Transcription of ST2A Encoding A Sulfotransferase Family Protein That Is Involved in Jasmonic Acid Metabolism Is Controlled According to the Circadian Clock- and PIF4/PIF5-Mediated External Coincidence Mechanism in Arabidopsis thaliana

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Plant elongation growth on a day-to-day basis is enhanced under specific photoperiod and temperature conditions. Circadian clock is involved in the temperature adaptive photoperiodic control of plant architecture, including hypocotyl elongation in Arabidopsis thaliana. In this regulation, phytochrome interacting transcriptional factors, PIF4 and PIF5, are activated at the end of night under short photoperiod or high temperature conditions, due to the coincidence between internal (circadian rhythm of the transcripts) and external (length of dark period) time cues. It is previously found that biosynthesis or metabolism of phytohormones including auxin, and their signal transduction-related genes are downstream targets of circadian clock and PIF4/PIF5 mediated external coincidence mechanism. Brassinosteroid and gibberellic acid played a positive role in the hypocotyl elongation of seedlings under light and dark cycle conditions. On the other hand, cytokinin and jasmonic acid played an opposite role. In this study, diurnal expression profile of a gene encoding a sulfotransferase family protein that is involved in the jasmonic acid metabolism, ST2A, was examined. It was found that transcription of ST2A is induced at the end of night under LD/22°C and SD/28°C conditions according to the external coincidence mechanism. The results of this study support the idea that the circadian clock orchestrates a variety of hormone-signalling pathways to regulate the photoperiod and temperature-dependent morphogenesis in A. thaliana.

Key words: Arabidopsis thaliana; circadian clock; external coincidence mechanism; elongation of hypocotyls; light response

It is generally admitted that growth and development are controlled through interactions between light and phytohormone signaling in plants.1,2 Circadian clock is also involved in the control of the plant growth and development on a daily basis under day and night cycle conditions.3 It is known that the lengths of hypocotyls are affected by an environmental time cue, photoperiod in Arabidopsis thaliana (A. thaliana). For example, Arabidopsis seedlings grown in long days exhibit a phenotype of short hypocotyls, while those in short days a phenotype of long hypocotyls. The critical photoperiod to cause elongation of hypocotyls in Arabidopsis seedlings is duration of around 12 h dark.4 Phytochrome-interacting bHLH transcription factors, PIF4 and PIF5 are involved in the photoperiodic control of hypocotyl growth. A pif4 pif5 double mutant almost, if not completely, abolishes the photoperiodic response of hypocotyl elongation.5,6 It is demonstrated that the interaction with photo-activated phyB induces rapid phosphorelation and subsequently leads to the 26S proteasome-mediated degradation of PIF4 and PIF5.6–8 It is also revealed that transcription of PIF4 and PIF5 is under the control of circadian clock, and expression level of their transcripts shows robust diurnal rhythm with the peak in the middle of the day under light and dark cycle conditions.9 PIF4 is expressed exclusively in light phase under long day conditions, but PIF4 protein is thought to be inactivated during the daytime (i.e., light phase) by phyB-dependent post-translational regulation described above (Fig. 1A). Especially under shorter photoperiodic conditions, PIF4 expression is observed at the end of night (i.e., dark phase) in addition to the daytime. Because it is only when transcription of PIF4 coincides with PIF4 protein stabilization, it was speculated that PIF4 is activated at the end of night specifically in short days, resulting in elongation of hypocotyls (Fig. 1B).5 Identification of PIF4-target genes and examination of their transcriptional regulation demonstrated that PIF4 is actually activated at the end of night specifically under short day conditions.10 The coordinated interplay between photoreceptor phyB and circadian clock makes it possible for seedlings to grow in such a way that elongation of hypocotyls is accelerated at the end of night specifically in short-days due to coincidence between circadian clock-dependent internal and photoperiod-dependent external time cues.

It is known that the lengths of hypocotyls are affected not only by photoperiods but ambient temperatures in A. thaliana.11,12 When seedlings are grown under relatively high growth temperature condition of 28°C, elongation of hypocotyls is stimulated even in long days in a PIF4-dependent manner. On the contrary, when

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Abbreviations: BA, 6-benzylaminopurine; BL, brassinolide; GA, gibberellin A₃; MeJA, methyl jasmonate
PIF4-Mediated External Coincidence Mechanism in A. thaliana

Preparation of RNA and qRT-PCR. Total RNA was purified from frost plant materials (the aerial part of 7 or 8-d-old seedlings) with the RNeasy plant mini kit (Qiagen, Venlo). To synthesize cDNA, RNA (1 μg each) was converted to cDNA with ReverTra Ace (TOYOBO, Osaka) and oligo-dT primer. The synthesized cDNAs were amplified with the SYBR Premix Ex Taq II kit (Takara Bio, Kyoto), analyzed by using a Stepone Plus™ Real-Time PCR System (Life technologies, CA). The primer sets used were 5′-ATCACATATGCGCCGTTAT-3′ and 5′-ATGGCACACCACCTACCATCTT-3′ for detection of JAI29, 5′-GACAAGTTGGCATGGTGTCACG-3′ and 5′-CCTGGTCACTCAGAAGCC-3′ for detection of CKX5, 5′-CTGGAGCCCTTATCTATACCG-3′ and 5′-CGACAAACCTCGTGGTGAC-3′ for detection of ST2A, 5′-GGTTCTCTCAATGAGTCCC-3′ and 5′-GACGGAATCC-3′ for detection of RGF6/GLV1, and 5′-CACGGAACTCTAGAAAGTAG-3′ and 5′-CGGGATATCTTGAGTCACAGG-3′ for detection of RGF9/GLV2. The following standard thermal cycling program was used for all PCR: 95°C for 120 s, 40 cycles of 95°C for 10 s, and 60°C for 30 s. CT value for individual reaction was determined by analysis of raw fluorescence data (without baseline correction) using the freely available software PCR Miner (http://www.miner.ewindup.info).35 Based on the comparative CT method, relative expression level was calculated. The APX3 gene encoding an ascorbate peroxidase isozyme was used as an internal reference.

Results and Discussion

PIF4/5-Mediated temperature adaptive photoperiodic control of hypocotyl elongation is affected by various kinds of phytohormones

We characterized a set of phytohormone-associated genes as PIF4/5-targets in plants grown under light and dark cycle conditions (i.e., in growth on a day-to-day basis) previously. It is found that not only auxin-associated genes (GH3.5, IAA19, IAA29), but also ones associated with other growth-regulating hormones such as brassinosteroids (BR6ox2), gibberellic acids (GAI), ethylene (ACS8), and cytokinins (CKX5) are under the control of circadian clock- and PIF4/5-mediated external coincidence mechanism.22 Recent genome-wide analysis revealed the direct target of PIF4 and PIF5 systematically.15,24 It is thought from the recent studies that biosynthesis and transport of auxin are important for rapid elongation of hypocotyls under shade and high temperature conditions.15,19,20,23,25,26 It is also known that brassinosteroids and gibberellic acids act cooperatively to enhance the hypocotyl elongation.27,28 The evidence prompted us to examine the effect of brassinosteroids and gibberellic acids on hypocotyl elongation under diurnal light and dark cycle conditions. When seedlings were grown on medium containing brassinolide (BL) and gibberellin A3 (GA), elongation of hypocotyls was stimulated significantly even in long days, namely, under non-induced conditions (Fig. 1C, F). The event was also observed in the pif4 pif5 mutant, although the effect of BL and GA was less than in wild-type (Fig. 1C, F). The result suggests that other regulatory networks in addition to the cooperative action of BL and GA signaling are implicated downstream of the circadian clock and PIF4/5 mediated external coincidence mechanism that is responsible for the photoperiodic control of hypocotyl elongation. Then we examined the effect of cytokinins and jasmonic acids on hypocotyl elongation under diurnal light and dark cycle conditions because both phytohormones are known to suppress hypocotyl elongation under various light conditions. We found that

Materials and Methods

Plant lines and growth conditions. Arabidopsis thaliana plants used in this study were all Col-0 background. The pif4-101 pif5-1 mutant was described previously.46 Seeds were surface sterilized and stratified at 4°C, germinated and grown on Petri dishes containing Murashige and Skoog medium, 1.0% (w/v) sucrose, and 0.3% (w/v) gelan gum, at pH 5.7, in climate-controlled growth chambers with continuous light, 16 h light/8 h dark, and 8 h light/16 h dark photoperiod cycles as constant, long day, and short day conditions, respectively, under neutral white fluorescent light (PFPD = 70 μmol m⁻² s⁻¹). In addition to optimum ambient temperature conditions of 22°C, seedlings were grown at 16°C and at 28°C as relatively high and low temperature conditions, respectively. A light emitting diode (I-em = 740 nm, half-value width = 25 nm, EYLEA) was used at light intensity of about 50 μmol m⁻² s⁻¹ for irradiation of far red light.

Changes in plant architectures.22 In this external coincidence model, suggesting that the mechanistic basis of the circadian clock- and PIF4-mediated coincidence mechanism is applicable to the temperature adaptive photoperiodic control of plant growth, based on the observation that the transcription of PIF4 occurs precociously at the end of night under high temperature conditions, and the expression of PIF4 at the end of night is repressed under low temperature conditions.13,14 Agreeing with this, target genes of PIF4 are also induced at the end of night under high temperature conditions in long days, and repressed under low temperature conditions even in short days.13,14 In agreement with this, target genes of PIF4 are also ones associated with other growth-regulating hormones such as brassinosteroids and gibberellic acids (GAI), ethylene (ACS8), and cytokinins (CKX5) are under the control of circadian clock- and PIF4/5-mediated external coincidence mechanism.22 Recent genome-wide analysis revealed the direct target of PIF4 and PIF5 systematically.15,24 It is thought from the recent studies that biosynthesis and transport of auxin are important for rapid elongation of hypocotyls under shade and high temperature conditions.15,19,20,23,25,26 It is also known that brassinosteroids and gibberellic acids act cooperatively to enhance the hypocotyl elongation.27,28 The evidence prompted us to examine the effect of brassinosteroids and gibberellic acids on hypocotyl elongation under diurnal light and dark cycle conditions. When seedlings were grown on medium containing brassinolide (BL) and gibberellin A3 (GA), elongation of hypocotyls was stimulated significantly even in long days, namely, under non-induced conditions (Fig. 1C, F). The event was also observed in the pif4 pif5 mutant, although the effect of BL and GA was less than in wild-type (Fig. 1C, F). The result suggests that other regulatory networks in addition to the cooperative action of BL and GA signaling are implicated downstream of the circadian clock and PIF4/5 mediated external coincidence mechanism that is responsible for the photoperiodic control of hypocotyl elongation. Then we examined the effect of cytokinins and jasmonic acids on hypocotyl elongation under diurnal light and dark cycle conditions because both phytohormones are known to suppress hypocotyl elongation under various light conditions. We found that
the PIF4/5-dependent hypocotyl elongation observed in short days is inhibited by exogenously added phytohormones, 6-benzylaminopurine (BA) or methyl jasmonate (MeJA) (Fig. 1D, E, G). The effect of BA and MeJA was no longer observed in *pif4 pif5* mutant background. These results suggest that PIF4/5 are important for JA/BA mediated hypocotyl regulation. In order to gain insight into roles of jasmonic acids in the diurnal growth of *A. thaliana*, we focused on identifying the jasmonate-associated gene that is under the control of the PIF4/5 dependent external coincidence mechanism.

Identification of jasmonate-associated candidate genes as PIF4-targets

For the identification, we adopted the same strategy that we used earlier. We re-inspected a number of public databases for genes possibly regulated by PIF4/5; (i) transcriptome for shade avoidance response under low R/FR conditions; (ii) transcriptome for end-of-days-FR-light (EODFR) treatment; (iii) transcriptomes for red light and shade responses, taking advantage of a *pif1 pif3 pif4 pif5* quadruple mutant; (iv) a set of genes the expression of which is regulated in a time of day-specific and a PIF4- and PIF5-dependent manner; (v) a set of genes induced by FR-light, investigated by means of RNA-sequencing; and (vi) a set of genes regulated by FR-light in a PIF4- and PIF5-dependent manner. The last dataset also provided us a list of genes, to which PIF5 would bind directly (i.e., the results of ChIP-sequence analyses). As a result, we found the *ST2A* gene encoding a hydroxyjasmonate sulfotransferase as a candidate for jasmonate-associated PIF4/PIF5-targets. We also found the *RGF6/GLV1* gene encoding a tyrosine-sulfated secretory peptide as another candidate of peptide hormone-associated PIF4/PIF5-targets. At first, light responsiveness of the candidate genes was characterized. *IAA29*, which encodes an auxin inducible transcriptional regulator, is a well-known PIF4 and PIF5 regulated gene, and was used as a reference gene. *IAA29* is rapidly induced upon exposure of light-grown seedlings to FR-light (Fig. 2A), while *IAA29* is severely repressed upon exposure of dark-grown seedlings to white-light (Fig. 2D). Induction of *IAA29* in response to FR-light was partially suppressed in *pif4 pif5* mutant seedlings (Fig. 2A). The result suggests that other PIF family proteins are involved under the shade avoidance conditions. This is in good agreement with the fact that transcription of *IAA29* of dark grown seedlings was partially inhibited in the *pif4 pif5* mutant (Fig. 2D). Then, we examined
changes in transcription of ST2A and RGF6/GLV1 in response to white and FR-light. Transcripts of ST2A were induced by FR treatment in wild type, but the response was considerably suppressed in the pif4 pif5 mutant (Fig. 2B). On the other hand, transcripts of ST2A in etiolated seedlings were repressed in response to light exposure, and involvement of PIF4 and PIF5 in the control of ST2A was also observed in the dark-grown seedlings (Fig. 2E). The essentially same responses were observed in transcription of RGF6/GLV1, although the responsiveness and dependency on PIF4 and PIF5 was lower than those of ST2A (Fig. 2C, F). Because the involvement of PIF4 and PIF5 in the control of ST2A and RGF6/GLV1 was confirmed, transcriptional regulation of the above two kinds of phytohormone related genes is investigated on the daily basis to gain new insights into the temperature-adaptive photoperiodic control of plant growth.

ST2A is induced at the end of dark phase under short day and/or high temperature conditions

It is well-characterized that PIF4 is activated at the end of every night specifically in short days due to the coincidence between internal (circadian clock) and external (photoperiod) time cues. In order to clarify whether transcription of ST2A is under the control of the circadian clock- and PIF4-mediated external coincidence mechanism, diurnal expression profile of ST2A was examined under long day and short day conditions. Since both BA and MeJA affected elongation of hypocotyls negatively under light and dark cycle conditions (Fig. 1D, E), expression of CKX5 encoding a cytokinin oxidase was examined as a reference for ST2A in this experiment. It was shown that diurnal expression of ST2A as well as CKX5 was induced at the end of night specifically in short days, although both the expressions were depressed to the basal level in long days (Fig. 3A, B). The induced expression at the end of night in short days was no longer observed in the pif4 pif5 mutant background (Fig. 3C). Recently we found that not only photoperiod but temperature signals are integrated into the external coincidence mechanism so that PIF4 could be activated at the end of every night even in long days under relatively high temperature condition of 28 °C, and be repressed even in short days under relatively low temperature condition of 16 °C (Fig. 3D). In good agreement with the external coincidence model, diurnal expression of ST2A is induced at the end of night at 28 °C even in long days (Fig. 3F). The induction was fundamentally dependent on PIF4 and PIF5 (Fig. 3G). On the contrary, short days-specific induction of ST2A transcription at the end of night is no longer observed at 16 °C (Fig. 3I). The essentially same event was observed for the diurnal expression profile of the control gene, CKX5 (Fig. 3E, H). Since diurnal expression profile of the ST2B gene encoding the paralog of ST2A was different from that of ST2A, it is likely that the property for time of day- and photoperiod/temperature-specific transcriptional regulation is specific to ST2A among hydroxyjasmonate-related sulfotransferase family genes (Fig. 3J).

RGF6/GLV1 is regulated according to the circadian clock- and PIF4-mediated external coincidence mechanism

RGF6/GLV1 belongs to a root meristem growth factor family including RGF1, RGF2, RGF3, and RGF4, which are involved in the maintenance of root stem cell...
Fig. 3. Characterization of Diurnal Expression Profile of the Phytohormone-Associated Genes under Light and Dark Cycle Conditions.

A and B, diurnal expression profile of the CKX5 (A) and ST2A (B) genes in 7-d-old wild-type seedlings under long day (LD) and short day (SD) conditions. C, comparison of diurnal expression profiles of ST2A under SD conditions between 7-d-old wild-type and pif4 pif5 mutant seedlings. D, external coincidence model that is responsible for temperature adaptive photoperiodic control of hypocotyl elongation. Diurnal expression profile of transcripts of PIF4, level of PIF4 activity, and diurnal expression level of transcripts of PIF4 target genes are shown under LD/22°C, LD/28°C, and SD/16°C conditions. E and F, diurnal expression profile of the CKX5 (E) and ST2A (F) genes in 7-d-old wild-type seedlings under long day (LD) and short day (SD) conditions between 7-d-old wild-type and pif4 pif5 mutant seedlings. H and I, diurnal expression profile of the CKX5 (H) and ST2A (I) genes in 7-d-old wild-type seedlings under SD/22°C and SD/16°C conditions. J, diurnal expression profile of the ST2B gene in 7-d-old wild-type seedlings under long day (LD) and short day (SD) conditions.

It was recently reported that RGF6/GLV1 modulated the distribution of auxin. In order to clarify whether the external coincidence mechanism is involved in the control of the secreted peptide, diurnal expression profile of RGF6/GLV1 was examined. Expression of an auxin-associated gene, IAA29, was also examined as a reference for RGF6/GLV1 in this experiment. It was found that RGF6/GLV1 was induced in a time of day- and a photoperiod-dependent manner. Namely, expression of RGF6/GLV1 was induced at the end of night in short days, while only a basal level expression was observed throughout the day in long days (Fig. 4B). Even under the long day conditions, expression of RGF6/GLV1 was induced at the end of night if growth temperature was relatively high at 28°C (Fig. 4E). On the contrary, transcriptional induction of RGF6/GLV1 observed at the end of night in short days was suppressed when growth temperature was relatively low at 16°C (Fig. 4H).
Fig. 4. Characterization of Diurnal Expression Profile of the Phytohormone-Associated Genes under Light and Dark Cycle Conditions.
A and B, diurnal expression profile of the IAA29 (A) and RGF6/GLV1 (B) genes in 7-d-old wild-type seedlings under long day (LD) and short day (SD) conditions. C, comparison of diurnal expression profiles of RGF6/GLV1 under SD conditions between 7-d-old wild-type and pif4 pif5 mutant seedlings. D and E, diurnal expression profile of the IAA29 (D) and RGF6/GLV1 (E) genes in 7-d-old wild-type seedlings under LD/22 °C and LD/28 °C conditions. F, comparison of diurnal expression profiles of RGF6/GLV1 under LD/28 °C conditions between 7-d-old wild-type and pif4 pif5 mutant seedlings. G and H, diurnal expression profile of IAA29 (G) and RGF6/GLV1 (H) genes in 7-d-old wild-type seedlings under SD/22 °C and SD/16 °C conditions. I, diurnal expression profile of RGF6/GLV1 gene in 7-d-old wild-type seedlings under long day (LD) and short day (SD) conditions.

Implications
The study described above suggests that circadian clock- and PIF4/PIF5-mediated external coincidence mechanism controls various kinds of phytohormone-associated genes for temperature-adaptive photoperiodic control of plant growth. Auxin, brassinosteroids, and gibberellins affect positively on elongation of hypocotyls of Arabidopsis seedlings. On the other hands, cytokinins and jasmonic acids do negatively. It is found that a cytokinin-associated gene CKX5 and a jasmonic acid-associated gene ST2A are regulated according to the external coincidence mechanism. CKX5 encodes cytokinin oxidase, which catalyzes the degradation of cytokinins.40) ST2A encodes hydroxyjasmonate sulfotransferase, which inactivates function of jasmonic acids.35,36) Two genes were induced under short day or high temperature conditions, namely, when elongation of hypocotyls is stimulated. Plant architecture including elongation of hypocotyls is modulated by various kinds of phytohormone networks. It is possible that reduction of active cytokinin and jasmonic acid levels is involved in the control of the temperature adaptive photoperiodic control of plant growth (Fig. 5). To understand the molecular mechanism how phytohormone signaling networks function downstream of circadian clock- and PIF4/PIF5-mediated external coincidence mechanism remains to be elucidated in the future.

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