Necessity of high temperature for the dormancy release of *Narcissus tazetta* var. *chinensis*

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\textbf{A B S T R A C T}

Winter dormancy has been extensively studied in many plants, while much less information is available for summer dormancy. *Narcissus tazetta* var. *chinensis* is characterized by a prolonged period of summer dormancy during the annual cycle. In the present study, we characterized the nature of dormancy in the controlled growth conditions and investigated the effects of temperature and ethylene on dormancy release. Cessation of growth and senescence of aerial tissues occurred even under conditions favorable for growth, suggesting an endo-dormancy process. Bulbs failed to sprout when they were exposed to low storage temperatures, while high temperature treatment preceding low storage temperatures, or heating interruption during low storage temperatures, could make bulbs sprouting. These results suggest that high temperatures are necessary for endo-dormancy release. Ethylene application during a later storage stage showed an obvious accelerative effect on bulb sprouting, whereas ethylene application during the middle stage had no effect on sprouting. The biological progression of dormancy was further studied through cytological and physiological analyses. Under natural conditions, the ethylene level was reduced to trace amounts with the transition and progression of dormancy. A transient peak in ethylene release was found when the plugged plasmodesmata (PD) began to re-open and flower initiation began. The most common PD possessed electron-dense deposits in endo-dormancy. These data indicate that endo-dormancy ends when flower initiation begins and ethylene peak occurs. Ethylene application had no effect on dormancy release, while it hastened sprouting only after the release from endo-dormancy by high temperature.

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\textbf{Introduction}

Dormancy is an adaptive response that evolves from the environment of origin of various species, enabling their survival during threatening seasons (Lang et al., 1987). While this behavior has been extensively studied in plant species that experience severe winter conditions, perennial plant species that survive summer drought have not been given much attention. Plant species exhibiting summer dormancy usually inhabit semi-arid regions with a Mediterranean type of climate. Such plants are characterized by a period of intensive growth and flowering during mild weather, rainy winter, and spring, followed by a prolonged rest period during the hot and dry summer (Ofir and Kigel, 2006). This resting stage increases the probability of plant survival during summer, allowing future growth and reproduction (Rinne et al., 2001; Phillips, 2010; van der Schoot and Rinne, 2011).

The definition of eco-dormancy as conditional dormancy or facultative growth suspension dormancy has been controversial (Rees, 1981; van der Schoot et al., 1995). However, the definition of endo-dormancy as the most stable trapped state of the meristem even under conditions conducive to growth has gained common acceptance (van der Schoot, 1996; Rinne et al., 2001; Horvath et al., 2003; Volaire and Norton, 2006; Rohde and Bhalerao, 2007). The strategy of eco-dormancy is favored where summer conditions are unpredictable, whereas endo-dormancy is advantageous in habitats where season conditions are predictable (Vaughton and Ramsey, 2001). The morphogenetic activity of the shoot meristem (SM) that changes during winter dormancy cycling in wood species is reflected by changes in cell–cell networking, including symplastic pathways created by plasmodesmata (PD) (Rinne et al., 2001, 2011; van der Schoot and Rinne, 2011). During endo-dormancy, the SM assumes a state of self-arrest by sealing off all PD at its orifices with callose-containing dormancy sphincter complexes (DSCs) and impregnating cell walls with as yet unidentified substances that

\textit{Abbreviations: SM, shoot meristem; PD, plasmodesmata.}

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impede the movement of water, water-soluble ligands, and other molecules (Rinne and van der Schoot, 1998; Rinne et al., 2001). DSCs are composed of an extracellular callose ring and an intracellular cytoplasmic plug built inside the PD entrance around the internal macromolecular complex. They can be inspected as electron-dense deposits by transmission electron microscopy (Rinne et al., 2001). Formation of DSCs on PDs impairs intrinsic signaling networks that integrate cellular functions and sustain SM behavior, resulting in a dormant state no longer reversible by growth-promoting conditions (Rinne et al., 2001). When the SM is released from endo-dormancy, PDs are restored by the breakdown of plasmodesmal DSCs (van der Schoot and Rinne, 2011). Cellular changes that appear in SM cells during the annual cycle of the plant exhibiting summer dormancy are unknown.

Considerable variation is present within multiple species regarding the timing of the onset and release of bulb summer dormancy (Phillips et al., 2008, 2010). Differences in the timing of the onset of dormancy within species are habitat-correlated and likely tied to differences in temperature, photoperiod, and/or soil moisture (Kamenetsky and Rabinoswitch, 2006; Phillips et al., 2008, 2010). Based on the limited literature available on bulb dormancy release, a period of low temperatures is required for breaking bulb dormancy in some species, such as Allium acuminatum, Allium brandegei, and Allium passyei (Phillips, 2010), whereas hot treatment stimulates dormancy release in Allium schoenoprasum (Folster and Krug, 1977). Many other aspects of dormancy, such as commonalities or variations in summer dormancy induction and release, require further study in many species.

Chinese narcissus (Narcissus tazetta var. chinensis) is a plant from family Amaryllidaceae that exhibits dormancy for approximately five months from late May to the end of September. Plants grow actively during winter and early spring. Aboveground parts of the plants senesce in late spring and early summer. Dormant bulbs are usually harvested and stored during the hot summer. Florenogenesis is initiated within larger sized bulbs during summer dormancy, and high summer temperatures trigger transition of the bulb SM from the vegetative to the reproductive stage (Noy-Porata et al., 2009). Compared with the abundant information on flowering, less knowledge about dormancy in narcissus is available (Kamenetsky, 2009). Different temperatures, photoperiods, and/or soil moisture stimuli induce dormancy release in different species (Kamenetsky and Rabinoswitch, 2006; Phillips, 2010; van der Schoot and Rinne, 2011). Hormonal control, which involves a gradual increase in the ratio of sprouting promoters to inhibitors, may underlie the loss of dormancy with time (van der Schoot and Rinne, 2011). Numerous reports on the effect of ethylene on breaking the dormancy of geophytes are available (Masuda and Asahira, 1980; Bulfer, 2009; Sutcliffe, 2009). Notwithstanding the conflicting scientific reports on its effects, the real function of ethylene on dormancy release is related to the application duration, conditions, or application timing (Sutcliffe, 2009). In agricultural production, ethylene is often used to advance narcissus flowering. However, the specific role of ethylene and other environment factors in the regulation of dormancy and sprouting of narcissus bulbs remains unknown.

In the present study, controlled growth conditions were adopted over three years to determine whether dormancy is imposed (eco-dormancy) or physiological (endo-dormancy) to address the cardinal question about the nature of summer dormancy in Chinese narcissus. Different temperature regimes were designed and combined with ethylene applications to address how dormancy is released and ascertain which environmental conditions and whether or not ethylene affects this process. Additionally, ethylene production during the annual cycle of Chinese narcissus was measured. The annual cycle was also analyzed at the cytological and physiological levels. This study not only ascertained the endo-dormant nature of Chinese narcissus but also showed that endo-dormancy ended in early August when flower initiation began. Heat treatment was necessary for the release of endo-dormancy, whereas ethylene hastened sprouting rather than release endo-dormancy.

Materials and methods

Plant materials and growth conditions

Narcissus tazetta var. chinensis bulbs were commercially obtained from Chongming, Shanghai, China. Healthy bulbs with similar sizes (one-year-old bulbs with 5 ± 1 cm circumference and three-year old bulbs with 15 ± 1 cm circumference) were grouped and stored in natural or controlled conditions. A dry and ventilated warehouse with ambient light and temperature was used as the natural conditions. The average temperatures in Shanghai are 20.8, 25.0, 29.2, 28.5, and 25.1 °C in May, June, July, August, and September, respectively. One-year-old and three-year-old bulbs were used as materials for ethylene measurement and dormancy release assays, respectively, to distinguish changes during the dormant state from those during flower differentiation.

Determining the nature of dormancy

Chinese narcissus plants were grown in a controlled greenhouse with favorable conditions, specifically, 10 h/14 h light/dark photoperiod at 20 °C and 70% humidity, to determine whether the nature of summer dormancy in the plant species is imposed (eco-dormancy) or physiological (endo-dormancy). Tests were conducted over three consecutive years.

Treatment method to break dormancy

Bulbs were stored under natural conditions and planted on different dates, specifically, 25 July, 15 August, 1 September, and 15 September (Nos. CK1, CK2, CK3, and CK4 in Fig. 1A), and sprouting rates were recorded to analyze differences in the release of dormancy between different bulbs. The experiment was repeated three times in the year of 2006, 2007 and 2008 respectively.

The effects of natural temperature, high storage temperature (30 °C), and low storage temperature (15 °C) (Nos. CK, 1, and 2 in Fig. 2A) on bulb sprouting were analyzed to determine whether high or low temperatures favor the release of dormancy. To test the effect of heating before or during low-temperature storage on sprouting, the treatment of 30 °C for 20 d with storage at 15 °C for 60 d (No. 3 in Fig. 2A), and the treatment of 15 °C for 60 d with heating at 30 °C for 20 d, and followed by 15 °C for another 30 d (No. 4 in Fig. 2A), were then conducted.

Results of storage at natural temperature with or without ethylene application just before planting (Nos. 5 and CK in Fig. 3A) were compared to determine the effect of ethylene on bud sprouting. Bulbs were initially subjected to high temperature (30 °C) for 40 d and then stored at room temperature until planting. Ethylene was applied either after high temperature treatment or prior to planting or not at all (Nos. 6, 7, and 8 in Fig. 3A) to measure the effects of the timing of ethylene treatment on sprouting rate. Three-year-old bulbs were used here and the experiment was conducted in triplicate with independent materials.

Temperature-controlled incubators were used for different temperature treatments. For ethylene treatment, bulbs were incubated for 8 h in 20 mg L⁻¹ Ethrel daily, and then dried at room temperature. The treatment lasted for 3 d. Unless otherwise stated, samples in each treatment were comprised of at least 40 bulbs. The detailed methods are illustrated in the figures. After treatments, all bulbs were planted in plastic pots (20 cm height, 15 cm diameter) filled with similar quantities of substrates (75% vermiculite, 10% perlite,
and 15% clay) in a controlled greenhouse with a short photoperiod (10 h/14 h light/dark) at 18–20 °C and 70% humidity. The salt solution of the macro-element in 1/5 Murashige and Skoog medium was applied in the soil bi-monthly after planting. The sprouting proportion was recorded every 4 d after planting.

Ethylene measurements

The ethylene level in the whole plant was measured at different developmental stages by sealing four plants or bulbs (during transition into dormancy) in airtight jars for 12 h at 22–23 °C, after which a 1 mL sample of the headspace was obtained and injected into a Hewlett-Packard 5890 series II gas chromatograph equipped with a flame ionization detector GC9800 (Guangzhou, China). Ethylene levels in bulbs were measured twice every month during the time course of dormancy. In the present study, data were presented as the mean of at least three samples of three-year-old plants (μL h⁻¹ g⁻¹ fresh weight).

Light and transmission electron microscopy

Shoot meristems (SMs) of three-year-old bulbs were collected weekly since their harvest. Apices were dissected under an anatomical lens as rapidly as possible, fixed in formaldehyde–acetic acid solution composed of 63% ethanol, 5% formaldehyde, and 6% acetic acid) at 4 °C overnight, and then dehydrated in an ethanol series. Median longitudinal paraffin sections (7 μm thick) were stained with 1% toluidine blue and investigated microscopically (Rinne et al., 2001).
Fig. 3. Effects of ethylene combined with different temperature regimes during storage on bulb dormancy release. (A) Schematic illustration of the different storage treatments of narcissus bulbs, in which temperature regimes combined with ethylene application are shown just below the corresponding date on top. Continuous lines represent periods of 22–25°C. (B and C) Effects of ethylene application at different times on sprouting rate. Detailed treatment regimes in (B) and (C) are shown in (A). Different lowercase letters indicate significant differences between different treatments at the given time point (Pearson’s Chi-square, χ²; P<0.05, n=30–50). (D) Ethylene release in bulbs at different times. Mean values are shown; vertical bars represent standard deviations and different lower-case letters indicate significant differences between values (P<0.05).

For transmission electron microscopy, SMs in one-year-old bulbs were collected every 2 weeks during their annual cycle and fixed in dual fixation solution. Samples were fixed for 4 h at 4°C in 2.5% (v/v) glutaraldehyde in 200 mM phosphate buffer (pH 7.4). The fixed tissue was washed in buffer and post-fixed overnight at 4°C in 1% (w/v) OsO₄ and then dehydrated in a graded ethanol series (Rinne et al., 2001). Samples were embedded in Spurr’s resin (Sigma). Ultra-thin sections (70 nm) were obtained from median longitudinal positions with an ultramicrotome (Leica, Nestzlar, Germany), stained with 2% aqueous uranyl acetate and Reynolds’ lead citrate, and then examined with a JEOL 1200 EXII electron microscope at 80 kV (Tokyo, Japan).

Fig. 4. The metabolism in narcissus bulbs showing dynamic changes. Transmission electron microscopy images of shoot apical cells during (A) active growth, (B) dormancy, and (C) dormancy release are shown. Amyloplasts are indicated by arrows, and N represents the nucleus. Scale bars = 1 μm. (D) The soluble sugar content in bulbs during dormancy progression. Mean values are shown; vertical bars represent standard deviations and different lower-case letters indicate significant differences between values (P<0.05).
The Results

17.0

Statistical analysis

Statistical analyses were performed with SPSS Statistics version 17.0 software. Pearson’s χ² test, ANOVA, and Student’s t-test were used to detect significant differences.

Significance of high-temperature treatment for dormancy release

The dormancy nature and variation in the release of bulb dormancy

In the three-year study, cessation of growth and senescence of aerial tissues occurred even under conditions favorable for growth, suggesting an endo-dormancy process. The later the planting date, the higher the sprouting percentage was during the same period starting from the time of planting (Fig. 1A and B). Up to 50% of the bulbs planted on September 15th sprouted within 25–30 d, and all narcissuses sprouted within 40 d. Statistical analysis showed no significant differences between bulbs planted on August 15th and September 1st. However, the sprouting percentages of bulbs planted on August 15th and September 1st at 28, 32, 36, 40, 44, and 48 d post planting were significantly different from those planted on September 15th. For bulbs planted before September 1st, 25.5 ± 2.5 d were required to reach 10% sprouting percentage, 36.3 ± 2.9 d were needed for half-number sprouting, and 53.3 ± 2.1 d were necessary to obtain the maximum sprouting percentage.
natural temperature. Thus, high temperatures appear to be necessary for bud sprouting.

**Ethylene treatment on bulb dormancy release**

Bulbs supplied with ethylene just before planting reached 10%, 50%, and final sprouting percentages significantly earlier than those without ethylene (No. 5 versus No. CK, Fig. 3B). The application of ethylene at different time intervals also had different effects on bulb sprouting. When ethylene was applied in the middle of the storage period, no significant difference in bulb sprouting rate was observed compared with those without ethylene application (No. 6 versus No. 7, Fig. 3C). However, when postharvest bulbs were subjected to ethylene treatment prior to planting, sprouting was advanced most significantly (No. 6 versus No. 8, Fig. 3C).

**Dynamic changes in the ethylene levels in the narcissus bulbs**

To discover the real role of ethylene treatment on the dormancy release, the ethylene level changes during the annual cycle were analyzed. Changing trends in the ethylene levels in both one-year-old (data not shown) and three-year-old bulbs were similar. When aboveground parts changed from active growth to wilting (i.e., from April to May before harvest), the ethylene level was reduced. Almost no ethylene could be detected in June. The ethylene level was increased gradually in July and a transient peak was observed in early August (Fig. 3D).

**Growth-cycle and dynamic changes in the sugar levels in the narcissus bulbs**

Soluble sugar content and the endocytes in the SM cells were monitored every month to determine the end of endo-dormancy. Amyloplasts in SM cells became larger with the transition to dormancy from active growth (i.e., from March to late May before harvest). The largest number and size of amyloplasts appeared in June and July (Fig. 4A and B). Amyloplasts began to decrease in number and size by the beginning of August (Fig. 4C). Consistent with this observation, the soluble sugar content in SM cells decreased to minimal levels in June and July (Fig. 4D).

Flower transition occurred in late July under natural conditions. Before mid-July, the longitudinal cut of the SM in three-year-old bulbs was sharp and cylindrically cone-shaped. The height of the SM was longer than its width, a typical characteristic of vegetative growth, and only the leaf primordia were differentiated around its edge (Fig. 5A). In late July, the longitudinal cut of the AM began to change into a flat shape, and its width gradually became longer. A spathe inflorescence formed, and flower meristems began to initiate in early August (Fig. 5B–D). Floral organ primordia soon initiated, and several flowers formed in one spathe inflorescence, with four whole organs forming within some of the top flowers by mid-August (Fig. 5E and F). In late August, the development of ovules began, and fully developed flower buds formed in early November with 3 to 8 flowers in an inflorescence (Fig. 5G–I).

The morphology and substructure of PDs in SM cells of one-year-old bulbs were inspected continuously for 12 months. Dynamic changes in the presence of electron-dense deposits in close association with the collar and neck region of PDs during the annual cycle were noted. From September (i.e., when the bulbs were planted and the SM was actively proliferating) to May, most PDs exhibited straight channels without obvious electron-dense deposits (Fig. 6A). Some PDs possessing electron-dense deposits began to appear from late May to early June. Electron-dense deposits were often in close association with the collar, resulting in PDs with funnel-shaped neck regions. In June and July, the most common PDs possessed electron-dense deposits (Fig. 6B and C). The number of PDs with funnel-shaped neck regions decreased gradually from August and then vanished completely by September (Fig. 6D).
Discussion

High temperatures are necessary for endo-dormancy release in Chinese narcissus

The onset of endo-dormancy is controlled by internal physiology, not by external conditions (Lang et al., 1987; Volaire et al., 2009). The summer dormancy in Chinese narcissus should be a distinct developmental stage, that is, endo-dormancy, given that growth arrest is maintained even during conditions favoring growth.

The summer dormancy of Chinese narcissus is activated gradually by summer heating. The reported critical growth temperature is 25 °C (Lin, 2002). In Shanghai, the mean temperature is 25.0–29.2 °C in June, July, and August. Under the present experimental controlled conditions, constant temperatures without day and night temperature differences were designed. The data showed that storage at 15 °C resulted in failure of bulb sprouting, whereas heating interruption during low-storage temperature or heating preceding low-storage temperature eventually induced sprouting (Fig. 2C). These results suggest that high temperatures are necessary for endo-dormancy release.

The time of the end of endo-dormancy in Chinese narcissus could be estimated by physiological and cytological changes. The maximum size of amyloplasts in scale cells has been observed at the dormancy stage (Zaffiryar et al., 2007). In the present study, a similar phenomenon was inspected in bulb SM cells in June and July. The size and number of amyloplasts began to decrease gradually at the beginning of August (Fig. 4C). At the same time, plugged PDs re-opened and flower initiation occurred (Figs. 5 and 6). These data suggest that early August may be the end of endo-dormancy in nature, followed by immediate flower initiation in three-year-old bulbs. Symplasmic pathways were shut down, resulting in suspension of symplastic communication and inhibition of SM function as an integrated whole, a crucial step in the establishment of winter dormancy (Rinne and van der Schoot, 1998). When the SM is released from dormancy by chilling, the reverse process may be required (Rinne et al., 2001). In contrast to winter dormancy release, the summer dormancy of Chinese narcissus is activated gradually by summer heating. This kind of summer endo-dormancy relaxation might be an adaptive strategy to determine the end of hot and dry summers.

Ethylene prompts sprouting rather than release endo-dormancy in Chinese narcissus

Ethylene may have a positive effect on sprouting after endo-dormancy release in Chinese narcissus. Ethylene application during later storage stages showed an obvious accelerative effect on bulb sprouting, whereas ethylene application during the middle stage had nearly no effect on sprouting (Fig. 3). Similar reports have been reported in freesia, which exhibits summer dormancy. The application of ethylene to the corms of freesia promotes sprouting only after storage at high temperature for a certain time (Masuda and Asahira, 1980; Khan et al., 2009). The peak of ethylene production was coincident with flower initiation and endo-dormancy release (Figs. 3D and 4–6). Similarly, dormancy removal in apple embryos involves the stimulation of ethylene production (Gniazdowska et al., 2007, 2010). Thus, ethylene level peak is suggested to be a signal of endo-dormancy release and ethylene advance sprouting only after the release from endo-dormancy by high temperature. The peak of ethylene production was transient and could not be maintained. So ethylene application during the middle storage stage had nearly no effect on sprouting. The timing of ethylene application combined with the growth condition was effective on bulb sprouting. Ethylene induces bulb sprouting, which may result from decreased abscisic acid (ABA) content or sensitivity to ABA.

In recent years, the antagonism between ABA and ethylene acting in parallel with the reciprocal regulation of their metabolism and signaling pathways has been reported in seed germination (Cheng et al., 2002, 2009; Subbiah and Reddy, 2010).

In conclusion, temperature is the most significant factor affecting dormancy release in Chinese narcissus. In natural conditions, dormancy transition occurs during the end of spring, and growth transition occurs during early autumn. Endo-dormancy lasted throughout June and July and ended in early August. The endo-dormancy state was defined by an alteration of the symplasm interconnections in meristem cells. Endo-dormancy release required high temperature, and ethylene advanced the sprouting after the endo-dormancy release.

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