

Gibberellin Signaling Is Required for Far-Red Light-Induced Shoot Elongation in *Pinus tabuliformis* Seedlings¹

Wei Li,² Shuang-Wei Liu,² Jing-Jing Ma, Hong-Mei Liu, Fang-Xu Han, Yue Li, and Shi-Hui Niu^{3,4}

Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, National Engineering Laboratory for Forest Tree Breeding, College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, People's Republic of China

ORCID IDs: 0000-0003-0805-6765 (W.L.); 0000-0001-6930-7965 (S.-W.L.); 0000-0002-6296-9373 (S.-H.N.).

Gibberellin (GA) is known to play an important role in low red/far-red (R:FR) light ratio-mediated hypocotyl and petiole elongation in *Arabidopsis* (*Arabidopsis thaliana*). However, the regulatory relationship between low R:FR and GAs remains unclear, especially in gymnosperms. To increase our understanding of the molecular basis of low R:FR-mediated shoot elongation in pines and to determine whether there is an association between low R:FR and GAs action, we explored the morphological and transcriptomic changes triggered by low R:FR, GAs, and paclobutrazol (PAC), a GAs biosynthesis inhibitor, in *Pinus tabuliformis* seedlings. Transcriptome profiles revealed that low R:FR conditions and GAs have a common set of transcriptional targets in *P. tabuliformis*. We provide evidence that the effect of low R:FR on shoot elongation in *P. tabuliformis* is at least partially modulated by GAs accumulation, which can be largely attenuated by PAC. GAs are also involved in the cross talk between different phytohormones in the low R:FR response. A GA biosynthesis gene, encoding entkaurenoic acid oxidase (KAO), was strongly stimulated by low R:FR without being affected by GAs feedback regulation or the photoperiod. We show that GA signaling is required for low R:FR-induced shoot elongation in *P. tabuliformis* seedlings, and that there are different regulatory targets for low R:FR-mediated GA biosynthesis between conifers and angiosperms.

Light, an essential energy source and informational signal, is one of the most important environmental factors for plants (Jiao et al., 2007). As sessile organisms, shade-intolerant plants have evolved the ability to sense shading or surrounding light competitors and respond rapidly with shade-avoidance responses (SARs; e.g. elongation of the stem, hypocotyl, or petiole; Jiao et al., 2007). Far-red light (FR) is known to be an important light cue during this process (Ballaré et al., 1990). In the past few decades, significant progress has been made toward understanding the molecular regulatory network of FR-mediated SARs (Carriedo et al., 2016). A small subset of basic helix-loop-helix transcription factors, known as phytochrome-interacting

factors (PIFs), act as a signaling hub in this process (Leivar and Quail, 2011).

A variety of plant hormones are known to be involved in the morphological adaptation response to low R:FR (de Wit et al., 2016). Gibberellin (GA) is known to be the dominant hormone in the promotion of stem elongation in plants. Therefore, it not surprising that GA plays an important role in the plant light response (Lau and Deng, 2010). Interestingly, as opposed to auxin, few genes involved in the GA signaling pathway are directly regulated by PIFs (Zhang et al., 2013). A recent study showed that DELLA proteins (the central negative regulators of GA signaling) can directly promote the degradation of PIFs (Li et al., 2016). Therefore, GA plays a role upstream of PIFs and auxin in FR-mediated SAR.

Over the past ten years, a molecular framework of the coordinated regulation of hypocotyl elongation by light and GA has been explored. Red light (R) and FR are both sensed by the phytochrome (phy) light receptor in plants. FR or low R:FR releases PIFs by inactivating phy, eventually leading to growth enhancement. In addition, the activity of PIFs is suppressed by DELLA proteins through a direct protein-protein interaction (de Lucas et al., 2008; Feng et al., 2008), demonstrating that the DELLA proteins block PIF transcriptional regulation activity and also mediate their degradation (Li et al., 2016). Thus, GAs regulate hypocotyl elongation by suppressing the suppressor through the GA-GID1-DELLA module (Harberd et al., 2009).

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²These authors contributed equally to this work.

³Author for contact: arrennew@126.com.

⁴Senior author.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Shi-Hui Niu (arrennew@126.com).

W.L. designed and revised the paper; S.-W.L. performed most of the experiment and revised the paper; J.-J.M., H.-M.L., and F.-X.H. participated in some parts of the research; Y.L. designed the research; S.-H.N. originally designed this research, analyzed the data, and wrote the paper.

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The current model integrates phy and GA signals with the regulation of hypocotyl elongation through PIFs, which is the central hub of the signaling network. PIFs function as negative regulators of phy-mediated light responses (Shin et al., 2009; Stephenson et al., 2009) and accumulate in the nucleus in darkness to promote hypocotyl elongation (Leivar et al., 2008). Light stimulates DELLA proteins accumulation by reducing GA levels (Achard et al., 2007). DELLA proteins can function as transcriptional repressors by blocking the activity of PIFs, resulting in inhibition of PIF-mediated gene expression and hypocotyl elongation (de Lucas et al., 2008; Feng et al., 2008). Under low R:FR conditions, the biosynthesis of GA is stimulated mainly through transcript levels of *GA20ox* and *GA3ox* genes in angiosperms (García-Martínez and Gil, 2001; Hisamatsu et al., 2005), targeting the DELLA proteins for degradation via the 26S proteasome pathway (Djakovic-Petrovic et al., 2007; Leone et al., 2014). This process releases PIF3/4 from the negative effect of DELLA proteins in the nucleus, leading to modulation genes involved in cell elongation to promote internode or hypocotyl elongation (de Lucas et al., 2008; Feng et al., 2008). These changes in light quality trigger a series of responses known as the shade-avoidance syndrome (SAS; Pierik and de Wit, 2014; Fraser et al., 2016; Ballaré and Pierik, 2017).

Previous studies have shown that a completely functional GA system is required for phyB-mediated stem elongation (Peng and Harberd, 1997) and that active GA level was upregulated by FR-enriched light, which has been observed in many species (García-Martínez and Gil, 2001). However, the underlying molecular mechanism of FR regulation of GA biosynthesis in shoot elongation remains unclear. Two studies have shown that the GA biosynthetic genes *GA20ox1* and *GA20ox2* in *Arabidopsis* (*Arabidopsis thaliana*; Hisamatsu et al., 2005) and *GA20ox* and *GA3ox* in *Rumex palustris* (Pierik et al., 2011) are upregulated by low R:FR. Nevertheless, this effect may be restricted to petioles. When compared with *Arabidopsis* and model crop species where considerable progress has been made in elucidating the molecular basis of the low R:FR response (García-Martínez and Gil, 2001), details of the molecular mechanism underlying low R:FR-GA interactions in conifers remain poorly understood. Several studies by physiological ecologists have clearly demonstrated a typical SAS induced by supplemental FR in conifers (de la Rosa et al., 1998; Razzak et al., 2017). Recently, transcriptome analysis has shown that the underlying mechanisms of shade avoidance may be diverse in gymnosperms in contrast with that in angiosperms (Ranade et al., 2019).

To understand the effect of low R:FR on the response to GA in conifers, here we investigated the molecular basis of low R:FR-mediated shoot elongation in pines and the role of GAs in low R:FR-stimulated shoot elongation. We explored the morphological and transcriptomic changes triggered by FR, GAs, and paclobutrazol (PAC), a GA biosynthesis inhibitor (Rademacher, 2000), in *Pinus*

tabuliformis seedlings. To account for errors caused by slow growth and the physiological response to light in conifers (Burgin et al., 1999), we analyzed shoots for an extended time (>100 d) rather than using a hypocotyl model system for 1 week (Fernbach and Mohr, 1990; Burgin et al., 1999; Ranade and García-Gil, 2013). We found that low R:FR action on shoot elongation in *P. tabuliformis* is at least partially modulated by GA accumulation. Furthermore, we show that the GA biosynthesis gene *PtKAO2* is regulated by low R:FR in *P. tabuliformis* without being affected by GA feedback regulation or the photoperiod. These results show that GA signaling is required in conifers for FR-induced shoot elongation, and increases our understanding of the connection between FR and GA in plants.

RESULTS

Both Low R:FR and GA Promote Shoot Elongation in *P. tabuliformis* Seedlings

We examined the effects of low R:FR, GA₃, GA_{4/7}, and PAC on the regulation of shoot elongation in fully developed *P. tabuliformis* seedlings. GA₃ and GA_{4/7} weekly irrigation caused a similar growth stimulation effect, suggesting that both are active GAs in pines. Low R:FR significantly promoted shoot growth in *P. tabuliformis* seedlings under control conditions over 15 weeks (Fig. 1). GA and low R:FR caused shoot elongation through an increase in stem unit length rather than stem unit number (Fig. 1). This result supports the occurrence of FR-mediated SAR in pines (Razzak et al., 2017).

Low R:FR Mediated Shoot Elongation Associated with GAs Synthesis Pathway

Under normal light conditions, PAC treatment resulted in dwarfing and a dark-green phenotype (Fig. 1), similar to other GA-deficient syndromes in angiosperm plants. Low R:FR rescued growth inhibition by PAC, whereas PAC was seen to partially antagonize low R:FR-induced shoot elongation (Fig. 1). We measured the gibberellin content of the seedlings using a liquid chromatography–mass spectrometry system. The results show that bioactive GA₄ and GA₁ are the highest abundance GAs in the *P. tabuliformis* seedlings, and the low R:FR treatment plants have a higher level of both GA₄ and GA₁ than control seedlings (Table 1). This suggests that the low R:FR and GA response networks are at least partially overlapping and that the GA signaling pathway is required for low R:FR-mediated shoot growth enhancement.

Low R:FR and GAs Have a Common Set of Transcriptional Targets in *P. tabuliformis* Seedlings

To explore whether there was overlap between the low R:FR and GA response networks, we analyzed global gene expression in *P. tabuliformis* in response to

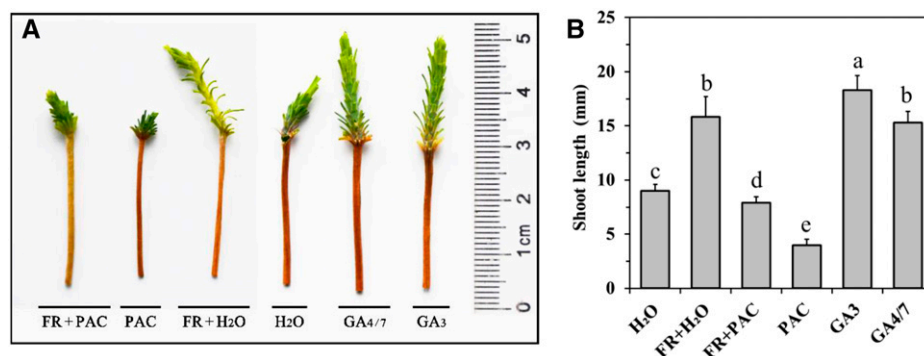


Figure 1. Effect of low R:FR, GA₃, GA_{4/7}, and PAC on shoot elongation in *P. tabuliformis* seedlings. A, Morphological changes of neoformed shoot elongation treated with low R:FR, GA₃, GA_{4/7}, and PAC for 15 weeks. FR, low R:FR light conditions. Needles were cut off before taking pictures; (A) was assembled from two photos which were taken at same time. B, Average shoot length of seedlings with different treatment for 15 weeks. This experiment was repeated at least four times, and data represent the means with SE of more than 16 seedlings. Significant differences between treatments are indicated with different letters.

low R:FR, GAs, and PAC. We found that 26.7% of the differentially expressed genes ($P < 0.01$) between low R:FR versus Control overlapped with those in GA₃ versus Control and GA₄ versus Control. However, because most GA-regulated genes have a weak expression response (Nemhauser et al., 2006; Goda et al., 2008; Kakei et al., 2015), we used a high-sensitivity threshold for screening differentially expressed genes as fold change > 1.2 and $P < 0.05$ compared with the GA-deficient conditions (PAC treatment). We identified 1,842 genes that responded to GAs and were differentially expressed between GA₃ versus PAC, GA_{4/7} versus PAC, and low R:FR versus PAC (Supplemental Fig. S1; Supplemental Table S1). We found that 60.9% of these genes were up-regulated by GAs and, at the same concentration, GA₃ treatment induced greater gene expression changes than GA_{4/7} (Fig. 2A). Expression changes of these genes were in the same direction and exhibited a very similar profile in the presence of GA₃ or GA_{4/7} (Fig. 2A), and 99.57% of genes were affected in the same direction under low R:FR conditions (Fig. 2B), supporting that they are GA-regulated genes. In addition, low R:FR conditions combined with PAC resulted in a GA-regulatory like gene expression shift, and the majority of these genes (98.53%) changed in the same direction as that observed under GA regulation (Fig. 2, C and D; Supplemental Fig. S2, heatmap). These results suggest that low R:FR shares a common transcriptional module with GA response networks.

Table 1. Concentrations of Bioactive GAs in *P. tabuliformis*

The GA concentration is given in nanograms GAs per gram of fresh needle. The values are the average of two biological replicates, with each sample containing all the needles of five seedlings.

Treatment	GA Concentration (ng/g)	
	GA ₁	GA ₄
Control	0.18 ± 0.02	0.30 ± 0.09
Low R:FR	0.28 ± 0.09	0.52 ± 0.02

GA-Mediated Plant Hormone Cross Talk in the Low R:FR Response in *P. tabuliformis* Seedlings

Previous reports have suggested that many plant hormones are involved in the FR response (Kebrom and Mullet, 2016). We analyzed genes involved in hormone responses that are affected by both GA and low R:FR (Fig. 3). Auxin is a key player activated by FR (Procko et al., 2016). Based on our results, auxin-response genes were primarily up-regulated by low R:FR and regulated by GAs, suggesting that GA plays a role upstream of auxin in FR-mediated SAR. Meanwhile, brassinosteroid (BR)- and salicylic acid-response genes were enhanced, whereas ethylene (ET)-response genes were repressed by both GAs and low R:FR. These results suggest that GA plays an important role in plant hormone cross talk in FR-mediated SAR.

A Putative GA-Biosynthesis Gene was Strongly Stimulated by Low R:FR in *P. tabuliformis* Seedlings

To explore the role of low R:FR in the GA signaling pathway, we analyzed the expression changes of 20 previously identified putative GA-metabolism genes (Niu et al., 2014), as well as genes encoding the GA receptor PtGID1 and two DELLA proteins (Du et al., 2017). We found that a putative ent-kaurenoic acid oxidase-encoding gene, *PtKAO2*, was strongly stimulated by low R:FR (Fig. 4). Moreover, the stimulation effect of low R:FR was not inhibited by PAC and showed a stable response over a relatively short period (Fig. 4).

The phylogenetic relationship between putative KAOs in *P. tabuliformis* and functionally identified KAOs in other plants was analyzed with the recently identified bacterial BdKAO (Nett et al., 2017) as out-group. We found that KAOs from monocotyledons and dicotyledons were separated into different subclusters, and *PtKAO2* is more similar to the angiosperm KAO

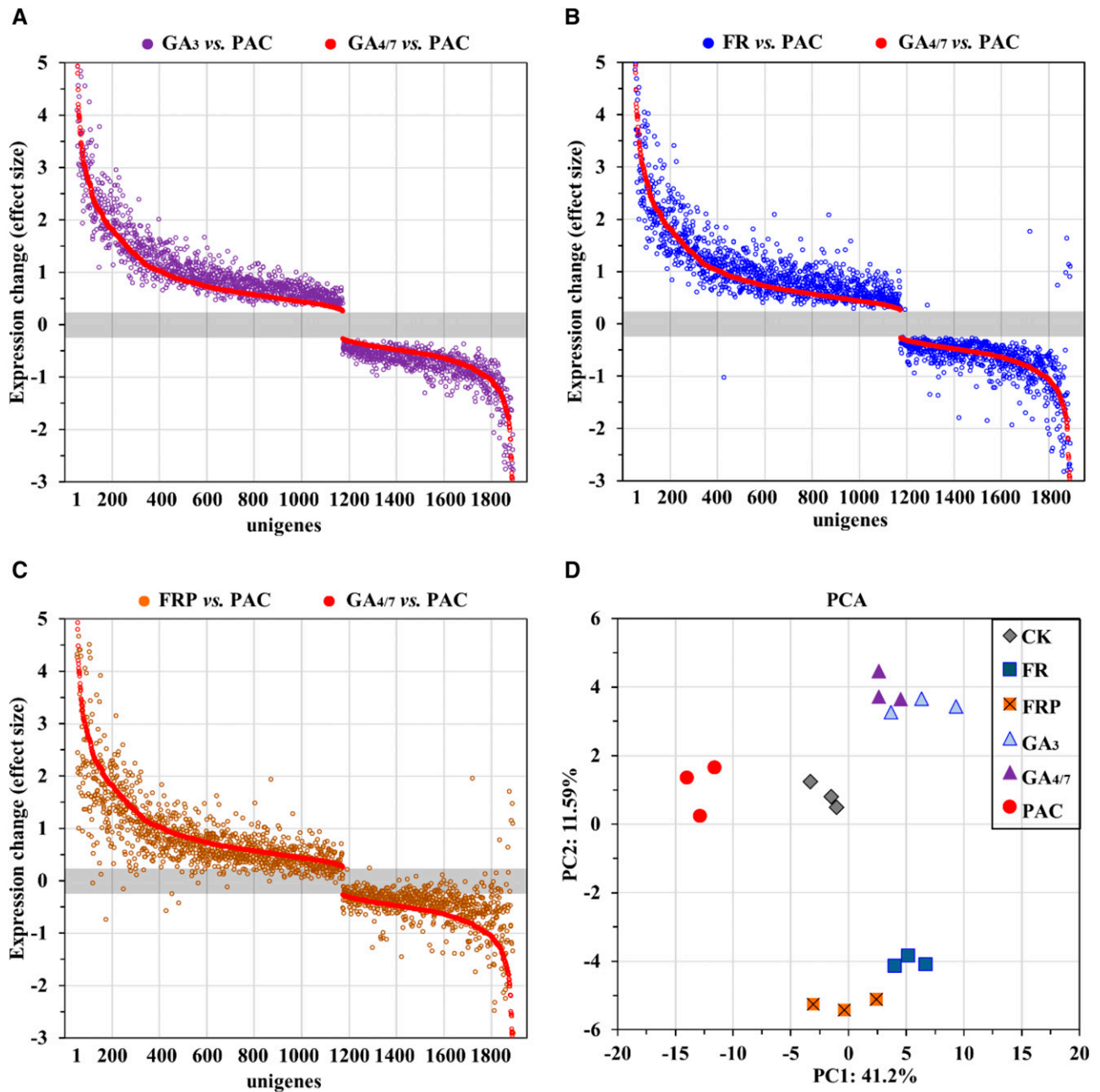


Figure 2. Transcriptomic changes in response to low R:FR, GAs, and PAC in *P. tabuliformis* seedlings. A total of 1,842 unigenes were shown, which were differently expressed between GA₃ versus PAC, GA_{4/7} versus PAC, and low R:FR versus PAC with an expression fold change > 1.2, $P < 0.05$. FR, low R:FR light conditions; FRP, simultaneous low R:FR conditions and PAC irrigation; CK, water control. Unigenes were rearranged in descending order according to the expression fold changes between GA₃ and PAC treatment. Data are mean values of three biological replicates. Corresponding expression changes of these unigenes between GA_{4/7} versus PAC (A), low R:FR versus FRP (B), and FRP versus PAC (C) are shown. D, Principal component analysis based on each of the 1,842 differently expressed genes. Each symbol represents a single sample ($n = 3$ replicate samples per treatment).

than PtKAO1 (Fig. 5). The amino acid sequence alignment suggests that KAO was conserved during the evolution of conifers and angiosperms. PtKAO2 shares 81% amino acid identity with at least one of the identified angiosperm KAOs in this study (Supplemental Fig. S3).

To explore the response pattern of *PtKAO2* to low R:FR, the expression of *PtKAO2* was detected by reverse transcription quantitative PCR at 0, 0.5, 1, 2, 3, 6, 9, 12, and 15 h (in darkness for 1 h) from the beginning of the light period during a 14:10-h photoperiod (Fig. 6). Expression of *PtKAO2* did not show a violent

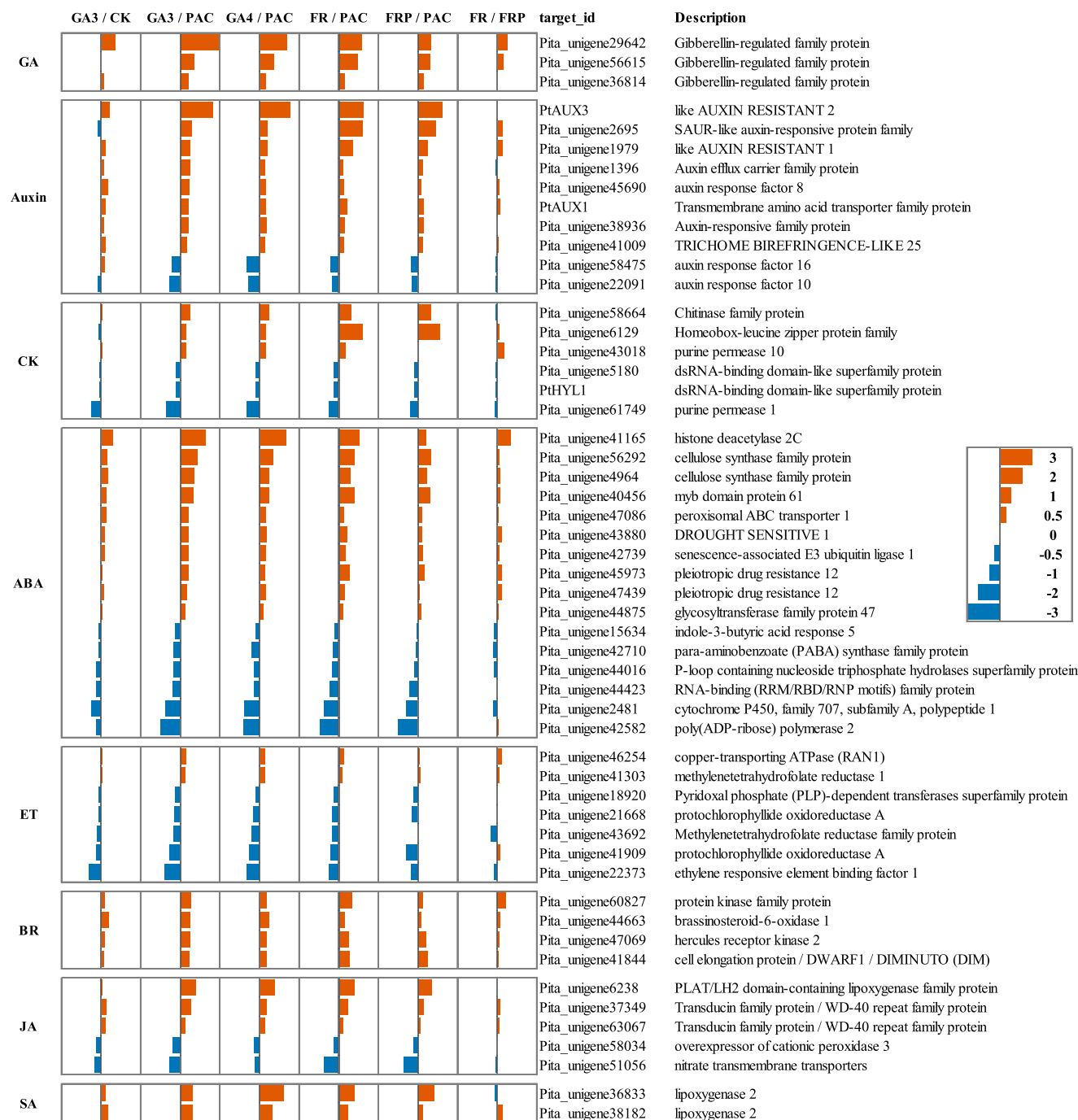


Figure 3. Differential expression of genes involved in the phytohormone response. Among the 1,842 unigenes regulated by low R:FR and GA, 53 genes with functions related to the response of phytohormones were selected and shown. FR, low R:FR light conditions; FRP, simultaneous low R:FR conditions and PAC irrigation; CK, cytokinin; ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid. The brown histogram (right) indicates genes that are expressed at higher levels under GA or low R:FR conditions and the stellblue histogram (left) indicates genes repressed by GA or low R:FR. The effect size (analogous to fold change) values were obtained from the sleuth software (Pimentel et al., 2017). Values represent the mean of three biological replicates.

oscillation pattern under regular conditions, and white light repressed this pattern throughout the day. *PtKAO2* showed a rapid response to both white light and FR, and the effect was significantly

attenuated following 1 h of darkness. However, the stimulation effect of low R:FR was gradually enhanced and peaked within 9 h. This result suggests that 7 h after exposed to low R:FR is an appropriate

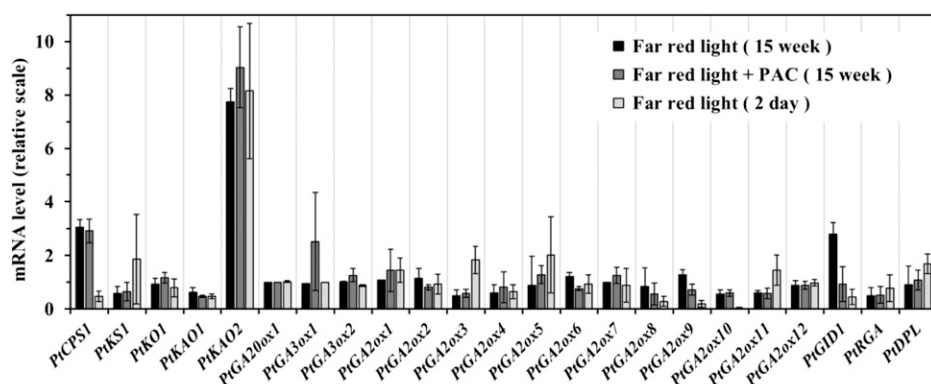


Figure 4. The expression response of genes involved in GA metabolism and signaling pathways to low R:FR in *P. tabuliformis* seedlings. The expression levels of each gene are relative to the value of the water control group (set at 1). Seedlings were grown under a 14:10 h photoperiod. FR, low R:FR light conditions. The mean of three biological replicates is plotted with SE.

time for sample collection for RNA-sequencing (RNA-seq) analysis in this study.

Expression Profile of *PtKAO2* in *P. tabuliformis*

To explore the regulatory and functional roles of *PtKAO2* in *P. tabuliformis*, we explored its expression under different conditions and during development. With the exception of pollen, it was ubiquitously expressed in all tissues (Fig. 7). Similar to *AtKAO* in Arabidopsis (Regnault et al., 2014), *PtKAO2* is more strongly expressed in reproductive organs. Interestingly, low R:FR induced *PtKAO2* expression in seedling needles to a high level, similar to that in female cones (Fig. 7). This activation was not affected by short-day or long-day photoperiods. When compared with the effect of low R:FR, GAs, and PAC, R only showed a minor regulatory role on the expression of *PtKAO2* (Fig. 7). These data confirm that low R:FR is an important regulator of *PtKAO2* expression in *P. tabuliformis*.

Overexpression of *PtKAO2* Complements Dwarfing in *kao1 kao2* Arabidopsis Plants

In order to confirm the biochemical function of *PtKAO2*, we overexpressed this gene in Arabidopsis because comparable genetic tools are still not available in *P. tabuliformis*. In Arabidopsis, ent-kaurenoic acid oxidase is encoded by two paralogous genes with functional redundancy, designated *KAO1* and *KAO2* (Regnault et al., 2014). Because *kao1* and *kao2* single mutants are indistinguishable from wild-type plants (Fig. 8A), we generated the *kao1 kao2* double mutant by crossing the corresponding single mutant lines. *kao1 kao2* plants exhibited typical nongerminating dwarf phenotypes, which were rescued by exogenous application of GA₃ (Fig. 8A). In the *kao1 kao2* genetic background, overexpression of *PtKAO2* also rescued the growth and germination defects of *kao1 kao2* plants and seeds (Fig. 8, B and C). These results suggest that *PtKAO2* plays a similar role as KAOs in Arabidopsis in the GA biosynthesis pathway.

DISCUSSION

GAs are pivotal growth-promoting regulators in plants. Therefore, it is not surprising that GAs play an

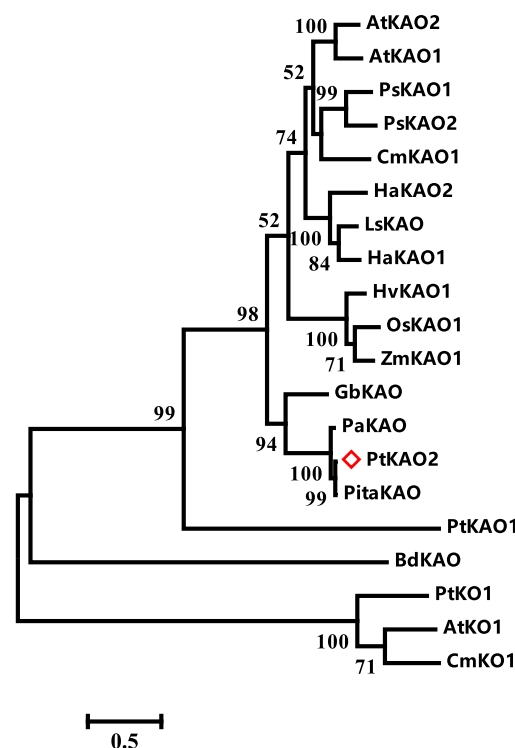


Figure 5. Phylogenetic relationship of *PtKAOs* in *P. tabuliformis* and KAOs identified from angiosperms. *PtKAO1*, -2, and *ZmKAO1* (Winkler and Helentjaris, 1995), *OsKAO1* (Sakamoto et al., 2004), *HvKAO1* (Helliwell et al., 2001), *LsKAO* (Sawada et al., 2008), *HaKAO1* and 2 (Fambrini et al., 2011), *PsKAO1* (Davidson et al., 2003), *CmKAO1* (Helliwell et al., 2000), *AtKAO1* and 2 (Helliwell et al., 2001), and the putative KAOs from other gymnosperms as *Ginkgo biloba*, *Picea abies*, and *Pinus taeda* were used to build the maximum likelihood tree. The bacteria *BdKAO* (Nett et al., 2017) and the related cytochrome P450 monooxygenase (CYP701A) *ent*-kaurene oxidases (KOs) were used as outgroup. The red diamond symbol indicates the *P. tabuliformis* gene that is identified in this study. The horizontal branch lengths are proportional to the estimated number of amino acid substitutions per residue. Bootstrap values were obtained from 1,000 bootstrap replicates.

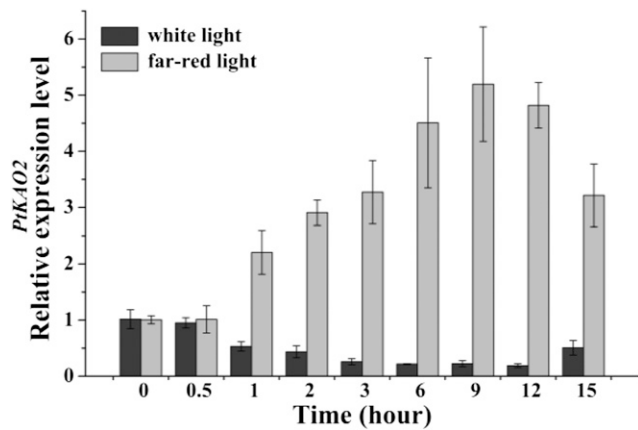


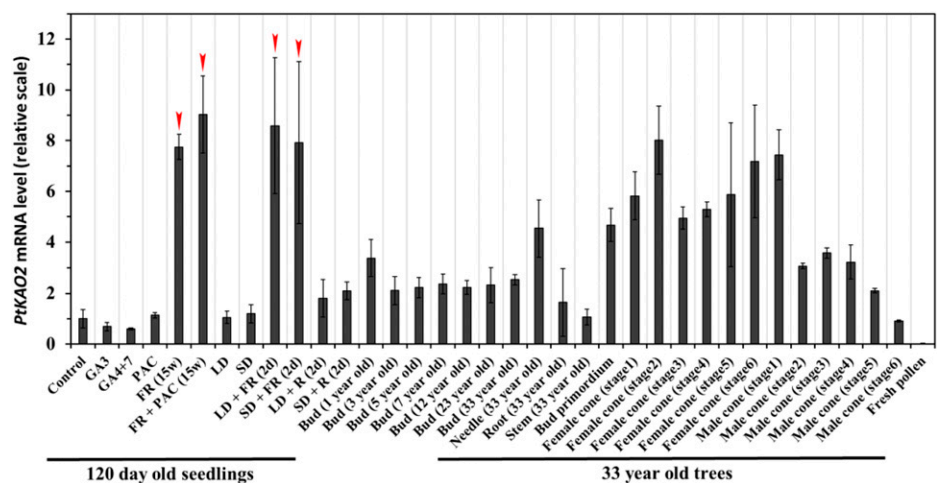
Figure 6. Expression pattern of *PtKAO2* under white light and low R:FR in *P. tabuliformis*. The expression level of each sample was relative to the value of the samples at 0 h, which were collected at the beginning of light treatment (set to 1). The mean of three biological replicates is plotted with SE.

important role in light-regulated growth (Kamiya and García-Martínez, 1999). Indeed, a completely functional GA system is required for phyB-mediated stem elongation (Peng and Harberd, 1997), and the active GA level is up-regulated by low F:FR conditions, which has been observed in many species (García-Martínez and Gil, 2001). However, the underlying molecular mechanism of low F:FR regulation of GA biosynthesis in shoot elongation remains unclear. Two studies have shown that the GA biosynthetic genes *GA20ox1* and *GA20ox2* in *Arabidopsis* (Hisamatsu et al., 2005) and *GA20ox* and *GA3ox* in *Rumex palustris* (Pierik et al., 2011) are up-regulated by low R:FR; however, this effect may be restricted to petioles. Genes associated with GA metabolism were analyzed based on transcriptome data in *Arabidopsis* (Supplemental Table S2), and no consistent differences were observed between the data sets. For example, no GA-biosynthesis genes were differently expressed more than 2-fold after 1, 3, or 24 h of low F:FR treatment (Leivar et al., 2012). By contrast,

AtGA20ox1 was significantly down-regulated in seedlings exposed to low F:FR for 4 h in the AtGenExpress data set (<http://jasp.weigelworld.org>). *AtGA20ox1* and *AtGA20ox2* showed no differential expression in cotyledons, hypocotyls, or roots under dark conditions (Ma et al., 2005). In another experiment, only *AtGA3ox1* showed a 2-fold change after exposure to low F:FR for 1 h, but was subsequently down-regulated after prolonged FR-rich treatment (Sessa et al., 2005). Under the monochromatic FR treatment, some *GA20oxs*, which contribute to bioactive GA reduction, were significantly up-regulated in both hypocotyls and cotyledons in *Arabidopsis* (Kirchenbauer et al., 2016; Supplemental Table S2). Recently, one study indicated that inactive GA₁₂ (synthesized by KAO) rather than active GAs is the major mobile GA in vivo (Regnault et al., 2015). These apparently contradictory results could be explained by the regulation of *GA20ox* and *GA3ox* genes downstream of GA₁₂ in a highly tissue-specific manner for the local synthesis of active GAs, which are sensitive to the feedback regulation of GAs (Hedden and Thomas, 2012). Moreover, the transcription of *GA20ox* and *GA3ox* are under circadian control and are very sensitive to environment cues (Wu et al., 1996; Xu et al., 1997), which results in complex observations. Overall, the association between FR and GA biosynthesis remains poorly understood.

In this study, we found that a putative GA biosynthesis gene, *PtKAO2*, was strongly stimulated by both short- and long-term low R:FR treatment in the conifer *P. tabuliformis*, and that this regulation was not affected by feedback regulation of GAs. We confirmed the biological functions of *PtKAO2* in *Arabidopsis*; its ectopic expression complemented the GA-defective phenotype of *kaol1 kaol2* plants, indicating that it encodes a functional ent-kaurenoic acid oxidase that functions in the GA biosynthesis pathway. Interestingly, in wild-type *Arabidopsis*, neither *KAO1* nor *KAO2* was consistently regulated by low R:FR in hypocotyls and cotyledons. However, KAO and normal GA synthesis are required for SAS in *Arabidopsis*, since the low R:FR response was significantly attenuated in *kaol1 kaol2* plant.

Figure 7. Expression profile of *PtKAO2* in *P. tabuliformis* under different conditions and during development. The expression level of each gene relative to the water control group (set to 1) is shown. The mean of three biological replicates is plotted with SE. FR, low R:FR light conditions; LD, long-day conditions; SD, short-day conditions; R: high R:FR light conditions. The arrows indicate the specific stimulation of *PtKAO2* expression by low R:FR conditions.



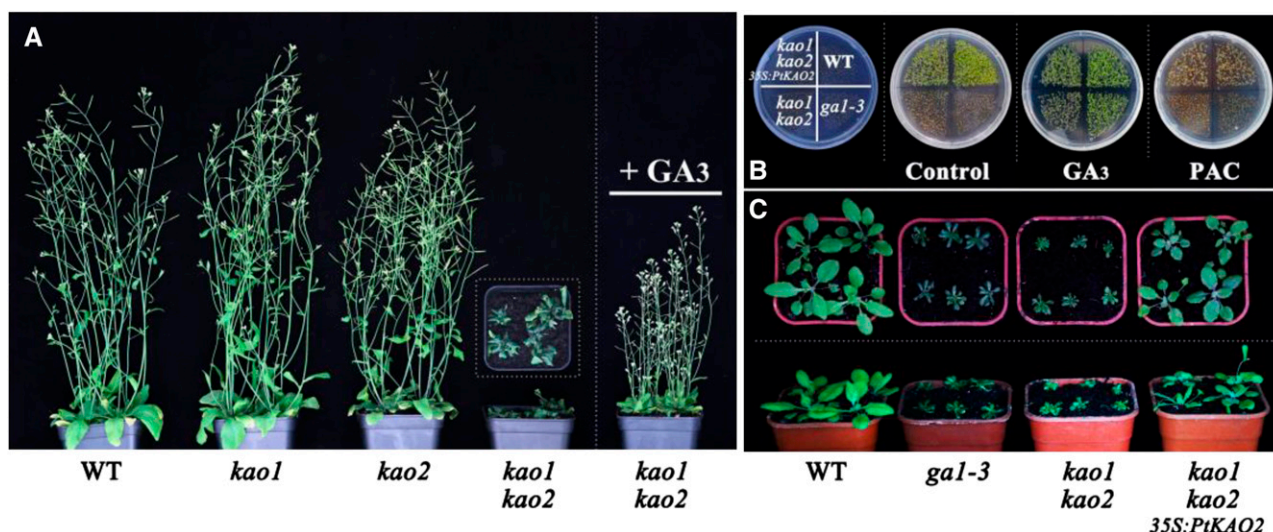


Figure 8. Overexpression of *PtKAO2* rescue the growth and germination defects of *kao1 kao2* double mutant in Arabidopsis. A, Arabidopsis *kao1 kao2* double mutant shows a severe dwarf phenotype that is partially rescued by 5 μM GA₃. B, *PtKAO2* transgenic lines in a *kao1 kao2* double mutant background display a normal germination phenotype similar to that of wild type (WT) under control, 5 μM GA₃, and 10 μM PAC treatment. C, Arabidopsis *kao1 kao2* double mutant exhibits a dark-green dwarf phenotype typical of *ga1-3*, which is partially complemented by *PtKAO2* overexpression. The parts of the figure were assembled from more than one photo, and the outlines of each original photo are indicate by dotted lines.

This suggests that GAs probably play an important role in both gymnosperms and angiosperms, but the regulatory mechanisms may differ.

In Arabidopsis, an annual plant with a very short life cycle, the response to low R:FR is a very rapid process (Tao et al., 2008; Leivar et al., 2012). After the start of low R:FR treatment, DELLA protein degradation and the corresponding petiole elongation were observed within 2 h (Djakovic-Petrovic et al., 2007). The expression changes of *PIF3-like 1* (*PIL1*) in response to low R:FR were detectable within 5 min and increased more than 30-fold within 1 h (Salter et al., 2003). Indeed, the phy response to light is significantly slower in conifers than angiosperms (Burgin et al., 1999). However, although phenotypic changes were barely detectable, the expression of *PtKAO2* was fully induced by low R:FR within 2 d in *P. tabuliformis* (Fig. 3). We further shortened the analysis time after the start of low R:FR treatment. Surprisingly, *PtKAO2* was rapidly up-regulated within 1 h and peaked within 9 h (Fig. 6), which is indicative of a highly responsive transcriptional network in conifers.

Many phytohormones play a role in SAR through interactions between different hormone pathways (Carabelli et al., 2007; Kebrom and Mullet, 2016). Under shade conditions, the biosynthesis of auxin, BRs, ET, and GAs are regulated by the interactions of phyB with PIFs in SAR (Ballaré and Pierik, 2017). Auxin is required for shade-induced growth in different plants (Procko et al., 2014; Müller-Moulé et al., 2016), and phyB plays a key role in the regulation of auxin signaling (Morelli and Ruberti, 2000). Previous studies have shown that auxin plays a major role in promoting hypocotyl elongation. In low R:FR shade, auxin is stimulated via the

TAA1-dependent pathway (Tao et al., 2008) and the Pr form of phyB-mediated PIFs accumulation and targeted transcription of *YUCCA* genes that catalyze the rate-limiting step in auxin biosynthesis (Hornitschek et al., 2012; Li et al., 2012; Müller-Moulé et al., 2016). Auxin-responsive defective mutations can suppress the constitutive SAR phenotype of *phyB*, leading to reduced hypocotyl growth (Tao et al., 2008). In a *phyB* mutant of sorghum (*Sorghum bicolor*), the auxin response was enhanced (Kebrom and Mullet, 2016). BR, another growth-promoting hormone, was shown to play a role in SAR in a nonredundant manner with auxin (Keuskamp et al., 2011). Interestingly, we show that the response of both auxin and BR was enhanced by GA in *P. tabuliformis*, indicating that low R:FR linked the actions of auxin and BR at least partly through the GA pathway. In tobacco (*Nicotiana tabacum*) and *Rumex palustris*, ET functions as a positive regulator in SAR, acting upstream of GA (Pierik et al., 2004; Pierik et al., 2011). However, in the sorghum *phyB* mutant, several ET-biosynthesis genes are down-regulated (Kebrom and Mullet, 2016). GA-induced hypocotyl or stem elongation is associated with regulation of PIFs activity independent of DELLA degradation (Djakovic-Petrovic et al., 2007; Leone et al., 2014). In this study, we identified ET-response genes that are repressed by low R:FR and GA. In contrast with auxin, ET signaling may not be a common factor in SAR across different species. In addition, past studies have shown that the phytohormone jasmonic acid plays a key role in the activation of plant immunity (Ballaré et al., 2012; Cerrudo et al., 2012; Cerrudo et al., 2017; Liu et al., 2019). In this study, several genes involved in cytokinin, abscisic acid,

jasmonic acid, and salicylic acid were regulated by both low R:FR and GA (Fig. 3), indicating that GA plays a role in the cross talk between different phytohormones during shade signaling.

SAR is undesirable in agricultural production because carbon resources are diverted to stem elongation rather than agronomically important tissues (Carriedo et al., 2016). However, SAR may be beneficial for tree breeding, specifically for trees with a very slow growth rate. In *P. tabuliformis*, a conifer tree, the stimulation of *PtKAO2* by low R:FR was not affected by photoperiod or GA-feedback regulation. Therefore, low R:FR could easily be applied under any conditions without strict control of illumination, and could be combined with exogenous GA application to promote seedling growth.

MATERIALS AND METHODS

Plant Material and Treatments

The seeds of *Pinus tabuliformis* were obtained from a primary clonal seed orchard located in Pingquan City, Hebei Province, China (40°99' N, 118°45' E, 560 m above sea level). Seeds were sown on sphagnum moss soaked with water and then germinated for 15 d in a growth chamber under conditions of 22°C 14-h/10-h light/dark (controls photosynthetically active radiation [PAR] = 30.438 $\mu\text{mol m}^{-2} \text{s}^{-1}$, light-emitting diode 50 W). Light quality was measured using an AvaSpec-ULS2048XL-EVO spectrometer (Avantes, <http://www.avantes.com>), which is able to measure wavelengths 200–1160 nm with a back-thinned CCD detector. The detector was held at the same height as needles to record the quality of light from the light-emitting diode source. Seedlings were then transferred in 8-cm diameter plastic pots with one individual and cultured in a mixture of prefertilized peat moss, perlite, and vermiculite (2:1:1, v/v) at 22°C with 14:10-h (normal), 16:8-h (long-day), or 10:14-h (short-day) photoperiods. The full developmental seedlings (Ranade and García-Gil, 2013) of *P. tabuliformis* were irrigated weekly with water, 50 μM PAC, 50 μM GA₃, and 50 μM GA₄₊₇ as well as low R:FR treatment (FR, peak wavelength at 723.21 nm, PAR = 15.440 $\mu\text{mol m}^{-2} \text{s}^{-1}$). In our previous experiments, we found that if the monochromatic far-red light treatment area was completely delimited by surrounding it with aluminum foil, the seedlings grew poorly or even died. Therefore, we provided supplementary white light (PAR = 5.707 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in subsequent far-red light treatments to ensure normal growth of the seedlings. The PAR of FR supplemented with white light was 21.147 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Therefore, FR treatment refers to low R:FR light conditions in this study for all experiments. Each treatment contained 16 seedlings with similar growth rates. The exact growth chamber and light conditions that were adopted in this study are shown in detail in Supplemental Figure S4.

The *Arabidopsis kao1* (Stock CS835691) and *kao2* (Stock CS827299) mutants were obtained from The Arabidopsis Information Resource (<http://arabidopsis.org>). We generated the *kao1 kao2* double mutants through crossing of the corresponding single mutant lines. PCR-based screening and sequencing were used for confirmation of double mutant progeny. For proliferation of nongerminating severe dwarf *kao1 kao2* double mutants, 5 μM GA₃ was applied to promote germination and growth.

Reverse Transcription Quantitative PCR Assays

The seedlings were grown for 3 weeks under normal conditions and were then transferred to low R:FR light conditions. Needles were sampled from the beginning of the light period at regular intervals. Total RNA was extracted using TRIzol reagent (Invitrogen) for gene expression analysis. Two micrograms of total RNA was used to synthesize single-strand complementary DNA using a M-MLV Reverse Transcriptase kit (Promega). qPCR assays were performed using a QuantiTect SYBR Green PCR Kit (Qiagen). PCR primers are listed in Supplemental Table S3.

RNA-Seq Analysis

Total RNA quantity and purity were assessed using the Nano Photometer spectrophotometer (Implen), and RNA concentration was measured using the

Qubit RNA Assay kit in Qubit 2.0 Fluorometer (Life Technologies). RNA integrity was assessed using the RNA Nano 6000 Assay kit of the Bioanalyzer 2100 system (Agilent Technologies). mRNA was fragmented into small pieces using divalent cations under increased temperatures. The cleaved RNA fragments were then reverse-transcribed to create the final cDNA library in accordance with the protocol for the mRNA-Seq sample preparation kit (Illumina). The average insert size for the paired-end libraries was 200–300 bp. The pooled libraries were sequenced on the Illumina HiSeq Xtenplatform (2 × 150 bp) using the paired-end module. The transcript abundances were estimated using kallisto software (Bray et al., 2016), and the differential expression analysis was performed using sleuth software (Pimentel et al., 2017).

Phylogenetic Analysis

The software ProtTest3 (Darriba et al., 2011) was used to determine the best-fit model of evolution. The “LG+G” model was finally selected, and the MAFFT tool (Katoh and Standley, 2013) was used for multiple sequence alignment. The maximum likelihood tree based on the LG+G model was obtained using PhyML 3.0 software (Guindon et al., 2010). Bootstrap values were obtained by 1,000 bootstrap replicates.

Plasmid Construction and Plant Transformation

The complete CDS of *PtKAO2* (GenBank: KJ158983) was amplified from the complementary DNA of *P. tabuliformis* needles by PCR. The products were cloned into pBI121, which contained the *nptII* gene as a selectable marker, which generated the *p35S::PtKAO2* construct. The construct was transformed into *Agrobacterium* GV3101 strain via the freeze/thaw method. *kao1 kao2* double mutant plants were transformed by floral dip. The transgenic lines were screened by kanamycin, and then confirmed by PCR and sequencing using primers that are listed in Supplemental Table S4.

Data Availability

The authors declare that all data supporting the findings of this study are available within the article and Supplementary Material online or are available upon request from the corresponding author.

Accession Numbers

The GenBank accession numbers of proteins mentioned in this paper are AHW42467 (PtKAO2), AHW42466 (PtKAO1), NP_001320198 (AtKAO1), NP_001189657 (AtKAO2), AAO23063 (PsKAO1), AAO23064 (PsKAO2), AAG41777 (CmKAO1), CBV36748 (HaKAO2), BAG71199 (LsKAO), XP_022022633 (HaKAO1), Q9AXH9 (HvKAO1), XP_015643774 (OsKAO1), NP_001105586 (ZmKAO1), AAT28221 (GbKAO), AAC28890 (BdKAO), AHW42468 (PtKAO1), NP_197962 (AtKAO1), and AAG41776 (CmKAO1). The RNA-seq data that support the findings of this study have been deposited in the China National GeneBank Sequence Archive (<https://db.cngb.org/cnsa/>) of China National GeneBank Database with accession number CNP0000737.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Overlap of genes between GA₃ vs. PAC, GA_{4/7} vs. PAC, and low R:FR vs. PAC.

Supplemental Figure S2. Heatmap of 1,842 GA-response genes under different treatments.

Supplemental Figure S3. Amino acid sequence alignment of PtKAO2 and ent-kaurenoic acid oxidases (KAOs) from angiosperms.

Supplemental Figure S4. The irradiance system and light qualities used in this study.

Supplemental Table S1. Expression level (TPM) and functional description of 1,842 GA-response genes under different treatments.

Supplemental Table S2. Expression data of GA-metabolism genes in response to low far/far-red light in *Arabidopsis*.

Supplemental Table S3. Primers used in reverse transcription quantitative PCR analysis.

Supplemental Table S4. Primers of PCR-based screening and sequencing for genetic background confirmation.

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