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Effect of Gibberellin on the Biosynthesis of Tocopherols in Oilseed Rape (*Brassica napus* L.) and *Arabidopsis*

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Supporting Information

ABSTRACT: Elevating the yield and altering the composition of seed tocopherols (Toc's) are important to rapeseed breeding and production. However, little is known about the biosynthesis of Toc's in response to environmental signals. In this study, we investigated the effects of exogenous gibberellin (GA_3) and paclobutrazol (PAC) on Toc biosynthesis. We also explored the interactive effects between the two plant growth regulators (PGRs) and other factors, such as PGR treatment duration, genotype, and growing location on the total Toc yield and composition in oilseed rape seed. GA_3 significantly enhanced the production of Toc's and elevated the α-/γ-Toc ratio in a time- and genotype-dependent manner. By contrast, PAC significantly reduced Toc yield. Genotypic differences were observed in the effects of GA_3 on Toc yield and composition in the seeds. GA_3 significantly increased the Toc yield and α-/γ-Toc ratio in Zheyou-50, a genotype with a low proportion of very long chain fatty acids (VLCFAs). However, GA_3 did not significantly influence these parameters in Jiu-Er-13Xi, a genotype with a high VLCFA proportion. The increased Toc yield induced by GA_3 was mediated by the upregulation of genes (*BnPDS1* and *BnVTE1*) that catalyze the production of Toc precursors. Therefore, applying GA_3 can improve rapeseed quality by increasing Toc yield and improving Toc composition.

KEYWORDS: *Brassica, Arabidopsis, gibberellin (GA_3), paclobutrazol (PAC), tocopherol content, tocopherol composition, gene expression*

INTRODUCTION

Oilseed rape (*Brassica napus* L., AACC, $2n$ = 38) in the Brassicaceae family has a high economic importance worldwide.[1,2] Oilseed rape oil is a vital dietary source of vitamin E in China, particularly in the Yangtze River valley region.[3,4] Tocopherols (Toc's) constitute a family of vital lipid-soluble antioxidants with several health benefits.[5−8] Protection of the photosynthetic apparatus from oxygen toxicity and lipid peroxidation in plants is attributed to α-tocopherol (α-Toc) enrichment in the chloroplast membrane,[9] whereas the prevention of polyunsaturated fatty acids from oxidation in seeds is enhanced by γ-tocopherol (γ-Toc).[10] The biological activities of the vitamin E components α-Toc, β-tocopherol (β-Toc), γ-tocopherol (γ-Toc), and δ-tocopherol (δ-Toc) are 100%, 30%, 15%, and 5%, respectively.[11] Canola oil, on the average, contains a high amount of vitamin E, with ∼64% γ-Toc, ∼35% α-Toc, and <1% δ-Toc.[1,12,13]

Plant growth regulators (PGRs) play important roles in various aspects of plant growth and development, including seed maturation and the accumulation of seed storage reserves.[14] Gibberellins (GAs) constitute a large group of tetracyclic diterpenoids that regulate seed germination, leaf expansion, stem and root extension, flower induction and development, and fruit expansion.[15−18] GA signals are received and transduced via the GID1 GA receptor/DELLA repressor pathway. *Arabidopsis* has five functionally overlapping DELLA proteins,[19−22] namely, GAI, RGA, RGL1, RGL2, and RGL3, that are nuclear negative regulators in the GA signaling pathway.[20,23,24] Paclobutrazol (PAC) or (2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol interacts antagonistically with GA by inhibiting its synthesis by blocking conversion of *ent*-kaurene to *ent*-kaurenoic acid. Exogenous PAC suppresses shoot growth, leading to reduced plant height, internode elongation, and leaf area as well as to increased lodging resistance, branch number, and seed yield in oilseed rape.[25,26]

Increasing the total Toc and/or altering the Toc composition (α-/γ-Toc ratio) are important to rapeseed breeding and commercial production. Previous studies on vegetable and maize seeds revealed interactive effects between genotypes and environmental factors on seed Toc's.[27−31] However, little is known about the response of seed Toc's to exogenous PGRs. Therefore, we conducted a field experiment to investigate the effects of gibberellic acid (GA_3) and its synthesis inhibitor PAC on seed Toc's. We also analyzed the expression levels of the

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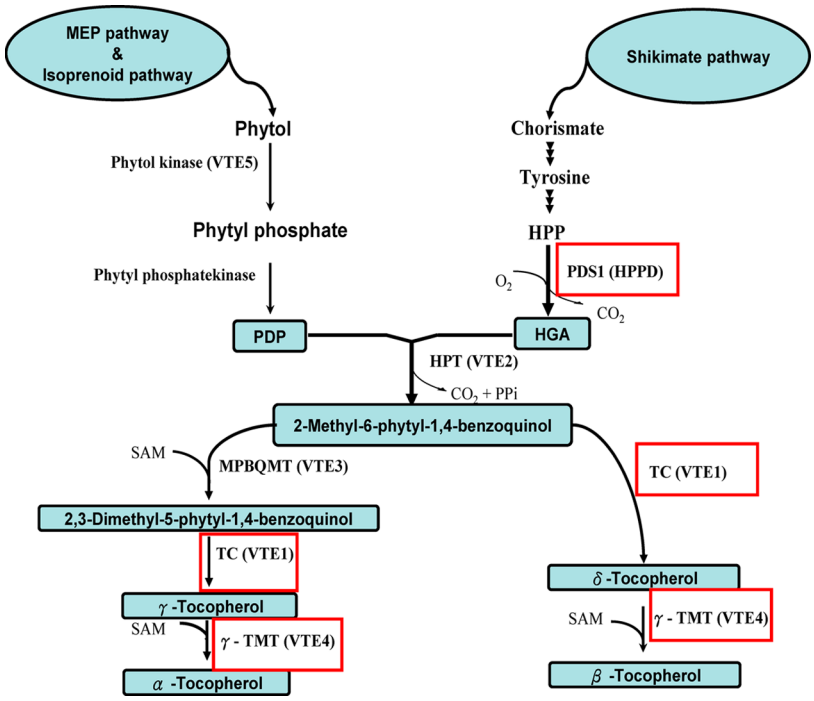

Figure 1. Tocopherol biosynthesis pathway in plants; a model from *Arabidopsis* adapted from Hussain et al.[8] HPP, *p*-hydroxyphenylpyruvate; HGA, homogentisic acid/homogentisate; PDP, phytyldiphosphate; HPPD (PDS1), HPP dioxygenase; HPT (VTE2), homogentisate phytyltransferase; MPBQMT (VTE3), 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase; TC (VTE1), tocopherol cyclase; γ-TMT (VTE4), γ-tocopherol methyltransferase. Genes highlighted and closed in red boxes were selected to study expression in response to exogenous GA_3 and/or PAC.

Table 1. Timing of PGR Applications to Rapeseed Plants (Year 2013)[a]

		Year 2013								
Location	PGRs Spray Timings	March 1	March 11	March 18	March 25	April 4	April 12	April 17	April 24	May 1
Hangzhou	S1									
	S2									
	S3									
	PGRs Spray Timings	Feb 29	March 9	March 14	March 22	March 27	April 2	April 12	April 20	April 24
Jinhua	S1									
	S2									
	S3									

[a]S1 = PGRs spray from bud formation to seed maturity; S2 = from flowering initiation to seed maturity; S3 = from flowering completion to seed maturity stage. Shaded boxes show the timings of PGRs spray.

PDS1, *VTE1*, and *VTE4* genes to monitor the synthesis of Toc precursors. *PDS1* plays a critical role in the conversion of *p*-hydroxyphenylpyruvate (HPP) to homogentisic acid (HGA). *VTE1* encodes a Toc cyclase that directly converts 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) into δ-Toc or 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DMPBQ) into γ-Toc.[32] *VTE4* is a transmethylase that catalyzes the production of α-Toc from γ-Toc and β-Toc from δ-Toc [33] (Figure 1). We also aim to understand the interactive effects between the PGRs and other factors, such as PGR treatment duration, genotype, and growing location.

MATERIALS AND METHODS

Plant Materials and Growth Conditions of *Arabidopsis*. The *Arabidopsis thaliana* ecotype Langdsberg *erecta* (*Ler*) (wild-type (WT)), the quadruple mutant (*gai-t6 rga-t2 rgl1-1 rgl2-1*) of wild-type background (*q-della/WT*), and the penta mutant (*ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1*) of *ga1-3* background (*p-della*), were used to evaluate the effects of GA_3 application on Toc's in *Arabidopsis* seeds.[24] The *q-della/WT* mutant was donated by Prof. Fu Xiangdong of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. The *p-della* mutant was provided by Prof. Jinrong Peng of the College of Life Science, Zhejiang University. All plants were grown under the same conditions (16 h light/8 h darkness) and harvested almost simultaneously. Seeds were stored in a dry cabinet (15% moisture level) until used.

Plant Material and Growth Conditions of Rapeseed. Field experiments were conducted during the winter canola growing season (2012−2013) at Hangzhou (location 1, L1) and Jinhua (L2). Both cities are in Eastern China, and are ∼250 km apart. Zheyou-50 (genotype 1, G1), a canola type with a high seed oil content of ∼50%, and Jiu-Er-13Xi (G2), a high erucic acid type with a low seed oil content of only 40%, were used. Pesticides and herbicides were applied following local cultivation management. One-month-old seedlings of uniform height were selected and transplanted from the nursery to the respective fields. The soil type of the fields at both locations was loamy clay. Before transplanting from nursery to field, urea at a rate of 150 kg ha^{-1} was applied as a basal dose fertilizer. At the end of January 2013, a second dose of urea was applied as topdressing at a rate of 75 kg ha^{-1}.

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The fields at both sites were not irrigated because rainfall was sufficient in Hangzhou (738.4 mm) and Jinhua (868.5 mm) during the rapeseed growth season. Plants were spaced 0.35 m apart between rows and 0.30 m within rows. The field experiment at both locations was performed following a randomized complete block design with three replications (blocks).

Application of PGRs to the Plants. GA_3 (plant growth regulator 1, P1) at 150 μmol L^{-1} and PAC (P3) at 150 mg L^{-1} were applied to rapeseed plants. Both PGRs were purchased from Sangon Company (Shanghai, China). Water was used as a control (P2). All PGRs were sprayed at 7−9 day intervals (no spraying on raining days) during one of the following stages: S1 (from bud formation to seed maturity), S2 (from flowering initiation to seed maturity), or S3 (from flowering completion to seed maturity). Timing and duration of PGR applications are listed in Table 1. S1, S2, and S3 included 9, 7, and 5 PGR spray events, respectively. Colored threads were tied to the main branch of rapeseed plants to tag the flowering initiation dates.

Analysis of Tocopherols. Toc's were extracted and analyzed as previously described with slight modifications.[2,34] In brief, ~75 mg of seeds were weighed and transferred into a 2 mL skirted screw-cap microtube containing 1.3 mL of *n*-hexane. The seeds were pulverized with a bead mill homogenizer (Bead Ruptor-24, Omni, Kennesaw, Georgia, U.S.A.) at 8 m s^{-1} for 30 s. The samples were stored in the dark for 15 h to extract Toc's. A 20 μL aliquot of the supernatant was analyzed on a normal-phase high-performance liquid chromatography (HPLC) apparatus (model 600, Waters, Milford, Massachusetts, U.S.A.) equipped with a Zorbax Rx-SIL column (4.6 mm × 250 mm × 5 μm; Agilent, Englewood, Colorado, U.S.A.) and a fluorescence detector (λ_{ex} = 295 nm; λ_{em} = 330 nm). The mobile phase of hexane/*tert*-butyl methyl ether (94:6, v/v) was delivered isocratically at 1 mL min^{-1}. Calibration curves of pure standards were used to quantify the Toc isoforms. References for α- (purity 99.90%, cat. no. 47783), β- (purity 99.00%, cat. no. 46401), γ- (purity 99.10%, cat. no. 47785), and δ-Toc (purity 95.50%, cat. no. 47784) were analytical standards and were obtained from Supelco (Bellefonte, Pennsylvania, U.S.A.). Liquid chromatography-grade hexane (Sigma−Aldrich, Shanghai, China) was used as the solvent. The peaks of the Toc standards (α-, β, γ-, and δ-Toc) were distinguished by their retention times. The standard curve for the quantification of the experimental samples was calibrated according to the corresponding peaks of individual Toc derivatives. The contents of Toc's were expressed in mg per kg of seed, and the total amount of Toc's was calculated as the sum of α- and γ-Toc.

Analysis of Gene Expression by Reverse Transcription Quantitative Polymerase Chain Reaction. Flowers were tagged with different colored threads to indicate days after pollination (DAP). Only seeds from the siliques on main inflorescences were harvested for RNA extraction. The RNA samples were isolated with the Invisorb Spin Plant RNA Mini Kit (Invitek, Berlin, Germany) following the manufacturer's instructions. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) amplification was performed as previously described.[35] All primer pairs used in the RT-qPCR analyses of *Arabidopsis* and rapeseed are listed in Supporting Information, Tables S1 and S2, respectively.

Statistical Analysis. For the analysis of gene expression, baseline and threshold cycles (CT value) were determined automatically by using Bio-Rad iQ Software (version 3.0; Hercules, California, U.S.A.). Relative amounts of RNA transcripts were calculated as previously described.[36] Field experimental data were classified with Win-Excel and subjected to ANOVA with the MStatc statistical package (version 7.05). Treatment means were compared for least significant differences at $P \leq 0.05$ or $P \leq 0.01$ (Table 2).

RESULTS

Response of Total Toc and Its Components to Exogenous PGRs and the Interactions between PGRs and Other Factors. ANOVA reveals that genotype (G), PGR (P), and number of PGR applications (S) significantly affected the total Toc and its composition. Growth location (L) only significantly affected α-Toc and the α-/γ-Toc ratio. Most of the interactions among these factors significantly affected the variation in total Toc and its components. However, the interaction between L × S and L × P × G exerted no significant effects on the α-Toc content, and the interaction between P × G exhibited no significant effect on the γ-Toc content (Table 2).

Table 2. ANOVA Showing the Effects of Location, PGR Type, Duration of PGR Treatment, and Genotype on Seed Tocopherol in Rapeseed[a]

factor	α-Toc	γ-Toc	total Toc	α-/γ-Toc ratio
location (L)	**	ns	ns	**
PGRs (P)	**	**	**	**
L × P	**	**	**	**
duration of PGR effect (or total spray times) (S)	**	**	**	**
L × S	ns	**	*	**
P × S	**	**	**	**
P × L × S	**	**	**	*
genotype (G)	**	**	**	**
L × G	**	**	**	*
P × G	**	ns	**	**
P × G × L	ns	**	*	**
S × G	**	**	**	**
L × S × G	**	*	*	**
P × S × G	**	**	**	**
L × P × S × G	**	**	**	**
error MS	229.411	341.067	974.096	0.001
CV (%)	7.10	5.93	6.10	4.07

[a]P, plant growth regulators (PGRs); G, genotype; L, location; S, duration of PGR effect (or total spray times); details are available in the Materials and Methods section. *Indicates significance at $P \leq 0.05$; **indicates significance at $P \leq 0.01$; ns = nonsignificant.

Effect of PGRs on Toc Content and Composition. Compared with the water control, GA_3 significantly increased the total Toc, α-Toc, and γ-Toc contents as well as the α-/γ-Toc ratio ($P \leq 0.01$). Meanwhile, PAC significantly reduced the total Toc but did not significantly influence the α-Toc and γ-Toc contents or the α-/γ-Toc ratio (Table 3).

Effect of the Interaction between PGR and Genotype on Seed Toc's. Highly significant variations in the seed total Toc, α-Toc, and α-/γ-Toc ratio ($P \leq 0.01$) were observed in response to the interaction between PGR and genotype (G), whereas no significant differences were observed in the seed γ-Toc (Table 3). Zheyou-50 plants treated with GA_3 produced higher total Toc (667.22 mg kg^{-1} seeds) and α-Toc (282.54 mg kg^{-1} seeds) than those treated with PAC or water. The lowest total Toc (380.74 mg kg^{-1} seeds) and α-Toc contents (160.00 mg kg^{-1} seeds) were observed in the Jiu-Er-13Xi plants untreated with PGR (Table 3). The Jiu-Er-13Xi plants treated with GA_3 had the highest α-/γ-Toc ratio (0.80), whereas the Zheyou-50 plants treated with PAC had the lowest α-/γ-Toc ratio (0.66) (Table 3).

Effect of the Interactions between PGR and Location on Seed Toc's. The interaction between PGR and location caused highly significant variations in the total Toc, α-Toc, and γ-Toc contents as well as in the α-/γ-Toc ratio ($P \leq 0.01$) (Table 2). The highest total Toc (538.35 mg kg^{-1} seed) and γ-Toc (315.32 mg kg^{-1} seed) contents were obtained after applying GA_3 at the Hangzhou site (L1). Conversely, the highest α-Toc content (238.38 mg kg^{-1} seed) and α-/γ-Toc ratio (0.82) were

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Table 3. Responses of Seed Toc Yield and Composition to PGR and the Interactive Effects between PGR and Location, Genotype, and Duration of PGR Effect (or Total Spray Times) In Rapeseed[a]

	Factors	Total-Toc	α-Toc	γ-Toc	α-/γ-Toc ratio	Factors	Total-Toc	α-Toc	γ-Toc	α-/γ-Toc ratio
PGRs (P)	P1	536.03 a	230.71 a	305.32 a	0.77 a	P1S1	625.50 a	263.62 a	361.88 a	0.74 bc
	P2	507.60 b	207.39 b	300.21 ab	0.70 b	P1S2	461.47 d	199.07 de	262.41 f	0.77 b
	P3	491.62 c	201.62 b	289.99 b	0.71 b	P1S3	521.12 c	229.44 b	291.68 de	0.81 a
	Sig. Level	**	**	**	**	P2S1	527.36 bc	213.22 b-d	314.14 bc	0.68 e
P x G interaction	P1G1	667.22 a	282.54 a	384.69	0.74 bc	P2S2	476.78 d	197.72 de	279.06 ef	0.72 cd
	P1G2	404.83 d	178.87 d	225.96	0.80 a	P2S3	518.68 c	211.24 cd	307.44 cd	0.70 de
	P2G1	634.47 b	254.78 b	379.69	0.67 d	P3S1	556.99 b	225.51 bc	331.49 b	0.70 de
	P2G2	380.74 d	160.00 e	220.74	0.73 c	P3S2	459.42 d	191.91 e	267.50 f	0.74 bc
	P3G1	597.08 c	236.64 c	360.43	0.66 d	P3S3	458.44 d	187.45 e	270.99 f	0.69 de
	P3G2	386.16 d	166.60 de	219.56	0.76 b	Sig. Level	**	**	**	**
	Sig. Level	**	**	ns	**					
P x L interaction	P1L1	538.35 a	223.03 b	315.32 a	0.73 b					
	P1L2	533.71 a	238.38 a	295.33 bc	0.82 a					
	P2L1	515.02 ab	207.89 c	307.14 ab	0.68 c					
	P2L2	500.18 ab	206.90 c	293.29 bc	0.72 b					
	P3L1	471.44 b	188.59 d	282.85 c	0.69 c					
	P3L2	511.80 ab	214.66 bc	297.14bc	0.73 b					
	Sig. Level	**	**	**	**					

[a]Sig. level shows the level of significance. The values of Toc content and composition are the means of three replications. P, plant growth regulators (PGRs); G, genotype; L, location; S, duration of PGR effect (or total spray times); details are available in the Materials and Methods section. Different letters to the figures in the same column following each category indicate significant differences at $P \leq 0.01$ (**) or 0.05 (*) as per ANOVA results.

Table 4. Responses of Seed Toc Yield and Composition to the Interactive Effects of PGR and Location, Genotype, and Duration of PGR Effect (Or Total Spray Times) In Rapeseed[a]

	Factors	Total-Toc	α-Toc	γ-Toc	α-/γ-Toc ratio	Factors	Total-Toc	α-Toc	γ-Toc	α-/γ-Toc ratio
P x L x S interaction	P1L1S1	673.9 a	269.79 a	404.12 a	0.69 f-h	P1SIG1	832.14 a	345.46 a	486.67 a	0.72 cd
	P1L1S2	452.15 ij	192.73 d	259.42 h	0.75 c	P1SIG2	418.86 e	181.77 e	237.09 fg	0.77 bc
	P1L1S3	489.01f-j	206.57 b-d	282.43 e-h	0.74 cd	P1S2G1	494.32 d	212.76 cd	281.56 e	0.76 bc
	P1L2S1	577.09 bc	257.45 a	319.65 cd	0.80 b	P1S2G2	428.63 e	185.37 e	243.23 f	0.78 b
	P1L2S2	470.80 g-j	205.40 b-d	265.40 h	0.79 b	P1S3G1	675.23 b	289.40 b	385.83 c	0.75 bc
	P1L2S3	553.24 c-e	252.31 a	300.93 d-f	0.88 a	P1S3G2	367.01 fg	169.48 e-g	197.53 i	0.87 a
	P2L1S1	555.90 cd	223.12 b	332.77 bc	0.67 g-i	P2SIG1	668.09 b	270.62 b	397.47 bc	0.68 d-f
	P2L1S2	479.87 g-j	197.78 cd	282.08 e-h	0.71 d-f	P2SIG2	386.63 e-g	155.82 f-h	230.81 f-h	0.69 d-f
	P2L1S3	509.31 d-g	202.75 b-d	306.56 c-e	0.66 hi	P2S2G1	558.18 c	222.82 cd	335.36 d	0.67 e-g
	P2L2S1	498.82 f-i	203.32 b-d	295.51 d-g	0.70 e-g	P2S2G2	395.38 ef	172.62 e-g	222.77 f-i	0.78 b
	P2L2S2	473.69 g-j	197.65 cd	276.04 f-h	0.73 c-e	P2S3G1	677.14 b	270.90 b	406.24 bc	0.67 e-g
	P2L2S3	528.04 d-f	219.72 bc	308.33 c-e	0.73 c-e	P2S3G2	360.21 fg	151.57 gh	208.64 hi	0.73 b-d
	P3L1S1	506.31 e-h	192.17 d	314.15 cd	0.64 i	P3SIG1	688.31 b	266.66 b	421.65 b	0.63 g
	P3L1S2	449.73 j	188.46 d	261.28 h	0.74 cd	P3SIG2	425.68 e	184.35 e	241.33 f	0.77 bc
	P3L1S3	458.27 ij	185.14 d	273.13 f-h	0.68 f-h	P3S2G1	532.04 cd	208.58 d	323.46 d	0.65 fg
	P3L2S1	607.67 b	258.84 a	348.83 b	0.75 c	P3S2G2	386.80 e-g	175.25 ef	211.55 g-i	0.83 a
	P3L2S2	469.10 g-j	195.37 d	273.73 f-h	0.74 c-e	P3S3G1	570.89 c	234.70 c	336.19 d	0.70 de
	P3L2S3	458.62 h-j	189.76 d	268.86 gh	0.70 e-g	P3S3G2	345.99 g	140.20 h	205.79 hi	0.68 d-f
	Sig. Level	**	**	**	*	Sig. Level	**	**	**	**
P x G x L interaction	P1G1L1	635.78 bc	259.65	376.13 ab	0.72 c-e	P2G1L2	654.71 b	263.36	391.35 a	0.67 f
	P1G2L1	440.92 e	186.40	254.51 d	0.73 b-d	P2G2L2	345.66 h	150.43	195.23 f	0.77 b
	P1G1L2	698.67 a	305.42	393.24 a	0.77 b	P3G1L1	561.59 d	213.28	348.31 c	0.62 g
	P1G2L2	368.75 gh	171.34	197.41 f	0.87 a	P3G2L1	381.29 g	163.89	217.39 ef	0.76 bc
	P2G1L1	614.23 c	246.2	368.03 bc	0.67 f	P3G1L2	632.57 bc	260.01	372.56 ab	0.70 d-f
	P2G2L1	415.82 ef	169.58	246.25 d	0.69 ef	P3G2L2	391.03 fg	169.31	221.72 e	0.76 b
	Sig. Level	*	ns	**	**	Sig. Level	*	ns	**	**

[a]Sig. level shows the level of significance. The values of Toc yield and composition are the means of three replications. P, plant growth regulators (PGRs); G, genotype; L, location; S, duration of PGR effect (or total spray times); details are available in the Materials and Methods section. Different letters to the figures in the same column following each category indicate significant differences at $P \leq 0.01$ (**) or 0.05 (*) as per ANOVA results.

achieved after applying GA_3 at the Jinhua site (L2). Application of PAC at the Hangzhou site (L1) produced the lowest total Toc, α-Toc, and γ-Toc contents as well as the lowest α-/γ-Toc ratio (Table 3).

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Table 5. Responses of Seed Toc Yield and Composition to the Four Factors Interactive Effects of PGR and Location, Genotype, and Duration of PGR Effect (or Total Spray Times) In Rapeseed[a]

	Factors	Total-Toc	α-Toc	γ-Toc	α-/γ-Toc ratio	Factors	Total-Toc	α-Toc	γ-Toc	α-/γ-Toc ratio
P x G x L x S interaction	P1G1L1S1	875.01 a	334.73 a	540.28 a	0.62 n	P2G2L1S1	448.62 j-l	174.44 k-o	274.20 g	0.64 mn
	P1G1L1S2	453.52 jk	205.73 g-k	247.79 gh	0.83 bc	P2G2L1S2	415.49 j-n	178.89 j-n	236.60 g-j	0.75 e-j
	P1G1L1S3	578.82 gh	238.50 d-g	340.32 f	0.70 h-m	P2G2L1S3	383.35 l-q	155.39 m-p	227.96 h-k	0.68 k-n
	P1G1L2S1	789.25 b	356.19 a	433.06 bc	0.82 b-e	P2G2L2S1	324.64 q	137.20 p	187.44 lm	0.73 f-k
	P1G1L2S2	535.11 hi	219.79 e-i	315.33 f	0.70 i-m	P2G2L2S2	375.27 m-q	166.34 l-p	208.93 h-m	0.80 c-f
	P1G1L2S3	771.63 bc	340.30 a	431.34 bc	0.79 c-g	P2G2L2S3	337.08 o-q	147.75 n-p	189.33 k-m	0.78 c-g
	P1G2L1S1	472.79 ij	204.84 h-k	267.95 g	0.76 d-i	P3G1L1S1	596.02 f-h	205.35 h-k	390.66 de	0.52 o
	P1G2L1S2	450.78 j-l	179.73 j-n	271.05 g	0.67 k-n	P3G1L1S2	529.83 hi	209.60 g-j	320.23 f	0.65 mn
	P1G2L1S3	399.19 k-p	174.64 k-o	224.54 h-l	0.78 c-g	P3G1L1S3	558.92 h	224.88 e-h	334.03 f	0.67 k-n
	P1G2L2S1	364.93 n-q	158.70 l-p	206.23 i-m	0.77 c-h	P3G1L2S1	780.60 bc	327.96 a	452.64 b	0.73 f-l
	P1G2L2S2	406.48 j-n	191.01 i-l	215.47 h-l	0.89 b	P3G1L2S2	534.24 hi	207.55 g-j	326.69 f	0.64 mn
	P1G2L2S3	334.84 pq	164.32 l-p	170.52 m	0.96 a	P3G1L2S3	582.86 gh	244.51 c-f	338.35 f	0.72 g-l
	P2G1L1S1	663.17 d-f	271.80 bc	391.37 de	0.70 i-m	P3G2L1S1	416.61 j-n	178.98 j-n	237.63 g-j	0.76 e-i
	P2G1L1S2	544.24 h	216.68 f-i	327.56 f	0.66 l-n	P3G2L1S2	369.64 m-q	167.31 l-p	202.32 j-m	0.83 b-d
	P2G1L1S3	635.27 e-g	250.11 c-e	385.16 e	0.65 mn	P3G2L1S3	357.62 n-q	145.39 op	212.23 h-l	0.69 j-n
	P2G1L2S1	673.00 de	269.43 b-d	403.57 c-e	0.67 k-n	P3G2L2S1	434.75 j-m	189.72 i-l	245.03 g-i	0.77 c-g
	P2G1L2S2	572.11 gh	228.96 e-h	343.15 f	0.67 k-n	P3G2L2S2	403.97 k-o	183.19 j-m	220.77 h-l	0.83 bc
	P2G1L2S3	719.01 cd	291.70 b	427.32 b-d	0.69 j-n	P3G2L2S3	334.37 pq	135.00 p	199.36 j-m	0.68 k-n
	Sig. Level	**	**	**	**	Sig. Level	**	**	**	**

[a]Sig. level shows the level of significance. The values of Toc yield and composition are the means of three replications. P, plant growth regulators (PGRs); G, genotype; L, location; S, duration of PGR effect (or total spray times); details are available in the Materials and Methods section. Different letters to the figures in the same column following each category indicate significant differences at $P \leq 0.01$ (**) or 0.05 (*) as per ANOVA results.

Effect of the Interaction between PGR and PGR Treatment Duration on Seed Toc's. The interaction between PGR and PGR treatment duration led to significant variations in the total Toc, α-Toc, and γ-Toc contents and in the α-/γ-Toc ratio ($P \leq 0.01$) (Table 2). The application of GA_3 (P1) from the green floral bud stage to seed maturity (S1) resulted in the highest total Toc (625.50 mg kg^{-1} seed), α-Toc (263.62 mg kg^{-1} seed), and γ-Toc (361.88 mg kg^{-1} seed) contents, whereas the application of GA_3 from flowering completion to seed maturity (S3) produced the highest α-/γ-Toc ratio (0.81). The application of PAC from flowering completion to seed maturity caused the lowest total Toc (458.44 mg kg^{-1} seed), α-Toc (187.45 mg kg^{-1} seed), and γ-Toc (270.99 mg kg^{-1} seed) contents and the α-/γ-Toc ratio (0.69) made statistic sense (Table 3).

Effect of the Interaction among PGR, Location, and PGR Treatment Duration on Seed Toc's. The interaction among PGR, location, and PGR treatment duration caused significant variations in the total Toc, α-Toc, and γ-Toc contents ($P \leq 0.01$) and a significant variation in the α-/γ-Toc ratio ($P \leq 0.05$) (Table 2). Among the 18 treatment combinations, the application of GA_3 from the green floral bud stage to seed maturity at the Hangzhou site (P1L1S1) resulted in the highest total Toc (673.90 mg kg^{-1} seed), α-Toc (269.79 mg kg^{-1} seed), and γ-Toc (404.12 mg kg^{-1} seed) contents. Conversely, the application of PAC from flowering initiation to seed maturity at the Hangzhou site (P3L1S2) led to the lowest total Toc (449.73 mg kg^{-1} seed) and γ-Toc (261.28 mg kg^{-1} seed) contents. The lowest α-Toc content (185.14 mg kg^{-1} seed) appeared following the P3L1S3 treatment combination. The highest α-/γ-Toc (0.88) was achieved by the P1L2S3 treatment combination, whereas the lowest α-/γ-Toc value (0.64) was obtained by the P3L1S1 treatment combination (Table 4).

Effect of the Interaction among PGR, PGR Treatment Duration, And Genotype on Seed Toc's. The interaction among PGR, genotype, and PGR treatment duration (PGS) gave rise to significant variations in the total Toc, α-Toc, and γ-Toc contents and the α-/γ-Toc ratio ($P \leq 0.01$) (Table 2). Among the 18 treatment combinations, GA_3 treatment from flowering initiation to seed maturity (P1S1G1) produced the highest total Toc (832.14 mg kg^{-1} seed), α-Toc (345.46 mg kg^{-1} seed), and γ-Toc (486.67 mg kg^{-1} seed) contents in the Zheyou-50 plants. The Jiu-Er-13Xi plants treated with PAC from flowering completion to seed maturity (P3S3G2) had the lowest total Toc (345.99 mg kg^{-1} seed), α-Toc (140.20 mg kg^{-1} seed), and γ-Toc (205.79 mg kg^{-1} seed) contents. The highest (0.83) and lowest (0.63) α-/γ-Toc ratios were produced by the P3S2G2 and P3S1G1 treatment combinations, respectively (Table 4).

Effect of the Interaction among PGR, Genotype, And Location on Seed Toc's. The interaction among the PGR, genotype, and location (PGL) caused a significant variation in the total Toc yield ($P \leq 0.05$) as well as significant variations in the γ-Toc content and α-/γ-Toc ratio ($P \leq 0.01$). However, the interaction did not produce a significant variation in the α-Toc content (Table 2). Among the 12 treatment combinations, GA_3 treatment at the Jinhua site (P1G1L2) produced the highest total Toc (698.67 mg kg^{-1} seed) and γ-Toc (393.24 mg kg^{-1} seed) yields in the Zheyou-50 plants. Conversely, the Jiu-Er-13Xi plants treated with PAC at Hangzhou (P3G2L1) resulted in the lowest total Toc (381.29 mg kg^{-1} seed) and γ-Toc (217.39 mg kg^{-1} seed) yields. The highest (0.87) and lowest (0.62) values of α-/γ-Toc were caused by the P1G2L2 and P3G1L1 treatment combinations, respectively (Table 4).

Effect of the Interactions among PGR, Location, PGR Treatment Duration, And Genotype on Seed Toc's. The interaction among all the four factors significantly influenced amount of Toc and composition ($P \leq 0.01$, Table 2). Among the 36 treatment combinations, GA_3 treatment from bud formation to seed maturity at the Hangzhou site (P1G1L1S1) produced the highest total Toc (875.01 mg kg^{-1} seed), α-Toc (334.73 mg kg^{-1} seed), and γ-Toc (540.28 mg kg^{-1} seed)

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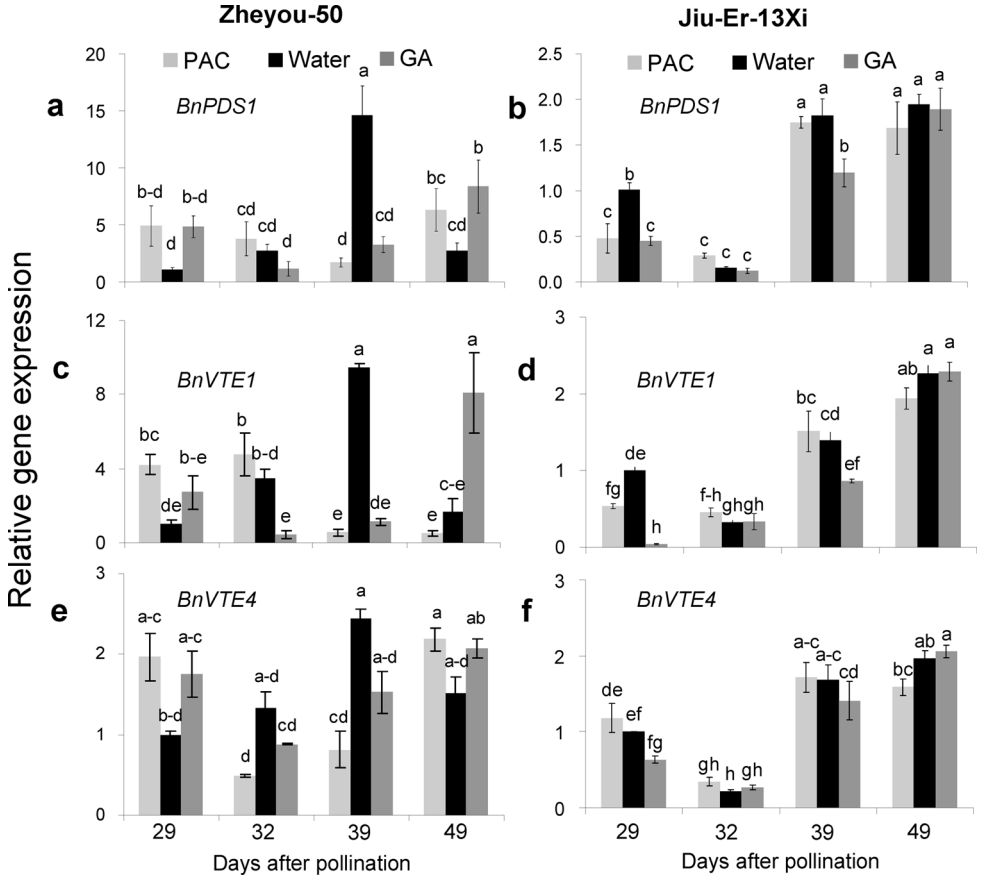

Figure 2. Relative transcription levels of tocopherol biosynthesis genes in GA_3, PAC, and water treated (control) plants of oilseed rape genotypes treated for different durations of GA_3 (or total spray times). Water, untreated/control plants; PAC, paclobutrazol. Bars on graphs show ±standard error.

contents in the Zheyou-50 plants. The Jiu-Er-13Xi plants untreated with PGR from flowering completion to seed maturity at the Jinhua site (P2G2L2S1) produced the lowest total Toc (324.64 mg kg^{-1} seed), and α-Toc (137.20 mg kg^{-1} seed) that was statistically at par to α-Toc content obtained with P3G2L2S3 treatment combination. The lowest γ-Toc (170.52 mg kg^{-1} seed) contents were produced by P1G2L2S3 treatment combination. The highest (0.96) and lowest (0.52) α-/γ-Toc ratios were obtained from the P1G2L2S3 and P3G1L1S1 treatment combinations, respectively (Table 5).

Transcriptional Changes in the Genes Regulating Toc Biosynthesis in Response to PGR Application. We investigated the effects of exogenous GA_3 and PAC on the expression levels of *BnPDS1*, *BnVTE1*, and *BnVTE4*, which are important in the Toc biosynthetic pathway (Figures 1 and 2). The RNA samples were harvested at 29, 32, 39, and 49 DAP, 7, 3, 4, and 1 day(s) after the sixth, seven, eighth, and ninth PGR applications, respectively. The result of RT-qPCR based on the RNA samples collected at 49 DAP, 1 day after the ninth application, indicated that GA_3 significantly upregulated the expression of *BnPDS1* and *BnVTE1* in Zheyou-50 (Figure 2a and c). No significant differences in gene expression were observed between the plants treated with GA_3 and water at 49 DAP in Jiu-Er-13Xi (Figure 2b, d, and f). The RT-qPCR results based on the RNAs collected at other time points (29, 32, and 39 DAP) from both genotypes did not show that GA_3 significantly upregulated the expression levels of *BnPDS1*, *BnVTE1*, and *BnVTE4*. Moreover, at 39 DAP striking inhibitory responses were observed in the expression of all three genes to PGRs in Zheyou-50 and to GA_3 in Jiu-Er-13Xi (Figure 2a–f).

Response of Toc Synthesis to GA Enhancement in *Arabidopsis*. To confirm whether or not GA enhancement can increase *PDS1*, *VTE1*, and *VTE4* expression and seed Toc content, we compared the expression levels of *VTE1*, *VTE4*, and *PDS1* between the WT (L*er*) and *q-della/WT* mutant (Figure 3d–f) as well as the seed Toc yield among the WT (L*er*), *q-della*, *p-della*, and wild-type plants treated with GA_3 (WT + GA_3) in *Arabidopsis* (Figure 3a–c).

The enhanced GA signaling in the *q-della/WT* mutant induced the upregulation of *VTE1*, *VTE4*, and *PDS1* at 4, 8, 12, and 16 DAP (Figure 3d–f). The expression levels of all three genes peaked at 12 DAP. *PDS1* expression showed the greatest difference between WT and the mutant. The transcript level of *PDS1* was 2-fold higher in the *q-della/WT* mutant than in the WT (Figure 3d).

The *q-della/WT* mutant, *p-della* mutant, and WT + GA_3 plants had considerably higher seed Toc yields than the WT plants (Figures 3a). Each 1 kg of *q-della/WT*, *p-della*, and WT + GA_3 seeds contained 412, 413, and 402 mg of Toc's, respectively, which were 11.9%, 12.2%, and 9.2% higher than the Toc in WT. The differences in the total Toc content between the genotypes were derived from the differences in γ-Toc content. No significant differences in seed α-Toc content were observed among the WT, *q-della/WT*, *p-della*, and WT + GA_3 plants, but the γ-Toc content was significantly higher in the *q-della*, *p-della*, and WT + GA_3 plants than in the WT plants (Figure 3a–c).

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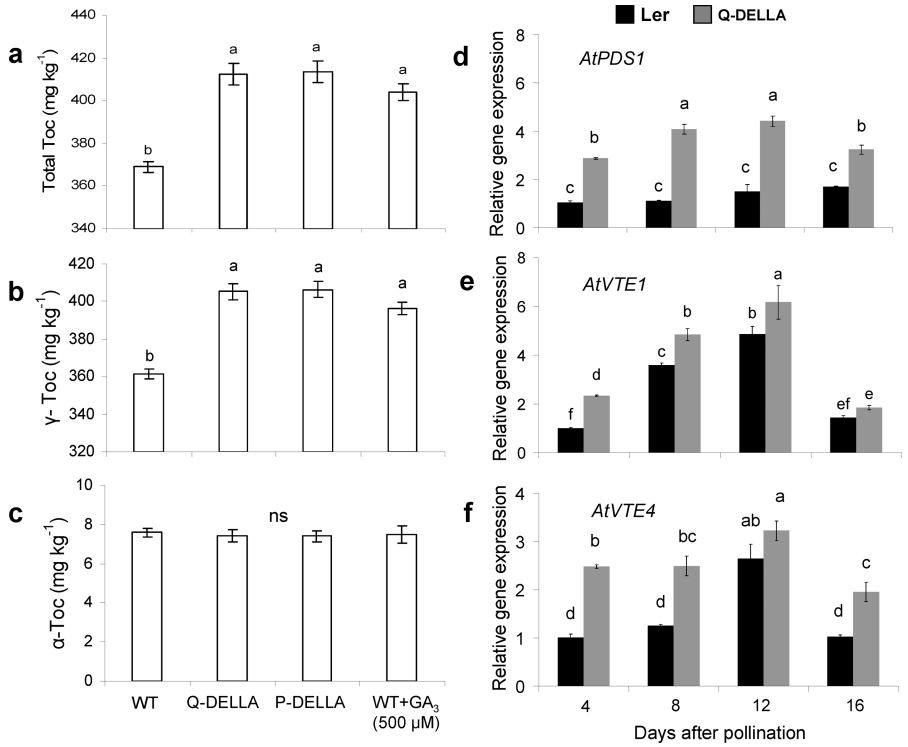

Figure 3. Comparison of α-Toc (a), γ-Toc (b), and total Toc (c) content among *Arabidopsis thaliana* WT, *q-della/WT*, *p-della* mutants and WT + GA_3 plants. Letters on the bars shows the comparison among treatment being significant at $P \leq 0.01$; ns = nonsignificant. Comparison of relative transcription levels of tocopherol biosynthesis genes (*AtVTE1*, *AtVTE4*, and *AtPDS1*) among *Arabidopsis* ecotype Langdsberg *erecta* [WT(*Ler*)] and *q-della/WT* mutant are shown in (d), (e), and (f), respectively, at different seed developmental stages. Bars on graphs show ±standard error.

DISCUSSION

GA signaling regulates a number of genes that are important for plant development processes, including seed germination, stem elongation, trichome, flower and fruit formation, and seed maturation.[15−18,37,38] We have reported recently that GA signaling promotes the expression of a group of GDSL-type genes that hydrolyze lipids.[24] We also observed that GA affects total Toc yield and the major Toc components in *Arabidopsis*. We postulated that a similar mechanism might exist in rapeseed, a major source of edible oil worldwide. Given that PAC is an inhibitor of GA synthesis and functionally antagonistic to GA,[39,40] we assumed that exogenous GA_3 and PAC may reversely affect seed Toc's in rapeseed. To test this hypothesis, we designed a field experiment and analyzed the PGR effects as well as any interactive effects between the PGR and factors such as genotype, PGR treatment duration, and planting location. Regardless of other factors, GA_3 increased total Toc yield 5.6% compared with the control. By contrast, the GA inhibitor PAC reduced the total Toc yield 3.3% compared with the control. Although the α-Toc and γ-Toc contents were both increased by exogenous GA_3, the increased α-Toc content (11.24%) was much higher than that for γ-Toc content (1.7%). Consequently, the value of α-/γ-Toc was significantly increased following GA_3 treatment (Table 3). In general, α-Toc is the predominant form of Toc in photosynthetic tissues, whereas γ-Toc is predominant in most plant seeds. A high α-/γ-Toc ratio is desired by rapeseed breeders because α-Toc has the highest vitamin E activity (100%), whereas γ-Toc has merely 8−19% relative activity.[41,42]

PGR treatment duration affected Toc production. The seed Toc yield increased as the PGR treatment duration was prolonged. The longest duration (S1) of GA_3 application (P1S1) caused the highest total Toc yield (625.5 mg kg^{-1} seed), whereas the shortest PAC duration (P3S3) led to the lowest total Toc yield (458.44 mg kg^{-1} seed) (Table 3, section P × S interaction). GA_3 signaling affects various processes of plant growth.[25,26] In the present study, early application of GA_3 (S1) caused undesirable changes in various agronomic traits, including increased plant height, reduced numbers of branches and pods per plant, and reduced seed yield (data not shown). However, late application of GA_3 (P1S3) did not significantly influence Toc yield compared with the control (P2S3). This dilemma can be overcome under some circumstances through particular genotype and location combinations. For example, the effect of GA_3 in elevating Toc yield was significantly greater than that for the control (P2G2L1S3) when GA_3 was applied during S3 to Jiu-Er-13Xi plants at the Hangzhou site (P1G2L1S3) (Table 5, section P × G × L × S interaction).

Genotype and PGR interaction significantly influenced the total Toc and Toc composition of the seeds. In general, the application of GA_3 to Zheyou-50 plants (P1G1) significantly increased Toc yield compared with the control (P2G1). However, the application of GA_3 to Jiu-Er-13Xi plants (P1G2 combination) did not significantly increase the total Toc yield as compared with the control (P2G2 combination) (Table 3, section P × G interaction). These results indicate the presence of genotypic differences in the response to exogenous GA_3. Zheyou-50 is a canola-type winter cultivar with a low content of erucic acid (<1%) but a high proportion of C_{18} fatty acids in seeds. By contrast, Jiu-Er-13Xi is a traditional Chinese "double high" cultivar with a high content of erucic acid (>40%) in seeds. Erucic acid (C22:1) is a very long chain fatty acid (VLCFA). In our previous study, we concluded that the amount of Toc, in particular α-Toc, significantly and negatively

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correlates with VLCFA in seeds.[2] Given such a correlation, we speculate that the increase in the Toc content of Jiu-Er-13Xi seeds as promoted by GA signaling enhancement is considerably limited.

The mechanism by which GA increases seed Toc yield can be partially explained by analyzing the transcriptional levels of the genes that are important to the Toc biosynthetic pathway (Figure 1). At the first day after the ninth application, the expression levels of *BnPDS1* and *BnVTE1* were 3-fold and 4-fold upregulated, respectively, by exogenous GA_3, which may account for the increased total Toc yield. *PDS1* is responsible for the synthesis of HGA, an upstream precursor of Toc, from HPP, whereas *VTE1* participates in the synthesis of γ-Toc from DMPBQ.[43−48] However, the upregulation of these two genes was observed only in Zheyou-50, which coincided with the finding that the Zheyou-50 plants treated with GA_3 exhibited increased total Toc (Table 3, section P × G interaction). Moreover, the genes were only upregulated at day one after the ninth application of GA_3 (Figure 2). The upregulation of genes in response to PGRs was not detected by RT-qPCR at other time points (29, 32, and 39 DAP). This might be due to (i) the long interval between PGR applications in terms of DAP and (ii) the delayed harvesting of seed samples for RNA extraction after PGR applications. To demonstrate the effect of endogenous GA_3 enhancement, we used the *Arabidopsis q-della/WT* mutant, in which most DELLA genes have lost their function and GA signaling is genetically enhanced. Under the given conditions, *AtPDS1* and *AtVTE4* were upregulated at all time points, whereas *AtVTE1* was upregulated at all time points except at 16 DAP. The upregulation of these genes increased the total Toc and γ-Toc contents but not the α-Toc content in the *Arabidopsis* mutants and WT + GA_3 plants (Figure 3a and b). This result was partially contradictory to that of rapeseed, where the upregulation of *BnPDS1* and *BnVTE1* was also associated with a greater amount of total Toc but was mainly due to the increase in α-Toc content (Table 3, section P sole effect and P × G interaction). Normally, in *Arabidopsis* seed, Toc composition predominately consists of γ-Toc (≥95%), δ-Toc (≤5%), and very small amounts of α-Toc (≤1%).[49−51] On the other hand, the most abundant types of seed Toc's on average in rapeseed are γ-Toc (46%) and α-Toc (35%), with a small proportion of δ-Toc (1%).[52,53,2,4,34] The difference between the α-Toc contents in both the species could be explained in terms of lower γ-tocopherol methyltransferase (γ-TMT) activity in *Arabidopsis*. Previously, Shintani and DellaPenna made an attempt to overexpress the γ-TMT in *Arabidopsis* seeds that led to the conversion of more than 95% of the γ-Toc to α-Toc, showing that the activity of γ-TMT could be the possible rate-limiting factor in the conversion of γ-Toc to α-Toc in *Arabidopsis*.[49]

In conclusion, exogenous GA_3 elevated total Toc yield, particularly the α-Toc content, in seeds of oilseed rape. However, the increased total Toc was dependent on the duration of GA_3 treatment, genotype, and planting location. Rapeseed plants responded to longer durations of GA_3 treatment with higher Toc yield. The genotype with a low VLCFA proportion responded to GA_3 with significantly higher Toc yield and α-/γ-Toc ratio, whereas the genotype with a high VLCFA proportion did not show a significant response to GA_3 in terms of the overall Toc yield or Toc composition. However, the molecular mechanisms by which GA signaling increases α-Toc content in rapeseed but increases γ-Toc content in *Arabidopsis* have yet to be elucidated.

■ ASSOCIATED CONTENT

Supporting Information

List of primer pairs used for RT-qPCR to amplify fragments of *VTE1*, *VTE4*, *PDS1*, and *ACTIN2* in *Arabidopsis* and list of primer pairs used for RT-qPCR to amplify fragments of *VTE1*, *VTE4*, *PDS1*, and *ACTIN7* in *Brassica napus* L. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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