Brassinosteroids-mediated regulation of $ABI3$ is involved in high-temperature induced early flowering in plants

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Abstract The interplay of plant hormones is one of the essential mechanisms for plant growth and development. A recent study reported that Brassinosteroids (BR) and ABSCISIC ACID (ABA) interact antagonistically in early seedling developments through the BR-mediated epigenetic repression of ABSCISIC ACID-INSENSITIVE 3 ($ABI3$). However, the other physiological roles of the BR-mediated regulation of $ABI3$ and ABA responses beyond early seedling developments remain largely unknown. Here, we showed that the activation of BR signaling by high temperatures promotes flowering time through the suppression of $ABI3$ expressions. Elevated ambient temperature induced early flowering in wild type Col-0 plants, but not in BR-defective bri1-116 mutant plants. Conversely, a hyper BR biosynthetic dwf4-D mutant displayed more sensitive thermomorphic long shoot elongation and early flowering. Both expression patterns and physiological responses supported the biological roles of $ABI3$ in the regulation of floral transition and reproduction under high temperature conditions. Finally, we confirmed that the lowered expressions of the transcript and protein levels of $ABI3$ brought on by elevated temperature were correlated with warmth-induced early flowering phenotypes. In conclusion, our data suggest that the BR- and warmth-mediated regulation of $ABI3$ are important in thermomorphic reproductive phase transitions in plants.

Keywords High temperature, Brassinosteroids, $ABI3$, Plant hormone, Flowering

Introduction

As sessile and autotrophic life cycles of terrestrial plants, they are always challengeable to cope with dynamic environmental changes. Phenotypic and developmental plasticity for adaptation to unpredictable external conditions have been broadly accepted in plant kingdom. With recent global warming issues, high temperature stress during plant growth and developmental processes is now considered as one of the most critical issues in ecological and agricultural fields. Prolonged ambient temperature affects to a broad spectrum of responses on plants, which are collectively referred to as thermomorphogenesis such as shoot elongation and early flowering (Quint et al. 2016). Flowering is initiated by complex developmental programs integrated with a variety of signaling cues including light, temperature, vernalization and plant hormones (Pin 2012; Song et al. 2015). The floral related signaling pathways are integrated into a central floral regulator, FLOWERING LOCUS T (FT) (Kardailsky et al. 1999). Many essential genetic and molecular signaling pathways have been well characterized for understanding floral organ initiations. Warmth-induced early flowering is tightly connected with a central regulator of ambient temperature signaling regulator, PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Koini et al. 2009). Also, several recent studies have revealed that phytochromes, circadian clock, and plant hormones including auxin, BR and GAs are cooperatively involved in temperature-mediated growth and developmental processes (Quint, Delker et al. 2016).

BR, a unique plant steroid hormone mainly regulates morphogenesis, cell growth, differentiation and flowering by complicated interactions with various external and internal signaling cues (Li et al. 2018). Consistently, BR biosynthesis and signaling pathways are dominantly involved in warmth-induced plant developmental plasticity such as shoot and root elongation (Iñiguez et al. 2018; Martins et al. 2017). According to the serval previous studies, control of thermomor-
phogenesis by PIF4 is dependent on BR activity (Ibañez, Delker et al. 2018). Also, the BR actions are importantly regulated for thermomorphogenesis with cooperation with Auxin and GA biosynthesis (Unterholzner, Rozhon et al. 2015). However, the physiological roles of BR and its signaling pathways in warmth-induced early flowering are still unclear.

**ABA INSENSITIVE 3 (ABI3)** is one of the essential transcription factors in ABA signaling responses such as seed dormancy and stress tolerance (Ooms et al. 1993; Parcy et al. 1994). The transcriptional regulation of ABI3 by ABA and BR-activated BES1 is likely critical for early seedling developments (Ryu et al. 2014, Kim et al. 2017). BES1-TOPLESS (TPL)-Histone deacetylase 19 (HDA19) repressor complex was directly recruited to the E-box sequences of ABI3 promoter regions and promoted the epigenetic histone 3 deacetylation (Ryu et al. 2014). These responses directly controlled the ABA responses by sequential upregulation of a major ABA signaling transcription factor, ABA INSENSITIVE 5 (ABI5). Although floral repressor roles of ABI4 and ABI5 functioned in another layer of transcription factors in ABA signaling have been reported (Shu et al. 2015; Wang et al. 2013), there are no direct evidences whether ABI3, a major up regulator of ABI5 is involved in flowering. Previous studies about ABI3-mediated plant developments were restricted to seed dormancy or maturation, ABA responses, and stress tolerances. However, the spatio-temporal expression patterns of ABI3 were observed in not only early seedlings, but also other developing tissues (Rohde et al. 1999), suggesting other biological functions of ABI3 beyond to seed dormancy and early seedling developments. In this study, we demonstrate that high temperature mediated early flowering is tightly connected with BR signaling pathways. Also, the repression of ABI3 transcript and protein levels by high temperature are critically involved in warmth-induced thermomorphic early flowering time in *Arabidopsis*.

**Materials and Methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana* ecotype Col-0 and Ler were used as wild-type controls as the genetic backgrounds of transgenic lines. *Arabidopsis* seeds were germinated on solid media (pH 5.7 – 5.8) containing 1/2 Gamborg B5 salts (Duchefa, Haarlem, The Netherlands), 1% sucrose and 0.8% plant-agar and all plants were grown in a greenhouse under long-day conditions (16-h light/8-h dark cycles) at 20 ~ 22°C. For high temperature condition, all plants were treated at 27°C. The mutant seeds (abi3-4 and brl-301) were ordered from ABRC stock center.

**Transgenic plants and immunoblotting assay**

To generate transgenic plants overexpressing HA-tagged ABI3 in the Col-0 background, genomic DNA fragment was cloned into pCB302ES containing the 3SS promoter and HA tag sequences as described previously (Ryu et al. 2010; Ryu et al. 2007). Electroporation was used to introduce the gABI3-PCB302ES vector into Agrobacterium *tumefaciens* strain GV3101. All transgenes were integrated into the Arabidopsis genome by Agrobacterium-mediated floral dipping methods. Transgene expression was verified by immunoblotting with a 1/2000 dilution of a monoclonal anti-HA antibody (Roche, catalogue number 12013819001). Total proteins from seedlings were extracted with protein extraction buffer (50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1× protease inhibitor cocktail (Roche), and 1% Triton X-100). Total protein (3 ~ 20 µg) was separated by SDS-PAGE (10% polyacrylamide), transferred to a polyvinylidene difluoride membrane and immunodetected using 1/2,000 dilution of peroxidase-conjugated high-affinity anti-HA (Roche) and anti-actin (MP Biomedicals, catalogue number 69100) antibodies.

**RNA extraction and Real time qRT-PCR**

Total RNAs were extracted from seedlings using a Total RNA extraction kit (Intron Biotechnology, Korea) according to the manufacturer’s instructions. Total RNA concentration and quality were measured using a K5600 Micro-spectrophotometer (Shanghai Biotechnol Co., China). A first-strand synthesis kit (Enzymomics, Korea) with oligo (dT) primers was used for cDNA synthesis from 1 µg of total RNA. The cDNA was then used for real-time quantitative PCR with a Quant Studio 3 (Applied Biosystems, USA) instrument using SYBR Green Real-time PCR Master Mix (Applied Biosystems). Primer lists are followed: **CPD**: 5’-ACGACAGGCCTCTCTAAATGTG-3’ and 5’-AGTACCAATCGCGGCGC-3’; **DFW4**: 5’-GGAGAAGTGGTAGTTTTTGCGAC-3’ and 5’-CAGAAATA CGAGAAACCCTTA, **ABI5**: 5’-TCATAGTCATATACTCC GACGTCATAAT-3’ and 5’-AGTTATTGTGTATTTGCTCTT TGGCGACTT-3’, **ABI5**: 5’-ATTGGAAGCTGAACTTAAC DWF4: 5’-CGAGAAACCCTTA, **ABI5**: 5’-TCATAGTCATATACTCC GACGTCATAAT-3’ and 5’-AGTTATTGTGTATTTGCTCTT TGGCGACTT-3’. Threshold cycle (Ct) values were used to calculate 2-ΔΔCt for expression analysis, where ΔΔCt for treated plants was determined as follows:

\[ ΔΔCt = \left( Ct_{\text{control}} - Ct_{\text{transgene}} \right) - \left( Ct_{\text{control}} - Ct_{\text{wild-type}} \right) \]
Results and Discussion

High temperature-activated BR signaling promotes floral transition of plants

Prolonged ambient high temperature affects to diverse developmental and physiological processes including thermomorphogenesis and floral phase transition in plants (Cho et al. 2017; Quint et al. 2016). Previous studies reported that BR signaling was dominantly involved in thermomorphogenesis including elongated aerial parts of plants (Ibañez et al. 2018; Martins et al. 2017). To confirm the warmth-activated BR signaling, we initially monitored the expression levels of BR responsive biosynthetic CPD and DWF4 in Arabidopsis seedlings under high temperature (27°C) conditions (Fig. 1A). As expected, the BR biosynthetic genes were greatly reduced in seedlings grown in 27°C, indicating that BR signaling was activated in this condition. Also, the high temperature induced early flowering (Fig. 1B) in a long day condition (LD), supporting that heat stress enhanced both BR signaling activity and floral transition in plants. To further investigate the relationship between BR signaling and thermo-induced early flowering, we then compared flowering time phenotypes of BR biosynthetic or signaling related mutants including dwf4-D (Kim et al. 2013) and bri1-301 (Xu et al. 2008) grown under different temperature (21 and 27°C) conditions. Hyper BR biosynthetic dwf4-D plants were more sensitive to elevated temperature conditions, but either BR insensitive or defective mutants displayed defects in promoting flowering time and shoot elongation (Fig. 1B, 1C) These results suggest that BRI1-initiated canonical BR signal transduction pathways are essentially involved in general thermomorphogenic developmental plasticity in plants.

High temperature-activated BR signaling regulates transcripts level of ABI3

Our previous studies have shown that BR signaling is in-

![Fig. 1](image-url) High temperature-activated BR signaling promotes thermomorphic floral transition. (A) Real-time quantitative RT-PCR analysis of the expression levels of the BR biosynthesis-related CPD and DWF4 genes in Col-0 seedlings treated at 21/27°C for 48 hours (n=3, ±S.E.M). An asterisk indicates a significant difference in expression levels (*P<0.05, **P<0.01, Student’s t-test). (B) Days to bolting and leaf number at flowering of Col-0, dwf4-D, and bri1-301 plants grown at 21/27°C under LD (n=14, ± S.E.M). Means and standard errors from over 14 plants were shown (P < 0.05; one-way ANOVA). (C) Images showing phenotype at flowering of Col-0, dwf4-D, and bri1-301 plants treated at 21/27°C under LD (DAG 20). Scale bars, 1cm
tegrated into BES1-TPL- HDA19 repressor complex to inhibit ABA-mediated early seedling developments by epigenetically suppression of ABI3 and ABI5 (Ryu et al. 2014). The expression of ABI5 was directly up-regulated by ABI3 activity (Lopez-Molina et al. 2002, Kim et al. 2016), and over-expression of the ABI5 resulted in severe late flowering phenotype (Wang et al. 2013). We therefore confirmed that the early flowering phenotype induced by high temperature was due to down-regulation of ABI3 by activated BR signaling. To elucidate whether high temperature could repress ABI3 expression, we carried out a qRT-PCR using Col-0 treated at 21/27°C for 48hour (Fig. 2A). We could then confirm that the expression of ABI3 and ABI5 was downregulated, and AIP2, which is known to induce ABI3 protein degradation (Zhang et al. 2005), were also downregulated (Fig. 2A). Because it was seen that the expression of ABI3 was downregulated at high temperature, it was confirmed whether the abi3-4 (Ler background), loss-of-function mutant of ABI3, affected the flowering time by high temperature (Fig. 2B). The flowering time of abi3-4 at 21°C was faster than Ler, wild type, and it also became faster at 27°C. These results suggest that heat stress may regulate the expression level of ABI3 by BR signaling activation, thereby affecting the flowering time. Taken together, heat stress activates BR signaling, and regulates transcripts level of ABI3, leading to early flowering phenotypes.

Ectopic expression of ABI3 shows late flowering phenotypes

The biological roles of ABI3 in plants have been focused on regulation of seed development and germination and stress responses (Oorns et al. 1993; Parcy et al. 1994). However, our results revealed that the low expression level of ABI3 was correlated with early flowering by heat stress, and previous study showed that BES1 epigenetically represses expression of ABI3. Therefore, we generated 35S:ABI3-HA T1 transgenic plants in Col-0 background in order to confirm that the phenotype of early flowering of activated stress signaling by BR was reduced in expression of ABI3, which negatively affects flowering (Fig. 3A, 3B). At 32 DAG, the Col-0 plant was already flowering, whereas 35S:ABI3-HA T1 transgenic lines continuously formed rosette leaves, but not bolting (Fig. 3B). The rosette leaf numbers at flowering were also increased in the ABI3 overexpression plants (Fig. 3C).
Heat stress reduces protein level of $ABI3$ and promotes flowering time

Our results showed that high temperature activates BR signaling and induce early flowering (Fig. 1). We confirmed that down-regulation of $ABI3$ was regulated by high temperature-activated BR signaling (Fig. 2) and we also observed late flowering phenotypes of $ABI3$ overexpression (Fig. 3). Because it was confirmed that the expression of $ABI3$ was downregulated by high temperature, we next evaluated whether the ectopic expression of $ABI3$ by a constitutive active $35S$ promoter affected the flowering time under high temperature (Fig. 4A). Surprisingly, the late flowering phenotypes of the $35S:ABI3-HA$ line grown at $27^\circ C$ was almost disappeared similar to wild type plants. These result supposed that high temperature could regulate both transcripts and protein levels of $ABI3$ to control floral transition. We therefore tested whether the protein levels of $ABI3$ were differentially regulated by heat stress (Fig. 4B). As expected, the $ABI3$ level at $27^\circ C$ was about half that of treated in $21^\circ C$ for 24 hrs. These results suggest that heat stress may regulate not only the expression level of $ABI3$ but also the protein level, thereby affecting the flowering time. Taken together, high temperature activates BR signaling, and regulates transcripts and protein level of $ABI3$, leading to early flowering phenotype (Fig. 4C).

Indeed, flowering is one of the most important processes in the success of reproduction as well as yield in plants and that should occur at the right time, such as when it is appropriate, seasons, interacts with the pollinators (Cho et al. 2017). Plant flowering is a process that becomes the starting point of the transition from vegetative to reproductive growth and is of agricultural importance. In the case of green vegetables in which the leaves of plants are used for edible purposes such as cabbage and lettuce, when flowering occurs, vegetative growth does not occur anymore and all the energy is concentrated on the fruit-bearing processes. Under stress conditions such as high ambient temperature,
drought, light stress, the floral transition is promoted or delayed (Cho et al. 2017; Kazan and Lyons 2015). Among the various stresses, heat stress by global warming is now one of the biggest environmental problems. Heat stress promotes flowering as it affects various developmental and physiological processes. If plants will be early flowering, the crop yields would be greatly reduced due to failure of reproduction and premature growth arise from reduction of vegetative growth periods. In this study, we reveal that ABI3 negatively regulates flowering by probably regulating the expression of flowering-related genes, and BR mediated regulation of ABI3 is involved in high-temperature induced early flowering in plants. Although our data could not provide detailed molecular basis mechanisms of ABI3-mediated late flowering, the ABI3 gene would be an important biotechnological material with its stress tolerance abilities (Bedi et al. 2016; Tamminen et al. 2001) as well as our novel finding for flowering. The pleotropic roles of BR in plant growth and developmental processes have been extensively revealed (Li et al. 2018). However, the functional roles of BR in thermomorphogenesis, especially early flowering are still unclear. With global warming issues, the molecular genetics and biological approaches of BR-mediated regulation of floral transition under prolonged high temperature will provide diverse layers for the physiological controls of plant life cycles.

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