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ORIGINAL ARTICLES

The Cold Awakening of *Doritaenopsis* 'Tinny Tender' Orchid Flowers: The Role of Leaves in Cold-induced Bud Dormancy Release

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Abstract Massive flowering of tropical *Phalaenopsis* orchids is coordinated by the cold-induced release of reproductive bud dormancy. Light and temperature are the two key factors integrated by the dormancy mechanism to both stop and reactivate the meristem development of many other angiosperm species, including fruit trees and ornamental plants. It is well established that leaves and roots play a major role in inducing flower development; however, currently, knowledge of molecular events associated with reproductive bud dormancy release in organs other than the bud is limited. Using differential gene expression, we have shown that the leaves of a hybrid of *Phalaenopsis* species, *Doritaenopsis* 'Tinny Tender', undergo major metabolic modifications. These changes result in the production of sucrose and amino acids, both of which can sustain bud outgrowth, and auxin and ethylene, which may play important roles in awaking the dormant buds. Intake of abscisic acid and synthesis of the hormone jasmonate may also explain the inhibition of vegetative growth that coincides with bud growth. Interestingly, many genes that were upregulated by cold treatment are homologous for genes involved in flower induction and vernalization in *Arabidopsis*, indicating that processes regulating flowering induction and those regulating reproductive bud dormancy release may use similar pathways and effector molecules.

Keywords Orchid · *Doritaenopsis* 'Tinny Tender' · Bud dormancy release · Differential gene expression · Cold-induced · Sugar metabolism · Plant genetics · Reproductive bud

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Introduction

Most flowering plants use photoperiod and temperature variations to detect seasonal shifts and reproductive periods (Boss and others 2004; Jaeger and others 2006; Penfield 2008; Adrian and others 2009; Amasino 2010; Olsen 2010). Vernalization and bud dormancy are two distinct mechanisms that allow temperature changes to affect flowering time (Chouard 1960; Penfield 2008). Vernalization has been studied extensively at the molecular and genetic levels, especially in the model plant *Arabidopsis*

thaliana (Amasino 2010). However, relatively little information is known about reproductive bud dormancy (Anderson and others 2010), as there is a lack of model species that can be used to study this process (Penfield 2008). Vernalization is the process by which a prolonged period of cold renders plant meristems competent to develop flowers (Chouard 1960). In temperate latitudes, some plants use vernalization to distinguish autumn and spring, which otherwise have similar photoperiodicity (Amasino 2010). Other species use a different mechanism in which partially developed buds become dormant (Lang 1987). Under specific temperature and photoperiod conditions, dormancy is released and reproductive buds develop into an inflorescence. Bud dormancy is used by many woody species over winter freezing periods and by tropical plants to coordinate their flowering time (Wilkie and others 2008). Here, we studied the reproductive bud dormancy release of an orchid species, *Doritaenopsis* 'Tinny Tender', which depends only on temperature to break dormancy (Chen and others 2008). This simple mode of release and the relatively rapid plant development make *Doritaenopsis* a convenient model in which to study reproductive bud dormancy. To our knowledge, this work constitutes the first study of reproductive bud dormancy release in a monocot species at the genetic level.

The orchid family, or Orchidaceae, comprises about 25,000 species and is the largest family of monocots, which encompasses approximately 70,000 species in total (Palmer and others 2004). *Doritaenopsis* are tetraploid hybrids between two orchid species of the *Phalaenopsis* genus; plants from this genus grow naturally in the tropical regions of Southeast Asia and North Oceania. They are among the most popular potted plants in flower markets around the world and a valuable resource in the cut flower industry due to their unique flower shape, various flower colors, and long flowering period (Griesbach 2002). Orchids from the *Phalaenopsis* genus develop two buds at each node that subsequently become dormant. When *Doritaenopsis* are incubated at a relatively low temperature (25/22°C, day/night) for 6–7 weeks, each bud grows into a stalk, which blooms about 3 months later (Fig. 1). Once the dormancy is released, it is irreversible and cannot be reinstated by a change to higher temperature. However, dormancy can be maintained if the orchids are constantly incubated at a higher temperature (28/25°C), corresponding to average temperatures of tropical zones (Cui and others unpublished). Figure 1 shows the different organs of *Doritaenopsis* and compares the phenotypes of a plant developing a flower stalk or in vegetative growth. Previous genetic studies of orchid flowers focused mainly on their morphological development and the synthesis of pigments (Yu and Goh 2001). For example, the differential expression of some MADS-box proteins, which interact with KNOX genes, was shown to be at the origin of floral morphogenesis in orchids (Yu and Goh 2000a). Although some investigations of flower development have been undertaken in orchids, the molecular mechanism of reproductive bud dormancy release remains to be elucidated.

Vegetative or reproductive bud dormancy regulations have been studied mainly at the genetic level in grapevine (Halaly and others 2008; Ophir and others 2009), potato (Law and Suttle 2004; Destefano-Beltrán and others 2006; Rodríguez-Falcón and others 2006), leafy spurge (*Euphorbia esula*) (Chao and others 2006; Horvath and others 2002, 2008, 2010), and poplar (Ruttink and others 2007; Olsen 2010). Two main conclusions were drawn from those investigations: first, that most of the bud genes associated with dormancy induction and release are also involved in flowering induction and vernalization, and second, that plant hormones participate in various aspects of the dormancy process. Nevertheless, knowledge of the molecular events associated with reproductive bud

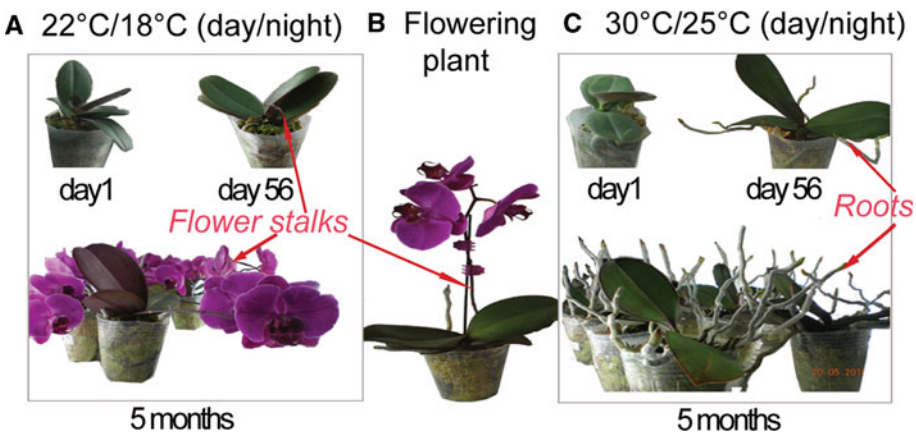

Fig. 1 *Doritaenopsis* 'Tinny Tender' plants at different stages. **a** *Doritaenopsis* plants after 1 day and 56 days low-temperature incubation (22/18°C, day/night). The day-56 plants develop flower stalks as indicated. **b** Flowering *Doritaenopsis* plant. **c** *Doritaenopsis* plants grow vegetatively when incubated at 30/25°C. The flower stalk grows from the base of the aerial part, along with the bottom leaf. *Doritaenopsis* 'Tinny Tender' plants start to flower about 3 months after the stalk becomes visible and is in full bloom after 5 months

dormancy release remains fragmented (Amasino 2010; Anderson and others 2010), possibly because most previous genetic investigations focused only on the bud. It is reasonable to postulate that other plant organs, excluding the bud, participate in the dormancy release process because it is well established that flower induction is primarily controlled by events occurring in the leaves (Bernier and others 1993; Amasino 2010), and the roots are involved in a later stage of floral development (Bernier and others 1993). In a preliminary experiment to this study, some young *Doritaenopsis* whose leaves were removed were unable to break bud dormancy (data not shown). In this context, our study of the events occurring in the leaves of an orchid that mainly responds to temperature to control bud dormancy release is particularly relevant. In this study we measured in *Doritaenopsis* leaves the variations of sugar content and identified genes whose expression is upregulated upon cold exposure. We then discussed the possible implications of the differentially regulated genes in temperature sensing and signal transduction on the basis of the three working mechanisms that have been hypothesized to be used by plants to detect temperature changes. An analysis of the upregulated metabolic pathways allowed us to identify possible metabolic changes in the leaves, including the production of sucrose and amino acids, which might help to sustain flower growth. We identified genes associated with the production of auxin and ethylene, both of which can potentially signal bud dormancy release, and the production of jasmonate and abscisic acid, which might be responsible for reduced vegetative growth. In this article we propose a working model for orchid flower bud dormancy release. Finally, we highlight differences with the vernalization/cold acclimatation mechanism in wheat, which is, like orchids, a monocot plant species.

Materials and Methods

Plant Material and Treatments

Orchid clones of *Doritaenopsis* 'Tinny Tender' (*Doritaenopsis* 'Happy Smile' × 'Happy Valentine') were vegetatively propagated. Seven-month-old plants were transplanted into 8-cm pots filled with sphagnum moss containing organic fertilizer and were incubated in a greenhouse at 24–28°C. The "low-temperature group," consisting of 120 clones, was transferred to growth chambers at 22/18°C (day/night, ±0.5°C) for flowering induction when the average length of the new upper leaves was about 1.5-2.0 cm. The two growth chambers were equipped with fluorescent tubes. The "control group," also comprising 120 clones, was incubated at 30/25°C. The average photosynthetic photon flux for both groups was 140 μmol m^{-2} s^{-1} and the photoperiod was set at 14 h of light and 10 h of dark. Plants were irrigated at an interval of 3–4 days, and nutrient solutions were supplied as previously published (Cui and others 2004). Plant phenotypic characters (width and length of leaves, flower stalk growth) were recorded once a week, and, at the same time, the second upper leaves from both groups were collected for analysis of sugar and starch content.

Determination of Sugar and Starch Content

Samples from ten second upper leaves were homogenized, extracted in distilled water at 50°C for 30 min, and centrifuged at 8000 rpm for 15 min. The extraction was repeated three times. The supernatant phases were collected, pooled, filtered on a Sep-Pak C18 column to discard pigments and other nonsugar compounds, and finally analyzed by a Waters 1500 HPLC (Waters Corp., Milford, MA, USA) equipped with a Sugar-Pak™ column. Starch was extracted from the pellet by washing with 80% methanol to remove remaining sugars, centrifuging at 5000 rpm for 30 min, dissolving in boiling distilled water for 30 min, and centrifuging at 5000 rpm for 30 min. The supernatant was collected and treated with two drops of a mixture of iodine (I_2) and potassium iodide (KI). The starch concentration was evaluated by measuring the absorbance at 660 nm and using a standard curve of absorbance (Mohotti and Lawlor 2002).

Total RNA Extraction and mRNA Purification

Between day 21 and day 28, 1-cm-diameter discs were punched in the second upper leaf of 30 plants in both the low-temperature and control groups. Plant materials were then stored at −80°C. Total RNA was extracted using the hexadecyltrimethyl-ammonium bromide (CTAB)-modified method described by Yu and Goh (2000b). RNA quality and purity were checked by formamide-formaldehyde denaturing agarose gel electrophoresis and a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PolyA mRNA was purified using an Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA). The quality and yield of the mRNA were checked by denaturing agarose gel electrophoresis.

Construction of Suppression Subtractive Hybridization cDNA Library and Sequencing

The purified mRNAs from the low-temperature and control groups were used to construct a suppression subtractive hybridization (SSH) library using a PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, USA). The cDNA from the low-temperature group was used as the

"tester" and the cDNA from the control group was used as the "driver." After selective amplification, the tester-enriched, size-separated, subtracted cDNA pool was randomly cloned into a pMD18-T vector (TaKaRa-Bio, Otsu, Shiga, Japan) and transformed in DH5α competent cells (Invitrogen, Carlsbad, CA, USA). The ampicillin-resistant colonies were picked and grown overnight in liquid Luria-Bertani medium containing ampicillin at 37°C. PCR selection by M13 forward primer 5′GTAAAACGACGGCCAG3′ and reverse primer 5′CAGGAAACAGCTATGAC3′ was carried out to estimate the average size of inserts. The clones were then sequenced using the M13 forward primer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Sangon, Shanghai, China).

Sequence Analysis

Raw sequences were trimmed of vector, adaptor, and low-quality sequence regions using SeqMan (Lasergene, DNASTAR Inc., Madison, USA). Sequences less than 100 bp were discarded and the remaining sequences were clustered into unigenes using SeqMan sequence assembly software with a minimum 90% match over 50-bp overlap.

Unigenes were aligned using the basic local alignment search tool (Altschul and others 1990) 2.2.24 (BLASTN) against the National Center for Biotechnology Information (NCBI) nonredundant nucleic acid (nt) sequence database (Benson and others 2010). Unigenes were also aligned using BLASTX against the UniProtKB database (Boutet and others 2007). Unigenes with no alignment presenting an expected value greater than 10^{-20} were considered as having no known homologous sequences and were annotated as having an unknown function. The unigenes were also aligned using the Smith and Waterman algorithm implemented in the FASTA programs (Pearson and Lipman 1988) to the *Arabidopsis* information resource (TAIR) database TAIR9 (20090619) (Swarbreck and others 2008). Alignments with an expected value greater than 10^{-30} and covering at least 70% of the unigene sequences were considered as significant of a possible functional homology. The unigenes were then functionally annotated by manually analyzing alignment hits in the UniProtKB, the NCBI nt database, and the TAIR databases. Classification of the unigene functions was performed using the Munich Information Center for Protein Sequences (MIPS) *Arabidopsis thaliana* database (MatDB) (Schoof and others 2004). All edited unigenes were submitted to GenBank dbEST (GenBank accession Nos. HO212438-HO213049).

Bioinformatics Analysis

The biochemical pathways possibly upregulated during reproductive bud dormancy release were predicted using two databases: the plant metabolic network (PMN) (Zhang and others 2005) and the Kyoto encyclopedia for genes and genomes (KEGG) (Kanehisa and others 2008). PMN was searched by aligning the unigenes using BLASTX against the enzyme sequences set of PMN Plant Cyc (20100621). KEGG was searched by submitting the unigene sequences to the KEGG automatic annotation server (KAAS) (Moriya and others 2007). KAAS was used first against the enzyme from plants only, then against a more general representative set of eukaryotes enzymes. Functional annotations obtained using the PMN Plant Cyc database and the KEGG database were compared to the annotations determined using TAIR, UniProtKB, and NCBI nt. Unigenes with inconsistent annotations were discarded from the metabolic pathway analysis. Information on some activated reactions and pathways are represented in Fig. 4.

A manual annotation approach was then used to determine and draw a map of the main activated metabolic pathways (Supplementary Fig. 1).

Gene Expression by Quantitative Real-time RT-PCR and Data Analysis

Based on functional annotation of the SSH library, 13 unigenes were selected and used to design real-time PCR primers using Primer3 software (Rozen and Skaletsky 2000). Corresponding primer sequences are shown in Table 1. A QuantiTect SYBR® Green PCR Kit (Qiagen) was used for the gene expression analysis. An actin gene, DhSL-50 (HO212487), extracted from *Doritaenopsis*, was taken as the standard because a preliminary analysis showed it to have a relatively constant transcription level in all the investigated plant materials. Three replicate measurements were performed for each sample. Transcription levels were computed relative to the transcription level in leaves at day 0. All data were analyzed using SPSS 14.0 (www.spss.com; SPSS, Inc., Chicago, IL, USA).

Results

Vegetative growth, starch, sucrose, and reducing sugar were monitored over a period of 50 days, with the first day noted as 0, as detailed below. This study allowed for the identification of the dormancy release period. A suppression subtractive hybridization (SSH) approach was used to isolate transcripts that are specific to dormancy release. The isolated transcripts were sequenced and analyzed using protein, nucleic acid, and metabolic databases, including UniProtKB (Boutet and others 2007), NCBI nt (Benson and others 2010), TAIR (Swarbreck and others 2008), PMN Plant Cyc (Zhang and others 2005), and KEGG (Kanehisa and others 2008). Finally, the level of 13 transcripts in the

Table 1 Primers of selected *Doritaenopsis* transcripts used for quantitative real-time RT-PCR

Name	Accession No.	Putative function	Forward primer (5′–3′)	Reverse primer (5′–3′)
DhSL-37	HO212474	Sucrose synthase	CCTCACGGCTACTTCGCTCAAG	TCTGGCAGCAATCTGGTCACAAT
DhSL-45	HO212482	Acetyl-CoA carboxylase	CTCAAGTGGCTCAGCTATGTTCCT	CGAGTTCTCAGGCGTGTATTCCA
DhSL-55	HO212492	UDP-glucose 6-dehydrogenase	AAGGGCTTGCTTGGCGACAA	ACGGCTGGAGGTGGATTGGA
DhSL-60	HO212497	Sucrose transporter	TTCGGTCCTCGGTGTTCCATTAG	CTCCTTGGTTGGCAGCTAGTTGT
DhSL-85	HO212522	Histone H3	AGGAGGCAGCGGAGGCATAT	ACGGCGGGCAAGTTGAATGT
DhSL-89	HO212526	Chlorophyll a/b-binding protein	CGAGCCACCTTCCTACCTCACT	CGCCGAACTTTACGCCATTGC
DhSL-92	HO212529	Arginine n-methyltransferase	CTGGCAGCAACAGAACTTCTATGG	TGTGTGGAATGAAGGTGGAGAGAC
DhSL-93	HO212530	Homospermidine synthase	GTTCATCAACACCGCTCAGGAATT	TACCAGCCACCAGCAGAGGAA
DhSL-112	HO212549	GIGANTEA	CAAGTGCCTCCGATCTTCTCCTT	CAGCGAACGGTAGCAGACAGT
DhSL-296	HO212733	Thioredoxin h	CGCCGATCTCGCCAAGAAGTT	CATTGCCTCCACCTTCCATTCTGA
DhSL-467	HO212904	IAA hydrolase	TGGATTCTCAGGTGGTGACTGTTG	AAGTGCCGCCAATGGTGACA
DhSL-493	HO212930	Sugar transporter	TCACAGGGCAACCAAGCGTTT	CCAAGCCTATCCACCACCAGAAC
DhSL-556	HO212993	Fructose-bisphosphate aldolase	GCCGTGGTCGCTTTCATTCTC	GTTGCTTCCGAGTTCGCCTTG

leaves was followed, after cold treatment, by reverse transcriptase polymerase chain reaction (RT-PCR) over a period of 35 days.

Flowering and Vegetative Growth Monitored at Different Temperatures

On day 0, 240 *Doritaenopsis* clones, aged 7 months, were divided into two groups and grown with identical light conditions but different day and night temperatures. The "low-temperature group" was kept at 22/18°C (day/night) and the "control group" was incubated at 30/25°C. Some flower stalks became visible on day 42 in the low-temperature group (Fig. 1) and the stalks bloomed 3 months later. All plants in the control group did not flower. The size of the upper leaf of all the plants was measured every week at regular intervals. The average leaf size of the low-temperature group was significantly smaller than that of the control group from, and beyond, day 14 ($p < 0.05$) (Fig. 2a). After day 28, the average leaf size of the control group was still increasing, whereas vegetative growth had stopped in the low-temperature group. This suggests that in the low-temperature group bud dormancy is progressively released until day 28 when the bud growth begins and vegetative growth halts. This timing is supported by the change of sucrose and starch content as described in the next subsection. Ultimately, the bud developed into a flower stalk that became visible at day 42 when it emerged from the epidermis of the stem.

Sucrose and Starch Content Changes in the Orchid Leaves

As shown in Fig. 2b, the starch content in the second upper leaves of the low-temperature group was significantly lower than that of the control group after day 21, remained low until day 42, and increased dramatically at day 49, reaching the same level as the control group. In the low-temperature group, the sucrose content increased until day 28 and then progressively declined until day 49. The opposite evolution of starch and sucrose content until day 28 at low temperature could indicate interconversion between starch and sucrose. After day 28, the sudden decrease of sucrose content is not followed by an immediate rise in starch content. Therefore, the sucrose may be used for purposes other than making starch or it is simply excreted by the leaves.

The evolution of both sucrose content and leaf size in the low-temperature group changed tendency at day 28, suggesting that bud growth should start shortly after this date. To study the genetic events associated with bud dormancy release, we constructed a SSH library between leaf material extracted from the low-temperature and control groups on days 21–28 after cold treatment. The resulting differential cDNA library was then sequenced.

Identification of Cold-induced Transcripts

From the SSH library, 2,360 clones were sequenced and clustered into 612 unigenes that are referred to here as

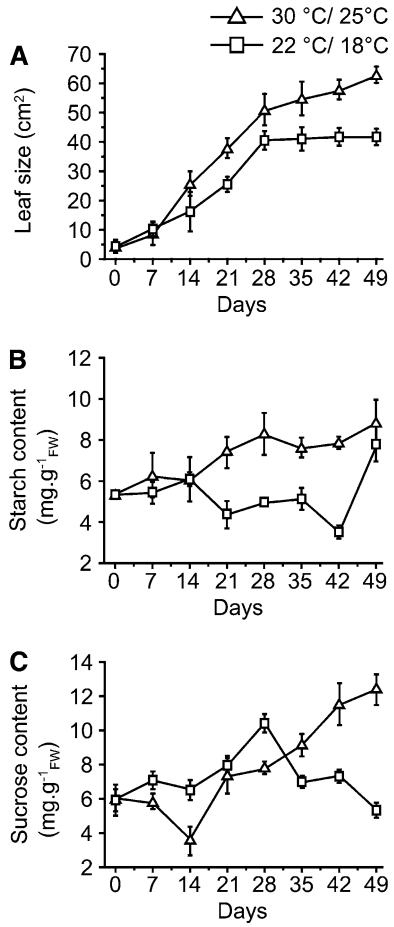

Fig. 2 Evolution of the leaf size and starch and sucrose content in *Doritaenopsis* leaves at different temperatures. Parameters were evaluated for a group of 240 *Doritaenopsis* 'Tinny Tender' plants grown at 22/18°C (day/night), and for a similar group grown at 30/25°C. **a** Variation of leaf size of the upper leaf in each group. **b**, **c** Variation of starch and sucrose content measured in the second upper leaves, respectively. Sucrose and starch content are given per mass of fresh weight (mg g^{-1} FW). All the plants were first grown at the higher temperatures, and then half of them were grown in a colder environment. Therefore, the plants that were continuously grown in higher temperatures are the controls

"transcripts." In this work, individual transcripts were named "DhSL-X," where X is the number of the transcript. The corresponding identifier in the NCBI EST database is provided in Supplementary Table 1. The transcript sequences were searched against UniProtKB (Boutet and others 2007) and NCBI nonredundant nucleic acid (nt) (Benson and others 2010) database entries using BLASTX and BLASTN (Altschul and others 1990), respectively. With an expected cutoff value of 10^{-20}, 189 transcripts did not have significant homology to any entry of the UniProtKB and nt databases, and those transcripts were annotated as having unknown functions. Only five transcript sequences significantly matched the *Phalaenopsis* database entries. By comparison, UniProtKB had only about 300 sequences from *Phalaenopsis* orchids. Our study therefore resulted in a tremendous increase in the number of published sequences extracted from species in the *Phalaenopsis* genus.

The *Arabidopsis* information resource (TAIR) database provides high-quality annotations of *A. thaliana* genetic information based on peer-reviewed publications (Swarbreck and others 2008). To further refine the transcript annotations, homologs were identified in the TAIR database, and the literature associated with each selected TAIR entry was used to complete the transcript annotations. The functional annotations of transcripts are detailed in Supplementary Table 1.

A classification of functional annotations is shown in Fig. 3 and was created using valid BLASTX hits in the MIPS *Arabidopsis thaliana* database (MAtDB) (Schoof and others 2004). According to this analysis, the upregulated transcripts are associated for 28% with metabolic processes ("metabolism" + "protein metabolism"), for 20% with responses to stress and stimuli ("response to stress" + "response to abiotic and biotic stimulus" + "signal transduction"), for 8% with cell growth processes ("development" + "cell organization and biogenesis"), and for 5% with the transport machinery. This analysis suggests that *Doritaenopsis* leaves respond to cold by modifying their metabolism, and this modification possibly results in the transport (import or export) of molecules. Some cell growth processes (growth or inhibition) also seem to be affected. The fact that a large part of the identified functions were related to metabolic processes prompted additional bioinformatics studies to identify pathways that were eventually upregulated.

Identification of Major Upregulated Metabolic Pathways

The plant metabolic network (PMN) database (Zhang and others 2005) and the Kyoto encyclopedia for genes and genomes (KEGG) database (Kanehisa and others 2008) were used to discover the main pathways activated by the upregulated transcripts. PMN provides common access to several databases specializing in model plants and was searched using BLASTX against its list of reference enzymes. The KEGG automatic annotation server (KAAS) (Moriya and others 2007) was used to search the manually curated KEGG entries of plant or eukaryote enzymes. Interestingly, the annotations provided by KAAS were nearly identical, whether using the plant sequence set or the KAAS preselected sequence set representative of eukaryote species. Functional annotations using PMN and KAAS were then compared to the annotations determined previously using UniProtKB, NCBI nt, and TAIR. Two entries were inconsistent with KAAS and PMN and were

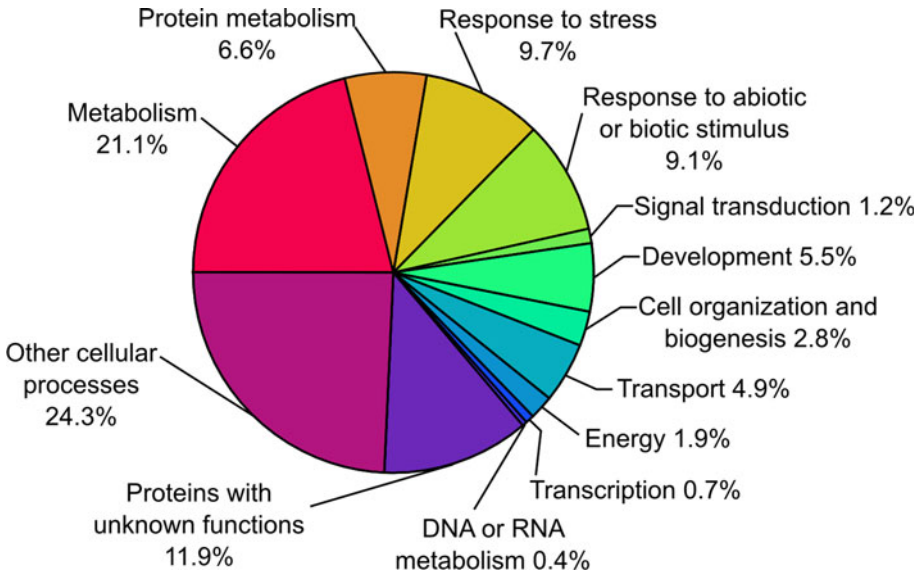

Fig. 3 Functional classification of differentially expressed genes during *Doritaenopsis* dormancy release. A subtractive hybridization (SSH) library was constructed using extracted cDNA from *Doritaenopsis* leaves during bud dormancy release and vegetative growth. The pie chart represents statistics on the functional classes predicted for each transcript using MAtDB (Schoof and others 2004)

discarded from the metabolic pathway analysis. Figure 4 and Supplementary Fig. 1 show an ensemble of metabolic reactions identified in PMN and KEGG and possibly upregulated in cold-treated *Doritaenopsis* leaves. The putative implications of 103 transcripts in the various metabolic pathways are shown in Supplementary Fig. 1. Additionally, 9 transcripts were identified as part of the spliceosome, 18 as part of ribosomes, 11 as part of the export machinery, and 30 as part of the electron chain transport in the mitochondria and the chloroplast thylakoids (Supplementary Table 1). As shown in Fig. 4, the main pathways predicted to be activated are the starch degradation and sucrose metabolism pathways, part of the glycolysis pathway, the C4 carbon fixation pathway, and the biosynthesis of fatty acids and lipids. Supplementary Fig. 1 also shows several other activated reactions that are connected to the main upregulated metabolic axes.

Transcriptional Changes of Selected Genes in Leaves During Floral Transition and in the Flower Stalks

Based on previous analyses, 13 transcripts were selected and their expression level compared by real-time RT-PCR at days 21, 28, and 35 following cold-induced bud dormancy release on day 0 (Fig. 5). In the period from day 21 to day 28, the bud dormancy release process is active (dormancy release phase), whereas at day 35, the bud is already growing (growth phase). The expression level of the transcripts was also measured in the immature flower stalk when it reached about 2–3 cm in length. The transcripts were chosen for their putative functions that could be related to different metabolism and cell regulation aspects: starch degradation was monitored through the evolution of a putative thioredoxin h (DhSL-296); sucrose synthesis and export through a fructose bisphosphate aldolase (DhSL-556), a sucrose synthase (DhSL-37), an UDP-glucose-6-dehydrogenase (DhSL-55), a vacuolar sugar transporter (DhSL-493), and a sucrose transporter (DhSL-60); genes associated with flower induction through an arginine N-methyl transferase similar to *A. thaliana* PRMT4B (DhSL-92), a transcript homologous to *A. thaliana* GIGANTEA (DhSL-112), and an histone H3 (DhSL-85); lipid synthesis through an acetyl-CoA carboxylase (DhSL-45); the photosynthetic machinery through a chlorophyll a/b-binding protein (DhSL-89); and genes associated with plant hormone synthesis through an homospermidine synthase (DhSL-93) and an IAA hydrolase (DhSL-467).

The transcripts associated with hormonal synthesis, that is, homospermidine synthase and IAA hydrolase, were continuously transcribed during dormancy release but their transcription dropped in the growth phase. These enzymes were also actively transcribed in the immature stalk. This suggests that hormones are produced in the leaves during dormancy release and that this production stops in the growth phase, during which the hormones are synthesized in the developing stalk. The transcription level of GIGANTEA and fructose bisphosphate aldolase increased during the dormancy release and continued to increase in the growth phase. Three transcripts associated with sucrose synthesis and transport—sucrose synthase, sucrose transporter, and UDP-glucose 6-dehydrogenase—showed only a slight decrease in transcription level during dormancy release and the following growth phase. Therefore, the production of sucrose in leaves seems to be globally maintained over the dormancy release and also in the growth phase. The vacuolar sugar transporter histone H3 and thioredoxin h, which is associated with starch degradation, had their highest level of transcription toward the end of the dormancy release. Interestingly, the transcription of chlorophyll a/b-binding is clearly upregulated in the growth phase compared to the dormancy

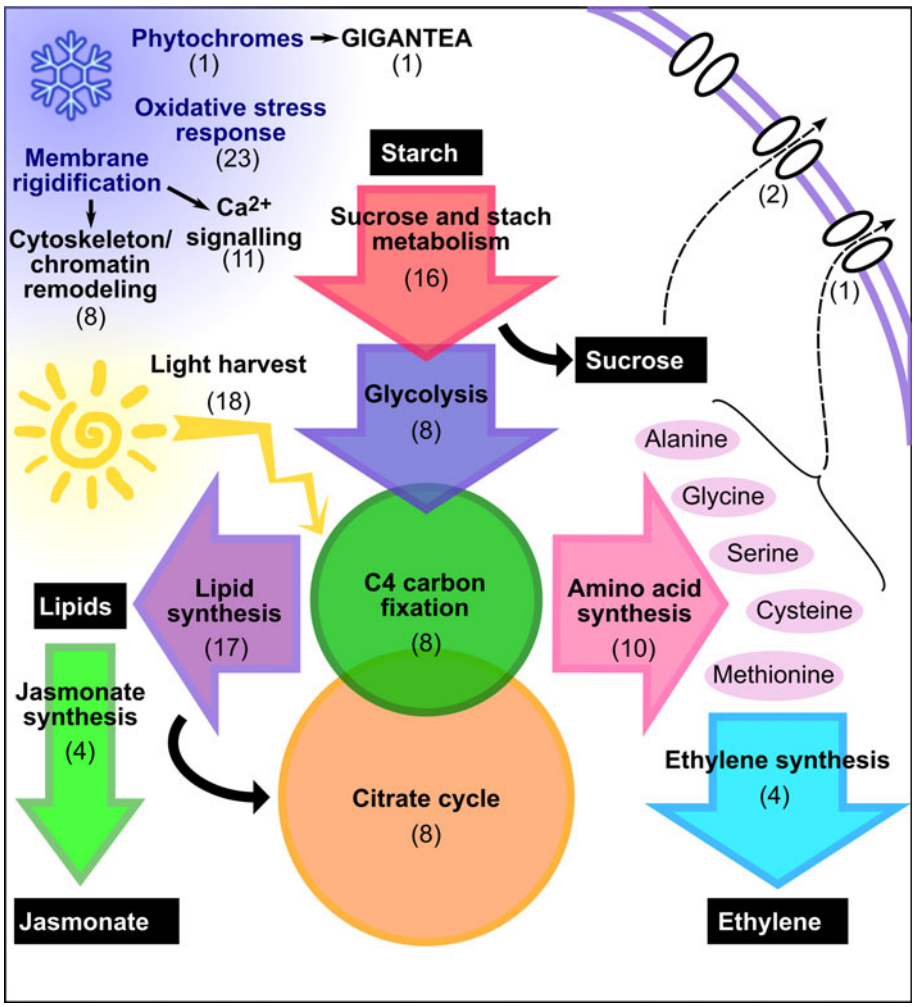

Fig. 4 Major metabolic pathways predicted to be upregulated in the leaves of *Doritaenopsis* during bud dormancy release and putative cold-sensing mechanisms. The number of transcripts that have been predicted to be associated with each pathway or sensing mechanism is given in parentheses. The metabolic pathways associated with transcripts specifically expressed during bud dormancy release in *Doritaenopsis* leaves were annotated using the KEGG (Kanehisa and others 2008) and PMN Plant Cyc (Zhang and others 2005) databases. The PMN Plant Cyc enzyme reference set was searched for homologous sequences to the transcripts and the corresponding pathways were identified in PMN. The KAAS annotation tool (Moriya and others 2007) was used to identify upregulated pathways in KEGG. Transcript accession numbers and their functional annotations are detailed in Supplementary Table 1. Supplementary Fig. 1 gives more details on the predicted metabolic reactions and the corresponding transcript identifiers for each metabolic pathway

release. These data suggest that starch degradation is slowed down in the growth phase, which is also shown in Fig. 2b, and, at the same time, photosynthetic synthesis of sugars is upregulated. Acetyl-CoA carboxylase, associated with lipid synthesis, has a minimal level of transcription at the end of the dormancy period. The transcription level of nine transcripts was significantly higher in the immature stalk ($p < 0.05$) than in any of the recorded expressions in the leaves. This possibly reflects the higher metabolic activity that could be expected in developing tissues than in plant organs, whose growth is inhibited. The three transcripts that had a lower transcription level in the immature stalk than in the leaves were fructose bisphosphate aldolase, chlorophyll a/b-binding protein, and vacuolar transporter. The flower stalks of *Doritaenopsis* are not photosynthetic organs, which explains the low level of chlorophyll. The metabolic consequences of the lower transcription level of DhSL-556 in the stalks compared to the leaves cannot be interpreted functionally because other transcripts with similar function, DhSL-423 and -556, have been isolated but their evolution has not be followed. The low expression of vacuolar sugar transporter in the stalks indicates that they may not store sugar in vacuoles and may instead be dependent on a constant supply.

Discussion

The molecular and metabolic consequences of a drop in temperature leading to reproductive bud dormancy release

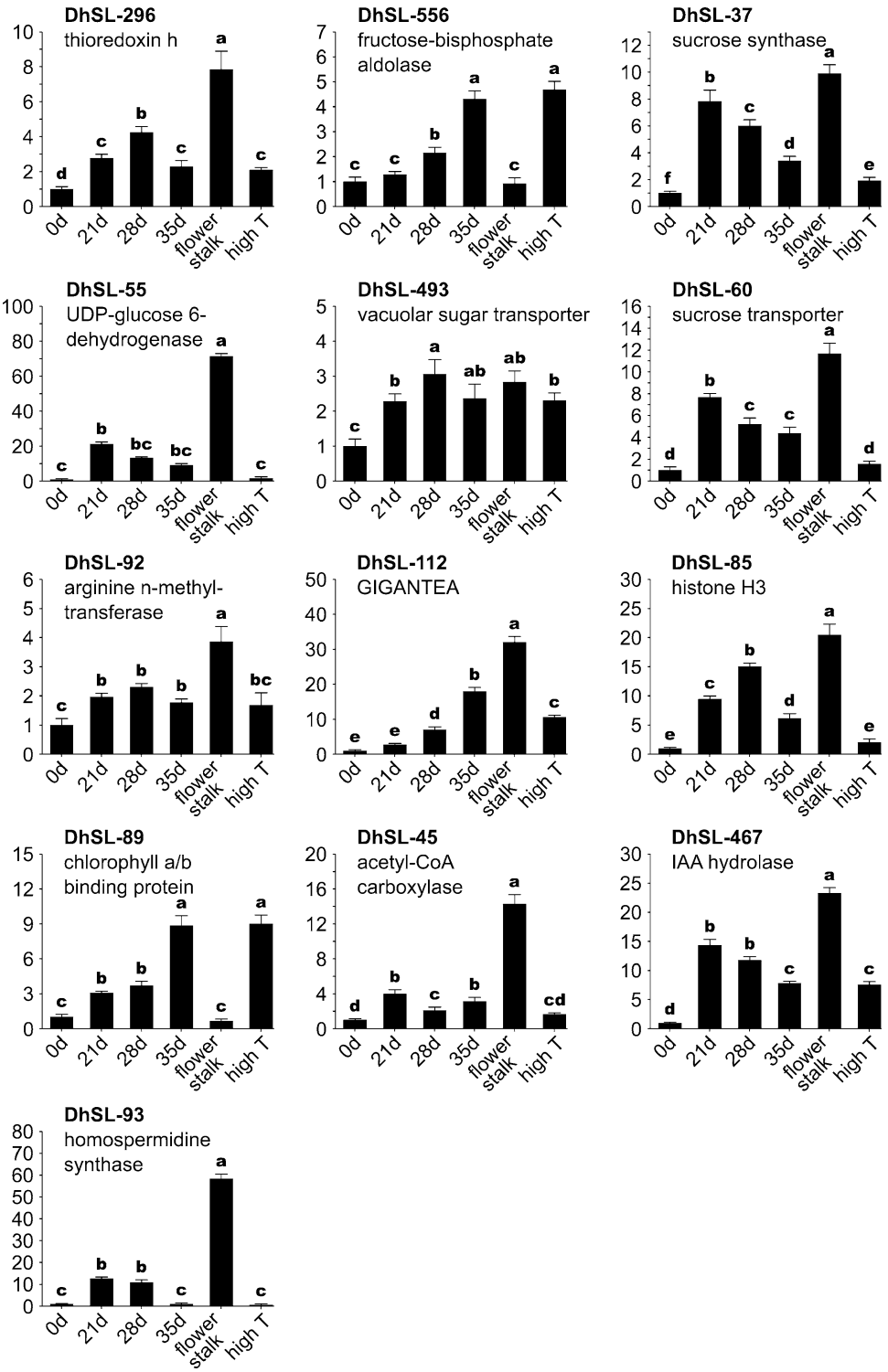

Fig. 5 Changes in the level of transcription of 13 genes during *Doritaenopsis* dormancy release. One transcript, DhSL-50, coding for an actin molecule, was used as an internal reference. Three replicates were carried out. The transcript quantifications at days 21, 28, and 35 in the flower stalks and at day 35 in the high-temperature group (high T) were calculated relative to the transcript level at day 0. For each graph, levels noted with the same letter are not significantly different according to Duncan's multiple comparison test ($p < 0.05$)

was investigated in the leaves of *Doritaenopsis*. During dormancy release, vegetative growth was decelerated and completely inhibited during the bud growth phase. Tremendous variation in sugar and starch content was also observed in the leaves. During the dormancy release phase, starch content decreased while sucrose concentration increased. A differential transcription analysis during dormancy release showed that an extended number of metabolic reactions were upregulated. The difference in transcription level of some transcripts during the dormancy release phase and growth phase and in the developing stalk suggested that some plant hormones may be produced during dormancy release and that leaves may provide sucrose to sustain bud development and growth. These

discoveries and hypotheses can be combined with knowledge of the molecular mechanisms in other plant species, especially *A. thaliana*, to begin to answer three key questions pertaining to the temperature-induced release of reproductive bud dormancy. First, how are temperature stimuli sensed by the leaves? Second, how are leaf cells responding to cold stress in the context of dormancy? And third, are some elements of leaf response to cold involved in the dormancy release of the buds?

Doritaenopsis Leaves Detect Temperature Changes

The mechanism by which plants monitor temperature changes is not well understood (Penfield 2008). Two main theories have emerged, implicating the effect of temperature on either membrane fluidity (Orvar and others 2000) or the electron transport chains in mitochondria and thylakoids (Suzuki and Mittler 2006). It has also been recently proposed that phytochromes can serve as temperature sensors because their photoequilibrium is sensitive to both light and temperature (Franklin 2009). Several elements possibly associated with these three theories were identified in our study, and the three corresponding mechanisms can potentially simultaneously influence dormancy release of *Doritaenopsis* reproductive buds.

Phytochrome A can integrate temperature and light signals Phytochromes A and B are two ubiquitous photoreceptors in plants and often have been associated with flowering time regulation (Amasino 2010). In our study, two upregulated transcripts, DhSL-112 and DhSL-336, are potentially associated with phytochrome A signaling. DhSL-336 has a high sequence identity with *A. thaliana* transcription factor PAT1, which mediates phytochrome A signal transduction (Bolle and others 2000). The sequence of DhSL-112 is homologous to *A. thaliana* GIGANTEA (GI), which has been studied extensively for its various roles in plant development, including in flower induction (Suárez-López and others 2001; Imaizumi and Kay 2006; Rubio and Deng 2007). GI is an evening component of the circadian clock and also influences the phytochrome A pathway (Oliverio and others 2007). In agreement with a possible role in cold-induced events in *Doritaenopsis* leaves, it has been shown in *A. thaliana* that GI expression increases when the temperature drops (Cao and others 2005) and that it plays an important role in seed germination (Penfield and Hall 2009).

Cold alters cell membrane fluidity Cold causes rigidification of lipid bilayers, which in turn provokes the opening of mechanosensitive calcium channels and a rearrangement of cytoskeleton (Sangwan and others 2001). It has been shown that cellular membrane rigidification controls the expression of a cold-inducible gene in *Brassica napus* (Sangwan and others 2001). This shows that a change in the mechanical properties of the cellular membrane is used by at least some plants as a sensor of temperature changes. Several transcripts were identified in our study of *Doritaenopsis* as potentially associated with calcium influx and signaling. Two transcripts whose proteins may be involved in the re-establishment of basal calcium concentration after calcium influx were upregulated in response to cold treatment: DhSL-525 was predicted to function as a calcium ATPase, and DhSL-70 was predicted to be a calcium/proton exchanger by sequence similarity with *A. thaliana* CAX1, which mediates the transport of calcium in tonoplasts (Catala and others 2003; Barkla and others 2008). The three main proteins that react to calcium are calmodulins, calcium-dependent protein kinases, and calcineurin B-like proteins (CBLs) (DeFalco and others 2010). One upregulated transcript in *Doritaenopsis*, DhSL-424, was predicted to function as a calmodulin. Other transcripts with a homolog in *A. thaliana* that were documented in TAIR to interact with a calmodulin and display a diverse range of functions include a protein of the proteasome 26S (DhSL-143), a protein of the photosystem I (DhSL-611), two elongation factors (DhSL-107 and DhSL-301), a peptidylprolyl isomerase (DhSL-548), and a chaperon (DhSL-585). CBLs are less well studied than the calmodulins (DeFalco and others 2010); they regulate the activity of plant-specific serine/threonine protein kinases (CIPKs), and the interactions of different CBLs and CIPKs have been proposed to modulate the cellular response to various external signals. Two predicted transcripts of CIPKs, DhSL-27 and DhSL-480, were induced by cold treatment. Additionally, in *A. thaliana*, a stress-activated CIPK was shown to target CAX1 (Cheng and others 2004), for which a close homolog, DhSL-70, was identified in our study.

The cold-induced change of mechanical properties of the cellular membrane may also cause a rearrangement of the cytoskeleton (Sangwan and others 2001). Modifications of the cytoskeleton require the destruction and creation of its structural components, and indeed some genes coding for actin and tubulin proteins were found in the SSH library. DhSL-50 and DhSL-561 transcripts encode actin molecules, which polymerize to form a network of filaments beneath the cellular membrane. Microtubules are the larger component of the cytoskeleton and are polymers of α-tubulin (DhSL-153, -508, and -519) and β-tubulin (DhSL-291 and -460) dimers. Changes at the membrane may also cause structural changes in the nucleus as the protein translated from the transcript DhSL-492 is homologous to *A. thaliana* SUN1, which is part of a complex that links cytoskeleton and nucleoskeleton (Graumann and others 2010).

Cold triggers plant oxidative stress response In mitochondria and thylakoids, a change of temperature disturbs the electron transport chain, resulting in the creation of reactive oxygen species, whose high reactivity can damage

cellular structures (Moller 2001). The oxidative stress response, which is activated to clear the reactive oxygen species, has been postulated to also serve as a mechanism to sense temperature changes (Suzuki and Mittler 2006). A number of transcripts were predicted in our study to be part of the oxidative stress response. Several genes coding for enzymes with peroxide-scavenging functions were found to be upregulated after cold exposure. They include some transcripts potentially coding for a glutathione-S-transferase (DhSL-348), some peroxidases (DhSL-72, -79, -273), a peroxiredoxin (DhSL-461), and two catalases (DhSL-61, -321). Other proteins implicated in the reduction-oxidation balance also had their gene upregulated, including thioredoxins (DhSL-100 and -296), a protein disulfide isomerase (DhSL-431), and a methionine sulfoxide reductase (DhSL-48). It has also been suggested that part of the protein-folding machinery could be upregulated by oxidative stress (Shapiguzov and others 2006). Indeed, two potential peptidyl-prolyl isomerases (DhSL-548 and -584), and ten chaperonin/heat-shock proteins (DhSL-96, -101, -159, -179, -284, -422, -488, -530, -551, and -585) were identified in our study.

Damage to the respiratory and photosynthetic systems should cause a rapid turnover of their molecular components. We indeed observed increased transcription of genes coding for proteins of the electron transport chain, listed in Supplementary Table 1, and for proteins specialized in their degradation. For example, DhSL-272 and DhSL-452 have high sequence similarity to *A. thaliana* FtSH1, which specifically catalyzes the proteolytic degradation of the D1 protein from photosystem II (Lindahl and others 2000). Another transcript, DhSL-121, was annotated as a Fe-S subunit of complex I of the electron transport chain in mitochondria, whose potential homolog in *A. thaliana*, FROSTBITE1, has been shown to be essential for cold stress response and for the induction of the oxidative stress response pathway (Li and others 2005). It is expected that DhSL-121 would be similarly essential for *Doritaenopsis* because DhSL-121 is the only upregulated transcript that was identified to be part of the mitochondrial complex I, whereas several redundant inorganic pyrophosphatases and elements of complex III are upregulated.

Another mechanism that may be adopted by *Doritaenopsis* to respond to photooxidative stress is the reduction in photosynthesis. This can be achieved by relocation of chloroplasts, and this mechanism is partly mediated in *A. thaliana* by the chloroplast outer membrane protein CHUP1 (DhSL-406), which binds actin filaments (Oikawa and others 2008). The synthesis of chlorophyll may also be downregulated in *Doritaenopsis* by DhSL-256, which has a sequence homologous to *A. thaliana* FLU (Meskauskiene and others 2001).

Metabolic and Epigenetic Modifications in *Doritaenopsis* Leaf Cells Coincide with Dormancy Release

Reproductive bud dormancy release in *Doritaenopsis* is associated with reduced vegetative growth and a modification of the starch/sucrose balance in the leaf. At the cellular level, those events coincide with the activation of several metabolic pathways, as shown in Fig. 4. Numerous genes related to biosynthesis of sucrose and amino acids, which are needed to sustain the growth of the buds, are predicted to be activated. An increase of temperature after dormancy break in *Doritaenopsis* does not stop floral development, which indicates that the flow of nutrients to the bud is preserved and becomes independent of temperature signals. Accordingly, we identified some transcripts that potentially indicate that the chromatin is modified, which is a mechanism able to durably alter gene expression pattern in leaves.

Doritaenopsis *leaves undergo epigenetic modification during the release of reproductive bud dormancy* The vernalization of *A. thaliana* involves chromatin remodeling, which occurs during extended cold periods corresponding to winter and renders the plant competent to flower in spring (He 2009). Dormancy release of *Doritaenopsis* presents some similarities with vernalization because a period of cold breaks dormancy and the subsequent flower development does not thereafter require low temperature to be maintained. Several elements associated with chromatin remodeling were identified in our study in the leaf. A previous study in potato had already shown that histone modifications occur in dormant organs during dormancy release (Law and Suttle 2004). To our knowledge, epigenetic changes in nondormant organs during dormancy release have never been described previously.

An indication that cold treatment causes chromatin modifications in *Doritaenopsis* is the upregulation of the transcript DhSL-246, which has a sequence homologous to *A. thaliana* FVE. FVE is an MSI1-like gene and a component of several complexes acting on chromatin (Hennig and others 2005). It has been implicated in the autonomous flowering induction pathway (He and others 2003). Known epigenetic modifications that control flowering induction consist of mainly modifications of histones and differential expression of histone variants, in particular H2A and H3 (He 2009). Accordingly, we found that the upregulated transcripts DhSL-85 and DhSL-337 encode for histones H2A and H3, respectively. Additionally, the transcript DhSL-92 has a sequence homologous to *A. thaliana* PRMT4B, which is involved in methylation of histone H3 and has been shown to influence flowering time (Niu and others 2008).

Cold-treated Doritaenopsis *leaves produce sucrose and amino acids* Developing and maintaining a flower is metabolically expensive, and *Doritaenopsis* leaves may support bud growth by releasing both sucrose and amino acids in the phloem. The differential transcriptome analysis showed simultaneous activation of the starch degradation and sucrose biosynthesis pathways, and therefore sucrose seems to be mobilized from starch, which is a mechanism already observed during the flower induction of *Sinapis alba* (Bernier and others 1993). DhSL-211 has a nucleic acid sequence homologous to *A. thaliana* SEX1, which phosphorylates glucose residues in amylopectin, one of the two molecules that constitute starch (Yano and others 2005). Phosphorylation improves the solubility of starch granules, which are consequently more accessible to degradation enzymes (Lloyd and others 2005). In *A. thaliana*, SEX1 has been shown to have an important effect on flowering time (Corbesier and others 1998). Amylopectin is a polymer of glucose residues that are linearly linked with α-1,4 bonds and irregularly branched by an α-1,6 bond. The starch-debranching enzyme pullunase specifically cleaves the α-1,6 linkages, and its enzymatic activity is increased four times by wheat thioredoxin h (Cho and others 1999), for which two transcripts with homolog sequences, DhSL-100 and DhSL-296, were upregulated in our study. The transcripts of several potential sugar transporters were also identified. Vacuolar sugar transporter (DhSL-493) mediates the translocation of glucose from the chloroplast stroma to the cytosol, where it can be further processed into sucrose. Potential sucrose transporters DhSL-60 and DhSL-527, both of which are homologous to *A. thaliana* SUT2, allow sucrose to be translocated through the cellular membrane into the phloem, where sucrose is passively transported to sink tissues (Imlau and others 1999).

As shown in Fig. 4, the transcription of some components of the biosynthesis pathways of several amino acids, including alanine, cysteine, glutamate, glycine, lysine, methionine, and serine, were upregulated. Additionally, DhSL-403 encodes a protein similar to *A. thaliana* bidirectional amino acid transporter BAT1, which has been suggested to take part in the transport of amino acids to sink tissues (Dündar and Bush 2009). Therefore, in response to cold treatment, *Doritaenopsis* leaves may become both a source of energy and of amino acids for the developing reproductive bud.

Doritaenopsis leaves communicate with other organs on their dormancy status using hormones We previously discussed how *Doritaenopsis* leaves may detect and be affected by temperature changes, but not how the information could be communicated to the rest of the plant and, more specifically, to the dormant buds. Sucrose could a priori be considered as a messenger of dormancy release as sucrose alone can induce flowering of *S. alba* (Bernier and others 1993). In the case of *Doritaenopsis*, sucrose is unlikely to play this role because it was shown in *P. amabilis*, a parent of *Doritaenopsis*, that sucrose cannot reach reproductive buds without hormonal activation (Chen and others 1994). Several plant hormones exist and their complex interplay regulates different aspects of plant development (Jaillais and Chory 2010). In our study of *Doritaenopsis*, several transcripts possibly associated with the synthesis and export of two positive-growth hormones, auxin and ethylene, were upregulated by cold treatment, and those hormones may be responsible for the release of reproductive bud dormancy. Additionally, the hormones abscisic acid and jasmonate are potentially involved in the reduction of *Doritaenopsis* vegetative growth.

Auxin and ethylene potentially induce Doritaenopsis *bud dormancy release* Auxin, or indole-3-acetic acid, plays an important role in the transition from dormancy to growth in the bud (Horvath and others 2002). It has a dual role because at low concentrations auxin promotes cell expansion, whereas at high concentrations it promotes cell division (Chen and others 2001a, b). In our study we found that genes involved in auxin synthesis and export are upregulated, and we postulate that leaves can influence bud growth using this hormone. Two transcripts, DhSL-466 and DhSL-467, are homologous to *A. thaliana* NIT4 (Bartel and Fink 1994) and IAA hydrolase (Rampey and others 2004), which synthesize auxin from indole-3-acetonitrile and amino acid amide-linked auxin, respectively. After synthesis, auxin is transported by PIN cargos (Schrader and others 2003). PP2A, which is homologous to DhSL-5, is a serine/threonine phosphatase that regulates PIN targeting (Michniewicz and others 2007) and has been shown to respond to abiotic stress (País and others 2009). *A. thaliana* AVP1, which is homologous to DhSL-176, is a proton-pyrophosphatase that also affects the expression of PIN molecules (Li and others 2005). DhSL-62 and DhSL-188 are homologous to *A. thaliana* SMT1 and SMT2, respectively, which are implicated in the synthesis of sterols (Carland and others 2010). Sterols mediate the cellular asymmetric localization of PIN molecules, which creates the cell polarity required for auxin export (Men and others 2008). SMT2, also called FRIGIDA-LIKE-1, is involved in the *A. thaliana* vernalization mechanism (Michaels and others 2004). Additionally, ten transcripts that possibly code for elements of the secretory machinery are upregulated at cold temperatures and are listed in Supplementary Table 1.

Auxin seems to have a regulatory effect in cold-treated *Doritaenopsis* leaves because two potential auxin-dependent transcription factors, DhSL-453 and DhSL-592, were found in our study (Gao and others 2004; Wang and others 2007). DhSL-453 is homologous to *A. thaliana* SCL21, which regulates the expression of histone

deacetylases and is therefore potentially involved in chromatin remodeling (Gao and others 2004). *A. thaliana* UCH2, which is homologous to DhSL-53, has been involved in protecting proteins involved in auxin signaling from ubiquitin-mediated degradation (Yang and others 2007).

Ethylene causes cell enlargement and has been associated with predormancy growth and dormancy release (Ophir and others 2009). For instance, ethylene was shown to be important for birch buds to set (Ruonala and others 2006), for the release of grape bud dormancy (Ophir and others 2009), and for the germination of sunflower seeds (Oracz and others 2009). In our study, four upregulated transcripts, DhSL-72, -150, -216, and -421, have been predicted to code for enzymes of the biosynthesis of ethylene from methionine. Ethylene and auxin are therefore potentially synthesized by *Doritaenopsis* leaves during bud dormancy release.

Abscisic acid and jasmonate potentially reduce Doritaenopsis *vegetative growth* How can *Doritaenopsis* leaves have their growth reduced and at the same time produce auxin and ethylene, which are positive-growth hormones? An important clue is provided by the transcript DhSL-224, which has a high sequence homology with *A. thaliana* BLH4, a transcription factor that inhibits leaf growth by repressing KNOX genes (Kumar and others 2007). Mechanisms using repression of developmental genes are often coordinated by hormones, and indeed transcripts associated with two hormones that cause growth inhibition, abscisic acid and jasmonate, were identified in our study.

In poplar, an increase in abscisic acid levels is correlated with the repression of bud cell proliferation that characterizes the entry to dormancy, and after reaching the dormant state, abscisic acid levels are low in the bud (Ruttink and others 2007). It was also shown that the level of abscisic acid in the *Phalaenopsis hybrida* leaves is slightly higher after bud break than during the dormant stage (Wang and others 2002). No gene associated with abscisic acid synthesis was identified in our differential transcription analysis, but one transcript, DhSL-435, shares high sequence homology with *A. thaliana* PDR12, which encodes for an ATP-coupled transmembrane receptor that mediates abscisic acid uptake (Kang and others 2010). Another *A. thaliana* gene, NPX1, which has high sequence homology to DhSL-289, downregulates enzymes of the abscisic acid synthesis pathway (Kim and others 2009). In *Doritaenopsis* abscisic acid may therefore be imported by cells within the leaf, which contributes to inhibiting their growth. The source of abscisic acid in the leaves during the release stage remains to be elucidated.

Jasmonate, or jasmonoyl-1-isoleucine, is a hormone that causes growth inhibition, and it has been implicated in several processes in *A. thaliana* and other plants (Katsir and others 2008; Acosta and Farmer 2010). An acyl-CoA oxidase (DhSL-161), two lipoxygenases (DhSL-326 and DhSL-451), and one jasmonate-amido synthetase (DhSL-362, homolog to *A. thaliana* JAR1) are essential enzymes for the biosynthesis of jasmonate and were found to be upregulated in our study. JAR1, which catalyzes the last step of jasmonate synthesis, has its expression under the control of auxin (Hsieh and others 2000). Jasmonate is therefore likely to be synthesized in the leaves, potentially in response to an increase in auxin.

Other hormones Two classes of plant hormones, cytokinins and gibberellin, are well documented for their involvement in plant growth (Jaillais and Chory 2010); however, we did not identify any transcripts specifically associated with either class. Cytokinins and auxin coordinate their activity to maintain a balance between differentiation and division of meristem cells (Nordström and others 2004). A study of flower induction in *S. alba* has shown that in response to signals sent by leaves, cytokinins were produced by the roots, which were subsequently transported in the shoot meristem (Bernier and others 1993). In the case of *Doritaenopsis*, auxin could therefore be produced by the leaves, while cytokinins could be synthesized in either roots or another organ.

Gibberellin has been shown to play a decisive role in bud dormancy release in *P. amabilis*, another orchid from the *Phalaenopsis* genus (Chen and others 1994). Treatment with gibberellin-3 was shown to break bud dormancy despite maintaining the plant at 30°C. Interestingly, in our study we did not find upregulation of genes involved in gibberellin synthesis. Because endogenous expression of gibberellin can be auxin-dependent (Ross and others 2000; Wolbang and others 2004), we postulate that reproductive bud cells can produce gibberellin molecules after receiving an auxin signal produced by the leaves. It has also been shown in *Phalaenopsis* that gibberellin-3 was required to allow the transit of sucrose from leaves to bud (Chen and others 1994). Based on this observation and on other elements discovered in our study, a working model for *Doritaenopsis* bud dormancy release can be proposed: (1) cold and light signals are first integrated in the leaves, which produce auxin, ethylene, and fructose; (2) auxin and ethylene activate the bud, which subsequently synthesizes gibberellin; and (3) gibberellin allows sucrose to reach the bud and to support floral development. This working model can form the basis for further investigations to understand the molecular events associated with reproductive bud dormancy.

Comparison to vernalization/cold acclimatation in wheat Wheat and orchids are monocot plant species, but, contrary to orchids, wheat uses vernalization to control flowering time. The cold response of wheat leaves was recently investigated at the proteome and transcriptome levels (Sarhadi and others 2010; Winfield and others 2010;

Rinalducci and others 2011). Upon cold exposure, the sucrose and starch contents in wheat leaves (Zeng and others 2011) follow the same tendency as those measured in orchid leaves during bud dormancy release. Two differentially expressed sucrose synthases (DhSL-376 and DhSL-574) and one fructose-bisphosphate aldolase (DhSL-556) in orchids have close homologs positively regulated by cold in wheat (Sarhadi and others 2010; Winfield and others 2010; Zeng and others 2011). Wheat and orchids both seem to activate similar sucrose biosynthesis pathways but probably for a different purpose, as simple sugars potentially act as cryoprotectant in wheat (Winfield and others 2010) but are probably produced by tropical orchids to sustain bud growth.

The cold-sensing mechanism used in wheat leaves is not well understood (Winfield and others 2010). The same category of oxidative stress defense proteins and genes (protein disulfide isomerase, peroxidase, peroxiredoxin, and glutathione) and of calmodulins and calmodulin-binding proteins are differentially expressed in wheat and orchids (Sarhadi and others 2010; Winfield and others 2010; Rinalducci and others 2011). Some metabolic pathways seem to be similarly activated in wheat, including the citric acid cycle (isocitrate dehydrogenase, malate dehydrogenase) and the metabolism of lipids (acyl-carrier, acyl-CoA binding) (Rinalducci and others 2011). Dehydration-related genes, coding for aquaporins and dehydration-responsive proteins, are also upregulated in both orchids (this study) and in wheat (Winfield and others 2010).

Major differences can be identified between the cold response in *Doritaenopsis* leaves and wheat leaves. No close homolog to vernalization-specific genes can be detected in our study. More specifically no genes homologous to VRN1 genes, COR genes, or WRKY transcription factor genes that are associated with cold in wheat were found (Sarhadi and others 2010; Winfield and others 2010; Rinalducci and others 2011). The VRN1 gene and specific COR and WRKY genes have been found in different plant species that use vernalization and cold acclimatation (Berri and others 2009; Greenup and others 2009), and their absence in our study is significant. Other differences include the upregulation of annexins and germin-like proteins in wheat (Winfield and others 2010). Some elements of our study support the potential activation of the ethylene and jasmonate synthesis pathways, which have never been associated, to the best of our knowledge, with cold induction of vernalization or cold acclimation in wheat.

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