Arabidopsis Circadian Clock Repress Phytochrome a Signaling

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The plants’ internal circadian clock can strongly influence phytochrome signaling in response to the changes in the external light environment. Phytochrome A (phyA) is the photoreceptor that mediates various far-red (FR) light responses. phyA signaling is modulated by FHY3 and FAR1, which directly activate the transcription of FHY1 and FHL, whose products are essential for light-induced phyA nuclear accumulation and subsequent light responses. However, the mechanisms by which the clock regulates phyA signaling are poorly understood. Here, we discovered that FHY1 expression is diurnally regulated, peaking in the middle of the day. Two Arabidopsis core clock components, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and TIMING OF CAB EXPRESSION1 (TOC1), repress FHY3/FAR1-mediated FHY1/FHL activation. Consistently, the specific expression pattern of FHY1 under diurnal conditions is altered in cca1-1, toc1-101, CCA1, and TOC1 overexpression plants. Furthermore, far-red induced gene expression and particularly nuclear accumulation of phyA are compromised in TOC1 and CCA1 overexpression seedlings. Our results therefore revealed a previously unidentified FHY1 expression pattern in diurnal cycles, which is negatively regulated by CCA1 and TOC1.

Keywords: FHY3/FAR1, FHY1/FHL, TOC1, CCA1, PHYA, clock

INTRODUCTION

The circadian clock regulates almost every aspect of metabolism and development in plants. The Arabidopsis circadian clock consists of a central oscillator loop that connects morning- and evening-phase circuits (Pruneda-Paz and Kay, 2010). The central loop is composed of three genes, two morning-expressed Myb transcription factors—CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) and an evening-expressed pseudoresponse regulator TIMING OF CAB EXPRESSION 1 (TOC1; Alabadi et al., 2001; Kamioka et al., 2016). CCA1/LHY and TOC1 repress each other’s expression, and thus constituting a negative feedback loop (Alabadi et al., 2001; Gendron et al., 2012). Besides the essential role of CCA1/LHY and TOC1 as central oscillators that control the activity of other clock components, this circularity module also regulates diverse output processes, including iron and ROS homeostasis, leaf senescence and photosensory pathway-mediated hypocotyl growth (Lai et al., 2012; Soy et al., 2016; Song et al., 2018; Xu et al., 2019).

Light controls the growth of plants through a network of photoreceptors. Arabidopsis has five red/far-red light-absorbing phytochromes (phyA, phyB, phyC, phyD, and phyE), of which phyA and phyB play the most predominant functions (Sharrock and Quail, 1989; Clack
et al., 1994). PhyA is the only photoreceptor mediating various plant responses to FR light compared with darkness (Quail et al., 1995). Upon light irradiation, phyA is activated and rapidly translocated into nuclear, representing a critical step of phyA signaling (Kim et al., 2000). Two small plant-specific proteins, FAR-RED ELONGATED HYPOCOTYL1 (FYH1) and its homolog FYH1-LIKE2 (FHL), are essential for nuclear accumulation of light-activated phyA and subsequent light responses (Hiltbrunner et al., 2005; Hall et al., 2001). The activation and repression of FYH1/FHL-phyA signaling are energetically demanding. FYH3 and FAR1, as a new type of transposase-derived transcription factors, activate FYH1/FHL gene expression directly, which in turn facilitates phyA nuclear accumulation on FR light irradiation (Lin et al., 2007). Mutant seedlings that lack FYH3 and FAR1 displayed elongated hypocotyls and impaired nuclear accumulation of phyA under continuous FR light (Lin et al., 2007). Thus, the regulators which modulate FYH3 and FAR1 activity inevitably affect the FYH1/FHL levels and ultimately the phyA signaling pathway. The function of the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) as a repressor for FYH1/FHL expression has been reported. HY5 negatively regulates FYH1/FHL expression via physical interaction with FYH3/FAR1 and interferes with their binding to the FYH1/FHL promoters (Li et al., 2010). Recently, the JA (jasmonic acid) signaling repressor JAZ1 was reported to interact with FYH3 and FAR1, and inhibit their transcriptional activity on FYH1/FHL expression (Liu et al., 2020a). The antagonistic interaction of FYH3/FAR1 with HY5 and JAZ1 may provide a mechanism for fine-tuning the phyA signaling pathway by light and hormone, respectively.

Daily changes of light, defining the diurnal cycle of everyday, are an essential input to the circadian clock. In plants, the photoreceptor phytochrome and cryptochrome set the clock by transducing the light signal to the central oscillator, which is called the input pathway. For instance, phyA and phyB are responsible for light-mediated entrainment of the circadian clock under FR and R radiation, respectively (Somers et al., 1998). On the contrary, like other clock-controlled output traits, the process of light input is rhythmic and regulated by the clock machine. Through a gating mechanism, the circadian clock modulates the light responsiveness of physiological outputs at different times of the day. For example, expression of the CAV genes that encode the chlorophyll a/b-binding proteins is not only induced by light but also controlled by the circadian rhythm, suggesting that the clock modulates the acute response to light (Millar and Kay, 1996). In addition, some key clock components, like PRR7, PRR9 and ELF3, are potentially involved in light input to the clock (McWatters et al., 2000; Farre et al., 2005). Despite this progress, how circadian clock components modulate phytochrome activity remains to be answered. A previous study has revealed that the action of the phyA signaling pathway is regulated at multiple levels. For example, the transcription level of PHYA is regulated by the circadian clock with peaking in the late afternoon (Hall et al., 2001). Importantly, under daily photoperiods of far-red light, the number of nuclei with phyA speckles is higher during daytime than during the night (Kircher et al., 2002), but the underlying mechanism remains obscure.

Here, our study revealed that the central oscillator components, CCA1 and TOC1, could suppress the function of FYH3/FAR1, which in turn inactivates FYH1/FHL expression and nuclear accumulation of phyA. Furthermore, we found that the action of CCA1 and TOC1 conferred the circadian expression pattern of FYH1, which might provide an adaptive mechanism for plant perception of far-red light under diurnal cycles.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild type Arabidopsis thaliana plants used in this study are of the Col-0 ecotype unless otherwise indicated. The fhy3-1, far1-2, fhy3-1 far1-2, 35S::Flag-FHY3-3A and 35S::FYH1-GFP have been described previously (Lin et al., 2007; Li et al., 2011; Chen et al., 2012). The cca1-1 and CCA1-OX were in Wassilewskija-2 ecotype (Wang and Tobin, 1998; Green and Tobin, 1999). 35S::FLAG-CCA1-1HA and 35S::FLAG-TOC1-1HA (TOC1-OX) were described previously (Li et al., 2011). Plants were grown on MS containing 2% sucrose and 0.75% agar under 12-h light/dark conditions (75 μmol m⁻² s⁻¹) in a Percival growth chamber (Percival Scientific).

Plasmid Construction

To generate FYH1p:LUC transgenic plants, the amplified FYH1 promoter was subcloned into the pPZP221-ELF4:Luc vector (Li et al., 2011) through PstI/BamHI sites.

Yeast Assays

Yeast one-hybrid assays were performed as described previously (Li et al., 2010).

Gene Expression Analysis

Total RNA was extracted from seedlings using Trizol (Invitrogen). The first-strand cdNA was synthesized from 1 μg of RNA using reverse transcriptase (Tiangen). The cdNA was diluted 1:10 and subjected to quantitative PCR using SuperReal PreMix Plus (Tiangen) and a 7,500 Real-Time PCR System (Applied Bio-systems) cycler. Gene expression levels were normalized to PP2A and are shown relative to the expression levels in wild type. Primers are listed in Supplementary Table 1.

Transient Expression Assay

Transient expression assays were performed as described previously (Li et al., 2011). The reporter and effector constructs were transformed into Agrobacterium strain EHA105. The Agrobacterium solutions containing the reporter or effector constructs were coinfected for 2 h and infiltrated into 3–4-week-old N. benthamiana leaves. Plants were incubated under continuous white light for 3 d after infiltration. The firefly LUC activity was photographed after spraying with 1 mM luciferin (Goldbio). For the dual-luciferase quantification assay, firefly luciferase and Renilla luciferase activities were assayed as described previously (Li et al., 2010).
Western Blot Analysis
For anti-FHY1 immunoblots, Arabidopsis seedlings were ground to a fine powder and resuspended in 200–500 μl of Lysis Buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.1% Tween 20, 1 mM PMSF, 40 mM MG132, and 1X complete protease inhibitor cocktail). Sample loading was made with 5X Laemmli’s buffer in 10% SDS–polyacrylamide gel electrophoresis gels. According to the manufacturer's recommendations after transference to PVDF membranes, the immunoblotting was detected with anti-GFP (1:1000) antibody (MBL; 598–7). For CCA1 and TOC1 immunoblots, proteins were detected with anti-FLAG (1:4000) antibody (MBL; M185-7). The secondary antibody used is HRP-conjugate (MBL; 1:8000).

Fluorescence Microscopy
For fluorescence microscopy analyses, seedlings were grown on MS medium for 5 d and then released to far-red light for 10 h. At least 10 independent lines for each cross combination were examined using a Zeiss LSM 510 multiphoton microscope.

RESULTS

FH Y1 Gene Is Clock Regulated
To investigate whether the expression levels of FH Y1 and FH L exhibit circadian rhythm, we first investigated these two genes in the public DIURNAL database (Mockler et al., 2007; Michael et al., 2008). Analysis of the microarray data from 12:1:12D time courses (Light:Dark hours = 12:12) revealed that expression of FH Y1 oscillated rhythmically, with a peak of expression occurring at Zeitgeber time 8 (ZT8; Figure 1A). FH L, the close homolog of FH Y1, lacked such rhythmical expression and kept steady around the whole day. Furthermore, the expression pattern of FH Y1 and FH L was confirmed by quantitative PCR (qPCR) analysis. Similarly, FH Y1 mRNA accumulated after dawn, reached a maximum at ZT8 and subsequently decreased toward the end of night (Figure 1B). Moreover, expression of FH L is not robustly regulated by the circadian clock (Figure 1C). To further confirm the circadian expression pattern of FH Y1, we generated FH Y1p:LUC transgenic line and examined its luciferase activity under continuous light conditions. As expected, the results are consistent with our qRT-PCR assay with FH Y1 expression peak around ZT8 (Supplementary Figure 1). As FH Y3 is essential for FH Y1 gene expression, we tested the role of FH Y3 on FH Y1 rhythm and found that the circadian rhythm of FH Y1 is lost in fh y3-4 mutant, while enhanced in the FH Y3 overexpression line (Supplementary Figure 2). To monitor whether FH Y1 protein levels oscillate, the transgenic line expressing FH Y1-GFP under the control of CaMV 35S promoter (35S::FH Y1-GFP) was used to detect the FH Y1 protein abundance under diurnal cycles. The results showed that FH Y1-GFP fusion protein accumulates after dawn, reaching a peak from mid-day to the afternoon (ZT4–ZT12) and a trough through the whole night (Figure 1D).

To determine whether the cycling expression pattern of FH Y1 was affected by the core clock components, we examined the FH Y1 expression pattern in TOC1 and CCA1 overexpression and mutant lines (TOC1-OX, CCA1-OX, toc1-101, and cc a1-1) under diurnal cycles. The results showed that the circadian expression pattern of FH Y1 changed in these lines compared with wild type. In cc a1-1 and toc1-101 mutants, the FH Y1 level increased compared with wild type (Figures 2A,B). In CCA1-OX transgenic plants, FH Y1 expression increased at night, and the peak shifted to dawn (Figure 2C). In addition, the FH Y1 peak in TOC1-OX at ZT8 disappeared and the circadian pattern was absent (Figure 2D). These results suggested that CCA1 and TOC1 affect FH Y1 expression and shape the precise middle-day-phased expression pattern of FH Y1.

Given that FH Y1-mediated phyA nuclear accumulation is essential for phyA signaling, it is interesting to test whether genes involved in phyA signaling are also clock regulated as well as FH Y1. Thus, we obtained time-course expression profiles of 224 phyA-induced genes (selected from Chen et al., 2014) from the DIURNAL database (Supplementary Dataset S1). Strikingly, we found that a vast majority of these genes display time-of-day specific phases under diurnal conditions. As expected, large numbers of genes peaked at noon (ZT8), coinciding with the rhythm of FH Y1 expression (Supplementary Figure 3). These findings indicated that phyA signaling pathway is regulated by the clock.

CCA1 and TOC1 Repress Transcriptional Activation Activity of FH Y3
A previous study showed that CCA1 could directly interacts with FH Y3/FAR1 and represses their transcriptional activating activity (Li et al., 2011). Recently, we have confirmed the interactions between FH Y3/FAR1 and TOC1 (Liu and Wang, 2020b). Therefore, we speculated that CCA1 and TOC1 might affect FH Y3/FAR1-mediated FH Y1 transcription. To begin to assess potential CCA1 and TOC1 repression of FH Y3 activity, we conducted the modified yeast one-hybrid assay to test the ability of FH Y3 to promote FH Y1 expression when CCA1 or TOC1 was introduced. As expected, our results showed that FH Y3 could activate FH Y1p:LacZ reporter gene expression. In contrast, the inclusion of CCA1 or TOC1 removed FH Y3’s activation activity, suggesting that CCA1 and TOC1 negatively regulate FH Y3/FAR1-activated FH Y1 expression (Figures 3A,B). In parallel, we performed a transient gene expression assay in N. benthamiana leaf to test the effect of FH Y3-CCA1 and FH Y3-TOC1 interaction on FH Y1 expression. Consistent with the results of Y1H assay, FH Y3 could effectively activate the FH Y1p:LUC reporter gene expression, whereas co-expression of CCA1 or TOC1 with FH Y3 significantly repressed the expression of the FH Y1p:LUC reporter (Figures 3C,D), indicating that both CCA1 and TOC1 can suppress the transcriptional activation activity of FH Y3 on FH Y1 transcription in planta.

CCA1 and TOC1 Repress Expression of FH Y1 and FH L
To investigate the role of CCA1 and TOC1 in regulating FH Y1 expression in vivo, we examined FH Y1 expression level in toc1-101, cc a1-1 mutants and the overexpression lines of TOC1-OX and CCA1-OX, compared with the wild type (Col-0 and
Ws ecotypes) and fhy3-1, far1-2, and fhy3-1 far1-2 mutants. Previous studies showed that FHY1 and FHL transcript levels displayed a declined expression pattern when dark-grown seedlings were transferred to far-red light (Lin et al., 2007; Li et al., 2010). qRT-PCR analysis revealed that FHY1 and FHL transcript levels were significantly reduced in the CCA1-OX and TOC1-OX plants, similar to the mutants of fhy3-1, far1-2 and fhy3-1 far1-2 (Figures 4A–D). In contrast, the FHY1 and FHL expression in cca1-1 and toc1-101 mutants were not significantly altered compared with wild-type plants.

To determine whether far-red light affects the activity of CCA1 and TOC1, we then examined the mRNA and protein levels of CCA1 and TOC1 in this time course. We found that, when dark-grown seedlings were exposed to far-red light, both the CCA1 mRNA and protein levels started to decrease, while TOC1 showed an increased pattern (Supplementary Figures 4, 5).

Given FHY1 and FHL are essential for phyA nuclear accumulation and subsequent far-red light signaling, we hypothesized that the expression of FR responsive genes in CCA1-OX and TOC1-OX transgenic plants might be compromised. To this end, we examined the expression of six FR responsive genes (HY5, β-AMY, PIL1, CAB2, CAB3 and HFR1) in TCO1-OX and CCA1-OX plants grown under dark to FR conditions. As shown in Figure 5, expression levels of these six FR responsive genes significantly declined in CCA1-OX and TOC1-OX plants compared with the wild type in some time points. Moreover, we tested the expression levels of these six genes in toc1-101 and cca1-1 mutants. Similar to the results of FHY1 expression, expression of these six genes did not differ dramatically from the wild type (Supplementary Figure 6). Together, these data suggested that CCA1 and TOC1 antagonize FHY3-mediated FHY1 expression and FR responsive gene expression.

Furthermore, we examined the phenotype of hypocotyl growth under continuous FR light conditions. The results showed that cca1-1 mutant displayed short hypocotyl, while CCA1-OX plant displayed long hypocotyl, which is consistent with the expression of FR responsive genes (Supplementary Figure 7A). However, hypocotyl of toc1 mutant seemed longer than wild type, and no noticeable difference was observed between TOC1-OX and wild-type plants, implying other unknown mechanisms existed in TOC1-mediated hypocotyl growth in FR light conditions (Supplementary Figure 7B).
Nuclear Localization of phyA Is Inhibited in TOC1-OX and CCA1-OX Plants

To gain insight into the mechanism by which CCA1 and TOC1 antagonize phyA signaling, we analyzed the activity of phyA accumulation into nuclear upon FR irradiation. We generated phyA-GFP/TOC1-OX and phyA-GFP/CCA1-OX plants by crossing. In dark-grown seedlings, phyA-GFP was homogeneously dispersed in the cytoplasm as previously described (Kircher et al., 1999; Hisada et al., 2000). Strikingly, when dark-grown seedlings were transferred into far-red light for 8 h, nuclear accumulation of phyA-GFP was significantly reduced in TOC1-OX and CCA1-OX plants compared with wild-type phyA-GFP seedlings (Figure 6). We divided the status of phyA-GFP nuclear accumulation into three types: standard (like wild type, more photobodies); A, few photobodies; and B, no photobodies. Quantitative analysis of these three types revealed that, in TOC1-OX and CCA1-OX plants, standard types are prominently reduced (only 29% in TOC1-OX; 40% in CCA1-OX), abnormal type A (56% in TOC1-OX; 38% in CCA1-OX) and type B (15% in TOC1-OX; 22% in CCA1-OX) appear and increase compared with wild type. These findings suggested that TOC1 and CCA1 repress phyA mediated FR signaling pathway via downregulation of FHY1 level and subsequent phyA nuclear accumulation.

DISCUSSION

In this study, we revealed a previously unidentified FHY1 expression pattern in diurnal conditions. The clock components TOC1 and CCA1 modulated FHY1 expression and conferred its circadian rhythm with peaking at the mid-day. Furthermore, we presented evidence to show that TOC1 and CCA1 inactivate phyA signaling via repressing FHY3/FAR1-activated FHY1 and FHL transcription. Given the reported interactions of FHY3-CCA1 and FHY3-TOC1 (Li et al., 2011; Liu and Wang, 2020b), we proposed a model in which CCA1 and TOC1 act as transcriptional repressors of FHY3, thereby reducing the FHY1 transcription level and dysfunction of phyA nuclear accumulation (Figure 7). In addition, CCA1 and TOC1 also limit and shape the FHY1 expression pattern under diurnal conditions (Figure 7B).

Besides CCA1 and TOC1, the bZIP transcription factor HY5 has been reported to repress FHY3-activated FHY1 transcription (Li et al., 2010). Unlike CCA1 and TOC1, HY5 can directly bind the ACE element in the FHY1 promoter. Due to the close location of FHY3 and HY5 binding sites on FHY1 promoter, HY5 interacts and interferes with FHY3 for binding to FHY1 promoter (Li et al., 2010). In addition, CCA1 can physically interacts with HY5, and they act synergistically on circadian genes expression.
Therefore, the role of HY5 in the clock-mediated *FHY1* regulation will be interesting to investigate in future studies.

We demonstrate that the protein and mRNA accumulation of *FHY1* followed a diurnal rhythm and exhibited maximum expression in the light phase (around ZT8). It has been reported that the circadian clock regulates promoter activity and/or mRNA accumulation of PHY and CRY genes (Tóth et al., 2001). Among them, phyA promoter reporter activity and phyA mRNA displayed a biphasic curve, with the first peak appearing just after the lights-on signal, which is very similar to *FHY1* expression. Thus, the inner coincidence of the photoreceptor phyA with the transfer conductor FHY1 might be critical for the effective transduction of far-red signaling. Because of this, the phyA signaling downstream genes (phyA-induced) showed a significant oscillation pattern similar to *FHY1* and phyA (Supplementary Figure 3).

As the core components of the circadian clock oscillator, evening gene TOC1 and morning CCA1 reciprocally repress each other in the clock network (Alabadi et al., 2001; Gendron et al., 2012). Actually, TOC1 and CCA1 do not always act oppositely in regulating the clock-output pathways. In some cases, they may play the same role. For example, both TOC1 and CCA1 can repress the flowering time (Niwa et al., 2007). In this study, we revealed another case in which both TOC1 and CCA1 acted negatively in regulating the phyA signaling pathway. Our results showed that both TOC1 and CCA1 were implicated in repressing FR signaling pathway via inhibiting FHY3-mediated FHY1 activation. It was noted that the hypocotyl growth of *cca1* mutant and *CCA1OX* plant in FR light conditions is consistent with the repression role of CCA1 on *FHY1* expression (Supplementary Figure 7A), while the hypocotyl growth of *toc1* mutant and *TOC1OX* plant seemed opposite with the molecular evidence of TOC1 (Supplementary Figure 7B). The hypocotyl growth is mainly controlled by the level of PIFs (Soy et al., 2012). Due to the direct repression of PIF3 by TOC1, the *TOC1OX* has a low amount of PIF3 and exhibits short hypocotyl (Soy et al., 2016). Thus, we speculated that, although TOC1OX lead to reduced
FIGURE 4 | CCA1 and TOC1 negatively regulate FHY1 and FHL expression. (A,B) qRT-PCR analysis of FHY1 (A) and FHL (B) expression in wild type (Col-0), fhy3-1, far1-2, fhy3-1 far1-2, toc1-101, and 35S:TOC1 seedlings. (C,D) qRT-PCR analysis of FHY1 (C) and FHL (D) expression in wild type (WS), cca1-1 and CCA1ox seedlings. Seedlings are grown in darkness for 4 d and then transferred to FR light for various time periods. Asterisks indicate significant differences from wild type plants (p < 0.05, Student’s t-test). Values are means ± SD; n = 3.

FIGURE 5 | Expression of FR responsive genes is reduced in CCA1-OX and TOC1-OX seedlings. qRT-PCR analysis of HY5, βAMY, PIL1, CAB2, CAB3 and HFR1 expression in wild type and CCA1-OX (upper panel), and TOC1-OX (lower panel) seedlings grown in darkness for 4 d and then transferred to FR light for various time periods. Asterisks indicate significant differences from wild type plants (p < 0.05, Student’s t-test). Values are means ± SD; n = 3.
A proposed model depicting the repression effect of TOC1 and CCA1 on FHY1 transcription and contributing to its circadian expression pattern.

(A) TOC1 and CCA1 repress the FHY3 transcriptional activity on FHY1, which impaired the nuclear transport of phyA and downstream FR signaling.

(B) In diurnal cycles, CCA1 and TOC1 repressed FHY1 expression levels in the morning and evening, resulting in peaked expression of FHY1 in the noon.
activity of PIF3 and FHY1 simultaneously, the dominant role of PIF3 in hypocotyl growth may mask the effect of FHY1 under FR light, thus leading to short hypocotyl phenotype in TOC1OX plant. In addition, the inconsistent circumstances of phenotype and gene expression have been described in TOC1-mediated flowering time regulation. The flowering repressor ELF4 is repressed by TOC1, yet inactivation of TOC1 displays early flowering time, similar to elf4 mutant (Kikis et al., 2005; Niwa et al., 2007). Thus, as a strong repressor that targets various important genes and pathways, TOC1-related phenotype analysis is complex and requires further attention.

In this study, we presented the first evidence that core clock components control photoreceptor nuclear accumulation. The light-induced phyA nuclear accumulation was impaired in CCA1-OX and TOC1-OX, indicating that the circadian clock regulates light signal input into plant organisms. To confer a selective advantage upon the organism, entrainment must be adaptable. Light signals, especially photoperiod, change with the seasons in temperate latitudes: the optimal phase for a plant to be adaptable. Light signals, especially photoperiod, change with the seasons in temperate latitudes: the optimal phase for a plant to be adaptable. Light signals, especially photoperiod, change with the seasons in temperate latitudes: the optimal phase for a plant to be adaptable.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YL and HW designed research. YL, YS, HY, YZ, and SC performed experiments. YL analyzed the data and wrote the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.809563/full#supplementary-material

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