2/jin1 mutants, suggesting that MYC2 expression may be reduced in syd (Figure 1A) [22,30]. Furthermore, as SWI/SNF class chromatin remodeling ATPases’s are primarily considered activators of transcription it is highly unlikely that SYD is acting directly to...
repress PRI expression in WT plants [4]. Taken together these data demonstrate that SYD is required within specific stress signaling pathways in response to pathogen infection.

The finding that SYD is required for gene expression within specific stress signaling pathways suggests that loss of SYD function may reduce tolerance to specific biotic stresses. To test this hypothesis we first examined the resistance of syd mutants to B. cinerea. For this experiment we tested two independent syd null alleles and compared them to their respective WT background. As shown in Figure 3A and 3B, syd mutant plants are more susceptible to B. cinerea infection. The increased susceptibility of the syd mutants to B. cinerea is likely due to altered ET and/or JA signaling impacting defence mechanisms. It should also be noted that the phytoalexin camalexin plays a role in B. cinerea resistance [26]. However no significant difference in camalexin levels were detected between syd mutant and WT plants after elicitor treatment (data not shown). This suggests that SYD affects B. cinerea resistance through ET/JA signaling independent of camalexin production. To determine if reduced resistance was specific to B. cinerea we inoculated syd mutants with virulent P. syringae, for which resistance is predominantly mediated via SA signaling [26]. In contrast to B. cinerea, syd mutants and WT plants show similar resistance to P. syringae (Figure 3C and 3D). These results demonstrate that chromatin remodeling via SYD is required for stress specific disease resistance.

SYD was originally implicated in stress responses by the observation that SYD transcripts accumulate upon wounding. [21]. Therefore we examined which aspects of the ET and JA signaling pathways are impacted in syd mutants following wounding. Furthermore, we wished to directly compare gene expression levels with SYD recruitment to specific promoters via chromatin immunoprecipitation (ChIP) assays. Wounding is therefore advantageous as it enables better synchronization of the stress stimulus and is a feasible treatment for the large amount of tissue required for ChIP. We first monitored the expression of ALLENE OXIDASE SYNTHASE, which is involved in JA biosynthesis, and found its transcription to be similar in WT and syd-2 plants before and after wounding (Figure S1B). Measurement of JA levels reveals that basal

Figure 2. SYD is required within specific stress signaling pathways in response to biotic stress. (A) RT-PCR analysis of select ET/JA responsive genes in Ler and syd-2 detached rosette leaves either mock (M) or B. cinerea (B.c) treated for 48 h. (B) RT-qPCR analysis of SA biosynthesis and signaling genes 0 and 8 hours post inoculation (hpi) with 2x10^8 CFU/ml virulent P. syringae pv. tomato (Pst) DC3000. Transcript levels were normalized to internal control genes measured in the same samples. Data are means of 3 independent biological replicates ±SEM. doi:10.1371/journal.ppat.1000237.g002

Figure 3. The chromatin remodeling ATPase SYD is required for resistance to B. cinerea. (A) Visual symptoms 4 d following spot inoculation with B. cinerea spores. (B) Lesion size 4 d after spot inoculation with B. cinerea spores. Data are means of 16 independent biological replicates ±SEM. Asterisks denote a significant difference from WT (P<0.05) as determined by t-tests. B. cinerea susceptibility assays were performed 3 times with similar results. Statistically different lesion size was also observed 3 d after inoculation. (C,D) Bacterial growth in WT and syd inoculated with 2x10^8 CFU/ml virulent P. syringae pv. tomato (Pst) DC3000. Data are means of 8 independent biological replicates ±SD. No significant differences were detected by t-tests. Pathogen assays comparing Ler versus syd-2 were repeated 3 times with similar results. doi:10.1371/journal.ppat.1000237.g003
and wound-induced JA biosynthesis is intact in syd-2 plants (Figure 4A). In agreement with B. cinerea treatment (Figure 2A), expression of ERF1 is similar in WT and syd-2 in response to wounding (Figure 4B). Furthermore, an additional ethylene response factor (ERF2), which when overexpressed results in enhanced PDF1.2a levels [31], was similar in WT and syd-2 (Figure S1B). These data collectively indicate that SYD activity is required downstream of ET and JA biosynthesis and ERF1&2 expression.

Downstream of ERF1 the expression of PDF1.2a is severely reduced, to similar levels, before and after wounding in syd-2 mutants (Figure 5A). SYD is also required downstream of JA biosynthesis for the expression of the bHLH Leu zipper transcription factor MYC2 (Figure 5B). The reduced expression of MYC2 suggests that the increased level of PRI may indeed be due to decreased MYC2 levels in syd plants. Consistent with the reduced level of MYC2 transcripts, the expression of VEGETATIVE STORAGE PROTEIN 2 (VSP2), a gene in the JA signaling pathway which requires MYC2 for expression [22,32–35], is severely reduced in syd-2 mutants (Figure 5C). Taken together these data show that while chromatin remodeling via SYD is not required for expression of ET and JA biosynthesis genes, SYD activity is required for expression of PDF1.2a, MYC2 and VSP2.

The finding that MYC2 transcript levels are reduced in syd-2 plants (Figure 5B), even though syd-2 is more susceptible to B. cinerea (Figure 3A and 3B), appears to conflict with published models of defense signaling where MYC2 acts as a negative regulator of PDF1.2a expression and resistance to necrotrophic pathogens (Figure 1A) [22,34]. However, the apparent discrepancy can be reconciled. The slight increase in resistance against B. cinerea of myc2/jin1 mutants is thought to be due to derepression of pathogen defense genes such as PDF1.2a [34]. In syd-2 mutants derepression of PDF1.2a does not occur even though the level of MYC2 is reduced, suggesting that the requirement of SYD for PDF1.2a expression precedes repression of PDF1.2a by MYC2. It is also possible that the level of MYC2 transcript reduction in syd-2 is not great enough to have a measurable biological impact. Additionally, increases in resistance to B. cinerea exhibited by the myc2/jin1 mutants, assayed by qualitative disease symptom rating, appeared to be subtle [34], suggesting that the myc2/jin1 effect could be masked in syd mutants. To better quantify the impact of MYC2 on resistance to B. cinerea, we measured lesion development on leaves of myc2/jin1 mutant plants following infection with multiple B. cinerea isolates. We found no significant quantitative difference in lesion formation between WT and myc2/jin1 mutants (Figure 6A and Figure S3A and S3C). We also measured defense-associated secondary metabolites, including camalexin and glucosinolates, in mock- and B. cinerea- treated WT and myc2/jin1 plants. Glucosinolates are associated with Arabidopsis defense against insect herbivores and pathogens and some are regulated by JA signaling [36]. Of the five measured metabolites known to be regulated by JA, camalexin and indole-3-yl-methyl were unaffected by the myc2/jin1 mutation in comparison to WT, 3-methylsulfinyl and 4-methylsulfinyl decreased only in mock treated myc2/jin1 while 4-methoxy-indole-3-yl-methyl was present at higher concentrations in only B. cinerea treated myc2/jin1 (Figure 6B and Figure S3B, S3D and S3E and Table S1). Together these data suggest that MYC2 has neither directionally consistent nor major impacts on all molecular JA responses. It is therefore not surprising that syd mutants are more susceptible to B. cinerea even though MYC2 levels are reduced in syd-2.

The requirement of SYD for the expression of select ET and JA responsive genes suggests that SYD may be directly recruited to the SYD specific antibody, which was previously used in ChIP experiments to show that SYD binds the WUSCHEL promoter to regulate stem cell fate [8]. Additionally, ChIP-qPCR was performed using IgG (negative control) and RNA polymerase II (POLII) (positive control for actively transcribed regions) antibodies. The background level of SYD binding to non-specific genomic loci

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**Figure 4. SYD activity is required downstream of ET and JA biosynthesis.** (A) Measurement of JA metabolite levels in non-wounded and wounded Ler and syd-2 plants. Data are means of 3 independent biological replicates ±SD. (B) Total RNA was extracted from non-wounded and mechanically wounded rosette leaves and subjected to RT-qPCR analysis. ERF1 transcript levels were normalized to At4g26410 measured in the same samples. Data are means of 3 independent biological replicates ±SEM.

doi:10.1371/journal.ppat.1000237.g004

**Figure 5. SYD regulates expression of ET and JA responsive defense genes.** (A–C) Total RNA extracted from non-wounded and wounded Ler and syd-2 rosette leaves was subjected to RT-qPCR analysis. Transcript levels were normalized to both At4g34270 and At4g26410 measured in the same samples. Data are means of 3 independent biological replicates ±SEM.

doi:10.1371/journal.ppat.1000237.g005
(dashed line in Figure 7A and Figure S4) was determined by ChIP-qPCR performed on the promoters of two seed specific genes, **OLEOSIN1** and **AT2S3**, which are subject to repressive histone H3 lysine 27 trimethylation and are not expressed in *Arabidopsis* leaf tissue [37]. Additionally, ChIP-qPCR was performed on *syd-2* tissue wounded for 12 h to further ensure that the results are SYD specific (Figure S5). Under the experimental conditions tested, SYD does not bind the promoter of either **PDF1.2a** or **ERF1** above the background level of detection (Figure 7A and Figure S4). To further verify the lack of SYD binding to **PDF1.2a** a second region of the **PDF1.a** promoter was assayed and SYD binding was not detected (Figure S4). As shown in Figure 7A, SYD binds the promoter of **MYC2** before and after wounding. Finally, SYD is recruited to the promoter of **VSP2** following wounding (Figure 7A).

Based on our findings we propose a model (Figure 7B) that summarizes the roles of SYD in response to wounding. Although
SYD is required for the expression of PDF1.2a we were unable to detect SYD enrichment at the promoter of PDF1.2a, suggesting that SYD may act indirectly through an unknown factor(s) to enable transcription of PDF1.2a. Additionally, SYD is bound to the MYC2 promoter, which is consistent with the reduced expression of MYC2 in ysd-2. The direct recruitment of SYD to the MYC2 promoter may also help explain the reduced transcription levels of VSP2, in non-wounded ysd-2, even though SYD binding to the VSP2 promoter region was only detected following wounding. Altogether these data suggest that the altered expression of ET and JA responsive genes in ysd-2 is likely a result of the loss of SYD acting both directly and/or indirectly on their promoters to regulate transcription.

Conclusions

Our results show that ATP-dependent chromatin remodeling is required for expression of specific genes within stress signaling networks. Additionally, this requirement is likely both direct and indirect as the chromatin remodeling ATPasE SYD binds several, but not all, of the stress responsive promoters examined in vivo. Loss of chromatin remodeling activity also results in increased susceptibility to B. cinerea but not P. syringae. These results provide biological evidence that chromatin remodeling complexes, which are evolutionarily conserved within eukaryotes, are required for stress tolerance not only in yeast but also multicellular organisms. Furthermore, the requirement of ATP-dependent chromatin remodeling complexes is pathogen-specific and not a result of a general reduction in fitness.

Materials and Methods

Plant growth conditions and treatment

Arabidopsis thaliana plants were grown in a 16 h light/8 h dark photoperiod at 22°C; except plants for pathogen treatments, which were grown in a 12 h light/12 h dark photoperiod. Wounding was performed as previously described [21]. All experiments were performed on 4 to 5-wk-old plants, which exhibited no disease symptoms or insect herbivory prior to treatment. Detached leaf assays were performed using the B. cinerea isolates DN, Grape, B05.10 and B05.12 [38]. Arabidopsis leaves were inoculated with 5 μl of spores at a concentration of 50,000 spores/ml [38,39]. For P. syringae bacterial growth assays Arabidopsis leaves were inoculated with 2×106 CFU/ml P. syringae pv. tomato (Pst) DC3000 by hand injection.

Expression analyses

Total RNA from rosette leaves was isolated by TRIzol extraction (Life Technologies, Grand Island, NY) and treated with DNaseI to control for DNA contamination. RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, California). PCR for RT-PCR were conducted in 25 μl reactions containing 20 ng cDNA, 1.3 mM MgCl2, 0.2 mM each dNTP, 0.05 μM each primer, and 1 U Choice-Taq Blue (Denve Scientific, Metuchen, NJ) and amplified for 29 cycles except for PDF1.2a in Figure 1B and ERF1 in Figure 2A, which were amplified for 34 cycles. Quantitative RT-PCR was conducted in 50 μl reactions containing 10 ng cDNA, 1× iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA), and 200 or 250 nM each primer. Amplification and data analysis were carried out as previously described [21]. The internal controls At4g26410 and At5g29410 previously described were used for transcript normalization [40]. Primers are listed in Table S2.

JA measurement

Extraction of JAs (MeJA and JA) were carried out as previously described [40]. Primers are listed in Table S2. The internal controls At4g26410 and At5g29410 previously described were used for transcript normalization [40]. The internal controls At4g26410 and At5g29410 previously described were used for transcript normalization [40].

Statistical analysis

To determine statistical significance of treatment effects comparing WT versus ysd t-tests were performed using Sigma Stat v3.5 (San Jose, CA). For comparison of WT versus myc1/jun1 factorial ANOVA performed within SAS (Cary, NC) was used to analyze the effects of genotype and treatment on measured phenotypes, with significance of differences determined via t-tests of pre-selected comparisons.

Accession numbers

PR1: At2g14610, PDF1.2a: At5g44420, UBQ10: At4g05320, ERF1: At3g23240, PAD4: At5g52430, ICS1: At1g74710, NPR1: 5973 network mass selective detector operated in electron ionization (EI) mode.
levels of detached rosette leaves either mock (M; black bar) or 3 d after spot inoculation with POLII (black bars; right axis) recruitment to the promoter of (A) the JA biosynthesis gene. Data are means of 8 independent biological replicates inoculation of Col and repeated with similar results. (C) Lesion size 3 d after spot isolate B05.10 for 3 d. Data are means of 8 independent biological replicates.

**Supporting Information**

**Figure S1** Expression of wound responsive transcripts in synd. (A) RT-PCR analysis of select rapid wound response genes in Ler and synd-2 plants in response to wounding (B) RT-PCR analysis of the JA biosynthesis gene AOS and the ET/JA responsive ERF2 in response to wounding. Found at: doi:10.1371/journal.ppat.1000237.s003 (0.62 MB EPS)

**Figure S2** RT-PCR expression analysis of genes involved in SA biosynthesis and signaling in response to P. syringae. Found at: doi:10.1371/journal.ppat.1000237.s002 (0.71 MB EPS)

**Figure S3** Role of MYC2 in response to B. cinerea. (A) Lesion size 3 d after spot inoculation with B. cinerea isolate 83-2. B) Camalexin levels of detached rosette leaves either mock (M; black bar) or B. cinerea isolate 83-2 (B); grey bar) treated for 3 d. (A and B) Two alleles were tested, jin1-8 and jin1-9. No significant differences between alleles were detected, therefore the two alleles were pooled (myc2/jin1). Data are means of 8 (Col) or 16 (myc2/jin1) independent biological replicates ± SEM. Experiments were repeated with similar results. (C) Lesion size 3 d after spot inoculation of Col and jin1-9 plants with B. cinerea isolate B05.10. Data are means of 8 independent biological replicates ± SEM. (D) Camalexin levels of detached rosette leaves treated with B. cinerea isolate B05.10 for 3 d. Data are means of 8 independent biological replicates ± SEM. (E) Camalexin levels of detached rosette leaves treated with 5 mM AgNO3 for 3 d. Data are means of 8 independent biological replicates ± SEM.

**Figure S4** SYD does not bind the promoter of PDF1.2a or ERF1. ChIP-qPCR analysis of SYD (grey bars; left y-axis) and POLII (black bars; right axis) recruitment to the promoter of (A) PDF1.2a (-836 to -604) and (B) ERF1 (-1035 to -942). Data presented are normalized to input DNA and expressed as fold enrichment of SYD or POLII relative to IgG. ChIP-qPCR was performed on non-wounded and wounded Ler plants. Data are means of 3 or 4 independent biological replicates ± SEM. The dashed line represents the mean SYD background fold enrichment assayed at the promoters of OLEOSIN1 and AT283.

Found at: doi:10.1371/journal.ppat.1000237.s006 (0.03 MB XLS)

**Table S1** Glucosinolate levels in myc2/jin1 plants. Found at: doi:10.1371/journal.ppat.1000237.s004 (0.57 MB EPS)

**Table S2** Primers used for expression and ChIP-qPCR analysis. Found at: doi:10.1371/journal.ppat.1000237.s007 (0.02 MB XLS)

**Acknowledgments**

We thank Simon Chan, Dior Kelley, and Peter Quail for comments regarding this manuscript and the UC Davis Genome Center Expression Analysis Core Facility for assistance with the ChIP protocol. We would also like to thank Siobhan Braybrook and John Harada for the OLEOSIN1 and AT283 primers.

**Author Contributions**

Conceived and designed the experiments: JW HCR KD. Performed the experiments: JW HCR YX EWC. Analyzed the data: JW HCR YX EWC. Contributed reagents/materials/analysis tools: DJK DW KD. Wrote the paper: JW.

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