Cell Wall Invertase Is Essential for Ovule Development through Sugar Signaling Rather Than Provision of Carbon Nutrients

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Ovule formation is essential for realizing crop yield because it determines seed number. The underlying molecular mechanism, however, remains elusive. Here, we show that cell wall invertase (CWIN) functions as a positive regulator of ovule initiation in Arabidopsis (Arabidopsis thaliana). In situ hybridization revealed that CWIN2 and CWIN4 were expressed at the placenta region where ovule primordia initiated. Specific silencing of CWIN2 and CWIN4 using targeted artificial microRNA driven by an ovule-specific SEEDSTICK promoter (pSTK) resulted in a substantial reduction of CWIN transcript and activity, which blocked ovule initiation and aggravated ovule abortion. There was no induction of carbon (C) starvation genes in the transgenic lines, and supplementing newly forming floral buds with extra C failed to recover the ovule phenotype. This indicates that suppression of CWIN did not lead to C starvation. A group of hexose transporters was downregulated in the transgenic plants. Among them, two representative ones were spatially coexpressed with CWIN2 and CWIN4, suggesting a coupling between CWIN and hexose transporters for ovule initiation. RNA-sequencing analysis identified differentially expressed genes encoding putative extracellular receptor-like kinases, MADS-box transcription factors, including STK, and early auxin response genes in response to CWIN-silencing. Our data demonstrate the essential role of CWIN in ovule initiation, which is most likely to occur through sugar signaling instead of C nutrient contribution. We propose that CWIN-mediated sugar signaling may be perceived by, and transmitted through, hexose transporters or receptor-like kinases to regulate ovule formation by modulating downstream auxin signaling and MADS-box transcription factors.

In higher plants, most carbohydrate making up the bulk of the biomass and crop yield originates from Suc, making Suc a crucial yield determinant (Ruan, 2014). Suc is predominately derived from photosynthetically active leaves and translocated through phloem to sink organs. Upon being delivered to sinks, it is degraded either by Suc synthase (Sus; EC 2.4.1.13) or invertase (INV; EC 3.2.1.26). Sus reversibly degrades Suc into uridine diphosphate (UDP)-Glc and Fru in the presence of UDP. INV, on the other hand, irreversibly hydrolyzes Suc into Glc and Fru. Based on their subcellular location, INV s are classified into three subgroups: cell wall invertase (CWIN), vacuolar invertase (VIN), and the structurally unrelated cytoplasmic invertase (CIN; Sturm, 1999, Wan et al., 2018). CWINs, also referred to as extracellular or apoplastic INV s, are insoluble proteins ionically bound to the cell wall with a pH optimum of 3.5 to 5.0 (Roitsch and González, 2004). Although symplasmic transport through plasmodesmata (PDs) is considered a common pathway for Suc unloading, Suc can only be transported apoplasmically across cell wall matrix and plasma membranes in cellular interfaces lacking the PD connection (Patrick, 1997). In the latter case, Suc is often unloaded from the sieve element-companion cell (SE/CC) to the surrounding cell wall matrix, and then taken up by recipient sink cells via Suc transporters (SUTs) or hexose transporters (HXTs) following hydrolysis of Suc by CWIN at the extracellular space (Braun et al., 2014; Li et al., 2017). The CWIN-SUT/HXT nexus could also operate in regions beyond the SE/CC unloading site for post-phloem delivery of assimilates in sink organs (Li et al., 2017). For example, as a common feature of all developing angiosperm seeds, the filial tissue (endosperm and embryo) is symplasmically isolated from the surrounding maternal seed coat or pedicel in dicot or monocot species, respectively. In this scenario, assimilates must be transported across the apoplastic compartment between the maternal and filial tissues to nurture the developing embryo, where CWIN could play a critical role in seed development (Patrick 1997; Weber et al., 2005). A classic example is the maize (Zea mays) miniature-seed...
phenotype as a result of mutation of a CWIN gene expressed in the endosperm transfer cell, the outermost cell layer of the filial tissue facing the pedicel (Miller and Choureay, 1992). Similarly, in fava bean (Vicia faba), CWIN activity at the innermost layer of the seed coat positively correlates with cell division of cotyledons, hence seed size (Weber et al., 1996). In developing tomato fruit (Solanum lycopersicum), a CWIN gene is mainly expressed in the phloem of placenta connecting the developing seed where unloading occurs apoplasmically (Jin et al., 2009; Palmer et al., 2015). Here, genetically elevating the endogenous CWIN activity increased seed weight (Jin et al., 2009), whereas silencing its expression resulted in stunted seed (Zanor et al., 2009). A similar role for CWIN has been reported in other species, including rice (Oryza sativa; Wang et al., 2008) and barley (Hordeum vulgare; Weschke et al., 2003).

Apart from its function in Suc unloading, CWIN also plays a role in sugar signaling (Ruan, 2012). For instance, the maize miniature seed phenotype of CWIN-deficient mutant could not be recovered by the provision of exogenous hexose (Cheng and Choureay, 1999), and enhancing CWIN expression in the shoot apical meristem accelerated flowering and increased inflorescence branching in Arabidopsis (Arabidopsis thaliana; Heyer et al., 2004). More recently, elevation of CWIN activity in tomato was found to induce the expression of R and PR genes in fruitlets (Ru et al., 2017) and to block programmed cell death under heat stress (Liu et al., 2016). These findings indicate that CWIN has a signaling function in development and defense, but they do not differentiate between that and its role in providing sugar nutrients.

Despite the extensive studies of CWIN as outlined above, it remains unknown whether and how CWIN may exert its potential role in the formation of seed precursors, ovules, at the prefertilization stage. Ovule initiation and establishment represent a crucial window that sets yield potential, as ovule formation determines seed number. Not only would progress on this frontier contribute to our understanding of the roles of CWIN-mediated Suc metabolism or signaling in prefertilization-reproductive development, it could also uncover promising targets for designing innovative approaches to enhance crop seed yield.

In this study, we reported that two CWIN genes, CWIN2 and CWIN4, were highly expressed in the placenta region of gynoecia where ovule primordia initiate. To determine their potential role in ovule development, we generated transgenic lines in which an ovule-specific SEEDSTICK promoter (pSTK; Kooiker et al., 2005) was used to drive artificial microRNA (amiRNA) specifically suppressing CWIN2 and CWIN4 during ovule formation (amiRNA:CWIN24). The pSTK:amiRNA:CWIN24 transgenic plants exhibited inhibition of ovule initiation and increased ovule abortion, which collectively resulted in >50% seed loss compared to the wild type. Surprisingly, measurement of the transcript levels of carbon (C) starvation reporter genes and manipulation of the source/sink ratio showed that the transgenic plants did not suffer from C starvation. This, together with the finding that as-similate is delivered symplasmically to the ovule primordia (Werner et al., 2011), shows that CWIN regulates ovule initiation through sugar signaling rather than provision of C nutrient. Further transcriptomic analyses support a model in which CWIN-generated hexose signals can be perceived by and transmitted through a cohort of plasma membrane HXTs or receptor-like kinases (RLKs) to modulate downstream candidate transcription factor (TF) and auxin signaling genes, thereby positively regulating ovule development.

RESULTS

CWIN2 and CWIN4 Were Highly Expressed in the Floral Meristem, Ovule Primordia, and Young Ovules in Wild-Type Inflorescence

Among the four CWIN paralogs in Arabidopsis, CWIN2 and CWIN4 are known to be predominately expressed in reproductive organs (Schmid et al., 2005). As a first step to address the potential involvement of CWIN in ovule development, we performed in situ hybridization experiments to examine whether CWIN2 and CWIN4 were expressed in the cellular sites of ovule initiation.

The analysis revealed that CWIN2 mRNA was detected in the inflorescence meristem and floral primordium (Fig. 1A), in comparison with the sense control (Fig. 1B). The signal intensity of the CWIN2 transcript in the floral primordium appeared to increase from early stages 2 and 3 to late stage 5, when the primordium gave rise to gynoecium and stamens (Fig. 1A). The transcript was also evidently detected in gynoecium and stamens at stage 7 (Fig. 1A). Noticeably, strong expression of CWIN2 mRNA was observed in the middle part of the gynoecium at stage 9 (Fig. 1C), where ovule initiation takes place (Smyth et al., 1990). The transcript was also detectable in the developing ovules at stage 11 (Fig. 1D) and in anthers (Fig. 1, C and D, versus Fig. 1E). Interestingly, abundant expression of CWIN2 was found in the stigma papillae at the top of the gynoecium at stage 11 (Fig. 1D).

CWIN4 mRNA exhibited an expression pattern similar to that of CWIN2 in the inflorescence meristem and floral primordia (Fig. 2). Evident expression of CWIN4 was observed in the inflorescence meristem, floral primordium, and early floral organ primordia from stages 1 to 7 (Fig. 2, A and B), including primordia of gynoecium and stamen, in comparison with the sense control (Fig. 2C). Strong CWIN4 mRNA signals were detected in the central part of the gynoecium when the ovule initiated at stage 9 (Fig. 2D), similar to the case for CWIN2 (Fig. 2C). Expression of the CWIN4 gene was also present in the developing ovules from stages 10 and 11 (Fig. 2, E and F) in comparison with the sense control (Fig. 2G). However, the CWIN4 mRNA signal strength became weaker in the developing ovules
compared to that in the early stage of gynoecium (Fig. 2B) and ovule initiation (Fig. 2D).

**pSTK::amiRNA<CWIN24 Transgenic Plants Displayed a Significant Reduction of CWIN2 and CWIN4 Transcript Levels at the Ovule Initiation Stage**

The data from the in situ hybridization experiments indicate a possible role of CWIN2 and CWIN4 in ovule development. We thus further investigated whether disruption of CWIN2 and CWIN4 expression would affect ovule development. To achieve this, we employed an amiRNA approach to silence the expression of the two CWIN genes using an ovule-specific pSTK (Kooiker et al., 2005).

Through transgene screening and selection analyses, we obtained five independent transgenic lines homozygous for the transgene pSTK-amiRNA<CWIN24 at the T3 generation. Among them, lines 4-3, 80-4, and 106-2 displayed a severe reduction in seed number and siliqua length compared to those of the wild type (Supplemental Fig. S1). These lines were subsequently selected for detailed analysis. We first examined the expression level of CWIN2 and CWIN4 using a pooled sample of the flower buds from stages 8 to 10, when ovules initiate from the placenta (Figs. 1 and 2; Smyth et al., 1990). In comparison with the wild type, the transcript levels of CWIN2 and CWIN4 were reduced by 40% to 45% and 30% to 54%, respectively, in the transgenic lines (Fig. 3, A and B). We further investigated whether the reduction of CWIN2 and CWIN4 transcripts had any effect on the expression of other members of the INV family (CWIN1, CWIN5, VIN1, and VIN2; Wang and Ruan, 2012). None of these genes displayed altered expression levels compared to the wild type (Fig. 3, C–F). The findings indicate specific suppression of CWIN2 and CWIN4 in pSTK::amiRNA<CWIN24 transgenic plants.

We next examined whether suppression of CWIN2 and CWIN4 led to a decrease in CWIN activity. Here, enzyme activity was assayed on gynoecia-enriched samples that were carefully dissected from the same floral buds used for reverse transcription quantitative PCR (RT-qPCR) analysis. The dissection was based on the consideration that the ovule-specific STK-driven amiRNA<CWIN24 would specifically silence CWIN2 and CWIN4 in the placenta region and ovule primordia rather than in the surrounding tissues, such as anthers, sepals, and petals, where CWIN2 and CWIN4 were also expressed (Figs. 1 and 2). The enzyme assay revealed that CWIN activity was reduced by ~40% in transgenic lines 4-3 and 80-4 and by 18% in line 106-2 (Fig. 3G).
Neither VIN nor CIN activities were affected in the transgenic lines (Fig. 3G).

Collectively, these data showed that the pSTK-driven amiRNA/CWIN24 specifically suppressed CWIN2 and CWIN4 expression, leading to a reduction of CWIN activity at the ovule initiation stage.

Silencing CWIN Inhibited Ovule Initiation and Induced Ovule Abortion

We then investigated whether silencing of CWIN2 and CWIN4 impacted ovule development. Phenotypic analyses revealed a significant reduction in silique length of the transgenic plants compared to that in the wild type (Fig. 4A) with average reduction of 50.8%, 62.6%, and 56% in seed number per silique in lines 4-3, 80-4, and 106-2, respectively (Supplemental Fig. S1; Supplemental Table S1).

To determine the developmental basis for the reduction in seed number, we counted the ovule number from cleared pistils dissected from flower buds at stages 10 to 11, prior to anthesis. The ovule number was significantly reduced in the transgenic lines to ~33 to 40 per pistil, as compared to ~59 in the wild type (Fig. 4B, D, and Supplemental Table S1). The pistil length was also reduced by 20% to 30% in the transgenic lines compared to that in the wild type (Fig. 4B).

The ratio of seed to ovule number was 0.95 in the wild type, but was reduced to ~0.62 to 0.74 in the transgenic lines (Supplemental Table S1), indicating aggravated ovule or seed abortion, manifested as gaps shown in the cleared siliques at stage 17 in the transgenic lines (Fig. 4A, red arrows). Under a dissection microscope, a substantial number of residual white structures were clearly observed in the transgenic siliques (Fig. 4C, red asterisks), a phenotype hardly observed in the wild-type siliques. The tiny white dots in the transgenic lines morphologically resembled ovule abortion (Ebel et al., 2004). However, we cannot rule out the possibility that some may be residuals derived from early seed abortion. On average, there were 10, 12, and 15 tiny white dots (designated as ovule or seed abortion) per silique in lines 4-3, 80-4, and 106-2, respectively, as compared to only three in the wild-type siliques, hence constituting a higher abortion rate in the transgenic lines (Fig. 4E).

Based on the knowledge of ovule number per pistil (Fig. 4B; Supplemental Table S1), the number of ovules blocked from initiation in the transgenic pistils was calculated by subtraction of the number of transgenic ovules from that of wild-type ovules. It was found that on average, 22, 26, and 20 ovules were blocked from initiation in lines 4-3, 80-4, and 106-2, respectively (Supplemental Table S1), accounting for ~60% to 70% of seed loss, with the remaining ~30% to 40% loss derived from ovule or seed abortion (Fig. 4D and F). These findings demonstrate that blockage of ovule initiation is the primary cause of reduction in seed number, with ovule or seed abortion contributing to additional seed loss in the transgenic plants.

Blockage of Ovule Initiation Was Not Caused by C Starvation in the CWIN-Silenced Plants

In Arabidopsis, Suc supply to young ovules follows a symplasmic pathway, as evidenced by the unloading of GFP and fluorescent tracers from the placenta SE/CC to the ovule primordia at floral stage 9 (Werner et al., 2011). Given that CWIN typically plays a role in
apoplastic phloem unloading and postphloem transport (e.g. Jin et al., 2009, Palmer et al., 2015), it is intriguing from a C nutrient perspective that silencing of CWIN2 and CWIN4 in pSTK:amiRCWIN24 transgenic lines. RT-qPCR analysis was conducted using a pool of flower buds from stages 8 to 10, when the ovule initiates from placenta (Smyth et al., 1990). Each value is the mean ± s.e. of at least four biological replicates for each independent transgenic line. One replicate was from a pooled sample comprised of ~10 flower buds at stages 8 to 10 harvested from one plant. Different lowercase letters over bars indicate significant difference (P ≤ 0.05, one-way ANOVA). G. Reduction of CWIN activity in the pSTK:amiRNA-CWIN24 transgenic plants compared to the wild type. Note that there is no difference in VIN and CIN activity between wild-type and transgenic plants. Each value is the mean ± s.e. of six biological replicates. Each replicate was comprised of pooled gynoecium samples that were dissected from flower buds at stages 8 to 10 from more than three individual plants. Different lowercase letters over bars indicate significant difference (P ≤ 0.05, one-way ANOVA).

To directly investigate whether the transgenic floral buds were under C starvation, we measured the transcript levels of three C starvation report genes, ATL8 (At1g76410), KMD4 (At3g59940), and OLEOSIN7 (At5g56100), that are strongly induced in the flower buds at stages 8 to 10 under sugar-depletion conditions (Lauxmann et al., 2016). RT-qPCR analysis revealed that none of these genes was induced in the transgenic plants (Fig. 6). The above findings indicate that pSTK-amiRNA-CWIN24 plants did not suffer from C starvation and the ovule phenotype did not arise from insufficient C supply.

RNA-Sequencing Analysis between pSTK-amiRNA-CWIN24 and Wild-Type Plants: Overall Quality and Reliability Assessment

To explore the underlying basis of CWIN-mediated ovule initiation, RNA-sequencing (RNA-seq) was conducted to compare the transcript profiles of flower buds
pooled from stages 8 to 10 in a representative transgenic line, 4-3, and the wild type. After trimming, an average of 58.11 million and 55.58 million clean reads were obtained from four biological replicates of the wild-type and transgenic plants, respectively. Approximately 90% of the clean reads were uniquely mapped to The Arabidopsis Information Resource genome reference (TAIR10; Supplemental Fig. S2A). Principal component analysis (PCA) of the transcriptome revealed a clear separation between the transgenic line and the wild type and showed that the biological replicates were clustered closely in each genotype, with no outlier detected (Fig. 7A). Hence, all replicates from each genotype were used for subsequent identification of differentially expressed genes (DEGs). A total of 1,940 DEGs were identified between transgenic and wild-type plants (Supplemental Table S2), of which 408 were upregulated and 1,082 were downregulated (Fig. 7B).
To validate the RNA-seq analysis, we selected a subset of genes for RT-qPCR analysis across the pSTKamiRNA24 transgenic line and the wild type using the same samples as for RNA-seq. The analysis revealed that the transcript fold change (FC) of the seven selected genes from RT-qPCR measurement was consistent overall with that of RNA-seq (Supplemental Fig. S2B). It was noted that the FC measured by RT-qPCR was generally lower than that from the RNA-seq analysis, probably due to RNA-seq being more sensitive and specific than RT-qPCR (Griffith et al., 2010). Importantly, correlation analysis of these two data sets revealed a $R^2$ value of 0.6756 (Supplemental Fig. S2C), which is within the range of reported

Figure 5. Increasing C nutrient availability to the newly formed floral buds by trimming two-thirds of the developed siliques did not recover the ovule phenotype in the pSTK:amiRNA24 transgenic plants. A, Thirty-five-day-old Arabidopsis plant before (left) and after (right) trimming. The newly developed siliques from the shoot apex (red circles) were counted and measured at ~5 d after anthesis, at flower stage 17, when the siliques were fully expanded. Scale bar = 0.4 cm. B to E, Statistical analysis of silique length (B), seed number per silique (C), ovule number per silique (D), and percentage of aborted ovule of total ovule per silique (E) before and after trimming in pSTK:amiRNA24 transgenic plants. Each value is the mean ± se, with data collected from at least six biological replicates; each replicate was comprised of counts from 5 to 10 fully expanded siliques harvested from one individual plant. The double asterisk indicates significant difference relative to the wild type (WT; **$P \leq 0.01$, Student’s t test).
correlations between RNA-seq and RT-qPCR results (Xu et al., 2011). Together, the analyses indicate the reliability of our RNA-seq data.

A Large Number of Early Auxin-Responsive Genes Were Downregulated in Response to CWIN Silencing

Given the increasing number of studies indicating that sugar-hormone interaction plays a role in modulating plant development (León and Sheen, 2003; Wang and Ruan, 2013) and the importance of hormones in ovule initiation (Cucinotta et al., 2014), we closely examined hormone-related DEGs from the transgenic and wild-type plants.

In total, 49 DEGs were classified in the hormone category, which was subclassed into six groups (Fig. 7C). Among them, genes involved in auxin, abscisic acid, and ethylene metabolism were the top three groups, representing 53%, 16%, and 14% of the total hormone genes, respectively. Genes involved in gibberellin (GA), jasmonate (JA), and cytokinin (CK) pathways were each <10% of the total hormone-related genes (Fig. 7C). These findings indicate that in comparison with other hormone pathways, auxin-related genes appear to be affected the most in response to CWIN suppression.

Among the 26 auxin-related genes, the majority (19 of 26) were early auxin response genes (Table 1). Remarkably, these early auxin response genes were all downregulated in response to CWIN suppression, which includes 14 SMALL AUXIN UP RNAs (SAURs), four AUX/IAAs, and one GH3. AUX/IAA encodes a short-lived nuclear protein that functions as a repressor against auxin-inducible gene expression, in which AUX/indole-3-acetic acid (IAA) forms a heterodimer with auxin response factors (ARFs) to repress the transcriptional activity of ARF genes (Hayashi, 2012). GH3 encodes an enzyme that catalyzes the conjugation of IAA with amino acids to yield an inactive storage form of IAA (Ludwig-Müller, 2011), thereby modulating the intracellular IAA level. Among the 14 SAURs downregulated in the CWIN-silenced samples, eight, SAUR1, SAUR4, SAUR12, SAUR21, SAUR29, SAUR64, SAUR67, and SAUR70, were auxin inducible, and one, SAUR6, was auxin repressive (Ren and Gray, 2015); functions for the remaining five SAURs remain unknown.

A Cohort of MADS-Box TFs Were Downregulated in pSTK:amiRNA24 Plants, and Overexpression of One of Them, STK, Partially Complemented the Silique and Seed Phenotype

A total of 76 DEGs encoding TFs (classified into 30 TF families) were identified from the transgenic samples (Supplemental Table S3), representing 5.1% of the 1,490 DEGs. This was done by searching the Arabidopsis transcription database (PlantTFDB), which contains 2,996 TFs classified into 58 families (Jin et al., 2017). Previous studies in Arabidopsis have found that most of the TFs that function in ovule formation belong to the MADS-box family (formerly MICK_MADS TFs, STK, and AGAMOUS (AG), sharing a common function in determining ovule identity and exhibiting overlapping expression patterns in ovule primordia (Pinyopich et al., 2003; Skinner et al., 2004). Indeed, RNA-seq analysis revealed that members of two subfamilies of the MADS family were significantly downregulated in response to CWIN suppression, including three MICK_MADS TFs, STK (formerly AGL11), AGL66, and AGL104, and one M_type_MADS TF, AGL94 (Table 2). These findings suggest that MADS TFs may function as downstream targets of CWIN-mediated ovule initiation pathways. Interestingly, among the three ovule identity genes, SHP1, SHP2, and STK, only STK was downregulated in response to CWIN silencing, suggesting that CWIN-mediated ovule initiation may be exerted, in part, through modulation of STK expression.

To test this possibility, STK, driven by its native promoter, was overexpressed in the transgenic background to test whether overexpression could complement the ovule phenotype. Here, a pSTK::STK (coding sequence [CDS]) overexpression construct was transformed into transgenic line 4-3. Two homozygous complementation lines, 2-9 and 7-7, were identified that exhibited partial recovery of the silique-length phenotype compared to the wild type and CWIN-silencing
The STK mRNA level was fully recovered and increased in complementation lines 7-7 and 2-9, respectively (Fig. 8B), leading to partial complementation of the silique-length and seed-number phenotypes (Fig. 8, C and D), due to partial recovery of ovule number and alleviated abortion in lines 2-9 and 7-7 (Fig. 8, E and F).

*pSTK*-amiRNA-Mediated Silencing of CWIN Altered the Expression of Genes Encoding RLKs and Small GTPases

As described above, a wide variety of hormone-related and TF genes exhibited differential expression in response to CWIN suppression. This, together with the findings that CWIN may regulate ovule initiation through sugar signaling (Figs. 5 and 6), suggests that alteration in sugar concentration or flux by modulating CWIN in the extracellular region must be sensed, probably by plasma membrane proteins (Ruan, 2014), for transduction of the signal into the intracellular space to regulate gene expression. Consistent with this view, 23 of 28 DEGs encoding RLKs, showed increased expression during ovule initiation in response to CWIN silencing (Table 3). RLKs are a group of transmembrane proteins with an N-terminal extracellular domain, a transmembrane span, and a cytosolic protein kinase domain that are involved in a wide range of signal perception and transduction activities (Wu et al., 2016). We also found 15 DEGs encoding small GTPase-related proteins; all of them exhibited decreased expression during ovule initiation in the CWIN-suppressed plants compared to wild-type plants (Table 3). Apart from the small GTPase genes, three genes encoding regulatory proteins of Rho GTPase were also observed (Table 3). These include Rho of plants (Rop) guanine nucleotide exchange factor 9 (ROPGEF9) and two Rho GTPase effector proteins, RIC1 and RIC3 (Vernoud et al., 2003).

We also noticed that two wall-associated kinase (WAK) genes, WAK1 and WAK2, were upregulated in response to CWIN silencing (Table 3). WAKs represent a unique class of RLKs that contain an extracellular domain that physically links with the pectin molecules of the cell wall and a cytoplasmic Ser/Thr kinase domain (Kohorn et al., 2006).

Hexose Transporter Genes Were Largely Repressed in Response to CWIN Silencing during Ovule Initiation

Suc hydrolysis by CWIN generates Glc and Fru in the extracellular space, which are then taken up across the plasma membrane by HXTs into the adjacent cells, with
Table 1. A large number of auxin signaling-related genes were downregulated during ovule initiation in pSTK-amiRNA24 transgenic plants compared with the wild type

RPKM values were derived from four biological replicates of floral buds at stages 8 to 10, when ovules initiate, with each replicate comprised of 10 to 15 buds from one plant.

| Name   | Average RPKM | Log₂ FC | Annotation                      |
|--------|--------------|---------|---------------------------------|
|        | Wild Type    | Transgenic |                               |
| IAA1   | 4.85         | 1.24    | −2.22 IAA5 (IAA Inducible 5)    |
| IAA19  | 15.25        | 7.62    | −1.11 IAA19 (IAA Inducible 19)  |
| IAA14  | 1.25         | 0.51    | −1.31 IAA14 (IAA Inducible 14)  |
| IAA1   | 20.22        | 9.24    | −1.22 IAA1 (IAA Inducible 1)    |
| SAUR64 | 1.40         | 0.64    | −1.19 Auxin-responsive protein, putative; auxin inducible |
| SAUR67 | 5.71         | 2.90    | −1.03 SAUR67; auxin inducible   |
| SAUR86 | 7.64         | 3.79    | −1.09 Auxin-responsive protein, putative; auxin repressive |
| SAUR12 | 10.83        | 5.57    | −1.08 Auxin-responsive protein, putative; auxin inducible |
| SAUR29 | 1.16         | 0.48    | −1.79 Auxin-responsive protein, putative; auxin inducible |
| SAUR47 | 1.37         | 0.08    | −4.42 Auxin-responsive protein, putative |
| SAUR39 | 3.55         | 0.21    | −4.2 Auxin-responsive protein-related |
| SAUR49 | 19.61        | 8.61    | −1.28 Auxin-responsive protein, putative |
| SAUR1  | 2.10         | 1.00    | −1.18 Auxin-responsive family protein; auxin inducible |
| SAUR3  | 2.55         | 0.87    | −1.7 Auxin-responsive family protein |
| SAUR4  | 2.93         | 1.40    | −1.13 Auxin-responsive family protein; auxin inducible |
| SAUR5  | 2.83         | 0.95    | −1.64 Auxin-responsive family protein |
| SAUR21 | 4.63         | 2.33    | −1.06 Auxin-responsive protein, putative; auxin inducible |
| SAUR70 | 8.38         | 3.81    | −1.21 Auxin-responsive protein, putative; auxin inducible |
| AT5G13335 | 3.99   | 0.58    | −2.88 Auxin-responsive CH3 family protein |
| YUC4   | 0.54         | 1.18    | 1.06 Auxin biosynthesis         |
| YUC2   | 5.04         | 2.39    | −1.15 Auxin biosynthesis        |
| AT1G54070 | 1.30   | 0.06    | −4.6 Dormancy/auxin associated protein-related |
| DMRI2 | 71.75        | 4.59    | −4.05 Dormancy/auxin associated family protein |
| AT5G47530 | 8.02   | 1.32    | −2.66 Auxin-responsive protein, putative |
| BIG    | 4.10         | 9.16    | 1.06 BIG, a positive regulator of polar auxin transport |
| AT2G04852 | 0.79   | 1.97    | 1.22 Locus overlaps with AT2G04850 |

Table 2. Genes encoding several MADS TFs were downregulated in pSTK-amiRNA24 transgenic plants compared with the wild type during ovule initiation

RPKM values were derived from four biological replicates of floral buds at stages 8 to 10, when ovules initiate, with each replicate comprised of 10 to 15 buds from one plant.

| TF Family | Gene Identifier | Gene Name | Average RPKM | Log₂ FC |
|-----------|-----------------|-----------|--------------|---------|
| MIKC_MADS | AT4G09960       | STK       | 9.82         | 0.60    | −4.37 |
|           | AT1G77980       | AGL66     | 2.65         | 0.60    | −2.26 |
|           | AT1G22130       | AGL104    | 6.95         | 1.65    | −2.17 |
| M-type_MADS | AT1G69540   | AGL94     | 5.05         | 1.68    | −1.71 |
By contrast, none of the DEGs found for Suc transporters was expressed in the CWIN-silenced plants.

The downregulation of nine HXT genes in response to CWIN silencing prompted us to further examine whether they are spatially coexpressed with CWIN2 and CWIN4 during ovule initiation. To achieve this, we performed in situ hybridization on sections of floral buds from stages 8 to 10 in wild-type plants. We selected SWEET8 and STP9 as representatives of the respective SWEET and STP subfamilies, because they displayed high transcript levels during ovule initiation in the wild type (Table 3). In comparison with the sense probe control (Fig. 9A), the STP9 mRNA signal was detected in sections of floral buds at stages 8 to 10 (Fig. 9, B–D). Its transcript signals were observed in the middle part of the gynoecium before ovule primordia initiation at stage 8, in the initiating ovule primordia at stage 9, and in developing ovules at stage 10 (Fig. 9, B–D). A similar expression pattern was observed for SWEET8 mRNA (Fig. 9, E–H). Overall, the expression patterns of STP9 and SWEET8 overlapped with that of CWIN2 and CWIN4 (Figs. 1 and 2).

DISCUSSION

CWIN Plays an Essential Role in Ovule Initiation through Sugar Signaling Rather Than Provision of Carbon Nutrients

CWIN-mediated Suc metabolism and signaling is central to plant development, including the formation of pollen fertility, seed and fruit set, and stress response (Ruan et al., 2010; Ruan, 2014). It remains unknown, though, whether and how CWIN plays a role in ovule formation, a prerequisite for seed production. Here, we provide molecular and developmental evidence that CWIN plays a critical role in ovule initiation and growth in Arabidopsis. Among the CWIN gene family in Arabidopsis, CWIN2 and CWIN4 are predominately expressed in reproductive organs (Schmid et al., 2005), in which the two CWINs may play complementary or additive roles. In situ hybridization experiments revealed high expression of CWIN2 and CWIN4 mRNAs in the placenta region, where ovules initiate (Figs. 1 and 2). Ovule-specific silencing of CWIN2 and CWIN4 using the amiRNA approach resulted in significant and specific reduction of CWIN2 and CWIN4 transcripts, leading to a decrease in CWIN activity without impacting VIN and CIN expression or activity (Fig. 3). This molecular intervention reduced the seed number by >50% compared to that in the wild type, mainly due to blockage of ovule initiation and, to a less extent, aggravated ovule abortion (Fig. 4). These findings demonstrate that CWIN is critical for ovule initiation and differentiation. The findings fill a major gap in the understanding of CWIN-mediated Suc metabolism and signaling in plant development.
Table 3. Genes encoding RLKs, small GTPases, and HXTs were differentially expressed in pSTK-amiRNA CWIN24 during ovule initiation compared with the wild type.

RPKM values were derived from four biological replicates of floral buds at stages 8 to 10, when ovules initiate, with each replicate comprised of 10 to 15 buds from one plant.

| Functional Category       | Gene Identifier | Average RPKM | Log2 FC | Annotation                                                        |
|--------------------------|----------------|--------------|---------|------------------------------------------------------------------|
| **Signaling RLKs**        |                |              |         |                                                                  |
|                          | AT5G59670      | 4.06         | 8.89    | 1.05 LRR protein kinase, putative                               |
|                          | AT2G07040      | 1.79         | 0.08    | -4.51 ATPRK2A, PRK2A | PRK2A;                                   |
|                          | AT5G07620      | 1.25         | 4.26    | 2 Protein kinase family protein                                  |
|                          | AT4G20790      | 1.25         | 0.52    | -1.36 LRR family protein                                        |
|                          | AT1G07650      | 11.63        | 27.88   | 1.17 LRR transmembrane protein kinase                            |
|                          | AT1G34340      | 6.6          | 15.24   | 1.1 LRR family protein/protein kinase family protein             |
|                          | AT1G56120      | 0.63         | 1.39    | 1.02 Receptor kinases LRR VII.VIII-2                             |
|                          | AT1G34420      | 0.68         | 1.47    | 1.31 LRR family protein/protein kinase family protein            |
|                          | AT1G09970      | 20.98        | 52.69   | 1.24 LRR XI-23 | LRR XI-23                                                        |
|                          | AT4G28490      | 2.84         | 6.41    | 1.08 RLK5 HAE (HAESA)                                           |
|                          | AT5G25930      | 1            | 3.04    | 1.52 LRR family protein/protein kinase family protein            |
|                          | AT1G35710      | 3.38         | 7.4     | 1.36 LRR transmembrane protein kinase                            |
|                          | AT1G54380      | 11.16        | 23.85   | 1.02 THE1 (THESEUS1); protein kinase                            |
|                          | AT4G21410      | 3.73         | 8.33    | 1.05 Protein kinase family protein                               |
|                          | AT4G213130     | 1.11         | 2.56    | 1.12 CRK5 (Cys-rich RLK5)                                        |
|                          | AT4G32140      | 0.76         | 2.07    | 1.34 CRK6 (Cys-rich RLK6)                                        |
|                          | AT4G32120      | 0.8          | 2.26    | 1.4 Protein kinase family protein                                |
|                          | AT1G66880      | 3.39         | 7.47    | 1.15 Ser/Thr protein kinase protein                               |
|                          | AT5G38240      | 1            | 2.96    | 1.45 Ser/Thr protein kinase family protein                       |
|                          | AT2G18470      | 3.56         | 0.48    | -3.02 Protein kinase family protein                              |
|                          | AT4G34440      | 1.32         | 0.49    | -1.54 Protein kinase family protein                              |
|                          | AT3G12000      | 9.18         | 4.25    | -1.18 S-locus-related protein SLR1, putative (S1)                 |
|                          | AT1G21250      | 7.22         | 21.76   | 1.49 WAK1 (Cell wall-associated kinase 1)                        |
|                          | AT1G21270      | 2.41         | 5.28    | 1.03 WAK2 (Cell wall-associated kinase 2)                        |
| **Small GTPases**         |                |              |         |                                                                  |
|                          | RIC1           | 1.48         | 0.23    | -2.61 Rop-Interactive Crib motif-containing protein 1            |
|                          | RIC3           | 4.92         | 0.34    | -3.3 Rop-Interactive Crib motif-containing protein 3             |
|                          | ROP1           | 7.91         | 3.02    | -1.48 Rho-related protein from plants 1; GTP binding             |
|                          | ROP7           | 3.89         | 1.48    | -1.44 Arabidopsis RAC-like 2; GTP binding                        |
|                          | AT1G08340      | 8.18         | 2.9     | -1.58 Rac GTPase activating protein, putative                    |
|                          | RABA2B         | 9.76         | 4.7     | -1.13 Arabidopsis rab GTPase homolog a2b                         |
|                          | RABA4D         | 2.69         | 0.74    | -1.99 RAB GTPase homolog A4d, GTP binding                        |
|                          | RABH1e         | 7.55         | 2.85    | -1.47 Arabidopsis Rab GTPase homolog H1e, GTP binding            |
|                          | RABA6a         | 4.8          | 2.38    | -1.11 Arabidopsis Rab GTPase homolog A6a, GTP binding            |
|                          | ROPGEF9        | 1.1          | 0.36    | -1.66 ROPGEF9, Rho guanyl-nucleotide exchange factor              |
|                          | PRA1.B5        | 2.85         | 1.32    | -1.57 Prenylated Rab Acceptor 1.b5                               |
|                          | AT2G37290      | 1.84         | 0.94    | -1.05 Rab GAP/TBC domain-containing protein                      |
|                          | AT5G09550      | 36.17        | 7.65    | -2.33 Rab GDP-dissociation inhibitor                              |
|                          | AT1G2070       | 3.04         | 0.34    | -3.28 Rab GDP-dissociation inhibitor family protein               |
|                          | AT1G62450      | 10.99        | 1.72    | -2.78 Rab GDP-dissociation inhibitor family protein              |
| **Sugar Transporters and Water Channel** |                |              |         |                                                                  |
|                          | SWEET3         | 33.58        | 17.98   | -0.98 HXT                                                          |
|                          | SWEET4         | 15.96        | 9.34    | -0.88 HXT                                                          |
|                          | SWEET5         | 9.96         | 0.99    | -3.41 HXT                                                          |
|                          | SWEET7         | 26.03        | 10.71   | -1.38 HXT                                                          |
|                          | SWEET8         | 132.86       | 57.57   | -1.3 HXT                                                            |
|                          | STP2           | 76.32        | 33.43   | -1.28 STP2, Glc:hydrogen symporter                                |
|                          | STP6           | 17.54        | 1.79    | -3.35 STP6, Glc:hydrogen symporter                                |
|                          | STP9           | 68.39        | 7.04    | -3.4 STP9, Glc:hydrogen symporter                                 |
|                          | TMT3           | 10.95        | 2.87    | -2.14 TMT3 (tonoplast monosaccharide transporter3)               |
|                          | TIP1-3         | 18.01        | 2.2     | -3.13 TIP1-3 (tonoplast intrinsinc protein 1:3)                   |

(Table continues on following page.)
knowledge gap in our understanding of roles of sugar metabolism or signaling in reproductive development and offer opportunities to potentially improve ovule development and hence seed yield.

CWIN could contribute to sink development by facilitating phloem unloading of Suc and converting it to Glc and Fru as major nutrients and energy sources. It is thus possible that the blockage of ovule formation in the CWIN-silenced plants may be due to starvation of C nutrient in the floral buds. However, this is highly unlikely based on the following analyses.

First, artificially increasing assimilate availability to the newly forming floral buds of transgenic plants by increasing source-to-sink ratio failed to recover the ovule blockage phenotype (Fig. 5). Moreover, none of the CWIN-silenced transgenic plants exhibited induction of the C-starvation reporter genes in the floral buds at stages 8 to 10, when ovules initiate (Fig. 6). These data show that the transgenic plants did not suffer from C starvation. Second, Suc unloading from the gynoecia SE/CC to ovule primordia follows a symplasmic pathway through interconnecting PD in Arabidopsis (Werner et al., 2011), and the amount of Suc required for ovule initiation would be very small in comparison with that for seed or fruit development (Palmer et al., 2015). Thus, CWIN is likely not a major player in delivering Suc as a C nutrient for ovule initiation. We therefore conclude that the essential and positive role exerted by CWIN for ovule development (Figs. 3 and 4) must be achieved through a signaling effect rather than provision of nutrients.

Plasma Membrane HXTs and RLKs May Sense and Transmit CWIN-Mediated Sugar Signaling for Ovule Initiation

Given that CWIN is a Suc-splitting enzyme functioning in the apoplasm, it is likely that any CWIN-induced changes in sugar concentration or flux would be sensed by and transmitted through plasma membrane proteins of the adjoining cells. In agreement with this prediction, a group of HXTs from the SWEET and STP family were downregulated in the pSTK:amiRNA24 plants relative to the wild type (Table 3). Among the HXTs examined, transcripts of two representatives, SWEET8 and STP9, were spatially colocalized with CWIN2 and CWIN4 during ovule initiation. Interestingly, although there are four Suc/H+ symporters and six clade III SWEETs for Suc also expressed in the wild-type floral buds, none showed changes in transcript levels in the transgenic plants (Supplemental Table S3). These data suggest that CWIN-mediated sugar signaling may be transmitted by plasma membrane HXTs, but not by Suc transporters, from the cell wall matrix into the cytosol for ovule development. Alternatively, the alteration in extracellular sugar dynamics may be perceived by membrane RLKs. To this end, 28 DEGs encoding RLKs were differentially expressed in the CWIN-silenced plants (Table 3). RLKs, a group of transmembrane proteins, have been recognized as sensory proteins, functioning in a wide range of signal perception and transduction activities in plants (Wu et al., 2016). Significantly, 8 of the 23 upregulated RLKs among the transgenic plants, all of which belong to the leucine-rich-repeat (LRR)-RLKs, were confirmed to be expressed in floral buds, including ovules, based on promoter::GUS analyses (Wu et al., 2016; Table 3). Collectively, the data suggest that these LRR-RLKs may be involved in CWIN-mediated ovule initiation and probably function as sensors to sense extracellular sugar levels or fluxes.

It is noted that genes encoding constituents of the RLK-mediated intracellular signaling pathway, Rop signaling, were also differentially expressed in response to CWIN silencing. As listed in Table 3, among those 15 small GTPase-related genes, the five genes involved in the Rop signaling pathway were all downregulated. These include two Rop genes and one Rop regulatory protein, ROPGEF9. There is evidence that LRR-RLK either phosphorylates RopGEF to activate Rop signaling to promote pollen growth (Miyawaki and Yang, 2014) or activates Rop signaling upon sensing auxin to promote the development of pavement cells in leaf epidermis (Xu et al., 2014) and root hair elongation in Arabidopsis (Duan et al., 2010). It will be of significance to examine whether the CWIN-responsive RLKs identified in this study (Table 3) may transmit extracellular sugar signals into intracellular space in a similar manner.

Apart from a large number of LRR-RLKs, WAK1 and WAK2, encoding a different class of RLKs, were also upregulated in response to CWIN silencing (Table 3). A wak2 knockout mutant exhibited a reduced mRNA level and activity of vacuolar INV in the root that led to arrested growth on Suc-deficient medium, thus
Figure 9. In situ hybridization analysis of STP9 and SWEET8 mRNA at the early stage of floral buds from stages 8 to 10 in wild-type (Col-0) Arabidopsis. A and E, In situ hybridization with a sense probe of STP9 and SWEET8 at stage 8 as controls. B to D, Sections of floral buds at stages 8 (B), 9 (C), and 10 (D) hybridized with the STP9 antisense probe. Note the STP9 mRNA signal detected in the middle part of the gynoecium, before ovule primordium initiation at stage 8 (dashed rectangle in B), and initiating o-vule primordia (op) at stage 9 (yellow arrow; C), as well as the developing ovule (ov) at stage 10 (white arrow; D). The transcripts were also detected in anther (an) and sepals (se) across all the floral bud stages from 8 to 10. F to H, Sections of floral buds at stages 8 (F), 9 (G), and 10 (H) hybridized with the SWEET8 antisense probe, showing SWEET8 mRNA signals in the middle part of the gynoecium, before op initiation at stage 8 (dashed rectangle; F), and initiating op at stage 9 (yellow arrows; G), as well as the developing ovule at stage 10 (white arrows; H). The transcripts were also observed in an and se across all the floral bud stages from 8 to 10, similar to that of STP9. Note that the SWEET8 signals were most evident in the developing ov (white arrow) and the an (red asterisk) containing developing microspores or male gametophytes (Sanders et al., 1999) at stage 10 (H). Floral stages 8 to 10 correspond to stages prior to (8), during (9), and after (10) ovule initiation, respectively (Smyth et al., 1990). Scale bars = 200 μm.
establishing a link between WAK and sugar signaling or metabolism (Kohorn et al., 2006). Our finding that silencing CWIN resulted in the upregulation of WAK1 and WAK2 indicates that expression of WAK genes themselves is subject to dynamics in extracellular sugar signaling. The extracellular domain of WAK is known to bind a polysaccharide, pectin (Kohorn et al., 2006). It will be of significance to test whether WAK binds Suc or hexose. If it does, it could open up a new direction to determine the mechanism by which CWINs relay extracellular signaling to intracellular compartments.

Surprisingly, a group of aquaporins including four plasma membrane intrinsic proteins (PIPs) and two tonoplast intrinsic protein (TIPs) was downregulated in the CWIN-silenced transgenic plants (Table 3). It is unknown how dynamics in sugar metabolism signaling may regulate aquaporin gene expression. At the protein level, supplying Suc to C-starved Arabidopsis seedlings induced phosphorylation of aquaporins at their C terminus, promoting pore opening (Niittylä et al., 2007), possibly through SIRK1, a RLK, upon interaction with two other kinases containing extracellular domains, QSK1 and QSK2 (Wu et al., 2019). Physiologically, the reduced PIP and TIP expression in response to CWIN silencing could disrupt water influx to the initiating ovules, contributing to their abortion.

CWIN-Mediated Signaling May Be Relayed Ultimately through Early Auxin Response Genes and MADS TFs to Regulate Ovule Formation

RNA-seq analyses also revealed that over half (26 of 49) of the hormone-related DEGs were involved in auxin signaling, with most of these (19 of 26) being early auxin response genes, including 14 SAURs, 4 AUX/IAAs, and 1 GH3 (Table 1). This indicates that the early auxin response genes, especially SAURs, may be closely

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**Figure 10.** A model of how CWIN-mediated extracellular sugar signaling may be sensed at the plasma membrane and transduced to the intracellular space to modulate downstream pathways to regulate ovule initiation. In Arabidopsis gynoecia, Suc is unloaded symplasmically through PD for ovule primordium formation. Some Suc must have escaped to the cell wall for hydrolysis by CWIN into Glc and Fru. Silencing of CWIN would result in changes in the extracellular sugar level or flux, thereby generating sugar signal, which may be sensed by (1) RLKs transmitted through a small GTPase (Rop) into the cytoplasm that regulate gene expression in the nucleus and (2) HXTs, including clade I and II SWEETs and H/Hex symporters, to control cytosolic hexose level and sugar homeostasis, which may then be sensed by hexokinase or other proteins to regulate downstream gene expression. In this study, the expression of CWIN gene was silenced in the ovule initiating region. This genetic intervention reduced the expression of hexose/H symporters and SWEETs for hexoses and likely their activities, thereby affecting intracellular sugar homeostasis and signaling. In parallel, expression of RLKs and Rops was increased and decreased, respectively, in response to CWIN silencing, suggesting a disturbance of the RLK-Rop-mediated signaling pathway. Both scenarios could disrupt downstream molecular pathways required for ovule initiation, including genes encoding MADS TFs, such as STK, and the early auxin signaling proteins, SAURs, leading to blockage of ovule initiation. Solid and dash-dotted lines indicate known and predicted pathways, respectively.
involved in CWIN-mediated ovule initiation. Relevant to this point, SAUR16 has been shown to positively regulate ovule number in Arabidopsis (van Mourik et al., 2017). SAUR16 shares 93% protein sequence similarity with one of the SAURs, SAUR12, identified in our DEGs (Table 1) suggesting that SAUR12 may function similarly to SAUR16 in controlling ovule number.

Apart from 14 SAURs, four AUX/IAA genes were also downregulated in the CWIN-silenced plants (Table 1). AUX/IAA is an essential component of the auxin signaling pathway, in which it functions as a repressor against auxin-inducible gene expression (Lau et al., 2008). Disruption of the AUX/IAA signaling pathway changes auxin signaling and homeostasis, and impairs cell division and root development (Vanneste et al., 2005; Yan et al., 2013). These findings, together with the importance of auxin for organogenesis such as lateral root initiation (Benková et al., 2003) and ovule formation (Galbiati et al., 2013), suggest that suppression of AUX/IAA in the CWIN-silenced transgenic plants may disrupt auxin signaling at the site of ovule initiation, resulting in blockage of ovule initiation. In this context, Glc could transcriptionally regulate 62% of IAA-related genes in Arabidopsis seedlings, including those involved in auxin signaling, such as SAUR, AUX/IAA, and GH3 (Mishra et al., 2009). Together, the analyses indicate that the regulation of CWIN-mediated sugar signaling in ovule initiation may be achieved in part through auxin signaling genes such as SAURs and AUX/IAAs.

It is unclear how downregulation of SAURs could lead to inhibition of ovule development. An increasing number of studies indicate that SAURs positively regulate cell expansion (Chae et al., 2012; Spartz et al., 2012), for example by repressing PP2C.D phosphatases, the D clade subfamily of type 2C protein phosphatases, to activate plasma membrane H+ ATPases (Spartz et al., 2014). Plasma membrane H+ ATPases pump protons to the apoplastic (Hager, 2003), which is essential to maintain cellular function for cell division and expansion characteristic of ovule initiation. These observations, together with the finding that two H+ATPase genes were also downregulated in the CWIN-silenced plants (Table 3), suggest that SAURs may contribute to ovule formation through positive effects on H+ ATPase.

In addition to the auxin-related genes, a group of MADS-box TF genes including STK (formerly AGL11), AGL66, AGL94, and AGL104 were downregulated in transgenic plants (Table 2). Many MADS TFs, such as AG, SHP1, and SHP2, function in ovule development (Skinner et al., 2004). Indeed, STK, which is phylogenetically close to AG and a homolog of SHP1 and SHP2, has been proven to be critical for establishment of ovule identity and development (Pinyopich et al., 2003). Although there is a lack of functional analysis for the other three MADS TFs in ovule development, expression-pattern and phylogeny analyses indicate that AGL66, AGL94, and AGL104 may also be involved in CWIN-mediated ovule initiation. For example, the AGL104 transcript was detected in the placenta and ovule primordia (Pářenčíková et al., 2003), and AGL66 and AGL94 share a monophyletic clade with AGL104 (Smaczniak et al., 2012). To test whether those differentially expressed MADS TF genes were involved in CWIN-mediated ovule development, STK was overexpressed in the transgenic background, which led to partial complementation of the ovule phenotype in the CWIN-silenced plants (Table 3), pointing to STK as a downstream target of the CWIN-mediated ovule initiation pathway. It remains to be established whether CWIN-mediated sugar signaling could regulate STK expression. Nevertheless, sequence analyses using the AtcisDB tool (http://arabidopsis.med.ohio-state.ed/AtcpDB) identified three ARF1 binding sites (TGTCTC) in the STK promoter (approximately −1,261 to 1,266, approximately −2,058 to −2,063, and approximately −3,292 to 3,297). The altered expression of auxin signaling genes (SAURs and AUX/IAAs) observed in the CWIN-silenced floral buds (see above in this section; Table 1) may disrupt the binding of ARF1 with the STK promoter for its transcription, leading to the reduction of STK transcript, a possible focus for future studies.

A Model of CWIN-Mediated Ovule Development through Sugar Signaling

Based on the above analysis, we propose a model of how CWIN may regulate ovule development (Fig. 10). Sugar signals generated by CWIN in the cell wall matrix may be sensed by and transmitted through plasma membrane-localized HXTs, to modulate cytosolic sugar homeostasis and sugar signaling, and/or RLKs, to potentially interact with small GTPase (Rop) pathways in the cytoplasm. The CWIN-originated signals are then relayed to the nuclei to regulate expression of genes encoding auxin signaling components and MADS-box TFs, thereby modulating ovule initiation and differentiation. The model provides directions for future studies to determine the identity and transmission pathways of the sugar signals generated by CWIN for ovule development.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 (Col-0) and the transgenic plants generated from this background were grown in growth cabinets under long-day condition (16 h light at 22°C and 8 h dark at 18°C, with illumination at 120–150 µmol s−1 m−2 and 60% to 70% relative humidity).

In situ Hybridization

In situ hybridization was carried out using our established protocol (Jin et al., 2009; Wang and Ruan, 2012) with minor modifications in sample fixation, where Arabidopsis inflorescences were fixed in formaldehyde-acetic acid fixative containing 50% (v/v) ethanol, 5% (v/v) glacial acetic acid, and 3.7% (v/v) formaldehyde at 4°C overnight. To generate gene specific probes, the most
diverse sequences at the 3′ untranslated region (3′ UTR) were selected for probe synthesis. For CWIN2, a 139-bp CWIN2 fragment ranging from 50 to 189 bp downstream of the stop codon, was amplified using CWIN2-3′ UTR in situ primers, whereas a 134-bp fragment, ranging from 27 to 161 bp downstream of the stop codon of CWIN4, was amplified using CWIN4-3′ UTR in situ primers for CWIN4 probe synthesis. Similarly, specific fragments for two HXIs (STP9 and SWEET18) were cloned from their corresponding 3′ UTR region and synthesized using their respective 3′ UTR in situ primers. All primers are listed in Supplemental Table S4. These fragments were cloned into the pGEM-T easy vector with BamHI and Xhol restriction enzymes and transcribed for synthesis of sense and antisense RNA probes using T7 and Sp6 RNA polymerases (Roche Diagnostics), respectively, labeled with digoxigenin-UTP (Roche Diagnostics).

RNA Extraction and RT-qPCR

Total RNA was extracted from a pooled sample of flower buds at stages 8 to 10, when the ovule initiates (Smyth et al., 1990), using TRIZol Reagent (Invitrogen) according to the manufacturer’s instructions. About 1 μg of RNA was treated with DNase (Promega) to remove the residual genomic DNA. DNase-treated RNA was reversely transcribed into complementary DNA (cDNA) using the SuperScript III system (Invitrogen) along with 50 μM oligo(dT)20 and 10 μM deoxynucleoside triphosphate according to the manufacturer’s protocol. RT-qPCR was performed with SYBR Green fluorescent dye (Invitrogen) and Platinum Taq DNA polymerase (Invitrogen) on a Rotor-gene Q instrument (Qiagen). Gene-specific primers used in this study are listed in Supplemental Table S5. The relative expression level (R) of each target gene is calculated based on the Pfaffi method (Pfaffl, 2001):

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R = \frac{E_{\text{target}}^{\text{Ct}}}{E_{\text{ref}}^{\text{Ct}}} = \frac{E_{\text{calibrator test}}^{\text{Ct}}}{E_{\text{reference test}}^{\text{Ct}}}
\]

In the above equation, \(E_{\text{target}}\) and \(E_{\text{ref}}\) (reference) are amplification efficiencies of the target and reference genes, respectively; \(Ct\) (target/calibrator test) is the cycle threshold (Ct) of the target gene in the calibrator minus the Ct of the target gene in the test sample; and \(Ct\) (calibrator/reference) is the Ct of the reference gene in the calibrator minus the Ct of reference gene in the test sample. Among the four Arabidopsis reference genes, namely the F-box protein gene FBX (At5g15710), polyubiquitin gene UBC23 (At5g25760), UBC9 (At1g27960), and the P2A catalytic subunit gene P2PA (At1g3320), FBX and UBC23 showed the most stable expression among wild-type and transgenic cDNA samples based on analysis using geNorm software (http://medgen.ugent.be/jvdesomp/genorm/). Therefore, these two genes were used as reference genes for qPCR analyses in this study.

Invertase Enzyme Assay

Gynoecia-enriched tissues were dissected out from the sample pool of flower buds at stages 8 to 10 for activity assay of CWIN, VIN, and CIN as previously described (Tomlinson et al., 2004).

Design and Synthesis of amiRNA against CWIN2 and CWIN4

Taking advantage of the high specificity conferred by amiRNA silencing technology (Tiwari et al., 2014), we designed amiRNA159CWIN2, which targeted a 21-nucleotide (nt) sequence, conserved in CWIN2 and CWIN4 but not in CWIN7 and CWIN5 (Supplemental Fig. S3A), upstream of the CWIN catalytic domain (WECPD; Supplemental Fig. S3B) to achieve a maximal silencing effect against CWIN functionality. The amiRNA159CWIN2 was chosen based on the selection rules for the amiRNA-target sequence (Eamens et al., 2014) to target a “shared” 21-nt sequence conserved between CWIN2 and CWIN4 from 278 to 299 bp downstream of the start codon AGT (Supplemental Fig. S3, A and B). The approach of amiRNA-mediated gene silencing exploits the intrinsic nature of the miRNA biogenesis pathway. Replacement of the miRNA/miRNA* sequence of endogenous presynthesized amiRNA precursor (pre-miRNA) with amiRNA/amiRNA* while maintaining the double-strand RNA hairpin stem-loop structure can generate mature miRNAs by key enzymes recruited for biogenesis of endogenous miRNA (Tiwari et al., 2014). Here, the endogenous miRNA159B biogenesis pathway was exploited, since miRNA159B is highly accumulated in floral tissues (Allen et al., 2007). We replaced the miRNA159B/miRNA159B* (guide and passenger strand, respectively) of the endogenous pre-miRNA159B with amiRNA159CWIN2/amiRNA159CWIN2* (Supplemental Fig., SCC). To mimic the pre-miRNA159B structure characterized with three mismatches at positions 12, 13, and 21 of the miRNA159B* passenger strand (Supplemental Fig. S3C), we introduced three mismatches at the same nucleotide positions of miRNA159CWIN2* passenger strand (Supplemental Fig. S3C). This ensures that the modified pre-miRNA159CWIN2 fragment is recognized and processed by the endogenous miRNA biogenesis pathway. The modified pre-miRNA159CWIN2 (Supplemental Fig. S3C) was synthesized (Integrated DNA Technologies) with sequence of pre-miRNA159CWIN2 list in Supplemental Table S5.

Construction of pSTK-amiRCWIN and Its Transformation into Arabidopsis

To achieve specific silencing of CWIN2 and CWIN4 at the ovule initiation site, an ovule-specific STK promoter that comprised the region from 1.8 kb upstream to 1.1 kb downstream of the translation start codon and contained the first exon and intron was used to drive the expression of amiRNA159CWIN2. The 2.9-kb STK promoter was cloned into the pART7rc vector using the primers listed in Supplemental Table S5. Sacl and Xhol restriction sites were added to its 5′ and 3′ ends, respectively. The pSTK was introduced into vector pART7rc by replacing the CaMV 35S promoter with restriction sites Sacl and Xhol. Subsequently, the 258-bp presynthesized amiRNA precursor (pre-miRNA159CWIN2) was cloned into the pSTK-ART7 vector with EcoRI and Xhol to form the pSTK-amiCWIN2-ART7rc vector. The gene cassette pSTK-amiCWIN2 was released by digestion with NotI, which was then cloned into the binary vector pBART7c. To complement the reduction of STK expression in the pSTK-amiCWIN2 transgenic plants, a complementary vector pSTK-STK(CDS)-pcAMBI1300 was constructed to drive the expression of a 771-bp STK CDS. Arabidopsis inflorescences were dipped in cell suspension of the Agrobacterium tumefaciens strain AGL-1 (Zhang et al., 2006), which carried the target binary vector, for 10 s with gentle agitation. Dipped plants were removed from cell suspension and drained for 3 to 5 s and then were covered with plastic film to maintain high humidity for 16 to 24 h before being moved to a glasshouse for recovery and growth under normal conditions.

Ovule, Seed, and Silique Phenotyping

For ovule phenotyping, flower buds at stages 10 to 11 (~2 to 1 d before flowering; Roeder and Yanofsky, 2006) were collected and fixed in ethanol/acetone (9:1) overnight, and then washed by 90% (v/v) ethanol, followed by a further wash with 70% (v/v) ethanol after a 30-min interval. Thereafter, flower buds were mounted in a mixture of chloralhydride/glacial acetic acid (8:1:2 v/v/v) and cleared overnight. Cleared flower buds were dissected to expose the gynoecium and observed under differential interference contrast with a Leica dissection microscope. For analyses of seed number and siliques length, fully expanded siliques at stage 17 (~5 d after flowering; Roeder and Yanofsky, 2006) were collected from the main inflorescences of each genotype. These siliques were cleared and mounted as described (Berketh and Jurgen, 1993). For seed number counting, siliques were fixed and clear mounted using the same method as for ovule phenotyping.

RNA Isolation and Illumina Sequencing

A pooled sample of flower buds was harvested at stages 8 to 10, which covers stages prior to (stage 8), during (stage 9), and after (stage 10) ovule initiation. RNA was extracted from the pooled sample of the wild type and transgenic line 4-3, which exhibits the most severe reduction in ovule number, using the Quage(R) RNA Plant Mini Kit, followed by an on-column DNase treatment to eliminate genome DNA by DNaseI digestion. Eight RNA extracts (four biological replicates for each of two genotypes), each containing at least 5 μg of total RNA, were sent to the Australia Genome Research Facility (AGRF) for RNA sequencing. RNA quality was determined by Agilent 2100 Bioanalyzer, and only samples with RNA integrity number > 7 were further processed for cDNA library preparation and sequencing. Sequencing of the cDNA libraries was performed using an Illumina HiSeq2500 with 125-bp pair end with an average sequencing depth of 30 million reads.

Sequencing Trimming and Mapping and Determination of DEGs

Raw reads were trimmed using CLC Genomics Workbench 10.1.1 (Qiagen) based on Ru et al. (2017) with some modifications. Briefly, raw reads were
trimmed as follows: (1) Removal of reads with quality scores <20 (base-calling error probability <0.01) and those with more than two ambiguous nucleotides; (2) adapter trimming; and (3) removal of reads with sequence length of <15 nucleotides. To estimate gene expression level, trimmed reads were mapped to the Arabidopsis reference genome sequence (TAIR10) using the CLC Genomics Workbench, which allows unique mapping with a maximum of two mismatches. Reads mapped to unigenes were counted and used for expression analysis. The number of mapped reads per kilobase per million reads (RPKM) was used as a measure of the expression level of each gene. Using the CLC genric RNA-seq differential analysis tool, DEGs of transgenic samples, as compared to wild-type samples, were identified based on the following criteria: (1) mean RPKM value ≥1 ± 1 at least one sample, in either the wild-type or transgenic group, where only genes exhibiting a mean RPKM ≥1 were considered to be expressed; (2) false discovery rate <0.05; and (3) log2FC >1 or less than –1. MAILMAN (Thimm et al., 2004) was used to identify functional categories of the DEGs. The RNA-seq data have been uploaded to the National Center for Biotechnological Information web site (https://www.ncbi.nlm.nih.gov/geo/; with data accession no. GSE39917).

Statistical Analysis

CLC Genomics Workbench 10.1.1 (Qiagen) was used for PCA of RNA-seq data. One-way ANOVA was performed for data analyses using JMP 11 software. For details, see figure legends and table captions.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Supplemental Tables S2 to S5.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Statistical analysis of silique length and seed number of five T3 homozygous pSTK amiRNA CWIN24 transgenic lines compared with wild-type plants.

Supplemental Figure S2. Overall assessment of the quality of RNA-seq data between wild-type plants and pSTK amiRNA CWIN24 transgenic lines (4-3).

Supplemental Figure S3. Custom design of amiRCWIN24.

Supplemental Table S1. Blockage of ovule initiation in pSTK amiRNA CWIN24 transgenic plants compared with the wild type.

Supplemental Table S2. A total of 1,490 genes differentially expressed in the pSTK amiRNA CWIN24 transgenic floral buds at stages 8 to 10 compared with the wild type.

Supplemental Table S3. A total of 76 TF genes differentially expressed in stage 8 to stage 10 floral buds of pSTK amiRNA CWIN24 transgenic plants compared with the wild type.

Supplemental Table S4. STP genes detectable in floral buds during ovule initiation in both wild-type and pSTK amiRNA CWIN24 transgenic plants, including DEGs and non-DEGs.

Supplemental Table S5. All oligos used in this study.

ACKNOWLEDGMENTS

The authors greatly appreciate Dr. Andy Eamens for his advice on the design of artificial microRNA construct. S.J.L gratefully acknowledges support from the University of Newcastle for providing a postgraduate scholarship.

Received April 3, 2020; accepted April 13, 2020; published April 24, 2020.

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