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Isolation and Characterization of a *Doritaenopsis* Hybrid GIGANTEA Gene, Which Possibly Involved in Inflorescence Initiation at Low Temperatures

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Abstract. In the *Doritaenopsis* hybrid, like most of the orchid species and hybrids, temperature is crucial for the vegetative-to-reproductive transition, and low temperature is required for bud differentiation. To understand the molecular mechanism of this process, an orchid *GIGANTEA* (*GI*) gene, *DhGI1*, was isolated and characterized by using the rapid amplification of cDNA ends (RACE) PCR technique. Sequence analysis showed that the full-length cDNA is 4,022 bp with a major open reading frame of 3,483 bp, and the amino acid sequence showed high similarity to GI proteins in *Zea mays*, *Oryza sativa*, *Arabidopsis thaliana* and other plants. Semi-quantitative RT-PCR revealed that *DhGI1* was expressed throughout development and could be detected in roots, stems, leaves, peduncles and flower buds. The expression level of *DhGI1* was higher when the plants were flowering at low temperature (22/18℃ day/night) than the other growth stages. Further analysis indicated that the accumulation of *DhGI1* transcripts was significantly increased at low temperature, and concomitantly, initiation of the peduncle was observed. However, *DhGI1* levels were low under high temperature (30/25℃) conditions, and flower initiation was inhibited. These results indicate that the expression of *DhGI1* is regulated by low temperature and that *DhGI1* may play an important role in inflorescence initiation in this *Doritaenopsis* hybrid at low temperatures.

***Additional key words*:** *DhGI1*, floral formation, orchid, RACE

Introduction

Flowering is one of the essential events in the life cycle of a plant. The transition from vegetative growth to flowering in plants occurs in response to the developmental status and multiple environmental cues (Levy and Dean, 1998). The major environmental factors that modulate flowering include photoperiod and growth temperature (Reeves and Coupland, 2000). The molecular and genetic basis of the photoperiodic induction of flowering has been deciphered in model pants, such as rice and *Arabidopsis* (Hayama and Coupland, 2004; Izawa et al., 2000). Flowering plants measure the length of the night through phytochrome or cryptochrome, as the active forms created by light during the daytime are consistent with the rhythms of the circadian clock (Hayama et al., 2003).

Although it is generally known that growth temperature modulates flowering time (Balasubramanian et al., 2006), understanding of the mechanism of growth temperature regulation of flowering remains enigmatic. Vernalization is a specific example of flowering regulation by temperature. In wheat, this process of vernalization is controlled mainly by the VRN1 and VRN2 genes (Tranquilli and Dubcovsky, 2000; Yan et al., 2003, 2004). Except for vernalization, temperature regulates the timing of flowering through other mechanisms, such as temperature compensation. Temperature compensation ensures that circadian clocks maintain robust and accurate

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timing over a broad range of physiological temperatures (Hecky and Muller, 2005). The ambient temperature has important effects on regulating gene expression related to circadian clocks, such as *GIGANTEA* (*GI*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) in Arabidopsis (Lee et al., 2010). The balance between *GI* and *LHY* is important in temperature compensation of the clock. *GI* plays a critical role in maintaining rhythmicity at higher and lower temperatures. Intermittent low temperature treatments have a greater effect on flowering time in *gi*-3 plants than in wild-type plants, which suggests that there may be a link between flowering time and low temperature response through *GI* in *Arabidopsis* (Cao et al., 2005). In rice, low temperature down-regulates the expression level of *OsGI*, which regulated *Hd3a*, resulting in lower expression levels. Additionally, the expression of *Hd3a* is repressed at low temperatures and heading time is delayed; the mechanism of which may be similar to that in *Arabidopsis* (Abe et al., 2008).

For most grown orchid species and hybrids, flowering is influenced by temperature and light. However, compared to photoperiod, low temperature is an important environmental signal than induced inflorescence in orchids. A relatively low temperature induces flowering, while a high temperature inhibits it. For many of these genera, an inductive temperature is about 12℃, but this is much higher (≤ 25℃) for *Phalaenopsis* (one of the parents of Doritaenopsis) (Roberto and Erik, 2005).

The requirement for low temperature in inflorescence initiation of the orchid species increases the cost of their industrial production. For example, the plants of phalaenopsis are usually grown at a temperature > 28℃ to inhibit flower initiation and a lower temperature (e.g., 25/20℃ day/night) to induce flowering (Chen et al., 1994; Lee et al., 1987; Sakanishi and Ishida, 1980). To solve the problem, growers transfer plants to the cool highlands at about 1,000 m elevation for inflorescence initiation, which results in greater cost of labor, time, and energy. Therefore, to determine the best method to reduce production cost of artificial regulation of flowering, understanding the physiological and molecular mechanism of flowering is necessary. However, to our knowledge, no genetic or molecular data have been reported showing how cold temperature regulates flower development in these orchid plants.

Doritaenopsis, abbreviated Dtps. in the horticultural trade, is an intergeneric hybrid between the orchid genera *Doritis* and *Phalaenopsis*. These hybrids are becoming more and more popular for their multiple flowering spikes, longer flowering period and more color than the traditional parents (Cui et al., 2004). In addition, the cold and disease resistance are much improved in these hybrids. The culture of *Doritaenopsis* hybrids is very similar to *Phalaeonopsis* orchids.

Our previous study identified more than 400 up-regulated unigenes from leaves of the *Doritaenopsis* plants during floral transition by using suppression subtractive hybridization (SSH). Among these unigenes, a 536-bp fragment encoding a putative *GI* designated *DhGI1* was identified to be involved in flowering initiation in some species. In the present study, *DhGI1* was further studied by isolation of the full-length cDNA sequence by the rapid amplification of cDNA ends (RACE) PCR technique. The transcript levels of *DhGI1* in different tissues at different growth stages were investigated under both high and low temperatures. The result showed that *DhGI1* was dramatically induced by low temperature, and the expression was up-regulated in all investigated tissues of the flowering plants at this temperature. These results may provide important clues for understanding the mechanism of temperature regulation of flowering in *Doritaenopsis* hybrids.

Materials and Methods

Plant Materials and Growth Conditions

Orchid clones of *Doritaenopsis* 'Tinny Tender' (Doritaenopsis Happy smile × Happy valentine) were vegetatively propagated, and 7-month-old plants were transplanted into 8-cm pots filled with sphagnum moss containing organic fertilizer and incubated in growth chambers. The average photosynthetic photon flux (PPF) for both groups was 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 14-h light/10-h dark cycle. Plants were irrigated with nutrient solutions at 3- to 4-day intervals according to our previous study (Cui et al., 2002).

Low temperature treatment was applied when the average length of the new upper leaves was about 1 cm. One group, consisting of 120 clones, was transferred to growth chambers at 22℃/18℃ (day/night) for flowering induction. The other 120-clone group continued growing at high temperature (30/25℃) as a control. Both groups shared the same light and photoperiodic conditions as described above. The roots, stems and leaves of plants from the low temperature-treated group (LTG) and control group (high temperature-treated group, HTG) were harvested at different time points after low temperature treatment: 0 d, 1 d, 3 d, 6 d, 12 d, 24 d, 29 d (initiation of peduncle), 36 d (longer peduncle) and 48 d (formation of flower bud). The different tissues of young plants were sampled before temperature treatment, and mature plant tissues were harvested in the two groups after the low temperature treatment for about 5 months, when 98% of the plants were flowering at low temperature. And for biological replication, each sample was got from one individual plant of each group, respectively, and 3 samples together for each time-point analysis. All samples were frozen in liquid nitrogen

and stored at -70℃ for further study.

RNA Extraction

Total RNA was extracted from Doritaenopsis samples with Trizol reagent (Invitrogen, USA). The samples were ground in liquid nitrogen in a 2-mL tube. About 1 mL of Trizol reagent was added, followed by 0.3 mL of chloroform, vigorous shaking and centrifugation. About 0.5 mL of the aqueous phase was transferred to a fresh tube and mixed with 0.5 mL isopropanol. The RNA was precipitated by centrifugation. Then, the RNA was dissolved with DEPC-treated water. Finally, the RNA was treated with RNase-free DNase I (TaKaRa, Japan) to remove genomic DNA contamination before reverse transcription.

3' and 5' RACE-based Cloning of DhGI1 cDNA

A 536-bp cDNA fragment was cloned using the SSH technique in a previous study (unpublished). Sequence analysis showed that this fragment shared high homology with the *GI* of *Oryza* sativa and Lolium perenne. Gene-specific primers were designed based on this fragment, and RACE-PCR was performed to isolate the complete sequence of this gene.

For 3’-RACE PCR, the first-strand cDNA was synthesized by avian myeloblastosis virus (AMV) reverse transcriptase using the adapter primer (AP). After first-strand cDNA synthesis, the original mRNA template was destroyed with RNase H. The amplification was performed with two gene-specific primers (3SP1, 3SP2) and the 3’-abridged universal amplification primer (3’-AUAP).

The 5’-RACE PCR was performed according to the manufacturer’s protocol (5’ RACE System for Rapid Amplification of cDNA Ends) (Invitrogen), with 5’ specific primers (5SGP1, 5SGP2, 5SGP3), the abridged anchor primer (AAP) and the abridged universal amplification primer (5’-AUAP). Finally, the predicted full-length cDNA of *DhGI1* was obtained through sequence combination. The primers used in this study are listed in Table 1.

Sequencing and Analysis

Sequence analysis was mainly analyzed by BLASTX based on the NCBI non redundant nucleic acid database. The predicted sequences of the *DhGI1* protein were used to search the NCBI database by BLAST to retrieve the putative *GI* homologs in other species. The sequences of *GI* proteins were in turn used to generate an alignment by ClustalX 1.83 for phylogenetic analysis (Thompson et al., 1997). The phylogenetic tree was constructed using PHYLIP 3.63 (Retief, 2000) through a series of programs including seqboot, protdist, maximum-likelihood, and consense. The MEGA 3.0 software (Kumar et al., 2004) was used to obtain a readable form of the phylogenetic tree.

Semi-quantitative RT-PCR

The reverse transcription was performed using the Oligo (dT)18 Primer and Reverse Transcriptase XL (AMV) (TaKaRa, Japan) at 42℃ for 60 min. The products of reverse transcription were used as templates for the RT-PCR analysis. The specific primers were *DhGI1F* and *DhGI1R*. The *ACT2* gene (Phalaenopsis sp. ‘True Lady’ actin-like gene, *ACT2*, AF246715) was used as an internal control, and the primers were ACT2F and ACT2R (Table 1). RT-PCR for *DhGI1* and *ACT2* was performed with the following cycles: 30 s at 94℃, 30 s at 55℃, 1 min at 72℃, extension at 72℃ for 8 min, repeated 21 times according to preparative experiments. PCR products were separated by electrophoresis in a 1.0% agarose gel. All the RT-PCR experiments presented in this study were repeated independently three times under identical conditions using two or more independent RNA preparations.

The relative expression of *DhGI1* mRNA was determined as the *DhGI1* to *ACT2* ratio and analyzed by densitometric measurement using Quantity One 1-D Analysis Software (Bio-Rad, USA). The average density for each band was calculated and expressed as the ratio of *DhGI1* to *ACT2*. Error bars indicate standard deviation values of RT-PCR analysis. The software package SPSS 10.0 was applied for

Table 1. The primers used in this study.

Abbreviation	Sequence (5′→3′)	Description
3GSP1	CTCAGCAAGGGTGGAGGCAGGTT	Gene specific primer; for 3’ RACE
3GSP2	GTTATTGATGCCCTCTGCGATGT	Nested gene specific primer; for 3’ RACE
5GSP1	CACCATCAACAAGCATCCCATCC	Reverse; for 5’ RACE
5GSP2	CCTCCACATTCTTTGACCTTG	Nested gene specific primer for 5’RACE
5GSP3	TCAATAACCTGCCTCCACCC	Nested gene specific primer for 5’ RACE
DhGI1F	AAGGAGAGAGCCAAGCCAG	Forward; for RT-PCR
DhGI1R	AAGGTCAAAGAATGTGGAGGA	Reverse; for RT-PCR
ACT2F	AAGGATGCTTATGTGGGAGA	Actin primer as internal control, Forward
ACT2R	GAATGTGCTGAGGGAGGC	Actin primer as internal control, Reverse

statistical analyses. The one-way analysis of variance with LSD (least significant difference) test was performed to evaluate the differences of treatment with the control. Differences were considered statistically significant for a P value less than 0.05 or extremely significant for a P value less than 0.01.

Results

Isolation and Characterization of the *DhGI1* Gene

To study the molecular mechanism that regulates flowering in the *Doritaenopsis* hybrid, we identified a homolog of *GI* from the SSH cDNA library of *Doritaenopsis* leaves and designated it *DhGI1*. The full-length cDNA of *DhGI1* was obtained through 5'- and 3'-RACE and is 4,022-bp with a

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AGGGGGGGGGGGGGGGGGGAGAGCAAAGTACCGCCTTCATGGTGTATTGAGTGCCCTGGATGCCATACCATCTTTCATTCAGAATTTGCAT
CGGAAGATAAAATCTCCACCCTCCACCTCTCTTTTTCCTCAGCGACGTTTATCCCATCGGCACAAACTCGCCGAAGTTCGGATAGCATAG
CTTCAGCAATAGCTGCTACACGAGTTCTACGCTTGTTCTGGTGCGCCGGGCGTGGTGGATGCTGTTTTGATTGGATCTTTTGGTTCCCAT
AGACAGAGATAAGAAGGGATTCAAACAAGGGGCATAAGATGTCTTCATCGTCTCAAAAGTGGATCGATGGCCTTCAGTTTTCCTCCTTGT
                                       M  S  S  S  S  Q  K  W  I  D  G  L  Q  F  S  S  L  L
TGTGGCCGCCACCTCAAGATGAGCAACAGAGACAGGTACAAATTATGGCCTATGTTGAGTATTTTGCTCAATTTACTTCGGAACAATTCC
  W  P  P  P  Q  D  E  Q  R  R  Q  V  Q  I  M  A  Y  V  E  Y  F  A  Q  F  T  S  E  Q  F  P
CCGAAGACATCGCTCAGCTAATCCAGAGACATTATCCTGTTAAGGAAAAACGTGTTCTGGATGATGTATTGGCAATATTCGTCCTCCATC
  E  D  I  A  Q  L  I  Q  R  H  Y  P  V  K  E  K  R  V  L  D  D  V  L  A  I  F  V  L  H  H
ATCCAGAGCATGGGCATGCCATTGTGCATCCAATTCTTTCATGTATAATTGATGGGACCTTGGTGCATGGCAAGAATAATCCGCCATTTA
  P  E  H  G  H  A  I  V  H  P  I  L  S  C  I  I  D  G  T  L  V  H  G  K  N  N  P  P  F  S
GTTCTTTCATATCTTTAATCGGCCAGACCACTGAGAAAGAATATTCTGAACAGTGGGCTCTAGCCTGTGGGGAGATTTTACGGGTCTTGA
  S  F  I  S  L  I  G  Q  T  T  E  K  E  Y  S  E  Q  W  A  L  A  C  G  E  I  L  R  V  L  T
CTCTCTACAATCGACCAATTTACAAATCTGAACACCACAATATTGAAGCAGAAAGAAGCAGTAGCGGCAGCCATGCTACAACAAGTGAAT
  L  Y  N  R  P  I  Y  K  S  E  H  H  N  I  E  A  E  R  S  S  S  G  S  H  A  T  T  S  E  S
CTATTGGAGGGGAATCCAGTAATAGTCCTGAGCGGAAGCCATTGCGTCCTTTGACTCCATGGATTACCGATATACTGCTTGCTGCACCTG
  I  G  G  E  S  S  N  S  P  E  R  K  P  L  R  P  L  T  P  W  I  T  D  I  L  L  A  A  P  V
TGGGAATACGGAGTGATTATTTTCGATGGTGTGGTGGAGTCATGGGGAAGTATGCTGCTGCTGGAGAACTAAAACCTCCAACAACTGCTC
  G  I  R  S  D  Y  F  R  W  C  G  G  V  M  G  K  Y  A  A  A  G  E  L  K  P  P  T  T  A  R
GTAGTCGACGGTCTGGAAAGCATCCTCAGCTTATGCCATCAACGCCAGGGTGGGCTGTCGCTAATGGTGCTGGTGTCATATTAAGCGTTT
  S  R  R  S  G  K  H  P  Q  L  M  P  S  T  P  G  W  A  V  A  N  G  A  G  V  I  L  S  V  C
GTGATGATGAAGTTGCTCGTTATGAGAGTGCAAACTTAACAGCAGCTGCTGTCCCTGCACTTTTATTACCTCCCCCTACAACTCCTTTAG
  D  D  E  V  A  R  Y  E  S  A  N  L  T  A  A  A  V  P  A  L  L  L  P  P  P  T  T  P  L  D
ATGAGCACCTAGTAGCAGGACTTCCAGCACTTGAGCCATATGCTCGTTTATTTCACAGGTACTATTCCATTGCTACTCCAAGTGCAACAC
  E  H  L  V  A  G  L  P  A  L  E  P  Y  A  R  L  F  H  R  Y  Y  S  I  A  T  P  S  A  T  Q
AGAGGCTTCTTCTTGGACTTCTTGAAGCACCTCCATCATGGGCTCCTGATGCACTTGATGCAGCTGTCCAGCTTGTAGAACTTCTCAGGG
  R  L  L  L  G  L  L  E  A  P  P  S  W  A  P  D  A  L  D  A  A  V  Q  L  V  E  L  L  R  A
CGGCTGAGGATTATGAGTCCGGCATGAGGCTTCCTCGAAACTGGATGCATTTACATTTTTTGCGTGCAATTGGGACTGCAATGTCAATGA
  A  E  D  Y  E  S  G  M  R  L  P  R  N  W  M  H  L  H  F  L  R  A  I  G  T  A  M  S  M  R
GGACGGGTATTGCTGCAGATGCTGCAGCGGCTTTGCTCTTTCGTATACTTTCGCAACCCATGCTACTTTTCCCACCATTGAGGCACACGG
  T  G  I  A  A  D  A  A  A  A  L  L  F  R  I  L  S  Q  P  M  L  L  F  P  P  L  R  H  T  E
AAGGAGTTGAAGTCCAGCATGAACCCTTGGCTGGCTACATATCATATAAGAAACAGCTGGAAGTACCTGCTGCTGAAGCCACTATTGAGG
  G  V  E  V  Q  H  E  P  L  A  G  Y  I  S  Y  K  K  Q  L  E  V  P  A  A  E  A  T  I  E  A
CAACTGCTCAAGGAATTGCCTCAATGCTATGTGCTCATGGCCCTGACGTGGAATGGAGGATCTGTACTATTTGGGAGGCGGCTTATGGCT
  T  A  Q  G  I  A  S  M  L  C  A  H  G  P  D  V  E  W  R  I  C  T  I  W  E  A  A  Y  G  L
TGCTTCCCCTGAGTTCATCAGCAGTTGATTTGCCTGAGATAGTAGTGGCAGCACCATTGCAACCTCCTGTATTGTCTTGGGGCTTGTACT
  L  P  L  S  S  S  A  V  D  L  P  E  I  V  V  A  A  P  L  Q  P  P  V  L  S  W  G  L  Y  L
TGCCTCTGCTAAAAGTGCTGGAGTACCTACCTCAAGGAAGTCCATCAGAGGCATGTCTTATGAGAATATTTGTTGCTATTGTTGAAGCGA
  P  L  L  K  V  L  E  Y  L  P  Q  G  S  P  S  E  A  C  L  M  R  I  F  V  A  I  V  E  A  I
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  L  R  R  T  F  P  H  V  T  S  I  E  Q  S  K  R  S  R  N  H  G  G  V  P  S  N  S  K  N  L
TTGCAGTAGCTGAGCTCCGCACAATGATTCATTCTTTATTTCTAGAGTCATGTGCATCTGAGGACCTTGCCTCTCGCCTATTGTTTGTAG
  A  V  A  E  L  R  T  M  I  H  S  L  F  L  E  S  C  A  S  E  D  L  A  S  R  L  L  F  V  V
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  L  T  V  C  I  S  H  E  A  L  P  S  G  T  K  R  S  I  G  T  A  P  S  S  G  E  V  A  D  E
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  L  Q  I  L  N  F  K  S  S  G  R  S  R  N  R  R  K  Q  G  P  A  A  T  F  D  S  Y  V  L  A
CTGCTATATGTGCATTGTCTTGTGAACTTCAATTGTTCCCTTTGATCACAAAAAACGGCTTTCATCTAGATTTTGAAACTCCTGTTGCAA
  A  I  C  A  L  S  C  E  L  Q  L  F  P  L  I  T  K  N  G  F  H  L  D  F  E  T  P  V  A  K
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  A  A  K  T  N  G  F  S  Q  K  I  Y  D  G  M  S  S  A  V  Q  H  T  R  R  F  L  K  I  L  E
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  N  G  S  M  Q  S  K  D  S  S  T  A  P  I  K  C  E  E  A  K  L  I  N  S  M  T  M  E  T  S
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  T  K  A  S  T  A  I  V  L  Q  A  E  R  D  L  Q  P  W  I  A  R  D  D  E  Q  G  Q  R  M  W
GGAGGATCAACCAGCGAATCGTGAAGTTGATTGTCGAACTCATGAGAAGTCAGGGAAGCCCCGAGGCACTAATAGTTATTGCAAGTGCCT
  R  I  N  Q  R  I  V  K  L  I  V  E  L  M  R  S  Q  G  S  P  E  A  L  I  V  I  A  S  A  S
CCGATCTTCTCCTTCGTGCCACGGATGGGATGCTTGTTGATGGTGAAGCTTGCACTCTACCACAGCTAGAGCTCCTGGAAGTGACAGCTA
  D  L  L  L  R  A  T  D  G  M  L  V  D  G  E  A  C  T  L  P  Q  L  E  L  L  E  V  T  A  R
GAGCAGTCCACTTAGTAGCAAATTGGGGAGAAATCGGTTTTGCAGTAGCTGATGGCCTTACAAATCTTCTCAAATGCCGACTGTCTGCTA
  A  V  H  L  V  A  N  W  G  E  I  G  F  A  V  A  D  G  L  T  N  L  L  K  C  R  L  S  A  T
CCGTTCGCTGTCTTTCTCATCCCAGCGCTCTTGTGAGAGCTCTAAGCATATCGGTTCTTCGAGATATTATGAATACCGGCCCCATCAATT
  V  R  C  L  S  H  P  S  A  L  V  R  A  L  S  I  S  V  L  R  D  I  M  N  T  G  P  I  N  S
CATCTAATTATACGAATACGGAGACACTACATTTGTACGATCCCTCATACCGAAGCCTGGGCATGACCAACTGGCATGCAAACATTGAGA
  S  N  Y  T  N  T  E  T  L  H  L  Y  D  P  S  Y  R  S  L  G  M  T  N  W  H  A  N  I  E  K
AGTGCATCAAATGGGAAGCTCGCAGCCGGCGAGCAACTGGCTTGGCTCTCTCCTTCCTTGGTTCTGCTTCAAAGGAGTTGGGCTGTCCTC
  C  I  K  W  E  A  R  S  R  R  A  T  G  L  A  L  S  F  L  G  S  A  S  K  E  L  G  C  P  L
TTCCTAGTTGACAGGCAATTGACATATTTTACGGCTTTTTACTTTTTTGTTTTCTTTTTGACATTATGTACCGTTGTCATTTGGTTGTAT
  P  S  *
TTAATGTTGTAATGACCCCAGTTGCTCATGTAGATATGAACAACAAACATTATTGGTTTTACATCAAATGACAACCTGCAATGTTAATGT
AAGTCATCATTGCCCCTCCTAAACATGTTCTACTACCAGGGATTGAACTATCAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of *DhGI1*. Full-length *DhGI1* (GenBank Accession No. HQ388416) nucleotide sequence and its deduced amino acid sequence are presented.

308-bp 5'-untranslated region, a 220-bp 3'-untranslated region and an 11-bp polyA tail (Fig. 1).

The gene has been submitted to the GenBank database with accession number HQ388416. Sequence comparison shows that the DhGI1 protein shares high homology with OsGI of rice, ZmGI of corn, TaGI1 of wheat, and GI of *Arabidopsis* with 74%, 74%, 73%, and 67% identity, respectively (Fig. 2).

To study the evolutionary relationships of the GI proteins in different plant species, a phylogenetic tree was generated

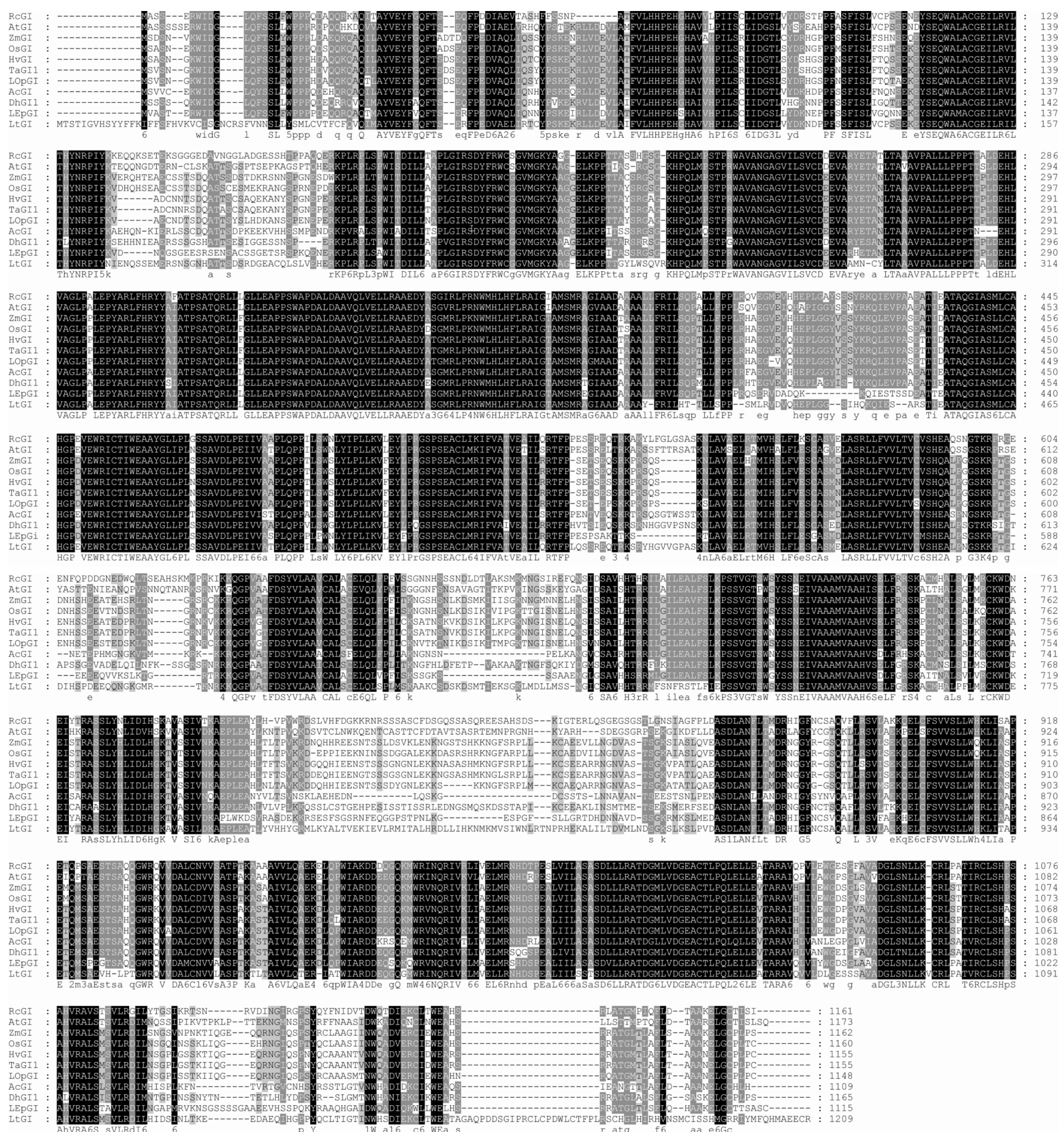

Fig. 2. Alignment of the DhGI1 amino acid sequences with GI from different plant species. DhGI1, *Phalaenopsis amabilis* (GenBank Accession No. HQ388416); ZmGI, gigantea 1A [*Zea mays*] (GenBank Accession No. DAA06172); OsGI, putative gigantea [*Oryza sativa* Japonica Group] (GenBank Accession No. BAD68052); HvGI, gigantea-like protein [Hordeum vulgare] (GenBank Accession No. AAW66945); TaGI1, gigantea [*Triticum aestivum*] (GenBank Accession No. AAT79487); LOpGI, GIGANTEA [*Lolium perenne*] (GenBank Accession No. CAY26028); AcGI, GIGANTEA [*Allium* cepa] (GenBank Accession No. ACT22764); RcGI, Protein GIGANTEA, putative [*Ricinus communis*] (GenBank Accession No. EEF38081); LEpGI, GI homologue 1 [*Lemna paucicostata*] (GenBank Accession No. BAD97864); AtGI, GIGANTEA [*Arabidopsis thaliana*] (GenBank Accession No. AAF00023); LtGI, gigantea-like protein [*Liriodendron tulipifera*] (GenBank Accession No. ACY30446).

using the protein sequences of 11 putative GI proteins, including DhGI1 and other 10 putative GI proteins from different species (e.g., *Arabidopsis*, rice, wheat and corn). Based on the phylogenetic relationship of the protein sequences, the 11 plant GI proteins were divided into two subfamilies, GI I and GI II (Fig. 3), belonging to monocotyledons and dicotyledons, respectively. An alignment of the GI protein sequences revealed highly conserved amino acid sequence among all GI proteins. All data above suggested that DhGI1 is a GI homolog.

Low Temperature Was Required in Inflorescence Initiation of the *Doritaenopsis* Hybrid

After the low temperature treatment for 29 days, the

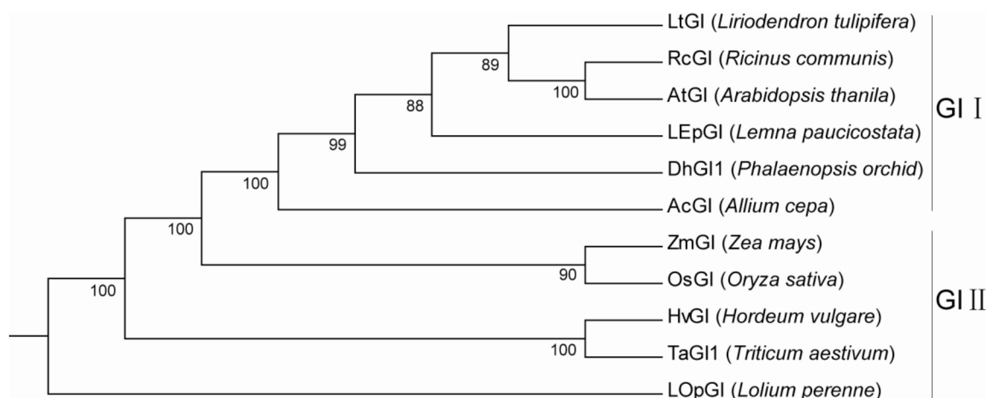

Fig. 3. The phylogenetic tree of the predicted *DhGI1* protein with GIs from different plant species. The phylogenetic tree was based on the genetic distance of the protein sequences constructed by the ClustalW method using MEGA3.0 software. The symbol in parentheses indicates the corresponding GenBank accession number. The numbers above the branches are the percentage of bootstrap trials that support the clade, and branches without numbers indicate less than 60%.

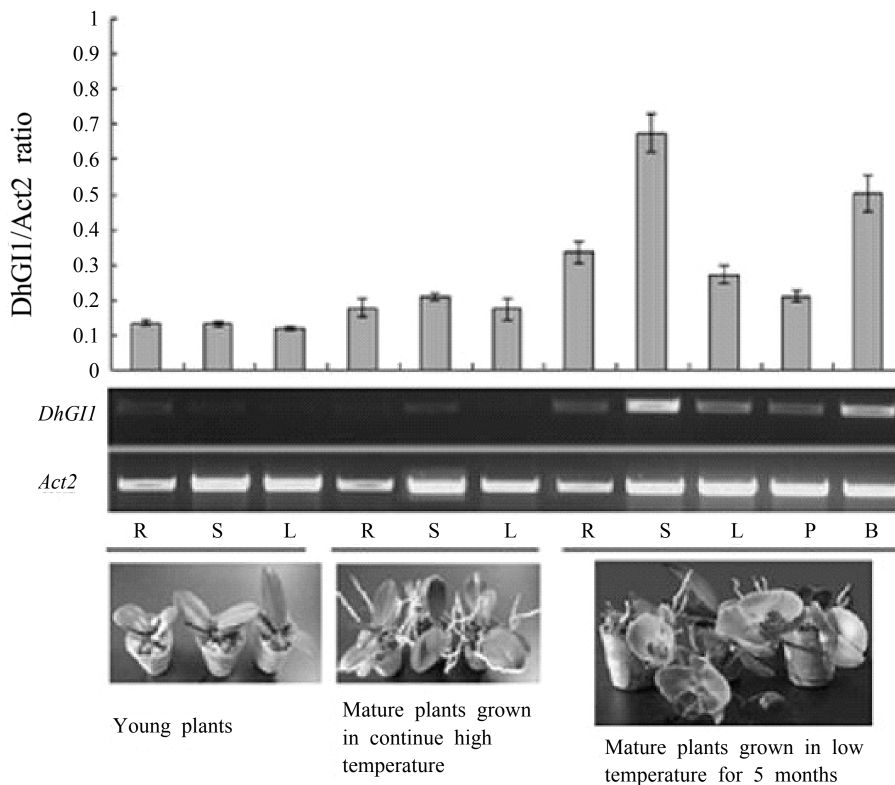

Fig. 4. Expression of the *DhGI1* transcript in different organs of *Doritaenopsis* plants at different growth stages. The pictures of *Doritaenopsis* plants at different growth stages and RT-PCR analysis with *ACT2* as the internal control. Relative expression of *DhGI1* by densitometric analysis using Quantity One 1-D Analysis Software (Bio-Rad, USA). The average density for each band was calculated and expressed as the ratio of *DhGI1* to *ACT2*. Error bars indicate standard deviation values of three independent RT-PCR replicates from a pooled sample. Total RNA was isolated from roots (R), stems (S), leaves (L), peduncles (P, mature plants of the low temperature-treated group) and buds (B, mature plants of the low temperature-treated group).

peduncles were initiated in some plants of the LTG (22/18℃), and after 48 days, some flower buds were formed. After 5 months, 98% of the plants were in flower. By contrast, the plants maintained vegetative growth and none of the plants had initiated an inflorescence in HTG (30/25℃) (Fig. 4).

Localization of *DhGI1* Transcripts in Different Tissues

To determine the tissue-specific patterns of *DhGI1* expression in *Doritaenopsis* plants at different growth stages, *DhGI1* transcripts were detected by semi-quantitative RT-PCR in different tissues of young plants and the mature plants including LTG (reproductive) and HTG (vegetative) after treatment for 5 months. As shown in Fig. 4, *DhGI1* was expressed throughout development and *DhGI1* transcripts can be detected in roots, stems, leaves, peduncles and flower buds. The expression levels of *DhGI1* were significantly higher in tissues of LTG than that in tissues of both young plants and the mature plants under high temperature, especially in stems and buds (Fig. 4).

The Expression of *DhGI1* Gene Was Regulated by Low Temperature

To investigate whether the *DhGI1* gene was induced by low temperature, *DhGI1* transcripts were amplified by semi-

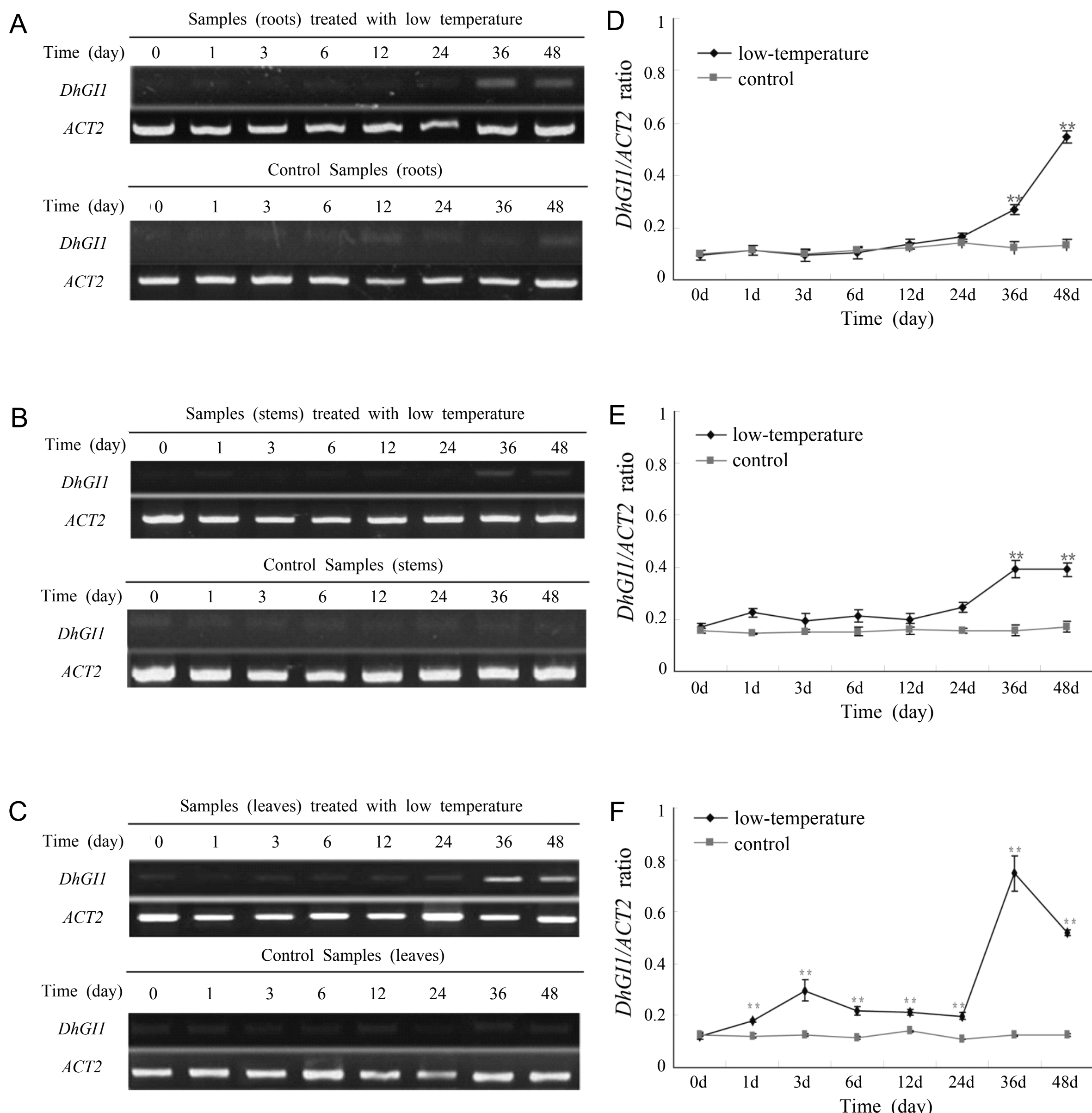

Fig. 5. Expression pattern of *DhGI1* in *Doritaenopsis* root, stem, leaves, treated with low temperature. (A-C) RT-PCR analysis with *ACT2* as the internal control. (D-F) Relative expression of *DhGI1* by densitometric analysis using Quantity One 1-D Analysis Software (Bio-Rad, USA). The mean density for each band was calculated and expressed as the ratio of *DhGI1* to *ACT2*. Error bars indicate standard deviation values of three independent RT-PCR replicates from a pooled sample. *Significant at P = 0.05, ** Extremely significant at P = 0.01.

quantitative RT-PCR from roots, stems and leaves of LTG at eight different time points (0 d, 1 d, 3 d, 6 d, 12 d, 24 d, 36 d, and 48 d). The HTG was used as a control. As shown in Fig. 5, the transcript levels of *DhGI1* were significantly increased after exposure to low-temperature for 24 days and reached the peak level at 36 days. In contrast, *DhGI1* gene transcripts in three tissues were at a very low level in control plants grown at high temperature (30/25℃) (Fig. 5).

Discussion

In our study, the cold treatments were performed under long day conditions, and it was found that low temperature treatment had a significant effect on flowering initiation in *Doritaenopsis* hybrid plants. Flowering initiation occurred in plants that were grown at a day temperature of 22℃ and a night temperature of 18℃ for about 1 month; after 5 months, 98% of the plants were in flower. On the contrary, none of the plants had initiated an inflorescence in the control group, which were grown at a day temperature of 30℃ and a night temperature of 25℃. The time of exposure to low temperature before inflorescence initiation may be associated with the maturity of the plants. Wang and Lee (1994) reported that plants that have not reached sufficient maturity could require cooler temperatures or longer exposure to initiate inflorescence than larger, more mature plants. This result showed that *Doritaenopsis* hybrids had the same requirement for inflorescence initiation as *Phalaenopsis* orchids, for which low temperature is the most important environmental signal that induces inflorescence (Blanchard and Runkle, 2006; Vaz et al., 2004).

To understand the molecular mechanisms of the process of inflorescence initiation of *Doritaenopsis* hybrids, the GIGANTEA gene *DhGI1* was isolated from the cDNA library of Doritaenopsis leaves, which was one of the up-regulated unigenes identified from the SSH library in our previous study. Sequence comparison (Fig. 1) indicated that it shared higher identity with GI from other plants, such as *Arabidopsis* and rice (Abe et al., 2008; Fowler et al., 1999). Our results indicate that the *DhGI1* is most likely an ortholog of the *GI* gene, and it was first isolated as an ortholog of *GI* from an orchid species.

The analysis of patterns of *DhGI1* expression in *Doritaenopsis* plants at different growth stages showed that *DhGI1* transcripts were significantly higher in tissues of LTG, which were flowering, than that in tissues of both young plants and the mature plants under high temperature. The result suggested that *DhGI1* may be involved in the transition from vegetative growth to flowering in *Doritaenopsis* hybrid. In *Arabidopsis*, the *GI* gene has been shown to be involved in several developmental processes, including photoperiodic flowering, circadian-clock function, transpiration and phytochrome signaling (Park et al., 1999; Sothern et al., 2002). *GI* encodes a nuclear protein involved in phytochrome signaling and is crucial for the control of circadian rhythms, photoperiodic flowering and the formation of complex. *GI* and *FKF1* are required for measurement of day length in *Arabidopsis*. *GI* regulates flowering through regulating the expression levels of *CONSTANS* (*CO*) and *FLOWERING LOCUS* (*FT*), important flowering genes that are downstream of *GI* (Jung et al., 2007; Tseng et al., 2004). During recent years, orthologs of *GI* have been cloned in various species, such as *OsGI* from rice (Hayama et al., 2002), *TaGI1* from wheat (Zhao et al., 2005), *HvGI* from barley and *BrGI* from cabbage (Dunford et al., 2005), and the function of these genes has been studied. In rice, *OsGI*, *Hd1* and *Hd3a* are identified as orthologs of *GI*, *CO* and *FT*, respectively, and are also one of the important regulators of flowering through a similar molecular mechanism (Hayama et al., 2002; Kojima et al., 2002). The wheat *TaGI1* is also involved in photoperiodic flowering (Zhao et al., 2005). Therefore, as *GI* and its orthologs, that play an important role in cross-talk between photoperiod and temperature for regulating flowering, *DhGI1* is presumably involved in the process of inflorescence initiation in *Doritaenopsis* hybrid.

Moreover, further study indicated that the expression of *DhGI1* was not significantly increased until low temperature treatment was conducted for 24 d. This result indicated that *DhGI1* was regulated by accumulation of low temperature, similar to *GI* in *Arabidopsis*, which is induced by cold stress. Cao et al. (2005) found that *GI* was involved in mediating the cold stress response. *GI* expression was induced by cold stress, but not by NaCl, mannitol, or ABA, suggesting that *GI* may play a role in freezing tolerance, which is consistent with the finding that *GI* transcripts were up-regulated in response to low temperature (Fowler and Thomashow, 2002). The *gi* mutations decrease constitutive freezing tolerance and impair cold acclimation, suggesting that *GI* is required for both constitutive freezing tolerance and cold acclimation in *Arabidopsis* (Cao et al., 2005).

Interestingly, the first peduncle was observed in a single plant after a 29-d incubation in low temperature, and then the peduncle of most investigated plants sprouted at about day 36 of cold treatment, while the *DhGI1* transcript level was significantly increasing (Fig. 5). It indicated that the expression of *DhGI1* was closely connected with initiation of peduncles. Therefore, it is possible that while it was induced by accumulation of low temperature, *DhGI1* would be involved in cold-induced flowering.

These results suggested that there may be a link through *DhGI1* between inflorescence initiation and low temperature

in the *Doritaenopsis* hybrid. Cultured similar to *Phalaeonopsis*, which is originated from tropical region and not sensitive to the photoperiod, 98% of *Doritaenopsis* plants of LTG flowered under long-day conditions (14-h day/10-h night cycle). Although, *Doritaenopsis* is commercially cultured in long-day photoperiod, it had been analyzing whether the *DhGI1* is involved in circadian rhythms in *Doritaenopsis*. Another further investigation, that overexpression or knockout of *DhGI1*, or our undergoing experiment of *Arobidopsis gi* mutant for complementation analysis, would help to confirm the function of *DhGI1* in transition from vegetative to reproductive growth at low temperature of *Doritaenopsis*, and elucidate the complicated mechanism of flowering in orchid plants.

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