GASA4, a GA-stimulated gene, participates in light signaling in Arabidopsis

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Abstract

Gibberellic acid-stimulated transcripts in Arabidopsis (*GASAs*) were previously isolated as GA-stimulated transcript 1 from tomato (*GAST1*) homologs and induced by GA. Here, we report that *GASA4* is differentially regulated by light and affected by photoreceptors and light signaling transducers in Arabidopsis. Interestingly, *GASA4* was down-regulated by a far-red (FR) light signaling regulator far-red insensitive 219 (*FIN219*) under all light conditions. *GASA4* T-DNA inserted mutant seedlings with undetectable *GASA4* transcripts exhibited reduced sensitivity to red (R) and FR light, and can be rescued by the ectopic expression of *GASA4*; the *gasa4* mutant also showed enhanced response to exogenous GA under the same conditions. Furthermore, the *gasa4* mutant showed up-regulated expression of several key GA metabolism genes such as GA 20-oxidase 1 (*GA20ox1*), GA 3-oxidase 1 (*GA3ox1*) and GA 2-oxidase 2 (*GA2ox2*), and displayed increased expression of vital flowering time genes, including *GI*, *CO*, *FT*, and *SOC1*, which leads to an early flowering phenotype under long-day conditions as compared to the wild type. Thus, *GASA4*, negatively regulated by *FIN219*, may play various roles to regulate hypocotyl elongation and flowering during Arabidopsis development.

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Keywords: Gibberellin; *GASA4*; *FIN219*; Light signaling; Flowering

1. Introduction

Searching for gibberellic acid (GA)-responsive genes is a way to understand the GA-mediated signaling pathway. The gibberellic acid-stimulated in Arabidopsis (*GASA*) gene family was first reported by Herzog et al. [1], and five *GASA* members (*GASA1*–*GASA5*) showed a tissue-specific expression pattern. *GASA1* and *GASA4*, rather than *GASA2* and *GASA3*, were induced by GA. *GASA1* is expressed in siliques and flower buds and *GASA4* in growing roots and flower buds, whereas *GASA2* and *GASA3* are exclusively expressed in dry siliques and seeds [1]. Furthermore, results of GUS promoter activity assay showed that *GASA4* is primarily expressed in meristematic regions, which implies a role in cell division [2]. Related proteins were also found in genes of various plant species such as tomato GA-stimulated transcript 1 (*GAST1*) [3] and root

system-induced gene 1 (*RSI-1*) [4], the gene family GA-induced protein (*Petunia GIP*) [5], *Gerbera hybrida* homolog of *GAST1* (*Gerbera GEG*) [6] and potato *Snaking-1* and -2 in *Solanum tuberosum* (*StSN1* and 2) [7,8]. The main features of these related proteins are (1) a putative signal peptide of 18–23 residues; (2) a varying length of highly divergent amino acid residues right after the putative signal peptide in the N terminus; (3) a conservative cysteine-rich (12 cysteines) C-terminal domain; and (4) a small peptide of 88 to about 112 amino acids.

The functions of these proteins are not so related. Tomato *GAST1* expression is induced by GA and inhibited by ABA. Transgenic plants with overexpressed or reduced *GAST1* expression did not show any phenotype [9]. The *Petunia GIP* expression pattern coincides with corolla and stem cell elongation [10], but *GEG* is expressed when cell elongation ceases [6]. As well, potato *StSN2* is an antimicrobial peptide induced by wounding and pathogen infection, which suggests its involvement in plant defense systems [8]. These data suggest that these related proteins share a similar biochemical/structural activity but are involved in different processes.

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Plant phenotypes are often the convergent results of interactions between environmental factors such as light and phytohormones. GA has been known to regulate various aspects of plant development, including embryogenesis, seed germination, stem elongation, flowering, tuber formation and fruit development [11,12]. Modulation of GA levels and the regulation of GA-mediated signaling components are important for plant developmental patterns. GA 20-oxidase (GA20ox) and GA3ox, involved in the last two steps of GA biosynthesis, as well as GA2ox, responsible for the deactivation of bioactive GA, are the key enzymes for GA levels in cells [13]. Especially, the expression of *GA3ox1* and *GA3ox2*, which catalyze the conversion of GA₂₀ into bioactive GA₁, is tightly regulated. Both *AtGA3ox1* and *AtGA3ox2* mRNAs are present in the cortex and endodermis of embryo axes of germinating Arabidopsis seeds; however, only *AtGA3ox1* expression is under feedback control by the GA response pathway [14]. Recently, Curaba et al. [15] reported that *AtGA3ox2* is negatively regulated during embryogenesis by *LEAFY COTYLEDON2 (LEC2)* and *FUSCA3 (FUS3)*, which indicates that GA biosynthesis is down-regulated in plant embryos.

Although *GASA4* is induced by GA and shown to have tissue-specific expression patterns, its physiological function in Arabidopsis remains unclear. Here, we describe our investigation of its involvement in light signaling in Arabidopsis, so implicated because *GASA4* displays 62% homology with a mungbean gene, gibberellin- and infrared light-stimulated transcript 1 in *Vigna radiate (VrGIR1)*, isolated from a study of infrared (IR) light-induced genes. Current results indicate that *GASA4* is differentially regulated under various light conditions, particularly red (R) and far-red (FR) light, under which its T-DNA knock-out mutant *gasa4* exhibits a long hypocotyl phenotype. Intriguingly, *GASA4* was negatively regulated by a FR signaling component far-red insensitive 219 (FIN219) under all light conditions and was able to repress the expression of several key GA metabolism genes as well as a group of flowering-time genes from flower buds under long-day conditions. Furthermore, the *gasa4* mutant also has an earlier flowering phenotype. Thus, *GASA4*, with a newly found function involved in light signaling, may play a vital role in crosstalk between light and GA signaling to regulate plant development such as hypocotyl elongation and flowering.

2. Materials and methods

2.1. Plant material and light treatment

Throughout this paper, the wild type is *Arabidopsis thaliana* ecotype Columbia. The T-DNA knock-out mutant *gasa4* (SALK_042431) was obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus). All other mutants used in this study were described previously [16]. Surface sterilization and cold treatment of Arabidopsis seeds and seedling growth conditions were described previously [16]. The light sources were described previously [17], and light intensities for FR, R, blue and white light were 8.36, 21.24, 3.47 and 77 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively.

2.2. RNA extraction, RNA gel blot and RT-PCR analyses

Total RNA was isolated from 4-day-old and different light-treated Arabidopsis seedlings by the Eppie-tube RNA isolation method [18]. Twenty micrograms of total RNA was separated on 1.2% agarose-formaldehyde gel, and then transferred to the positively charged nylon membrane (Roche Diagnostics). DIG-labeled cDNA probes were synthesized by PCR. Hybridization and washing conditions were performed at 65 °C with DIG-labeled cDNA probes following standard procedures according to the manufacturer's procedure (Roche Diagnostics). For all blots, equal loading was confirmed by ethidium bromide staining of ribosomal RNAs (25S and 18S). All RNA gel blot analyses were biologically and independently repeated twice for consistent results. Quantification of the mRNA levels was by use of the LAS-3000 FUJI image system (Fuji Photo Film Co. Ltd.). For RT-PCR analysis, RNA samples were treated with DNase I (Invitrogen) before reverse transcription. Primers used in this study were as follows: for *ent-kaurene synthase (KS)*: sense, 5'-AAGGCTGCTCAAAGTTATCCACAT-3', and antisense, 5'-ATAAAGTTGCAGCCCCAGAGAAAT-3'; for *ent-kaurenoic acid oxidase 1 (KAO1)*: sense, 5'-CTGACTCCTTCACTCGC-3', and antisense, 5'-CCTGAGACGCTTGTTGTT-3'; for *GA20ox1*: sense, 5'-CTCATGAATACACGAGCC-3', and antisense, 5'-TGATACACCTTCCCAAATG-3'; for *GA3ox1*: sense, 5'-CGATTTCGGTAAACTTTGGC-3', and antisense, 5'-ATCCATTGGATAGGATGTGG-3'; for *GA2ox2*: sense, 5'-TTTCCGTGAGTCGGTGG-3', and antisense, 5'-CTCCGCC-TCTTCCTCCG-3'; for *GAI*: sense, 5'-GTTGTGGCTTG-TGATGGA-3', and antisense, 5'-GGATTTAGTTTGGCTTCG-3'; for *RGA*: sense, 5'-CTCTCCGATACTCTTCA-GATG-3', and antisense, 5'-CAAGCCACCAGATTACAA-3'; for *GIGANTEA (GI)*: sense, 5'-GAGCTGTCTTTCTCCGT-TGTTT-3', and antisense, 5'-CTTCAATAGATTGGA-TAAACCGTC-3'; for *CONSTANS (CO)*: sense, 5'-AACAGT-GACAGATCCAGAGAACAG-3', and antisense, 5'-TTCTCTG-CATACGCTTTTCTTGAA-3'; for *FLOWERING LOCUS T (FT)*: sense, 5'-CCTGCTACAACCTGGAACAACCTTT-3', and antisense, 5'-GCTATATAGGCATCATCACCGTTTCGTTACTC-G-3'; for *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*: sense, 5'-GTTTCTGAAGAAAATATGCAGCATT-3', and antisense, 5'-GAACAAGGTAACCCAATGAACAA-3'.

2.3. Genomic DNA isolation and Southern blot analysis

Genomic DNA was isolated from rosette leaves of wild type and the *gasa4* mutant grown under white light condition (16 h light and 8 h dark) [18], digested with appropriate restriction enzymes, separated on 1% agarose gel, transferred to positive charged nylon membranes (Roche Diagnostics Corp., Mannheim, Germany), and hybridized at 65 °C with *GASA4* genomic DNA fragment labeled with digoxigenin-11-dUTP. The probe was amplified by PCR with *GASA41* primer, 5'-TCTGGATC-CATGGCTAAGTCATATGGAGCT-3' and *GASA42* primer, 5'-TCTGAATTCAGGGCATTGTTTGGTCCACCCTC-3'. The signal was detected by the chemiluminescent reaction with CSPD (disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tri-

cycle [3.3.1.1]decan)-4-yl) phenyl phosphate) (Roche) according to manufacturer's instruction.

2.4. Complementation test of the *gasa4* mutant

To overexpress *GASA4* in the *gasa4* mutant, a *Bam*HI–*Eco*RI DNA fragment containing the full-length *GASA4* cDNA was obtained by PCR amplification with the following primer pairs: sense, 5'-TCTGGATCCATGGCTAAGTCATATGGAGCT-3', and antisense, 5'-TCTGAATTCAGGGCATTGTTGGTC-CACCCTC-3' (underline indicates the built-in *Bam*HI (GGATCC) and *Eco*RI (GAATTC) sites, respectively. The purified PCR product was ligated into the binary transformation vector pCambia1390-cMyc. The resulting constructs were introduced into the *Agrobacterium* GV3101 by a standard method [19] and then transformed into the *gasa4* mutant by floral dipping [20]. Transgenic plants containing transgenes were selected with 25 µg/ml hygromycin. Approximately 30 independent T1 transgenic plants were selected and grown to the T2 generation. Phenotypic analysis was carried out with at least 10 independent lines.

2.5. Response of the *gasa4* T-DNA inserted mutant to exogenous application of GA

GA₃ was added to agar plates containing MS-medium plus 0.3% sucrose to a final concentration of 50 µM. Equal amounts of solvent, 95% ethanol, were added as the control. Wild type and mutant seeds were sterilized and sown on the plates containing 50 µM GA₃ as described previously [16]. Hypocotyl length of the seedlings grown for 4 days under FR and R light was measured as described previously [16].

2.6. Determination of flowering time for the *gasa4* T-DNA inserted line

Wild-type *Arabidopsis* and homozygous T-DNA-inserted lines of *gasa4* were grown under long-day (16 h day and 8 h dark) conditions. Flowering time was recorded by use of two different indexes: the days from the seedlings sown to the soil to appearance of the first inflorescence and leaf number at bolting. More than 10 plants for each case were investigated.

3. Results

3.1. Expression of *GASA4* is differentially regulated by light

GASA4 was previously shown to be induced by GA and developmentally regulated, and its expression among the members of the *GASA* gene family in *Arabidopsis* was the most abundant in young seedlings [1]. However, its physiological function in *Arabidopsis* remained to be elucidated. Moreover, *GASA4* also shows 62% homology with a mungbean gene, *VrGIR1*, that is regulated by IR and FR light (data not shown), which implies its regulation by light. Thus, we found the level of *GASA4* mRNA isolated from 4-day-old seedlings grown

Fig. 1. RNA gel blot analysis of the expression of *GASA4* under various light conditions. Total RNA was isolated from 4-day-old wild-type seedlings treated with different light, and 10 µg total RNA was loaded for gel blot analysis. EtBr-stained rRNA is shown below as a loading control. The numbers below the blot indicate normalized values based on the signal intensity in the samples and 25S rRNA by using the LAS-3000 FUJI image system, with white light (W) set at 1. B: blue light, R: red light, FR: far-red light, D: dark.

under different light conditions more than two-fold increased in darkness compared to white light and down-regulated in other light conditions, especially R and FR light (Fig. 1). So, *GASA4* expression was differentially regulated in response to various quality of light.

3.2. *GASA4* is regulated by photoreceptors and light signaling transducers

Since *GASA4* was regulated by different light, we wondered whether it was involved in light signal transduction. RNA samples of various light signaling mutants, including photoreceptors grown under continuous FR light (cFR) for 4 days, were isolated and underwent RNA gel blot analysis.

Fig. 2. RNA gel blot analysis of the expression of *GASA4* in light signaling mutants under different light conditions. Total RNA isolated from the mutant seedlings of photoreceptors and light signaling components grown in continuous far-red light for 4 days (A), or continuous white (W), blue (B), red (R), and far-red (FR) light and dark (D) (B), was used for gel blot analysis of the expression of *GASA4*. Ethidium bromide-stained ribosomal RNA was shown as a loading control. The numbers below the blots indicate normalized values based on the signal intensity in the samples and 25S rRNA by using the LAS-3000 FUJI image system, with WT in panel (A) or white light (W) for WT in panel (B) set at 1.

Fig. 3. Phenotypic investigation of the *GASA4* T-DNA inserted mutant seedlings. (A) Schematic diagram of the *GASA4* gene structure with location of T-DNA insertion. Boxes represent exons and thin lines are introns. The boxes with slash: translated regions, open boxes: untranslated regions. The line is in scale. (B) Genomic Southern analysis of the *gasa4* T-DNA inserted mutant. Ten micrograms of genomic DNA isolated from wild type and the mutant seedlings grown in white light (16 h light, 8 h dark) were digested with restriction enzymes shown above the figure and subjected to genomic Southern analysis under stringent conditions. The probe used in the hybridization is a *GASA4* genomic DNA fragment. WT: wild type, *gasa4*: *gasa4* T-DNA inserted mutant. (C) RNA gel blot analysis of *GASA4* expression in the *gasa4* T-DNA inserted mutant seedlings grown in far-red light for 4 days. Ten micrograms total RNA was loaded for gel blot analysis. EtBr-stained *rRNA* is shown below as a loading control. Duplicated T-DNA inserted lines (*gasa4*) were used in the analysis. Phenotypic analysis of the seedlings of the *gasa4* T-DNA inserted lines grown under red light (D, left panel) and far-red light (D, right panel) conditions for 4 days. WT: wild type. (E) Quantitative measurement of the hypocotyl lengths of wild type and the *gasa4* mutant seedlings grown in various light conditions for 4 days. The number of the seedlings measured is 30 ($N = 30$). Error bars represent standard deviation. D: dark, FR: far-red, R: red light, B: blue light, W: white light.

After normalization with loading control of rRNA levels, the expression of *GASA4* was found to be substantially reduced in the mutants *phyB*, suppressor of phytochrome A 1 (*spa1*), constitutive photomorphogenic 1 (*cop1-5*), a null allele of *COP1*, and slightly decreased in several photoreceptor mutants such as *phyA* and *cry1* as well as the *fly1* mutant involved in FR signaling and the *cop1-4* mutant, a weak allele

of *COP1* containing the N-terminal 282 amino acids of *COP1* (Fig. 2A). In addition, since *FIN219* has been shown to be a suppressor of *COP1* and involved in the phyA-mediated signaling pathway [16], we further tested whether *FIN219* was involved in the control of *GASA4* expression. Surprisingly, *GASA4* expression level was greatly increased about three-fold in the *fin219* mutant compared to the wild type

Fig. 4. Complementation of the *gasa4* mutants. (A) Phenotypic examination of the *gasa4* transgenic seedlings harboring a 35S promoter-driven *cmcy-GASA4* fusion construct in the T2 generation grown in the dark (a), blue light (b), white light (c), far-red light (d), and red light (e) conditions for 4 days. 4-4 and 4-7 in (d) and (e) represent different transgenic lines. The segregated seedlings with long hypocotyls of the lines 4-4 and 4-7 shown in (d) and (e) are similar to the background mutant *gasa4*; the short seedlings are rescued phenotypes. (B) Quantitative measurement of the hypocotyl lengths of wild type and transgenic seedlings shown in (A). The number of the seedlings measured is 30 ($N = 30$). Error bars indicate standard deviation. *G4OE/g4-S*: *GASA4* overexpressor/*gasa4* mutant with short hypocotyls; *G4OE/g4-L*: *GASA4* overexpressor/*gasa4* mutant with long hypocotyls. (C) RNA gel blot analysis of *GASA4* expression in transgenic seedlings. Total RNAs 10 μ g isolated from 4-day-old seedlings of three rescued transgenic lines grown under far-red light were loaded to each lane and subjected to RNA gel blot analysis. The probe is a Dig-labeled *GASA4* cDNA. An ethidium-bromide stained gel in the bottom was shown for RNA equal loading. *G4OE/gasa4*: *GASA4* overexpressor/*gasa4* mutant.

under all light conditions (Fig. 2B), which indicates that FIN219 negatively regulates *GASA4* expression. Taken together, these data suggest that the *GASA4* in Arabidopsis may act in a downstream manner and is regulated by light signaling components.

3.3. The null mutant of *GASA4* exhibits a hyposensitive phenotype under R and FR light

To further understand the function of *GASA4* involved in light signaling, T-DNA insertion mutants were obtained from the Arabidopsis Biological Research Center (ABRC, Ohio) and underwent phenotypic examination under various light conditions. The mutant was derived from a T-DNA putatively inserted in the first exon of the *GASA4* coding region that is composed of four exons and three introns (Fig. 3A). To further confirm whether the T-DNA was indeed inserted into the *GASA4* locus, the genomic DNA isolated from wild type and the *gasa4* mutant was digested with either *EcoRI* or *XhoI* and subjected to genomic Southern analysis with *GASA4* gene as a probe. The result revealed that the genomic DNA digested with either *EcoRI* or *XhoI* did show polymorphism between wild type and the *gasa4* mutant (Fig. 3B), indicating that the T-DNA was inserted in the *GASA4* gene, leading to barely detected *GASA4* transcripts in the *gasa4* mutant (Fig. 3C). Moreover, homozygous 4-day-old seedlings of the *gasa4* mutant examined under different light conditions displayed a hyposensitive phenotype, with long hypocotyls under continuous R light (cR) and cFR (Fig. 3D and E), respectively, but no phenotype under blue, white light and dark conditions (Fig. 3E), which implies that *GASA4* plays a role in the control of hypocotyl elongation under both R and FR light.

3.4. The null mutant of *GASA4* can be rescued by ectopic expressions of *GASA4*

To further understand whether *GASA4* is mainly responsible for the phenotype of the *gasa4* mutant shown under cR and cFR, the *GASA4* cDNA was driven by 35S cauliflower mosaic virus promoter and introduced into the *gasa4* mutant. The resulting transgenic lines were examined under various light conditions. Eleven out of 18 lines examined were found to have rescued phenotypes with short hypocotyls in T2 segregated seedlings under cR and cFR, and two representative lines are shown here (Fig. 4A(d and e) and B); but they have no phenotype under other light conditions as well as in the dark (Fig. 4A(a–c) and B). To examine whether the rescued phenotype was due to the expression of the transgene *GASA4*, a RNA gel blot analysis of *GASA4* in the rescued transgenic lines was carried out. It revealed that *GASA4* was highly expressed in the rescued transgenic seedlings under cFR when compared to the genetic background *gasa4* mutant (Fig. 4C), which indicates that *GASA4* is indeed responsible for the mutant phenotype, involved in the control of hypocotyl elongation in response to R and FR light.

3.5. *GASA4* represses several key enzyme-corresponding genes involved in GA metabolism

Although *GASA4* was induced by GA [1], how it affects GA-mediated responses remains unknown. To investigate whether *GASA4* influences components involved in GA biosynthetic or signaling pathways, wild type and *gasa4* mutant seedlings grown under cFR for 4 days underwent RT-PCR analysis of the 4 GA biosynthetic genes encoding the enzymes *ent-kaurene synthase* (*KS*), *ent-kaurenoic acid oxidase 1* (*KAO1*), GA 3-oxidase 1 (*GA3ox1*) and GA 20-oxidase 1 (*GA20ox1*); 1 GA deactivation enzyme, GA 2-oxidase 2 (*GA2ox2*) and 2 GA-signaling negative regulators, GA-insensitive (*GAI*) and repressor of *gal-3* (*RGA*). *GA20ox1*, *GA3ox1* and *GA2ox2* mRNA levels were up-regulated in the seedlings of the *gasa4* mutant, especially *GA3ox1* (Fig. 5). However, *KS* and *KAO1*, the early GA biosynthetic genes, and *GAI* and *RGA*, the GA signaling components, showed little change between the mutant and wild-type seedlings under cFR, which implies that *GASA4* might modulate GA levels through negatively controlling the key enzymes-corresponding genes in GA metabolism.

3.6. The *gasa4* mutant exhibits increased response to exogenous GA

The *gasa4* mutant exhibited a hyposensitive phenotype under cFR (Fig. 3) and the expression of several key enzyme-corresponding genes involved in GA metabolism was also

Fig. 5. RT-PCR analysis of the expression of GA biosynthetic and signaling genes in 4-day-old seedlings of the *gasa4* mutant grown under far-red light. *UBQ*: ubiquitin 10 as an endogenous loading control. WT: wild type, *GAI*: GA-insensitive, *RGA*: repressor of *gal-3*, *KS*: *ent-kaurene synthase*, *KAO1*: *ent-kaurenoic acid oxidase 1*, *GA20ox1*: GA 20-oxidase 1, *GA3ox1*: GA 3-oxidase 1, *GA2ox2*: GA 2-oxidase 2.

Fig. 6. Effect of exogenous application of gibberellins on hypocotyl phenotype of wild type and *gasa4* mutant under far-red and red light. Wild type (WT) and *gasa4* mutant seeds were sown on the GM plates with (+GA) or without (control) 50 μ M GA under far-red (FR) and red light (R) conditions for 4 days. $N = 30$.

altered (Fig. 5). To further understand whether the long hypocotyl phenotype of the *gasa4* mutant will alter its response to exogenous GAs, we tested the effects of exogenous GA on hypocotyl lengths of the *gasa4* and wild-type seedlings grown under cFR and cR. An amount of 50 μ M GA₃ could increase the hypocotyl length of wild-type seedlings by 20% as compared with the wild type without the addition of GA under both light conditions, whereas the hypocotyl length of the *gasa4* mutant increased up to 41% and 37% in the presence of GA under cFR and cR, respectively, as compared to the *gasa4* mutant without GA at the same conditions (Fig. 6), which indicates that the *gasa4* mutant increases response to GA. Thus far, the results suggest that GASA4 may affect GA metabolism and also modify responses to exogenous GA.

3.7. The *gasa4* mutant shows an earlier flowering phenotype under long-day conditions

Since both light and GA play crucial roles in the regulation of flowering time in Arabidopsis, the expression and function of

GASA4 are associated with both factors, and GASA4 transcripts were detected in flower buds [1,2], we then asked whether GASA4 regulated flowering under long-day conditions by investigating the flowering time of the wild type and the *gasa4* mutant. As compared to the wild type, the *gasa4* mutant showed an earlier flowering phenotype in terms of days to flowering and leaf number at bolting (Fig. 7A). To further explore how GASA4 altered flowering time, total RNA samples isolated from the floral buds of the wild type and *gasa4* mutant grown under long-day conditions underwent RT-PCR analysis for several genes known to control flowering in the long-day pathway. All 4 genes analyzed, *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), especially *GI*, *CO* and *SOC1*, were up-regulated in the *gasa4* mutant (Fig. 7B), which is consistent with the earlier flowering phenotype of the *gasa4* mutant shown in Fig. 7A. Thus, GASA4 can repress flowering by inhibiting key regulators of flowering in Arabidopsis under long-day conditions.

4. Discussion

GASA4, a member of the GASA gene family in Arabidopsis, was previously reported to be induced by GA. Here, we show that GASA4 function is associated with light-mediated inhibition of hypocotyl elongation and may modulate GA levels through controlling the expression of the genes for key enzymes in GA metabolism, as well as with control of the expression of major regulators of flowering time in Arabidopsis under long-day conditions. Therefore, GASA4 might play a crucial role to regulate hypocotyl elongation and flowering in response to the integration of light signaling and GAs.

At least 17 GASA members, forming a GASA gene family, are present in the Arabidopsis genome. GASA1–GASA4 were first reported to be induced by GA [1] and related to the tomato *GAST1*. GASA members have shown tissue-specific expression patterns, such as GASA1 mainly in siliques and flower buds, GASA2 and GASA3 in dry seeds and GASA4 in flower buds [1,2]. These expression patterns suggest that the members might play roles in the development of reproductive tissues.

Fig. 7. Analysis of flowering time and the expression of several key flowering time genes in wild type (WT) and the *gasa4* mutant. (A) Wild type and GASA4 T-DNA inserted mutant *gasa4* were grown under long-day conditions (16 h day and 8 h night). Flowering time was recorded on the basis of the days from seedlings sown in soil to appearance of the first inflorescence and the leaf number at bolting. Ten plants for each case were investigated. (B) RT-PCR analysis of the expression of flowering-time genes in the flower buds of WT and the *gasa4* mutant. UBQ, ubiquitin 10 as an endogenous loading control.

Here, we found that *GASA4* was regulated by different wavelengths of light (Fig. 1), which was further supported by multiple photoreceptors and light signaling components affecting *GASA4* transcripts under cFR light (Fig. 2A). In particular, *GASA4* transcripts were up-regulated in the *fin219* mutant, a positive regulator involved in the phyA-mediated FR signaling pathway, under all light conditions (Fig. 2B), which implies that FIN219 can repress the expression of *GASA4* in light signaling. *GASA4* expression was highly reduced in the mutants of key modulators of light signaling such as COP1 and SPA1, which can regulate phyA activity [21–23]. Intriguingly, *GASA4* transcripts were differentially expressed in the *cop1-4* and *cop1-5* alleles. Since the T-DNA-tagged *cop1-5* is null [24] and has no COP1 protein, which resulted in barely detected *GASA4* transcripts, whereas the *cop1-4* with only the N-terminal 282 amino acids caused a reduced expression of *GASA4* transcripts, implying that COP1 may positively regulate *GASA4* expression. However, COP1 has been shown to have E3 ligase activities that mediate protein degradation by 26S proteasome [21–23]. Whether COP1 regulates *GASA4* expression at the protein level remains to be elucidated. As well, the T-DNA knock-out mutant of *GASA4* displayed a long hypocotyl phenotype under both cR and cFR light (Fig. 3) and ectopic expression of *GASA4* can rescue the *gasa4* mutant to the wild type like phenotype under the same conditions (Fig. 4). Thus, *GASA4* is involved in the control of hypocotyl elongation in response to R and FR irradiation.

Furthermore, the photoreceptors *phyB* and *cry1/cry2* mutants grown under FR light showed reduced expression of the *GASA4* transcripts (Fig. 2A), which suggests that the interactions between phyA and phyB (the photoreceptor for R perception) as well as cry1 and cry2 (the photoreceptors for blue light perception) may be mainly responsible for the regulation of *GASA4* expression. Since the control of hypocotyl elongation by interactions among various photoreceptors has been documented [25–27], the synergistic action for hypocotyl growth and cotyledon unfolding is conditional. Phytochrome A in the high-irradiance response mode can positively regulate phyB signaling [28], but phyA in the very-low-fluence-response mode negatively regulates phyB signaling [29]. So, phyB in a continuous FR condition positively affects *GASA4* expression regulated by phyA (Fig. 2A). Similarly, cry1 and cry2 may work redundantly in FR to act synergistically with phyA to control *GASA4* expression (Fig. 2A).

The early steps in the GA biosynthetic pathway are controlled by single-copy genes or small gene families. Two *ent*-kaurenoic acid oxidases (*KAO1* and *KAO2*), *ent*-copalyl diphosphate synthase (*CPS*) and *ent*-kaurene synthase (*KS*) are encoded by single-copy genes in Arabidopsis [30]. By contrast, enzymes catalyzing the later reaction in this pathway are each encoded by a multiple gene family: *GA20ox* and *GA3ox* are encoded by four genes and *GA2ox* by six [30]. Regulations of the bioactive GA levels, whether under feedback or developmental regulation, are primarily determined by the enzymes in these three gene families. GA-mediated responses can be regulated through controlling specific biosynthetic genes. Recent studies provided evidence that the *KNOX* genes are

involved in modulating GA levels in the SAM region by repressing the expression of *GA20ox1* [31]. Furthermore, the levels of *GA₁* and *GA20ox* mRNA in the *KNOX*-overexpressed plants were reduced as compared with that in the wild type. In addition, the embryonic regulators LEC2 and FUSCA (*FUS3*) are involved in multiple aspects of Arabidopsis seed development by inhibiting the expression of *GA3ox2* to modulate GA levels [15]. In comparison, gene expression and enzyme activities are relatively steadier in CPS, KS, and KAOs [32]. Overexpressing either CPS or KS in Arabidopsis increases upstream gibberellin precursors but not bioactive GAs [33]. These findings are in accordance with our results, because *GASA4* controlled the expression of only the later biosynthetic enzymes *GA3ox1*, *GA20ox1* and *GA2ox2* and did not alter *KS* and *KAO1* expression (Fig. 5). These three later biosynthetic enzymes were used as the index in our study because they are the major enzymes expressed in each gene family at the Arabidopsis young seedling stage. All the three were up-regulated when *GASA4* is mutated (Fig. 5), so the *gasa4* mutant may show a greater GA flux from GA precursors to active GAs and then to inactive catabolites.

In addition, during de-etiolation, the responses of plants to the endogenous pool of *GA₁* are reduced in continuous light compared with dark-grown plants [34]. The *gasa4* mutant increased exogenous GA response under R and FR light (Fig. 6), which suggests that *GASA4* negatively regulates GA signaling under de-etiolation condition. The *gasa4* mutant may alter expression levels of the GA-responsive genes. Alternatively, the *gasa4* mutant might increase sensitivities to exogenous GA, leading to even longer hypocotyls in the presence of GA under both FR and R conditions.

GAI and RGA are two of the five DELLA members controlling GA signaling in Arabidopsis. Their regulation is primary at the protein level through the GA-stimulated 26S proteasome degradation pathway [35,36], so it is not surprising that *gasa4* mutation did not alter RNA levels of *GAI* and *RGA* (Fig. 5). The interaction between light and GA-regulated hypocotyl elongation has been extensively investigated [11,34,37]. However, the relation between light and GA in photomorphogenesis has been a long-standing controversy; in particular, is phytochrome-mediated photomorphogenesis the net result of a reduction in GA content or GA sensitivity of the stem? De-etiolation of pea involves a rapid decrease of *GA₁* level to about 25% in apical shoots upon transfer to white light and an increase in *GA₁* content in plants transferred to the dark [37]. Recently, Alabadí et al. [38] reported that GA can repress photomorphogenesis in darkness, which indicates that both the chemical and genetic reduction of endogenous GA level derepresses photomorphogenesis in part in darkness. Thus, the GA-deficient *gal-3* mutants display a much shorter hypocotyl phenotype than the wild type in darkness. In addition, some evidence also indicates that phytochromes regulate GA levels to control stem elongation and that photoperiod modulates GA content. For instance, GA levels decrease in PHYA-overexpressed tobacco plants and increase in antisense PHYB transgenic potato; long-day treatment actually regulates two steps of GA biosynthesis – *GA₅₃* to *GA₄₄* and *GA₁₉* to

GA₂₀ – leading to lower GA₁₉ and higher GA₂₀ content upon transfer from short- to long-day conditions [39,40]. The latest evidence indicates that FR-rich long days or end-of-day FR conditions can stimulate petiole elongation by regulating the expression of GA20ox biosynthetic genes through PHYB-type PHYs and not PHYA [41]. However, the cross-talk modulator linking FR light and GA responses remains unclear. Here, we showed that the *GASA4* in Arabidopsis can repress the GA biosynthetic genes *GA3ox1*, *GA20ox1* and *GA20ox2* (Fig. 5). Whether *GASA4* accordingly affects GA levels or GA signaling remains elusive. Furthermore, the *gasa4* mutant exhibits a long hypocotyls phenotype and an earlier flowering, implying that endogenous active GA levels in the mutant may be higher than that in wild type. Our preliminary data reveal that the level of active GA₄ in the *gasa4* mutant grown under cFR is almost two-fold increase compared to wild type, which supports the long hypocotyl phenotype of *gasa4* mutant observed under cFR. As well, *FIN219* is gradually decreased under dark-FR transition (unpublished data), leading to *GASA4* increase, thus reducing GA levels that result in photomorphogenic phenotype. This notion is consistent with the results shown in this study. In contrast, in the dark condition the photomorphogenic development will be repressed by a group of repressor proteins COP/DET/FUS [42]. How *GASA4* and *FIN219* are implicated in the repression of the photomorphogenic development in the dark remains to be answered. Thus, *GASA4* acts as a vital crosstalk between FR signaling and GA metabolism under the control of *FIN219* to regulate hypocotyl elongation and flowering.

GASA4 has been shown to display a tissue-specific expression pattern, with highly expressed in the meristematic regions [2]. Our result showed the *gasa4* mutant caused increased expression of flowering time genes such as *GI*, *CO*, *FT* and *SOCI*; leading to an early flowering phenotype (Fig. 7), which is consistent with previous reports that overexpression of flowering time genes resulted in early flowering phenotype [43,44]. This result also indicates that *GASA4* functions as a negative regulator of Arabidopsis flowering under long-day condition, which is further supported by the fact that the *fin219* null mutant exhibits a delay flowering phenotype under both long- and short-day conditions (our unpublished data). Since *FIN219*, a positive component in phyA-mediated FR signaling pathway negatively regulates *GASA4* expression (Fig. 2B), the *GASA4* expression increases in the *fin219* null mutant, leading to reduced expression of flowering time genes as described in the above and a delay flowering phenotype. Therefore, a new finding is that *GASA4* plays a role in light signaling and may function as a positive regulator of hypocotyl elongation of the seedlings as well as a negative regulator for the reproductive development of Arabidopsis.

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