Effect of Low Temperature on Changes in Endogenous Hormone Level and Plant Development in *Lilium* and *Tulipa*

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Abbreviations

ABA	Abscisic acid
BHT	Di-t-butyl-4-methylphenol
BP	Basal plate
DAF	Days after flowering
DAP	Days after planting
EDC	1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
MeOH	Methanol
MMCs	Microspore mother cells
PVP	Polyvinylpyrrolidone
S-1	Outermost scale
S-2	The 2nd scale layer from outer side
S-3	The 3rd scale layer from outer side
S-4	The 4th scale layer from outer side
Sht	Shoot
UV	Ultraviolet

Chapter 1

General introduction

Backgrounds and aims of the present study

Bulbous crops can develop dormancy to survive long periods of unfavorable conditions during the life cycle. Growth and development are temporarily suspended during the dormant period (Lang *et al*., 1985; Junttila, 1988). Environmental conditions during the dormancy period trigger the developmental processes leading to dormancy breaking (Bewley, 1997). Growth is resumed when the conditions become favorable (Rees, 1992). The physical environment exerts a marked influence on dormancy, which is usually broken by a period of cold treatment, depending on plant species.

In *Lilium*, dormancy is deep. Cold requirement must be satisfied to overcome the dormancy (Rees, 1992). Temperature and period needed for dormancy breaking differ between species and genotypes (Beattie and White, 1993). *Lilium longiflorum* requires a 6 week cool-moist period at 1.5-7.0 °C for rapid flowering (Miller, 1993; Rees, 1992). Asiatic and Oriental hybrids are stored at 1.0-2.0 °C for 6-9 weeks to break dormancy (Beck, 1984; Beattie and White, 1993). Cold requirement of bulbs in *Tulipa gesneriana* can be satisfied with 6-18 weeks of precooling at 5.0-9.0 °C, dependent on cultivars and whether bulbs will be special precooled or standard forced (Le Nard and De Hertogh, 1993). In both *Lilium* and *Tulipa*, external active growth is invisible in dormant bulbs, whereas there is hardly any true physiological dormancy internally (Rees, 1992; Miller, 1993).

Although low temperature is indispensably needed for breaking dormancy in

Lilium and *Tulipa*, it affects shoot elongation and flowering in a way that is still not well understood. It is generally assumed that storing bulbs at cold temperature (usually about 4 °C) triggers the increase of the promoters and/ or the decrease of inhibitors that result in rapid shoot emergence (Wang and Roberts, 1970). As assumed as one of the promoters, gibberellin-like substances have been identified in wheat (Suge and Osada, 1966) and bulbs of *Allium cepa*, *Hyacinthus orientalis*, Wedgwood Iris, *Lilium longiflorum*, *Narcissus tazetta*, *Tulipa gesneriana* (Aung *et al*., 1969) and *Lilium speciosum* (Ohkawa, 1977). However, GAs are not responsible for either the induction of dormancy or the release from dormancy (Karssen and Lacka, 1986; Rebers *et al*., 1996; Yamazaki *et al*., 2002). IAA-like activity was detected in tulip bulbs (Ito *et al*., 1960; Syrtanova *et al*., 1973). However, appreciable changes in auxin level were not found even in bulbs in *T. alberti* that had received eight weeks of a low temperature at 4 °C. Moreover, definite identification of auxin and detection of changes in auxin level in *Tulipa gesneriana* during bulb dormancy breaking have not been reported. Evidence shows that endogenous abscisic acid (ABA) is involved in the dormancy in *Allium wakegi* bulbs and *Lilium speciosum* bulblets generated *in vitro* (Kim *et al*., 1994; Djilianov *et al*., 1994; Yamazaki *et al*., 1995, 1999a). ABA is generally regarded as one of the inhibitors that negatively affect shoot emergence of bulbs. In *Tulipa*, ABA level decreased in the basal plate and scales but increased in bulblets after bulbs were stored at 5 °C for several weeks; a change in ABA level was found in the shoot but the change is cultivar dependent (Aung and De Hertogh, 1979). Since dormancy breaking of bulbs in tulip directly affects shoot elongation after planting of bulbs, ABA level in the shoot might be a signal of dormancy release of bulbs at the end of cold treatment. Thus, further experiments are needed to elucidate the changes in ABA level especially in the shoot.

Due to the low concentration of hormones in plant, extraction of hormones from plant organs or tissues is a hard work and large amount of plant organs are needed for one analysis. In addition, results obtained finally might be more or less affected by internal and/ or external factors. Appreciable changes in auxin level in tulip bulbs during the process of cold treatment have not yet been detected because the changes, if any, might be small or lower than the detectable level. New methods for *in situ* detection of IAA in plant tissues (Dewitte and Van Onckelen, 2001) have already been used in maize (Shi *et al*., 1993; Kerk and Feldman, 1995), peanut (Moctezuma, 1999; Moctezuma and Feldman, 1999), sunflower (Thomas *et al*., 2002) and *Arabidopsis* tissues (Aloni *et al*., 2003). Now, the site of IAA production or its transport path can be easily detected with these methods. In addition, a small number of plants suffice one detection.

The shoot elongates immediately following dormancy breaking of bulbs, when bulbs are planted under warm conditions. Numerous studies with application of exogenous IAA to the top of the stem after removal of the flower showed that IAA is the major hormone that controls stem elongation (Saniewski and De Munk, 1981; Gabryszewska and Saniewski, 1983; Banasik and Saniewski, 1985; Saniewski and Okubo, 1997, 1998a, b; Saniewski *et al*., 2005). However, the putative IAA has not been undisputedly identified in the floral stalk. In addition, it was suggested that the flower and leaves are production sites of auxins controlling elongation of the floral stalk of tulip after planting (Op den kelder *et al*., 1971; Hanks and Rees, 1977; De Munk, 1979; Saniewski and De Munk, 1981). However, these studies have been done by removal of the flower, flower organs or leaves with lanolin paste applied on the cut surface. The surgery, removal of the flower, flower organs or leaves, causes irreversible damage to tulip plant, which possibly triggers complex reaction below the cut surface on the top of the stem. Therefore, further study with different methods is necessary for a better understanding of the factors controlling stem elongation of

tulip plants.

Aims of the present study were to investigate the effect of low temperature on changes in the level of endogenous ABA and IAA and on plant development, as related to dormancy breaking of bulbs in *Lilium* and *Tulipa*.

Outline of the thesis

Dormancy breaking with low temperature is important for shoot elongation and flowering of bulbous plants. However, how low temperature affects dormancy of bulbs is still not well understood. Research on the changes in hormone level and plant development as affected by low temperature in *Lilium* and *Tulipa* would elucidate the mechanisms of dormancy and dormancy breaking of bulbs.

It was suggested that ABA level is closely related to dormancy in *Allium wakegi* (Yamazaki *et al*., 1995, Yamazaki *et al*., 1999a) even if it is not the only factor involved in dormancy development (Djilianov *et al*., 1994; Kim *et al*., 1994). Quantitative analysis of ABA during storage of *Lilium* bulbs at cold is important for a better understanding of the mechanism of dormancy, which is a vital factor that has to be considered in the production of lily flowers. Therefore, in Chapter 2, changes in ABA level during dormancy breaking of bulbs were investigated in *Lilium rubellum*, an important lily species native to Japan. In general, it is known that carbohydrate in the bulb is a source of energy for the growth of shoots and roots. However, whether the metabolism of sugars during low temperature treatments is related to dormancy release is not well understood. Therefore, soluble sugars in bulb scales of *Lilium rubellum* were also analyzed in addition to detections of ABA. In Chapter 3, changes in ABA level in different layers of scales, different parts of the shoot and basal plate of tulip bulbs stored at 4 °C or 20 °C for weeks were analyzed in details. In Chapter 4,

effect of cold treatment on the development of microspores in anthers was studied.

Tulip bulbs stored at 20 °C for a long time developed bud deterioration. In Chapter 5, GA_3 was injected into bulbs and the role of GA_3 in overcoming bud deterioration was examined. IAA was detected in the stem of shoots in 4 °C bulbs, 20 °C bulbs and 20 °C bulbs injected with GA_3. Without or with short period of cold treatment, tulip bulbs produce not harvestable flowers with low percentage of viable pollens at flowering. In Chapter 6, IAA was detected in the stem, leaves and flower organs with an immnocytochemical method. In Chapter 7, diffusates from different organs of tulip plants were collected for IAA analysis. Results obtained showed that top of the top internode might be the most important production site of auxins accounting for rapid elongation of the flower stalk in tulip. In Chapter 8, the relations between changes in hormone level in *Lilium rubellum* and tulip with the development of shoots and flower organs during dormancy breaking and after bulbs were planted, as affected by low temperature, were discussed

Chapter 2

Effect of low temperature on changes in concentration of ABA and soluble sugars and dormancy breaking of *Lilium rubellum* bulbs

2.1 Introduction

Lily bulbs usually sprout and grow in spring and flower in summer or early autumn depending on *Lilium* species. Most lily bulbs generally develop dormancy to survive unfavorable environmental conditions from late autumn to winter. Dormancy of bulbs can be broken by low temperature (Langens-Gerrits *et al*., 2003). Cold treatment triggers the metabolic activity within bulblets of lily (Shin *et al*., 2002) leading to the sprouting and plant development. It seems that temperature is the main factor in dormancy induction and breaking (Delvallée *et al*., 1990; Langens-Gerrits *et al*., 2001). In addition, it was reported that the levels of endogenous abscisic acid (Djilianov *et al*., 1994; Kim *et al*., 1994; Yamazaki *et al*., 1995, 1999a, b, 2002) and sucrose (Nowak *et al*., 1974; Hobson and Davies, 1977, 1978; Aguettaz *et al*., 1990) in bulbs are associated with dormancy development and release.

In recent years, a year-round program for flower production of *Lilium rubellum* has been established by earlier and later forcing through manipulating chilling temperatures, storage duration, and planting time (Ikeda, 1997). Flower bud formation in *Lilium rubellum* starts in mid September (Ikeda, 1971; Niimi and Oda, 1989) and completes around November to December (Niimi and Oda, 1989). Moreover, *Lilium rubellum* flowers earlier than most other lily species (Ohkawa, 1989). These characteristics make *Lilium rubellum* a very attractive species for horticultural use. However, the production of both cut flowers and

potted plants is very limited, due to the fact that the mechanism of dormancy breaking of bulbs by low temperature is not well understood. Dormancy breaking is an important process in commercial flower production. Moreover, procedures for breaking dormancy are often complex and cannot be transferred from one species to another (Dole, 2003). Thus, the present study was started aiming at clarifying changes in the levels of abscisic acid and soluble sugars during storage and determining their role in dormancy breaking of bulbs in *Lilium rubellum*.

2.2 Materials and methods

2.2.1 Plant materials

Commercial bulbs of *Lilium rubellum* were purchased from the Tsukida plantation in Fukushima prefecture. The bulbs were selected with an even size of ca. 20 g and stored in moist sawdust at 8 °C from middle of October until being used in November, during which time flower bud differentiation reached stage G (Niimi and Oda, 1989). On November 4, 2004, flower bulbs were transferred to a cooling room with temperature controlled at 4 °C. At the end of the 0th, 6th, 10th and 14th weeks after bulbs were stored, 30 bulbs each were randomly collected for analyses of both ABA and soluble sugars. To observe the growth of plants after planting in greenhouse, at the end of the 10th (Jan 13, 2005) and 14th (Feb 13, 2005) weeks from the beginning of bulb storage, 30 bulbs from each treatment were planted in 3 plastic planters (50 × 30 × 30 cm) in a 1: 1 (soil: leaf-compost) mix and placed in a greenhouse for forcing. Planters were randomly placed in a greenhouse. The temperature in the greenhouse was maintained at 25 ºC/ 20 ºC (day/ night).

2.2.2 Free ABA extraction and purification

Scales of each of the selected 20 bulbs were removed from the basal plate, carefully cleaned, quickly dried and frozen with liquid nitrogen after fresh weight was measured. Samples were extracted and purified according to Kojima (1996) with some modifications as follows: the sample was stored at –20 °C for 24 h in a polyethylene bottle with an equivalent volume of 80 % methanol (MeOH), to which polyvinylpyrrolidone (PVP, K-30, 0.6 g L^{-1}) and Di-t-butyl-4-methylphenol (BHT, 0.2 g L^{-1}) were added; the samples were homogenized, stored at –20 °C for another 48 h. The homogenized samples were then centrifuged at 4 °C for 20 min at 3000 rpm. And the supernatant was collected and filtered through a 0.2 μm membrane filter. An aliquot of the MeOH extract equivalent to 30 g FW was used for ABA analysis. After [$^2H_3$]-ABA was added as internal standard, the methanol phase was evaporated away with a vacuum evaporator at 40 °C. The pH of the residual aqueous phase was adjusted to 2.8 with hydrogen chloride solution and filtered through the membrane filter (0.2 μm) again. The aqueous filtrate was partitioned three times against diethyl ether and evaporated to dryness. The sample obtained finally was dissolved in 40 % methanol and fractionated on an HPLC system equipped with a UV-detector (UV-8010, Tosoh, Japan), an LC-10 AD pump (Shimadzu, Japan) and a DGU-14A degasser (Shimadzu, Japan). The column was Inertsil ODS-3 (10 mm × 250 mm; particle, Ø5 μm; GL science). The sample eluate was collected according to retention time of the authentic ABA, dried again, dissolved in 50 % methanol and fractionated on a second HPLC system equipped with a Diode Array Detector (DAD, L-7455, Hitachi, Japan). The second column was a series connection of two Inertsil ODS-3 columns (6 mm × 250 mm; particle, Ø 5 μm; GL science). Column temperature was maintained at

40 °C. The sample was eluted with 50% ethanol solution at a flow rate of 0.5 ml/ min. To the eluent used throughout the fractioning process on HPLC, 2% acetic acid was added. The sample eluate was collected according to retention time of the authentic ABA, dried and methylated with diazomethane.

2.2.3 ABA analysis

A gas chromatograph linked to a quadrupole mass spectrometer (Hitachi M-9000, GC/ 3DQMS system) was used for quantification of ABA in the selected-ion monitoring (SIM) mode. The MS electron multiplier was operated at 1500 eV and 250 °C. The GC-MS interface was at 230 °C. A capillary column (CP-Sil 8 CB, varian, USA) was used. Carrier gas was helium at a flow rate of 1.0 ml min^{-1}. Column temperature was programmed to change linearly from 50 °C to 250 °C at a rate of 20 °C min^{-1} during 10 min after being held at 50 °C for 5 min, then maintained at 250 °C for 5 min. The methylated sample was dissolved in acetate ether and injected. Split ratio was 30 in 0.01 min.

The four most abundant ions, 162 m/z and 190 m/z for methyl-ABA and 166 m/z and 194 m/z for methyl-[$^2H_3$]-ABA, were monitored. The calculation of ABA concentration was based on the ratio of peak areas of m/z 190 to m/z 194 and the internal standard added. Calibration was conducted routinely with each batch of samples by methylating a mixture consisting of a known amount of authentic isotope-labeled ABA and unlabeled ABA. A regression equation, derived from each standard curve, was referred to in calculating ABA concentration.

2.2.4 Soluble sugar extraction and analysis

Ten bulbs were used for one analysis of soluble sugars. The scales of each bulb were removed from the basal plate and analyzed separately. Samples were homogenized with a food-cutter. An evenly mixed sample of 4 g was placed into a flask, with 20 ml of 80 % ethanol added, and extracted in a water-bath maintained at 80 °C for 20 min. The extraction process was repeated twice. The supernatant was collected and stored at −20 °C overnight. After being filtered through a filter paper (No. 6, Whatman, Japan), the supernatant of each sample was evaporated to dryness with a rotary-evaporator, dissolved in 0.02 M HCl solution, filtered through the filter paper again, and the final volume was determined before being filtered through a membrane filter (pore size, 0.45 μm). Quantification of sucrose, fructose and glucose was performed on an Intelligent HPLC system (JASCO PU-980: Degasser, JASCO DG-980-50, 3-line degasser; Sampler, JASCO AS-950-10, intelligent sampler; Column, JASCO CO-965; Detector, JASCO RI-930, intelligent RI detector; Software, intuitive chromatography software, BORWIN version 1.20). Mobile phase was distilled water (filtered through 0.1 μm membrane filter before use) and flow rate was controlled at 1.0 ml min^{-1}. Maximum column pressure was set to 50 kg cm^{-2}, and column temperature was maintained at 80 °C. Along with each batch of samples a series of authentic sucrose, fructose and glucose were measured for calibration and referred to for calculation of the concentrations of soluble sugars.

2.3 Results

2.3.1 Shoot emergence and flowering as affected by chilling of bulbs

Bulbs stored for longer durations at 4 °C showed more synchronous shoot emergence. As shown in Fig. 2.1, 20 % of bulbs chilled for 10 or 14 weeks sprouted within 3 weeks after planting. Four weeks after planting, 60 % of bulbs chilled for 10 weeks and 90 % of bulbs chilled for 14 weeks sprouted, respectively. All bulbs chilled for 10 or 14 weeks sprouted 6 weeks after planting. Flowering was achieved in all 30 bulbs chilled for 14 weeks within 7 days from the first flowering of the plant, whereas flowering of all bulbs chilled for 10 weeks was completed within 19 days (Fig. 2.2).

2.3.2 Plant characteristics as affected by chilling of bulbs

More leaves per plant and longer stems developed in the 14-week cold treatment than in the 10-week cold treatment (Table 2.1). There was no difference in flower number per plant between the 10- and 14-week chilling treatments. However, flower size was smaller in the 14-week than in the 10-week chilling treatment.

2.3.3 Concentrations of free ABA and soluble sugars as affected by chilling duration at 4 °C

Concentration of endogenous free ABA in bulbs decreased as chilling duration increased (Fig. 2.3). Free ABA was detected at 11.6 pmol g FW^{-1} at the beginning of storage at 4 °C. The concentration decreased to 5.6 pmol g FW^{-1} at

the end of the 6th week and to 3.0 pmol g FW^{-1} at the end of the 10th week. Afterwards, the ABA concentration in bulbs chilled for 14 weeks remained at almost the same level as in bulbs chilled for 10 weeks.

Storing bulbs at 4 °C for 14 weeks induced 1.5-, 1.8- and 1.9- fold increase in concentrations of sucrose, glucose and fructose, respectively (Fig. 2.4). Sucrose increased steadily up to 10 weeks at 4 °C and decreased afterwards. Glucose and fructose both increased as the chilling continued for 14 weeks.

2.4 Discussion

Bulbs chilled at 4 °C for 10 weeks sprouted slowly after being planted in a greenhouse, whereas the bulbs stored at 4 °C for 14 weeks developed shoots more rapidly within a shorter period (Fig. 2.1). These results indicate that dormancy was broken in part of bulbs at the end of the 10th week but in most bulbs at the end of the 14th week. Dormancy breaking of bulbs is related to the decline in ABA concentration in bulb scales, though a time lag of 4 weeks exists between the decline of ABA (end of the 10th week) and full dormancy release (end of the 14th week). The relation between the decline of ABA and dormancy release of bulbs in *Lilium rubellum* was consistent with results of the studies on *Polianthes tuberosa* (Nagar, 1995) and *Allium wakegi* (Yamazaki *et al*., 1995, 1999a, b, 2002). Bulbs chilled for 14 weeks flowered more synchronously than did bulbs chilled for 10 weeks (Fig. 2.2). This result further suggests that full dormancy breaking might have occurred in bulbs stored at 4 °C for 14 weeks. In grasses, ABA and coumarin are the most frequently isolated inhibitors of germination, others such as catechins, tannins and phenols may also be present (Adkins and Bellairs, 1995; Adkins *et al*., 2002). Khan (1975) suggested that free ABA level alone does not necessarily inhibit growth in dormant seeds and

bulbs. In lily bulbs, some unknown factors other than ABA might also be involved in dormancy release. The time lag between the decline in ABA concentration and full dormancy release of bulbs might be caused by some unknown factors.

Treatment of bulbs with low temperature resulted in an increase in sucrose and reducing sugars in the scales (Fig. 2.4), a phenomenon also found in bulbs of Easter lily (Miller and Langhans, 1990), hyacinth and tulips (Algera, 1936). In Easter lily, bulbs can be induced to accumulate soluble sugars and yet be delayed in flowering, therefore, soluble sugar concentration of bulbs plays no direct role in controlling the rate of flowering. It is a consequence of low temperature exposure rather than a controlling factor in vernalization (Miller, 1993). As an essential soluble carbohydrate for plant growth, sucrose also serves as the primary transport carbohydrate in most plants (Arnold, 1968). The accumulation of sucrose in bulb scales could influence the sprouting and growth of the plant. Since both glucose and fructose are required for sucrose synthesis and could be directly used in the respiratory metabolism, accumulation of glucose and fructose also could influence the sprouting and plant growth. Sprouting behavior of *Lilium* bulblets cultivated under low temperature conditions is accompanied by rapid starch hydrolysis, and accumulation of sucrose and reducing sugars (Matsuo and Mizuno, 1974; Miller and Langhans, 1990). Therefore, the accelerating effects of 14 week chilling on shoot emergence and flowering in *Lilium rubellum* might be combined effects of the decline in the level of free ABA, accumulation of soluble sugars and a decline or increase of some unknown factors in bulbs. In *Allium wakegi* plants, ABA concentration is higher in innermost scales than in outer scales of bulbs (Yamazaki *et al*., 1995), and is lower in buds than in whole bulbs (Yamazaki *et al*., 1999a). This might also be true in lily bulbs. To fully understand bulb dormancy breaking in *L. rubellum* at low temperature, it will be necessary to

determine the changes of both ABA and soluble sugars in different organs of bulbs.

For the earliest forcing, Asiatic and Oriental hybrid lilies need to be stored for 6-9 weeks at 1-2 °C (Beck, 1984; Larson, 1992). For breaking dormancy of in vitro regenerated bulblets of *Lilium rubellum*, neither the treatment of 10 week chilling at 4 °C nor less than 16 week chilling at 8 °C were effective, compared with 14 week chilling at 4 °C (Niimi *et al*., 1988). Therefore, based on the previous and the present results, it can be concluded that dormancy breaking of commercial bulbs of *L. rubellum* is attained by storing bulbs at 4 °C for 14 weeks, during which time free ABA declines and sucrose and reducing sugars increase in bulbs.

Table 2.1 Leaf number, stem length, flower number per plant and perianth length at flowering as affected by chilling bulbs in *Lilium rubellum*

Weeks at 4 °C	Leaf number	Stem length (cm)	Flower number per plant	Perianth length (cm)
10	25 ± 1	29.4 ± 1.8	3.3 ± 0.3	6.20 ± 0.08
14	29 ± 2	31.8 ± 1.8	3.3 ± 0.2	5.62 ± 0.16

30 bulbs were planted per treatment. Data were mean of 30 plants in each treatment (± SE)

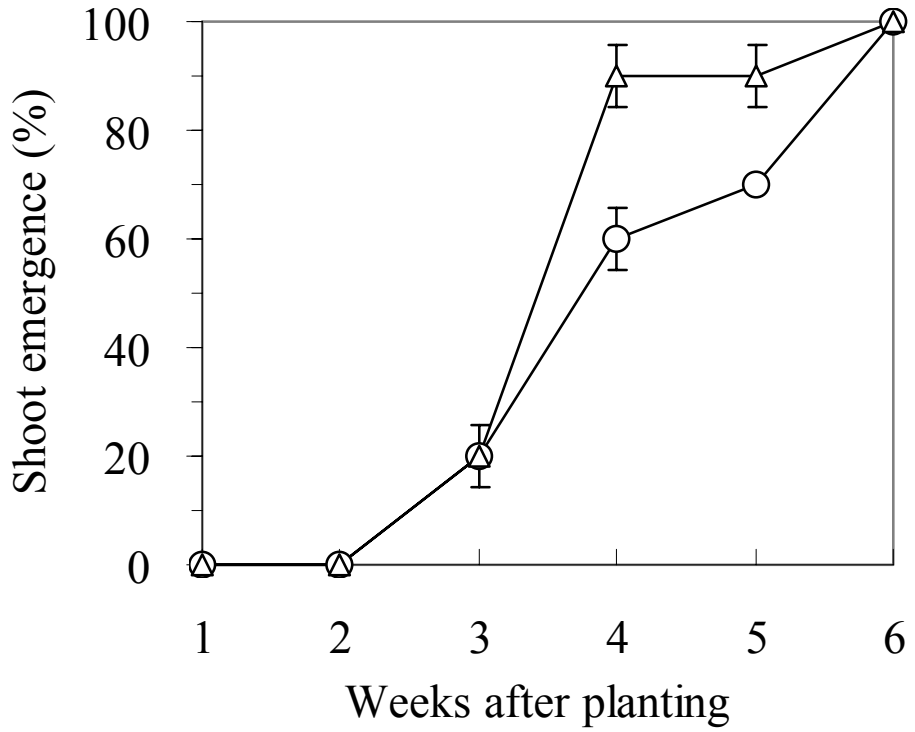


Fig. 2.1 Shoot emergence of bulbs chilled at 4 ºC for 10 or 14 weeks in *Lilium rubellum*. 4 °C-10 week (O), planted on January 13, 2005; 4 °C-14 week (Δ), planted on February 13, 2005. Vertical bars indicate SE (n = 30)

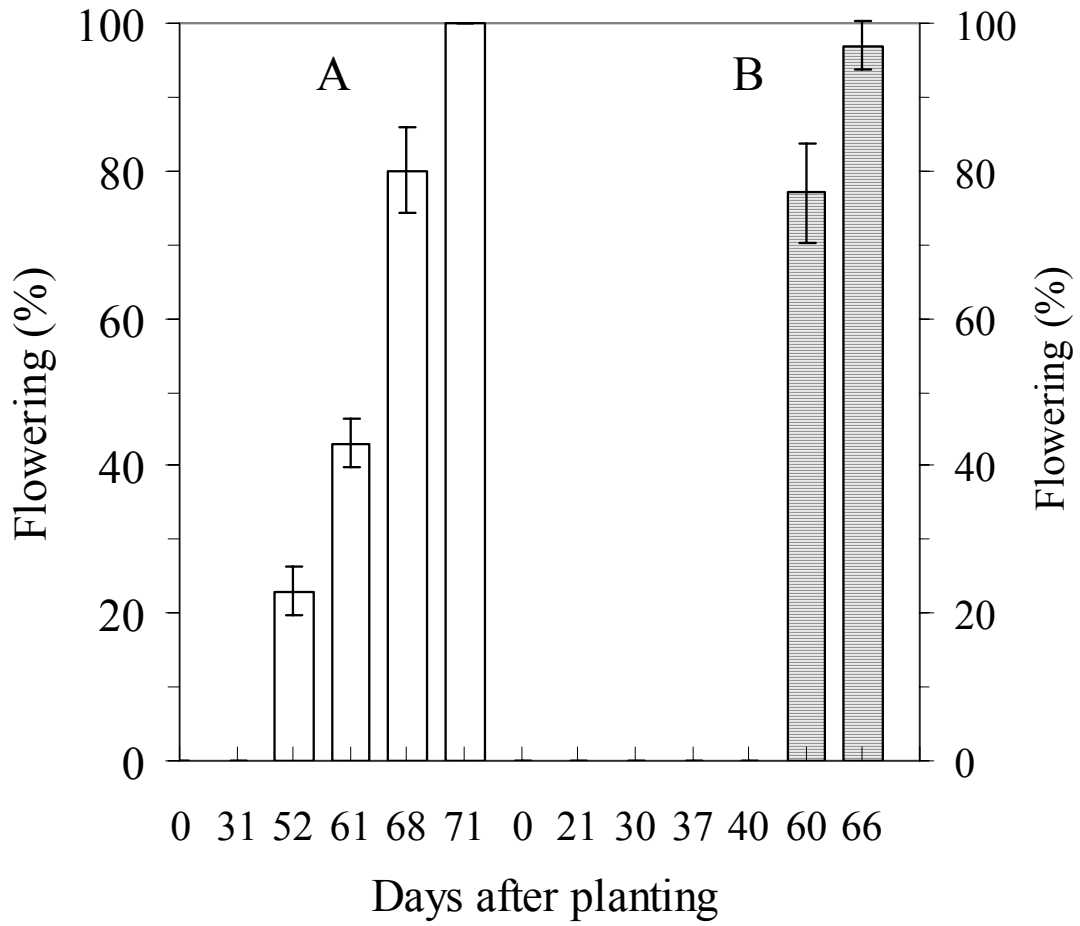


Fig. 2.2 Flowering of bulbs chilled at 4 ºC for 10 or 14 weeks in *Lilium rubellum*. 4 °C-10 week (**A**): planted on January 13, flowering, Mar 6 to 25, 2005; 4 °C-14 week (**B**): planted on February 13, flowering, Apr 14 to 20, 2005. Vertical bars indicate SE (n = 30). One of the bulbs stored at 4 °C for 14 weeks rotted after planting

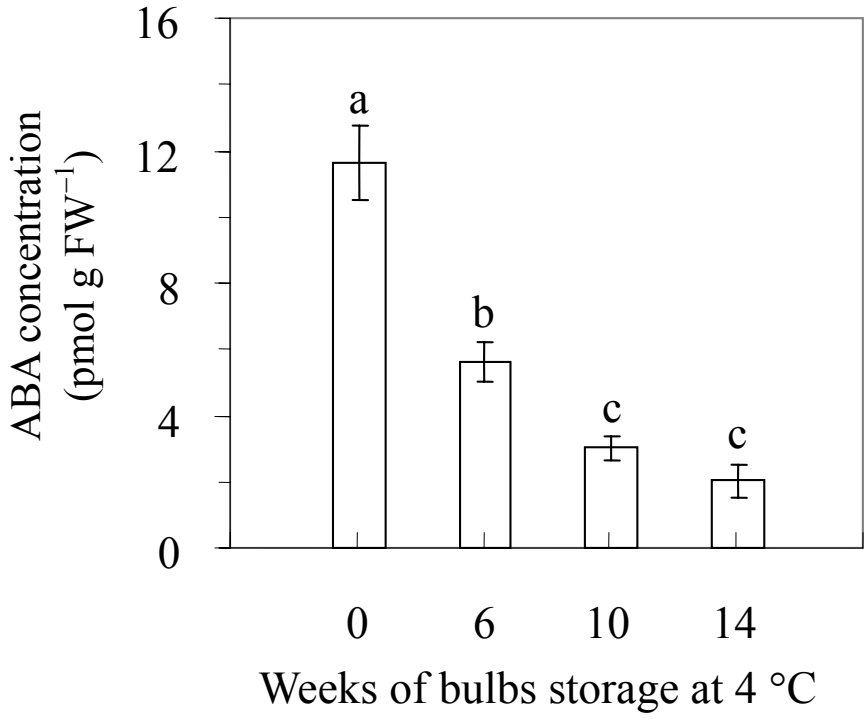


Fig. 2.3 Dynamic change in concentration of free ABA in scales of bulbs chilled at 4 °C in *Lilium rubellum*. Different letters show significant difference at 5 % level. Vertical bars indicate SE (n = 5)

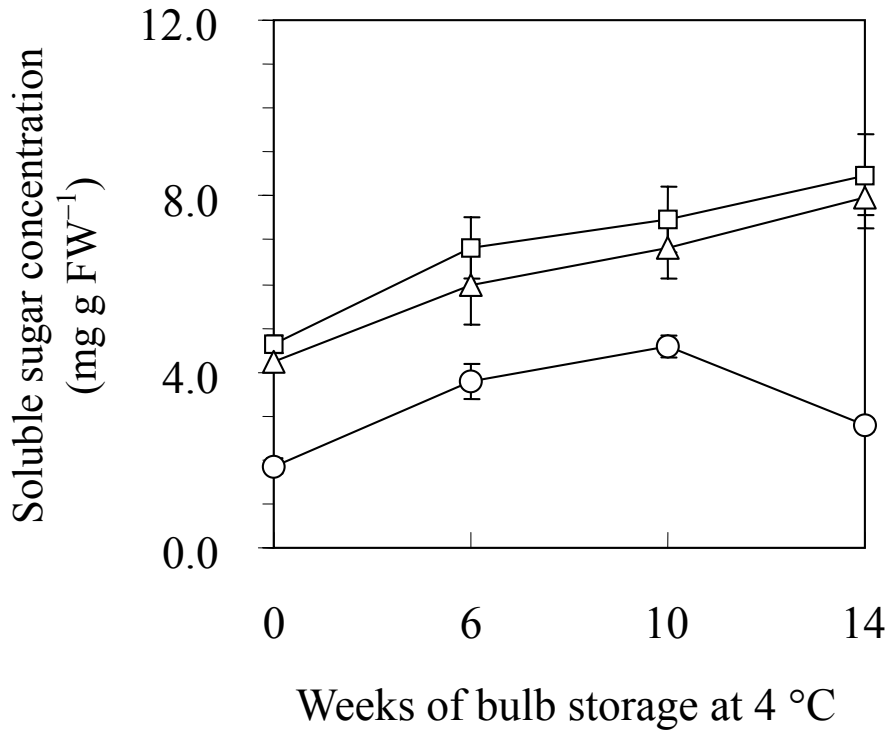


Fig. 2.4 Dynamic change in concentration of soluble sugars (mg g FW^{-1}) in bulbs chilled at 4 °C in *Lilium rubellum*. □, glucose; △, fructose; ・, sucrose. Vertical bars indicate SE (n = 10). Where vertical bars are not shown the limits are within the dimensions of the symbols

Chapter 3

Effect of low temperature on changes in ABA concentration and dormancy breaking of tulip bulbs

3.1 Introduction

Tulip bulbs, with terminal buds containing a complete flower, require 12-16 weeks of low temperature to break dormancy for floral stalk elongation (Kamerbeek *et al*., 1972). Changes in concentration of ABA-like compounds were detected in the scales, shoot, basal plate, bulblets and roots during the process of cold treatment of bulbs at 5 °C. The changes in ABA-like concentration are cultivar dependent and not obviously related to dormancy release of bulbs in varieties of 'Golden Melody' and 'Paul Richter' (Aung and De Hertogh, 1979). The ABA-like substance was identified as ABA (Terry *et al*., 1982). However, further analysis with HPLC combined with fluorescence detection did not show changes in ABA concentration during 12 weeks of storage at 5 °C in *Tulipa gesneriana* cv. 'Apeldoorn' (Franssen and Voskens, 1992). Since then, no other studies have been done on the endogenous ABA in tulip bulbs and the mechanism of dormancy breaking with low temperature in *Tulipa gesneriana* is still not well understood. ABA is one of the vital factors related to dormancy in seeds (Walker-Simmons, 1987; Wang *et al*., 1995; Bhargava, 1997; Suzuki *et al*., 2000; Corbineau *et al*., 2002; Jacobsen *et al*., 2002; Ali-Rachedi *et al*., 2004), grass (Ofir and Kigel, 1998) and bulbs (Djilianov *et al*., 1994; Kim *et al*., 1994; Nagar, 1995; Yamazaki *et al*., 1995, 1999a, b). More studies on changes in ABA concentration during cold treatment of tulip bulbs would further elucidate the mechanism of dormancy breaking,

especially the role of ABA, in bulbous plants. Thus, the present study was started aiming at elucidating the relation between ABA and dormancy breaking of tulip bulbs by studying in details the changes in ABA concentration in different organs of bulbs during cold storage.

3.2 Materials and Methods

3.2.1 Plant materials and collection of samples

Commercial bulbs of tulip (*Tulipa gesneriana* L., cv. 'Ile de France') with a circumference of 11 cm were stored at 18-20 °C until used. Experiments were performed for two years. In 2004, treatments of bulbs were started on September 23. In 2005, treatments of bulbs were started on October 11. In both years, period for cooling at 4 °C was 10 weeks following 3 weeks (2004) or 1 week (2005) of pre-cooling at 15 °C. The bulbs stored at 20 °C were used as control in both years.

Quantification analysis of ABA in the scales, basal plate, and whole shoot was performed using 20 bulbs at intervals of 4 weeks following one analysis did at the beginning of experiment. Analysis of the concentration of ABA in various parts of the shoot was conducted using 100 bulbs at the beginning and end of the 10 week storage period.

Four scales labeled from the outermost to the innermost as S-1, S-2, S-3 and S-4, basal plate (BP), and shoot (Sht) of each bulb were collected separately. The shoot was cut into 4 sections: the basal (1st) internode, top (2nd-4th) internodes, leaves and flower. Same type of organs were collected together as one sample. All samples were quickly frozen with liquid nitrogen, weighed and then stored at –20 °C until analyzed.

Water content of bulbs was measured with 10 bulbs at intervals of 2 weeks during the 8 week storage period. Both 4 °C and 20 °C bulbs were planted in a 15 °C/ 10 °C (day/ night) greenhouse at the end of the storage period. Stem length (stem top off the basal plate) of 4 °C or 20 °C bulbs was recorded during storage and after bulbs were planted. The experiments were performed with 3 replications.

3.2.2 ABA extraction, purification and quantification

Samples were extracted and purified according to Kojima (1996) with the following modifications.

(1) $^{2}H_{3}$-ABA was directly added into the samples as internal standard after the samples were homogenized in 80 % methanol.

(2) Instead of being filtered, homogenized samples were centrifuged twice at 0 °C at 4000 rpm for 15 min for collection of the supernatant. Centrifugation was firstly performed after the samples were stored at –20 °C for 48 h following homogenization in 80 % methanol. Residues were re-extracted in 80 % methanol at –20 °C for another 24 h. Then the samples were centrifuged again.

(3) Aqueous residues, after methanol was evaporated away at 35 °C, were dissolved in 50-100 ml distilled water with 50 mg l^{-1} ascorbic acid and stored at –20 °C for 24 h. Then the aqueous samples were defrosted and filtered through a 0.45 μm membrane filter on ice. (4) An aliquot of the prepared samples equivalent to 20 g FW was used for one analysis, after pH adjusted, partitioned against diethyl ether and fractionated with an HPLC system.

Quantification with a gas chromatograph-mass spectrometer system (Hitachi M-9000, GC/3DQ MS system) was performed after the samples were methylated with cold diazomethane.

3.3 Results

3.3.1 Changes of water content in the scales of bulbs stored at 4 °C or 20 °C

Water content in the scales of 4 °C bulb declined gradually from 59.3 % to 54.2 % during the 8 week storage period, whereas water content in the scales of 20 °C bulb decreased sharply to 41.2 % at the end of storage (Fig. 3.1).

3.3.2 ABA concentration in scales, shoot and basal plate of bulbs stored at different temperatures for different durations

The initial concentration of ABA in different organs of bulbs followed an order of that BP > Sht > S-4 > S-1, S-2 and S-3 (Fig. 3.2). The ABA concentration in the BP of both 4 °C and 20 °C bulbs declined steadily during 8 weeks of storage. ABA concentration declined slowly in the BP of 4 °C bulbs, but sharply in the BP of 20 °C during 8 weeks of storage. ABA concentration in S-4 of both 4 °C and 20 °C bulbs declined during 8 weeks of storage (Fig. 3.2). The concentration of ABA in S-4 of 20 °C bulbs decreased more sharply than in 4 °C bulbs during the storage. The concentrations of ABA in S-1, S-2 and S-3 of 4 °C or 20 °C bulbs were generally low, and no steady decrease or increase was observed during the 8 week storage period (Fig. 3.2).

ABA concentration in the Sht of 20 °C bulbs declined markedly during the initial 4 weeks and decreased further during another 4 weeks (Fig. 3.2A), whereas the ABA concentration in the Sht of 4 °C bulbs did not decrease during 8 weeks of storage (Fig. 3.2B).

3.3.3 ABA concentration in different parts of the shoot in 4 °C bulbs before and after 10 weeks of storage

The ABA concentration in the basal internode, top internode and leaves of the 4 °C bulbs declined after 10 weeks of storage, whereas the ABA concentration in the flower increased (Fig. 3.3). The ABA concentration in leaves was higher than in other organs at the beginning, whereas the concentration of ABA in the flower was highest at the end of the 10 week storage (Fig. 3.3).

3.3.4 Shoot elongation as affected by storage of bulbs at 4 °C or 20 °C

Stem elongation of both 4 °C and 20 °C bulbs was observed after the initial 4 weeks of storage. The stem extended rapidly during another 2 weeks and then elongation slowed down. The rate of elongation of the stem of 4 °C bulbs was higher than that of 20 °C bulbs during 10 weeks of storage (Fig. 3.4).

The stems of 4 °C bulbs elongated sharply after the bulbs were planted in a 15 °C/ 10 °C (day/ night) greenhouse. However, elongation of the stems of 20 °C bulbs was slow (Fig. 3.4).

3.4 Discussion

Stem elongation and ABA concentration were both affected by temperature for bulb storage (Figs. 3.2, 3.3, 3.4). The stems hardly elongated during the initial 4 weeks of storage at 4 °C or 20 °C but afterward the stems extended length obviously until the end of the 6th week. ABA concentration is higher in the organs connected to the shoot directly, such as in the basal plate (BP) and

innermost scale (S-4). These results indicate that dormancy breaking of the bulbs, or actually dormancy breaking of the shoot, is probably determined by these organs rather than by the outer scales. The concentration of ABA in the BP and S-4 in 4 °C or 20 °C bulbs decreased to a lower level before the rate of elongation of the stems increased. It indicates that the decrease in ABA concentration was involved in dormancy breaking of the bulbs.

Storage at 4 °C resulted in complete dormancy release of bulbs, as exhibited by the rapid stem elongation after planting (Fig. 3.4). However, the concentration of ABA was not reduced to a lower level in 4 °C bulbs than in 20 °C bulbs. The dormancy of tulip bulbs is not deep (Rees, 1992). The shallow dormancy is more easily broken by environmental factors other than temperature. The metabolic activities of substances such as hormones in bulbs might be more easily affected in shallow dormant plants than in deep dormant plants. During the storage period, water content decreased 5 % in 4 °C bulb scales but 18 % in 20 °C bulb scales during 8 weeks of storage (Fig. 3.1). Normal metabolic activity of hormones might be damaged by the sharp decrease of water in 20 °C bulbs.

Storage of bulbs at low temperature maintains normal water content in bulbs with higher relative humidity, whereas it can not be achieved in bulbs stored at 20 °C, because high temperatures with high humidity increase disease incidence.

Report of Geng *et al*. (2005) showed that flowering can be achieved in some *Tulipa gesneriana* cultivars without storage at cold if storage period at 20 °C is longer than that usually maintained for early flowing production. The initiation of stem elongation was observed in the 20 °C bulbs during storage period in the present study. Both of these results probably indicate that the shallow dormancy of tulip bulbs could be, even if not completely, broken by storing bulbs properly at around room temperature for a longer period.

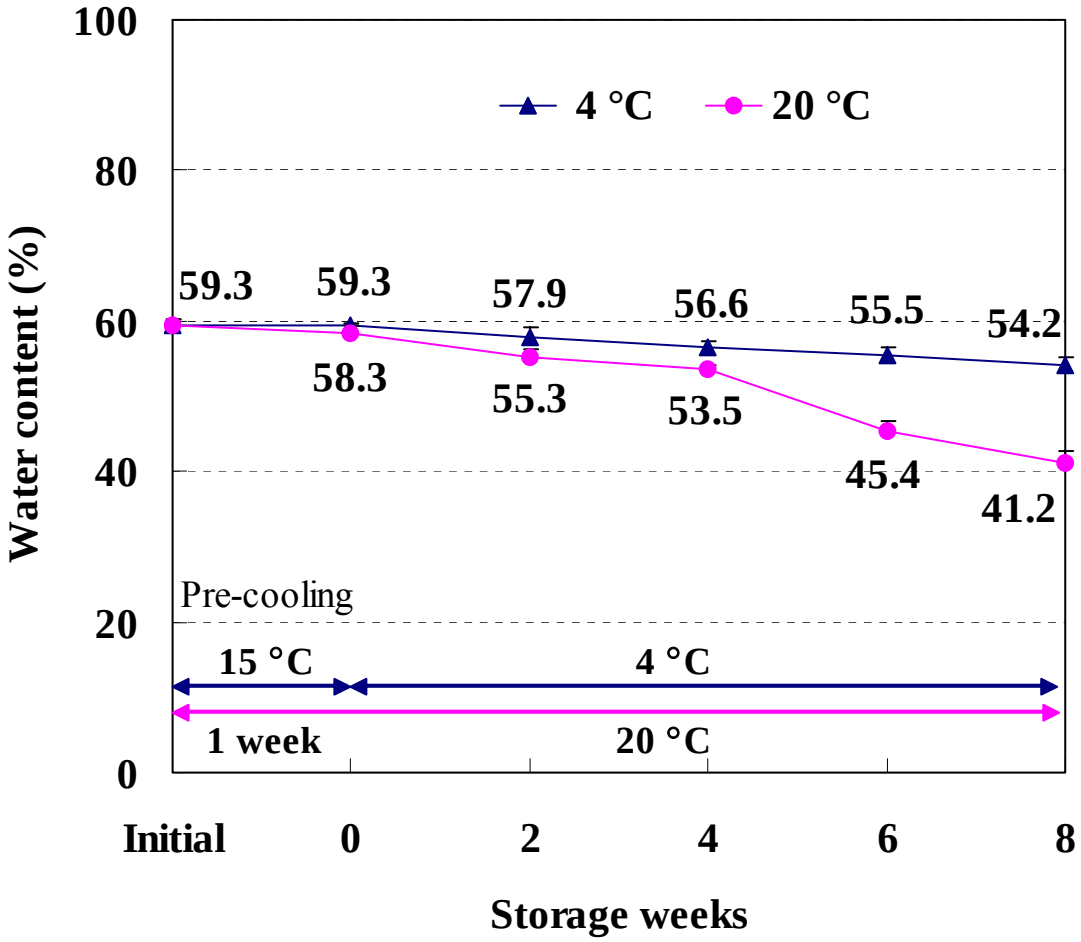


Fig. 3.1 Changes in water content (%) in the scales of bulbs stored at 4 °C or 20 °C in *Tulipa gesneriana* L., cv. 'Ile de France'. Data were means (± SE) of three determinations. Ten bulbs were used for each determination

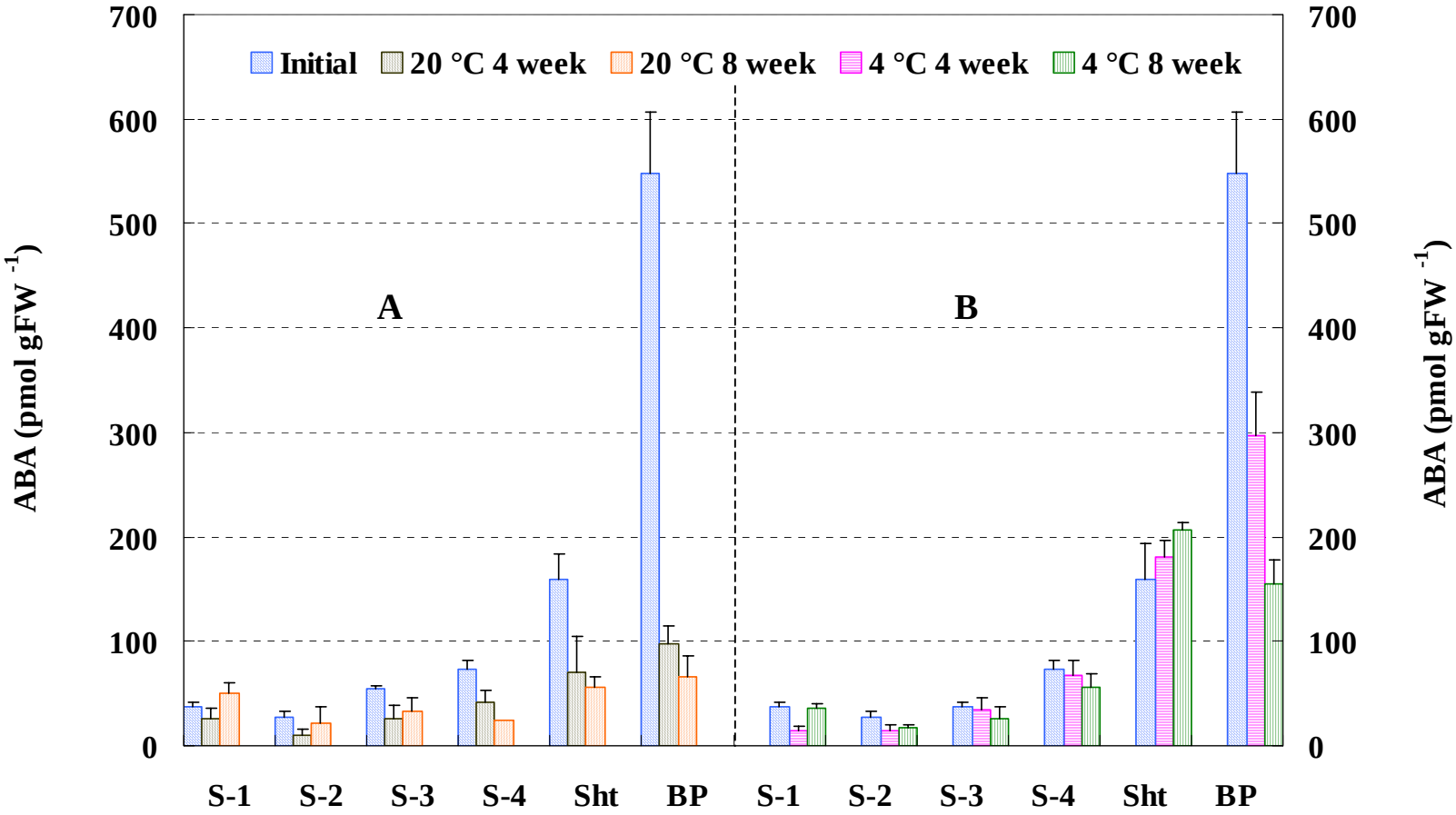


Fig. 3.2 ABA levels in scales, shoot and basal plate of bulbs stored at 4 °C or 20 °C for 0, 4, 8 weeks in *Tulipa gesneriana* L., cv. 'Ile de France'. **A**, Storage at 20 °C; **B**, Storage at 4 °C. Data are means (± SE)

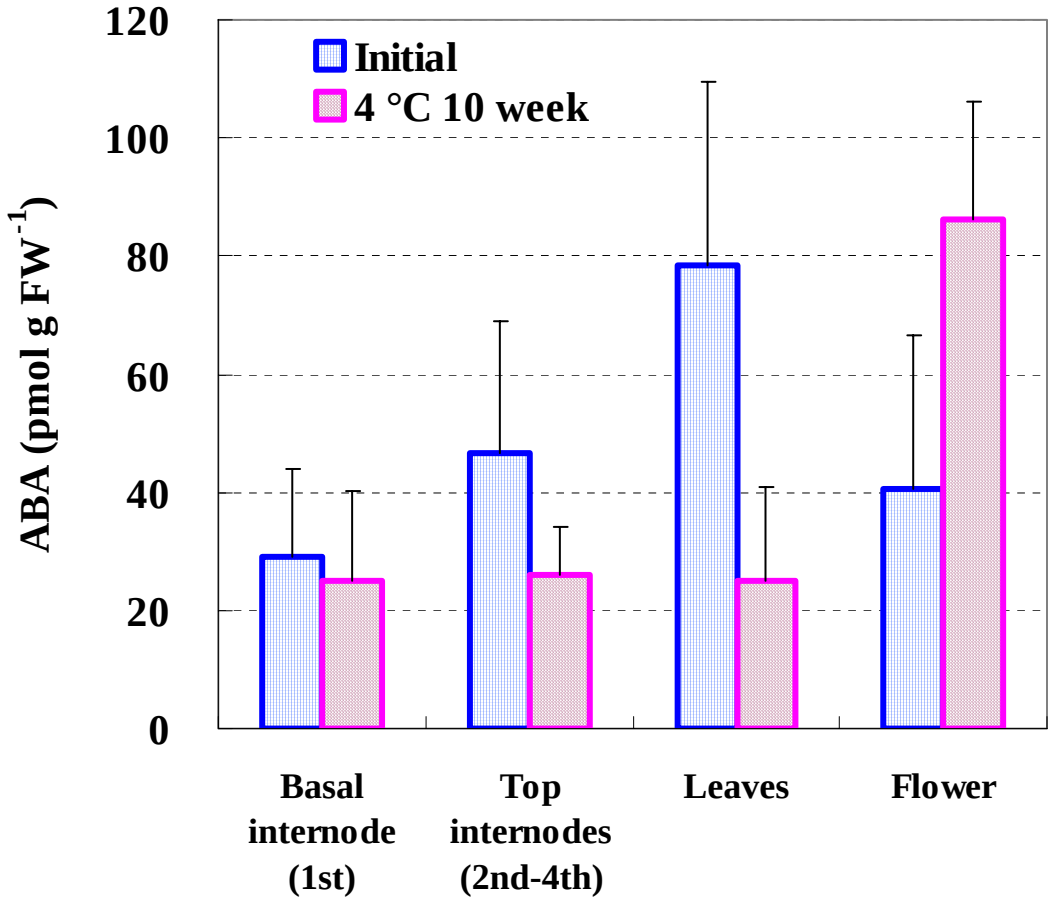


Fig. 3.3 ABA levels in different parts of the shoot in bulbs stored at 4 °C for 0 and 10 weeks in *Tulipa gesneriana* L., cv. 'Ile de France'. Data are means (± SE)

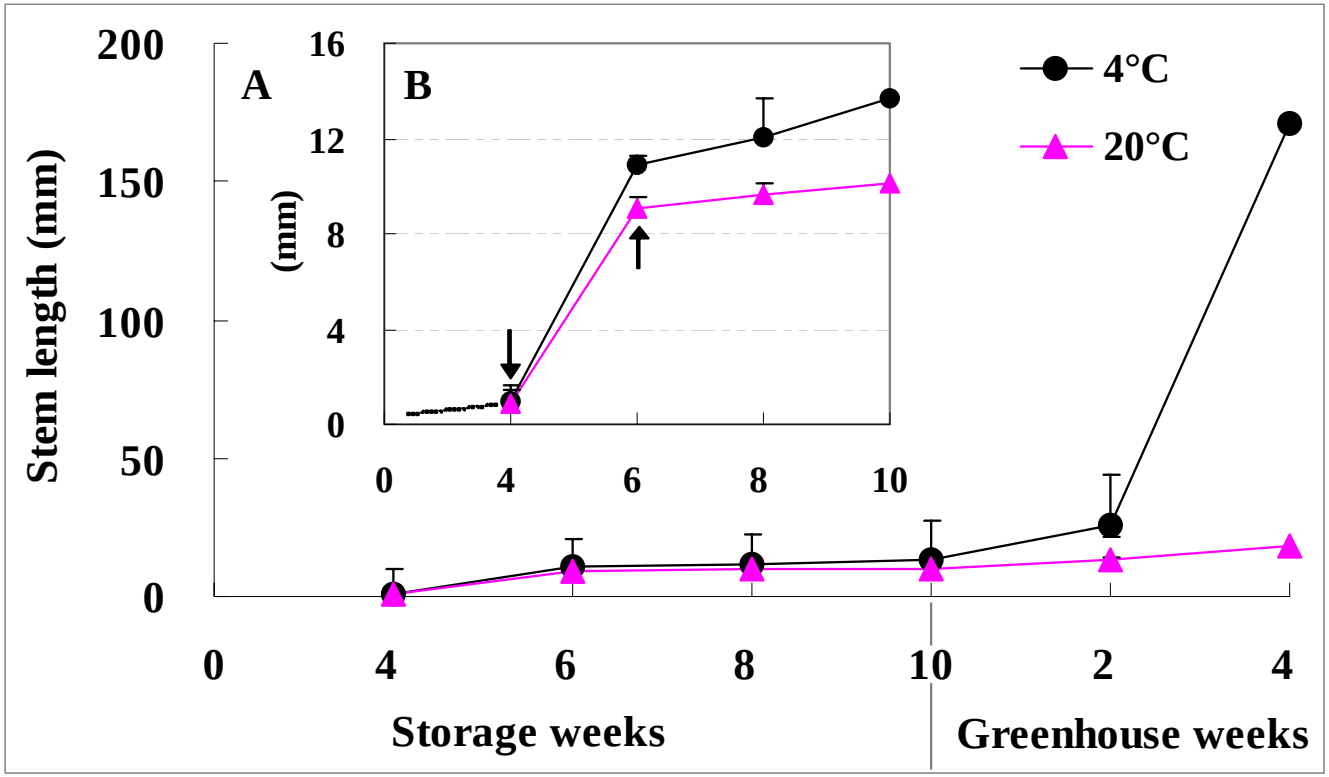


Fig. 3.4 (**A**) Stem elongation during bulb storage and in greenhouse, as affected by storage at 4 °C and 20 °C in *Tulipa gesneriana* L., cv. 'Ile de France'. (**B**) Magnified figure showing the stem elongation during 10 weeks of storage at 4 °C or 20 °C. Data are means of 30 bulbs (± SE)

Chapter 4

Effect of temperatures on microspore development and endogenous IAA level in pollen sacs in tulip

4.1 Introduction

Tulip bulbs, with a complete flower, have a kind of dormancy (Kamerbeek *et al*., 1972). A period of 12-16 weeks of low temperature requirement must be satisfied to induce floral stalk elongation and flowering (De Hertogh, 1974). Without cold treatment, stem extension is inhibited and the flower usually shows severe petal deformation and anther abortion (Rees, 1992). Many studies have been done on the effect of cold treatment on floral stalk elongation in tulip (Gabryszewska and Saniewski, 1983; Saniewski and Okubo, 1997, 1998a, b), whereas little attention has been given to the development of flower organs. The mechanism associated with development of the flower as affected by low temperature during dormancy breaking of tulip bulbs is still not well understood. Since characteristics of flower organs are important aesthetic aspects of a flower, attractiveness of a tulip flower would increase if desirable flower size, flower shape, flower color and fully developed flower organs are achieved in bulbs that released completely from dormancy. Researches on the development of the anthers as affected by low temperature would provide useful information for a thorough understanding of dormancy breaking in tulip bulbs, which necessitates this study.

4.2 Materials and Methods

4.2.1 Plant materials and treatments of bulbs

Commercial tulip bulbs of 6 cultivars (*Tulipa gesneriana* L. cv. ‘Aladdin’, ‘Balerina’, ‘Ile de France’, ‘Negrita’, ‘Queen of Night’ and ‘Spring Green’), 11-12 cm in circumference, were used. On arrival, bulbs were stored dry at 20 °C. With developmental stage ‘G3’ confirmed in the flower bud, bulbs were cooled at 4 °C following 3 weeks of pre-cooling at 15 °C.

Experiments were performed in the year of 2003 and 2005. In 2003, bulbs were cooled at 4 °C for 10 weeks for the examination of microspore development in ‘Aladdin’ and ‘Ile de France’. The 4 °C cooling of bulbs was started on Sep 8, Sep 22, Oct 6, Oct 20 and Nov 3 respectively to examine the effect of starting date of low temperature on microspore development in ‘Aladdin’. In 2005, bulbs were cooled at 4 °C for 12 weeks for the examination of IAA in anthers of the flower bud in ‘Ile de France’. The experiment was started on December 6, 2005. In both years, bulbs stored at 20 °C constantly were used as control.

In 2003, bulbs of the 6 cultivars were planted in a 25 °C/ 15 °C (day/ night) greenhouse at the end of the 10th week of storage at 4 °C on November 4, 2003. The flowering date was determined when the perianth of a flower was fully colored. The percentage of viable mature pollens was calculated from the ratio of pollens that were stained with 1 % aceto-carmine solution on the day when the petals of the flower opened. One calculation was the average of ten counts of views of pollens collected from 10 flowers on a light microscope (Microscope, DMLB-100, Japan).

4.2.2 Recording developmental stages of microspores in ‘Aladdin’ and ‘Ile de France’

Developmental stages of microspores were examined in ‘Aladdin’ during storage period. Five bulbs were collected from 4 °C or 20 °C bulbs respectively at intervals of 2 weeks. Stamens of the flower bud were fixed in an FAA solution (formalin: acetic acid: 70 % ethanol = 5: 5: 90, v/v/v), dehydrated in a graded ethanol series, embedded in paraplast (Oxford, U.S) and sectioned into 10 μm slices. After dried at 40 °C, sections were deparaffinized and hydrated in an ethanol-water graded series. Then the sections were stained, dehydrated, covered with cover glass, observed with a light microscope (Microscope, DMLB-100, Japan) and photographed with a digital camera connected to the microscope (Canon S80, Japan).

After planting of bulbs, the developmental stages of the microspores were examined in ‘Aladdin’ and ‘Ile de France’ at intervals of about 2 weeks. The experiment days for 4 °C bulbs were −1 (one day before planting), 12, 24, 38 and 52 days after planting (DAP) in ‘Aladdin’ and −1, 10, 24, 38 and 52 DAP in ‘Ile de France’. The experiment days for 20 °C bulbs were the same as for 4 °C bulbs in both cultivars, except the final examinations which were conducted 59 DAP in ‘Aladdin’ and 57 DAP in ‘Ile de France’. The colloid-like substance in the anther was squashed out, stained with 1 % aceto-carmine solution, observed on a light microscope (Microscope, DMLB-100, Japan) and photographed.

4.2.3 Immunolocalization of IAA in anthers in ‘Ile de France’

Immunolocalization of IAA in anthers was performed at the beginning of bulb storage and after bulbs were stored at 4 °C or 20 °C for 4, 8 and 12 weeks.

For each sampling, ten bulbs were randomly collected from 4 °C or 20 °C storage. Anthers of the flower bud were collected. The anthers were immediately pre-fixed in a 2 % (w/v, g ml^{-1}) aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide (EDC, Fluka, Switzerland) for 2-3 h, then post-fixed in a solution containing 4 % paraformaldehyde and 2.5 % glutaraldehyde at 4 °C for 24 h. After dehydrated at vacuum in a graded ethanol series, the samples were embedded in a polyester wax (melting point: 37 °C) and sectioned into 10 μm slices. After dried at 25 °C, the sections were dewaxed and hydrated in an ethanol-water series, and processed according to Hou and Huang (2005) with the following modifications.

(1) 100 μl 1:20 dilution of the primary IAA antibody (1 mg ml^{-1}) (Agdia, Elkhart, IN, USA) was added to each slide and the slides were incubated at 20 °C for 5 h in darkness in a humidity saturated chamber.

(2) 100 μl 1:100 dilution of the secondary antibody (1 mg ml^{-1}), an anti-mouse IgG-alkaline phosphatase-conjugate (1 mg ml^{-1}, Promega, USA), was added to each slide. Slides with secondary antibody were incubated at 20 °C for 12 h in darkness in a humidity saturated chamber.

(3) When the IAA signal, shown as a blue-green color, was observed and became steady on the sections, the slides were rinsed in water, dehydrated and mounted with a cover glass using a mounting medium (Eukitt, O. Kindler, Germany). After dried at 40 °C, the slides were observed with a light microscope and photographed.

Three controls were included to verify the effectiveness of the immunolocalization technique by omitting the primary antibody and/ or the secondary antibody incubation from the detection procedure.

4.3 Results

4.3.1 Microspore development as affected by temperature during storage of bulbs in ‘Aladdin’

The development of the microspore mother cells (MMCs) was initiated during pre-cooling at 15 °C, before transferred to 4 °C cooling (Fig. 4.1). With enlarged nuclei, the tapetum cells were distinct from the outer pollen sac walls and inner MMCs at the end of the 3 week pre-cooling (Fig. 4.1A). Then the callose surrounding the MMCs dissolved and the MMCs underwent meiosis during the following 6 weeks. The microspores of tetrads formed at the end of the 6th storage week at 4 °C (Fig. 4.1C, E, G) and remained attached to each other during the following 4 weeks until the end of the 10th storage week (Fig. 4.1I, K).

Pollen sacs were inactive during the first 9 weeks in bulbs stored at 20 °C (Fig. 4.1B, D, F, H). While the callose surrounding the MMCs did not dissolve, the microspores of tetrads formed in the thick callose sheath at the end of the 11th storage week at 20 °C (Fig. 4.1J). Deformed tetrads were observed at the end of the 13th storage week at 20 °C (Fig. 4.1L).

The development of the MMCs was not delayed by postponing the starting day for 4 °C cooling. The MMCs started development during pre-cooling at 15 °C (Fig. 4.2B, E, H, K, N), and the callose covering the MMCs dissolved at the end of 2 weeks at 4 °C (Fig. 4.2C, F, I, L, O).

4.3.2 Microspore development in 4 °C and 20 °C bulbs after planting of bulbs in ‘Aladdin’ and ‘Ile de France’

The MMCs developed through tetrad microspores, microspores, vacuolated pollens and engorging pollens, to mature pollens in 4 °C bulbs in ‘Aladdin’ and ‘Ile de France’ (Figs. 4.3A, C, E, G, I; 4.4A, C, E, G).

While the MMCs in 20 °C bulbs developed into tetrad microspores at the end of storage as did in 4 °C bulbs in ‘Aladdin’, the tetrad microspores did not separate from each other at later stages (Fig. 4.3B, D, F, H, J). The callose surrounding the tetrads dissolved but the four microspores of each tetrad were still attached on the 12th day after planting of bulbs (Fig. 4.3D). Later, the four microspores continuously clustered together and the tetrads became deformed (Fig. 4.3F, H, J). While development continued in a portion of microspores, they did not finally develop into viable mature pollens, due to the failure of separating from other microspores in the same tetrad (Fig. 4.3H, J). Some microspores were engorged but could not be stained with 1 % aceto-carmine solution (Fig. 4.3J).

The MMCs in 20 °C bulbs developed into tetrad microspores at the end of storage in ‘Ile de France’ (Fig. 4.4B). However, the tetrad microspores did not become separated but clustered together and severely deformed (Fig. 4.4D, F, H). Some tetrads comprised microspores with transparent cytoplasm (Fig. 4.4D, F).

4.3.3 Flowering and the percentage of viable pollens as affected by storage temperatures

A higher percentage of flowering was achieved in bulbs stored at 4 °C in ‘Balerina’, ‘Ile de France’, ‘Negrita’, ‘Queen of Night’ and ‘Spring Green’ but a

lower percentage in ‘Aladdin’ (Table 4.1). In addition, most flowers in bulbs stored at 4 °C were harvestable in each cultivar. The 20 °C bulbs achieved flowering too but did not produce harvestable flowers in all the cultivars.

A higher percentage of viable mature pollens were attained in bulbs stored at 4 °C in ‘Ile de France’, ‘Queen of Night’, and ‘Spring Green’ but a lower percentage in ‘Aladdin’, ‘Balerina’, and ‘Negrita’ (Table 4.1). There were no viable mature pollens produced in 20 °C bulbs in 5 cultivars, except in ‘Queen of Night’, in which 58.5 % pollens were stained with 1 % aceto-carmine solution.

4.3.4 IAA production in anthers as affected by temperatures and durations for bulb storage in ‘Ile de France’

The strength of IAA immunolocalization staining in the anthers varied in bulbs stored at different temperatures for different durations. At the beginning of bulb storage, the pollen sacs showed a very weak IAA signal (Fig. 4.5A). However, after bulbs stored at 4 °C for 4-12 weeks, a stronger immunolocalization staining appeared in the pollen sacs, especially at the tapetum (Fig. 4.5C-E). IAA signal was not localized in the pollen sacs in 20 °C bulbs stored for 4 weeks (Fig. 4.5B). Moreover, immunolocalization of IAA was not successfully achieved in bulbs stored at 20 °C for 8-12 weeks because bud abortion occurred due to long time of storage at 20 °C.

The immunolocalization procedure controls showed that the specificity of this immunohistochemical method for IAA detection was assured by the reaction between the primary and secondary antibodies, without which the final successful staining could not be achieved (Fig. 4.5G, H). Although tissue sections could be stained even if the primary antibody was omitted from the

procedure (Fig. 4.5F), the light grey-blue color on the control section was very different from the IAA signal, shown as the blue-green color, of the complete detection (Fig. 4.5A, C-E).

4.4 Discussion

Without low temperature treatment, the callose originally deposited on the surface of the MMCs in 20 °C bulbs did not dissolve during the developmental process (Fig. 4.1). Tetrad microspores formed at the end of storage at 20 °C but were severely deformed (Fig. 4.1L). The deformed tetrad microspores did not develop into individual microspores due to the failure to separate from each other. Microspores of the tetrads remained attached together during the whole greenhouse period and finally resulted in non-viable pollens at flowering (Figs. 4.3, 4.4). The deformed tetrad clusters were observed in 20 °C bulbs in 5 cultivars except in 'Queen of Night', which is less dependent on cold treatment for induction of shoot elongation and flowering (Xu *et al*., 2005). In contrast, mature pollen grains were produced in 4 °C bulbs in all 6 cultivars (Table 4.1). In addition, the development of the MMCs was not delayed by postponing the starting day for 4 °C cooling but was initiated during the pre-cooling at 15 °C (Fig. 4.2B, E, H, K, N). Moreover, the callose covering the MMCs dissolved at the end of the 2 week cooling at 4 °C (Fig. 4.2C, F, I, L, O). These results indicate that development of the microspores depends on low temperature.

The enlargement of the anthers during cold treatment of bulbs and development of the microspores at later stages need food reserves. Exposing bulbs to low temperature can satisfy these requirements, because low temperature induces higher level of starch in the anthers than does high temperature (Gorin and Heidema, 1985).

The tapetum of the pollen sacs has several secretory and nutritive functions related to pollen development (Lersten, 2004). The enlargement of the tapetum cells was one of the firstly initiated activities in the pollen sacs. It is possible that low temperature induced accumulation of some unknown substances in the tapetum or promoted the substances flow to the inner MMCs to initiate their development. There is evidence that the enzymes that produced in the tapetum such as the β-1,3 gluconase in two species and hybrid cultivars of lily (Stieglitz, 1977) can dissolve the callose. It is possible that the hormones are also involved in the metabolism in the pollen sacs and development of the microspores. The immunolocalization of IAA in anther pollen sacs, especially at the tapetum, in bulbs stored at 4 °C indicates that IAA was biosynthesized in bulbs during storage at cold. The IAA might play a vital role in the development of the microspores.

Table 4.1 Flowering percentage, harvestable flower percentage and percentage of viable pollens of flowers in 6 *Tulipa gesneriana* cultivars as affected by 10 weeks of storage at 4 °C or 20 °C

Group [1]	Cultivar	Flowering percentage (%)		Harvestable flower percentage [2] (%)		Viable pollen percentage (%)	
		4 °C	20 °C	4 °C	20 °C	4 °C	20 °C
L	Aladdin	56.8	20.0	54.1	0	47.3 ± 1.8 [3]	0
L	Balerina	94.4	4.0	94.4	0	38.5 ± 3.4	ΔΔ
SL	Ile de France	85.7	73.1	80.0	0	60.6 ± 3.3	0
T	Negrita	91.9	32.0	91.9	0	58.5 ± 4.9	Δ
SL	Queen of Night	90.9	44.0	66.7	0	89.4 ± 1.4	58.5 ± 2.1
V	Spring Green	94.3	50.0	85.7	0	70.3 ± 2.1	ΔΔ

[1] L, Lily flowered; SL, Single late; T, Triumph; V, Viridiflora

[2] Flowers with a short flower stalk, deformed perianth, or abnormal color were graded as not harvestable, according to the <Grading standards for the shipment of cut flowers in Japan, 1995>

[3] Means ± SE

Δ Pollens attached together

ΔΔ Petals did not open

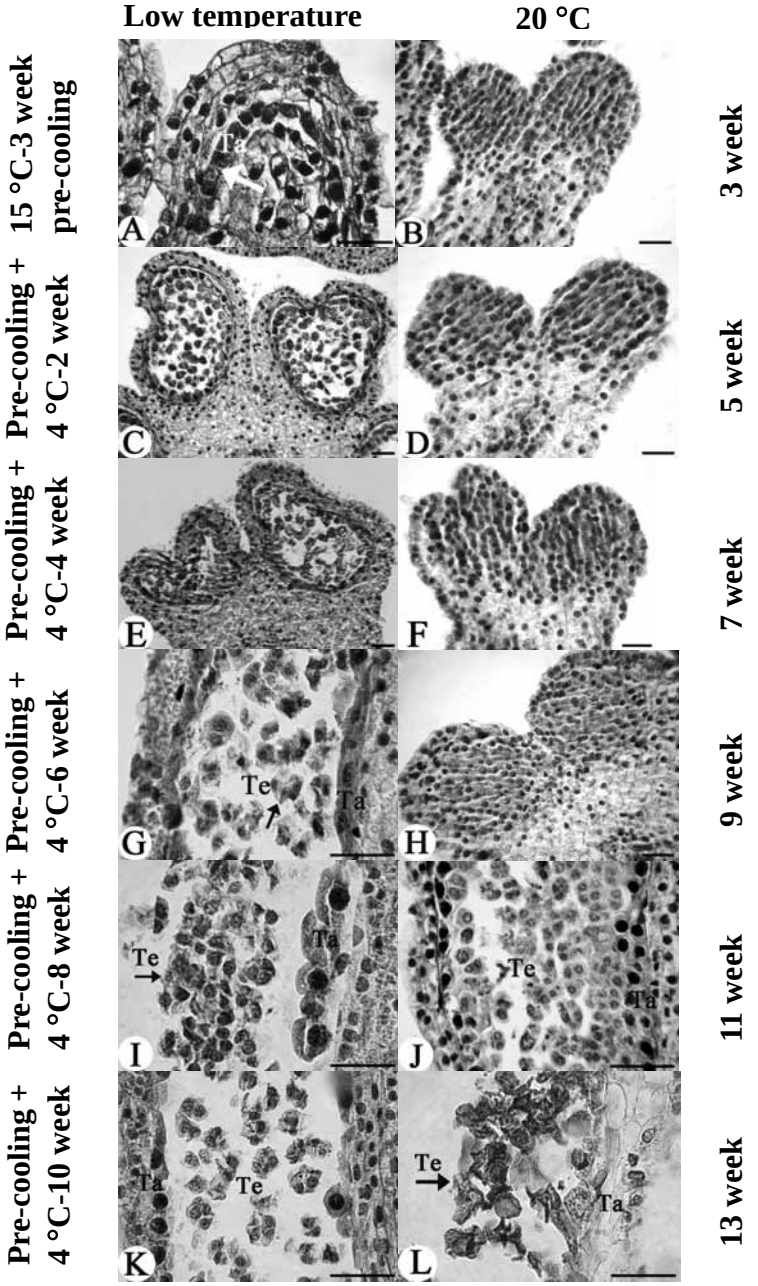


Fig. 4.1 Anther and pollen development as affected by storage at 4 °C or 20 °C in *Tulipa gesneriana* L. cv. 'Aladdin'. **A, C, E, G, I, K**, Pollen sacs of anthers after bulbs pre-cooled at 15 °C for 3 weeks followed by 2, 4, 6, 8 and 10 weeks of 4 °C cooling. **A**, The MMCs stage with distinct and enlarged tapetum. **C**, The meiosis stage with callose wall surrounding the MMC dissolved. **E**, The meiosis stage following **C**. **G**, The tetrad stage with degenerating tapetum. **I,** Tetrad microspores flowing in the pollen sac. **K**, Tetrad microspores attached together. **B, D, F, H, J, L**, Pollen sacs of anthers after bulbs stored at 20 °C for 3, 5, 7, 9, 11 and 13 weeks. **B, D, F, H**, Development of pollen sacs not initiated. **J**, Tetrad microspores embedded in the callose sheath. **L**, Tetrad microspores deformed and clustered together. **Ta**, Tapetum; **Te**, Tetrad. Arrow heads show the tetrads. Scale bar = 50 μm

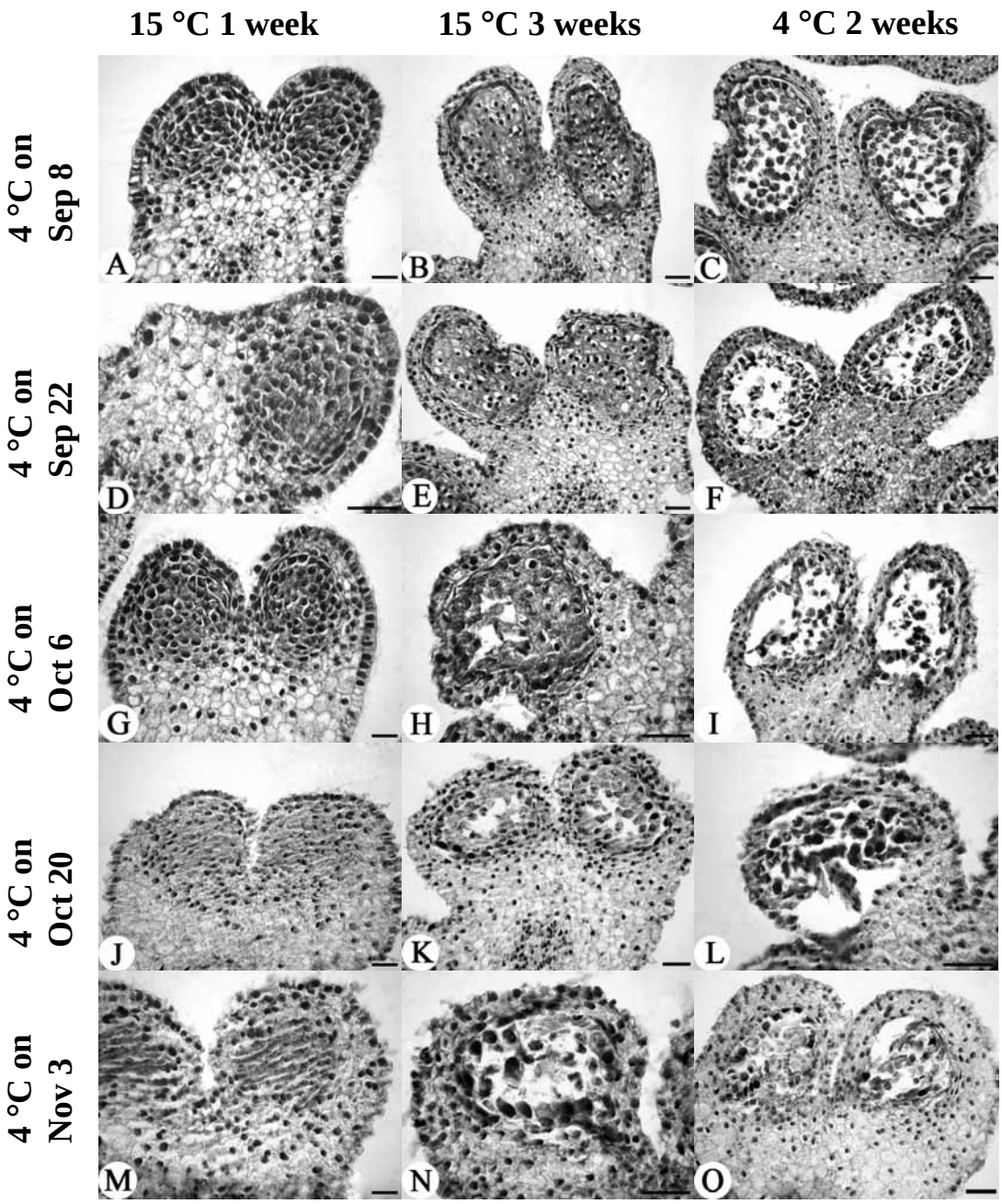


Fig. 4.2 Initiation of microspore development in flower buds as affected by 15 °C pre-cooling and 4 °C cooling that started on different days in *Tulipa gesneriana* L. cv. 'Aladdin'. **A, D, G, J, M**, After one week at 15 °C. **B, E, H, K, N**, After 3 weeks at 15 °C. **C, F, I, L, O**, After 2 weeks at 4 °C following 3 weeks at 15 °C. Scale bar = 50 μm

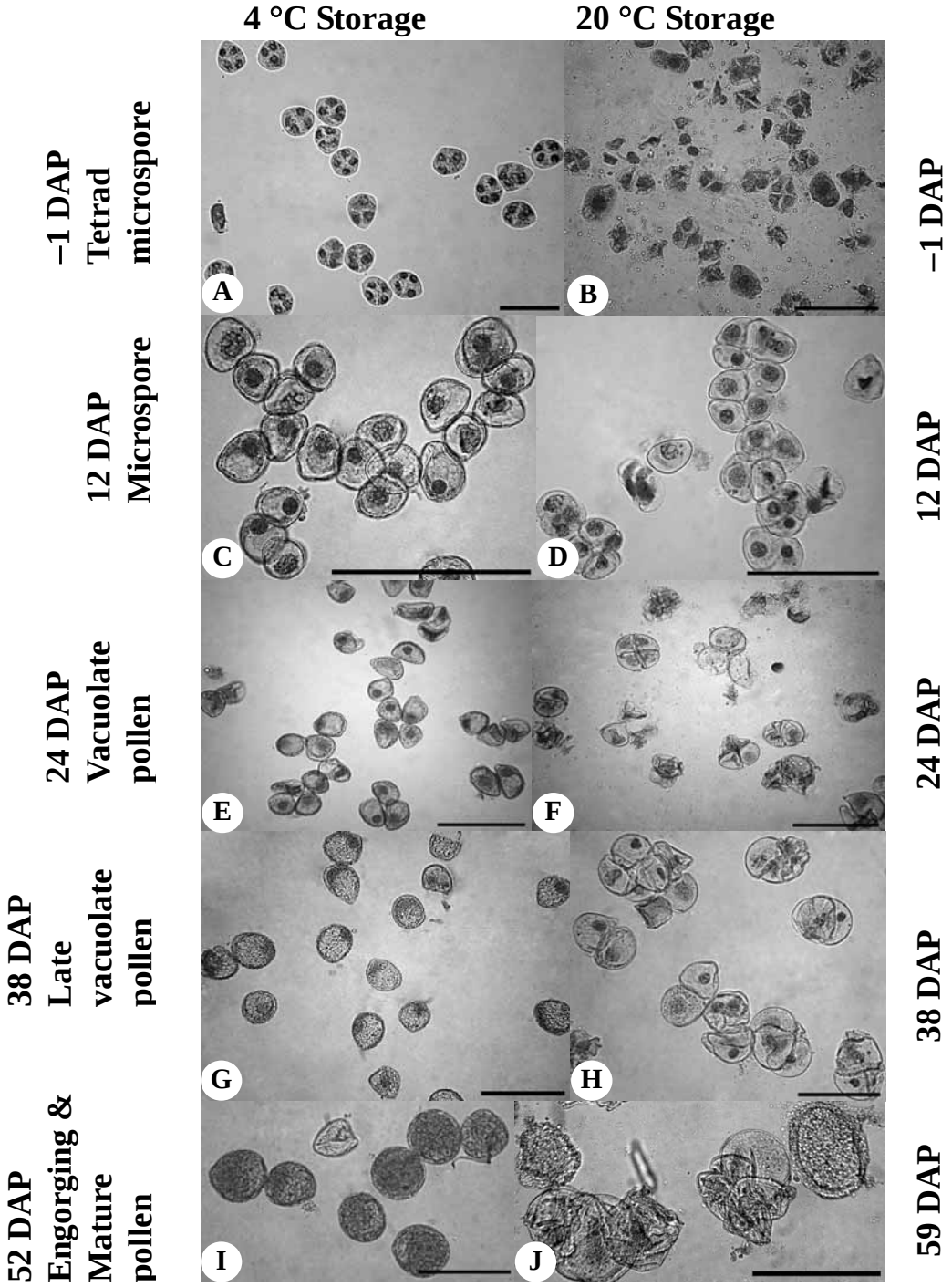


Fig. 4.3 Microspore development as affected by storage of bulbs at 4 °C or 20 °C in *Tulipa gesneriana* L. cv. 'Aladdin'. **A, C, E, G, I**, –1, 12, 24, 38, 52 days after bulbs were planted in a greenhouse, following 3 week pre-cooling at 15 °C and 10 week cooling at 4 °C. **A,** Tetrad stage. **C**, Microspores. **E**, Vacuolated microspores. **G**, Engorging pollens. I, Stainable mature pollens. **B, D, F, H, J**, –1, 12, 24, 38, 59 days after bulbs were planted, following 13 week storage at 20 °C. The tetrad microspores attached together till before flowering. DAF, Days after planting. Scale bar = 50 μm

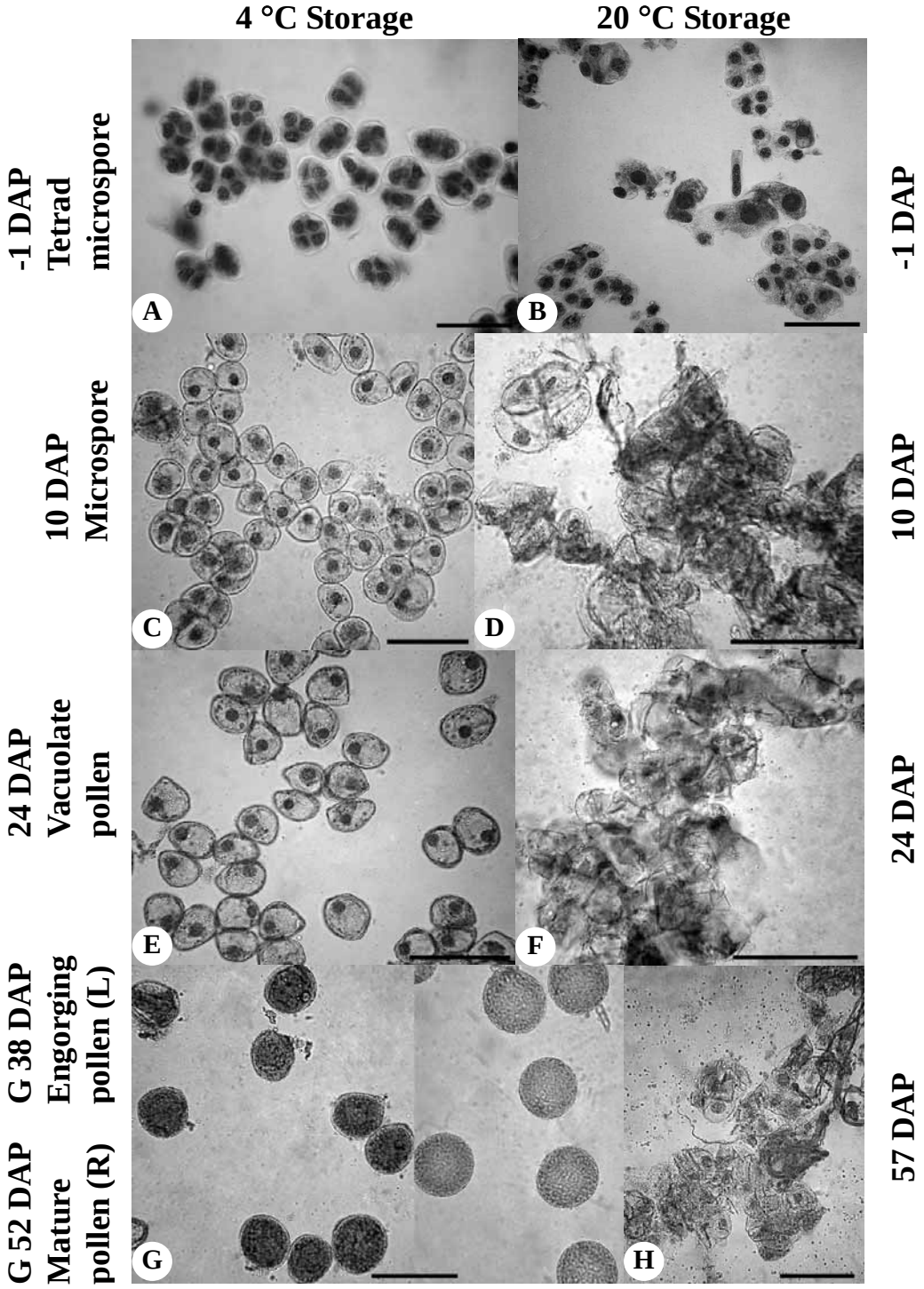


Fig. 4.4 Microspore development in bulbs stored at 4 °C or 20 °C in *Tulipa gesneriana* L. cv. 'Ile de France'. **A, C, E, G, H**, −1, 10, 24, 38, 52 days after bulbs were planted, following 3 weeks at 15 °C and 10 weeks at 4 °C. **A**, Tetrad stage. **C**, Microspores. **E**, Vacuolated microspores. **G**, Engorging pollens at later stage (left) and viable mature pollens (right). **B, D, F, H**, −1, 10, 24, 57 days after bulbs were planted, following 13 week storage at 20 °C. The tetrad microspores deformed and clustered together till before flowering. **DAF**, Days after planting. **L**, Left. **R**, Right. Scale bar = 50 μm.

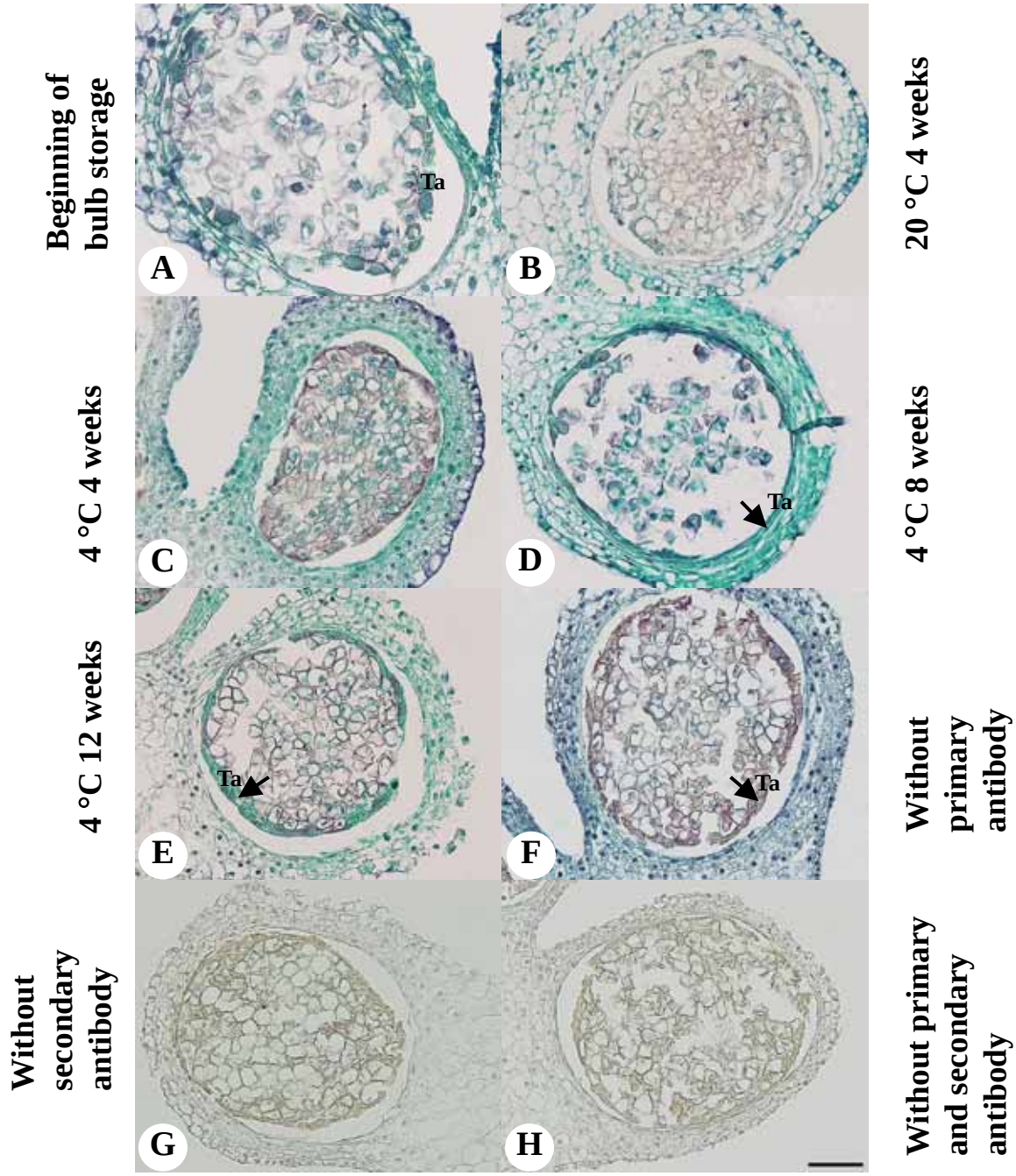


Fig. 4.5 Immunolocalization of IAA in anther pollen sacs in bulbs stored at 4 °C or 20 °C in *Tulipa gesneriana* L. cv. 'Ile de France'. The blue-green color in **A**, **C**, **D**, **E**, was the IAA signal. **A**, Beginning of bulb storage. **B**, After stored at 20 °C for 4 weeks. **C, D, E**, After stored at 4 °C for 4, 8, 12 weeks. **F**, Without primary IAA antibody. **G**, Without secondary antibody. **H**, Without primary and secondary antibodies. Other procedures of the immunolocalization process proceeded as usual. No detection was made in bulbs stored at 20 °C for 8 and 12 weeks, for bud abortion occurred due to long time of storage at 20 °C. **Ta**, Tapetum. Scale bar = 50 μm

Chapter 5

Bud deterioration during dry storage of tulip bulbs and exogenous GA_3 overcomes bud deterioration by promoting endogenous IAA activity in the internodes

5.1 Introduction

Tulip bulbs develop bud abortion during a long period of dry storage at high or low temperatures or during forcing in the greenhouse (De Munk and Hoogeterp, 1975; Le Nard and De Hertogh, 1993). It is a result of the cessation of the development of the flower bud, in the worst case, the deterioration of the flower bud. This disorder shows a range of symptoms from shriveled floral organs to wilted, papery and discolored leaves (De Munk, 1973). Unsuitable temperature regimes during the period of bulb management (De Munk and Hoogeterp, 1975; Le Nard and De Hertogh, 1993) and exposure to ethylene (De Munk, 1973; De Munk and Hoogeterp, 1975; Moe, 1979) give rise to this disorder. Water deficit imposes stress on the young shoot located inside (Van Kilsdonk *et al*., 2002) and is the cause of increased ABA level (Upreti *et al*., 1997/98; Lopez-Carbonell *et al*., 1994), which in turn results in a weakened sink of the flower bud. The sink of the flower bud can also be weakened by ethylene but strengthened by GA or kinetin (De Munk and Gijzenberg, 1977; Moe, 1979). Therefore, it was suggested that the natural balance between growth substances controls the flower bud development (De Munk and Gijzenberg, 1977). Unbalanced hormonal activity might cause the bud abortion in tulip.

Exogenous application of gibberellin prevents ethylene-induced flower-bud blasting in tulip (De Munk and Gijzenberg, 1977; Moe, 1979). However, it

remains unknown how tulip bulbs respond to the exogenous gibberellin on a hormone level. Since exogenous gibberellin has effects on other developmental activities of tulip by interacting with endogenous auxin (Saniewski *et al*., 1999), we presumed that exogenous gibberellin decreases bud abortion probably also by influencing the activity of endogenous auxin. The objective of the present work was to study this assumption and the effects of exogenous GA_3 on bud deterioration of tulip bulbs.

5.2 Materials and Methods

5.2.1 Plant materials and bulb treatment

Tulip (*Tulipa gesneriana* L. cv. 'Ile de France') bulbs with 11 cm circumference, produced in Niigata Prefecture, Japan in 2005, were used in this study. After lifting, the bulbs were stored at 20 °C, well-ventilated, before and after arrival to our laboratory. Total storage period was five months and the bulbs were in a healthy state before the start of bulb treatment on December 4, 2005. The bulbs were treated as follows (Table 5.1): (1) 4 °C treatment: the bulbs were pre-stored at 15 °C for three weeks before transferred to and stored at 4 °C for 4, 8 and 12 weeks. The 4 °C treatment was used as control. (2) 20 °C treatment: the bulbs were stored at 20 °C for 4, 8 and 12 weeks. (3) 20 °C with GA_3 treatment: the bulbs were stored at 20 °C and injected with pure GA_3 (Sigma, Germany) solution at 0.8 mg ml^{-1} at the beginning of storage; storage periods were 4, 8 and 12 weeks. Lateral injection was performed to avoid damage to the shoot of bulbs by using a syringe with a shorter needle. Each bulb was injected with 1.0 ml GA_3 solution to ensure that the bulb was filled with the liquid. The bulbs injected with GA_3 were kept with the nose straight upward for

24 h after injection for a better absorption of the solution by the bulbs.

5.2.2 Grading of bud deterioration during dry storage of bulbs

Thirty bulbs from each treatment were randomly collected at the end of the 4th, 8th and 12th weeks after treatment was started. Four bud deterioration degrees were roughly determined, by which Damage degree 0 was defined as normal flower bud without symptoms of deterioration and the other three degrees of damage were related to bud deterioration based on the symptoms reported by De Munk (1973) (Fig. 5.1): Damage degree 1, stamens, filaments and perianth leaves shriveled (stages 1-3 according to De Munk); Damage degree 2, outer leaves of the bud partially wilted, the top internode wilted and the innermost leaf and all floral organs wilted and yellowish brown (stages 4-6 according to De Munk); and Damage degree 3, all internodes wilted and all leaves wilted, papery and discolored (stage 7 according to De Munk). On the day when samples were collected, three batches of bulbs from the 4 °C, 20 °C and 20 °C with GA_3 treatments respectively were planted, with three replications, in a greenhouse (15 °C/ 10 °C, day/ night). The bulbs were planted in plastic planters (44 cm × 25 cm × 40 cm) with a mixed soil (leaf mold: compost: red clay = 1: 1: 1). The flowering date was determined when the flower buds were fully colored. Harvestable flowers were graded referring to the <Grading standards for the shipment of cut flowers in Japan, 1995>.

5.2.3 Immunolocalization of IAA in internodes of bulbs

Ten bulbs each were randomly sampled from each treatment after 4 weeks. Bulb scales were removed and the young stems of shoots were divided to the 1st

and the 2-4th internodes. The internodes were cut longitudinally to two sections. For the 1st internode, the top half part of one section was sampled. For the 2-4th internodes, one longitudinal section was collected. Sample sections were trimmed to 0.5 cm long. Then they were immediately pre-fixed at vacuum for 3-4 h in a 2 % (g ml^{-1}) aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl) -carbodiimide (EDC, Fluka, Switzerland), then post-fixed for 24 h at 4 °C in a solution containing 4 % paraformaldehyde and 2.5 % glutaraldehyde. After dehydrated at vacuum with a graded ethanol series, the samples were embedded in a mixture of ethanol and polyester wax (melting point: 37 °C) and sectioned to 10 μm. The sections were fixed onto glass slides after spread with distilled water at 25 °C on a hot plate.

Dried sections were dewaxed with xylene and hydrated in an ethanol-water series, then processed according to Moctezuma (1999) and Hou and Huang (2005) with the following modifications: (1) 100 μl 1:20 (mg ml^{-1}) primary IAA antibody (1 mg ml^{-1}) was added to each slide (Agdia, Elkhart, IN, USA) and the slides were incubated at 20 °C for 5 h in a humidity saturated chamber; (2) 100 μl secondary antibody, 1:100 (mg ml^{-1}) dilution of the anti-mouse IgG-alkaline phosphatase-conjugate (1 mg ml^{-1}, Promega, USA), was used; (3) the slides with secondary antibody were incubated at 20 °C for 12 h in a humid chamber; (4) when the blue-green color appeared and became steady, the sections were rinsed in water, dehydrated and mounted with a cover glass using xylene-based mounting medium (Eukitt, O. Kindler, Germany), dried at 40 °C and photographed .

5.2.4 Control sections and locations on the cross and longitudinal sections for photographing

Four controls were included to further verify the effectiveness of the technique as used by Moctezuma (1999) and Hou and Huang (2005). In each of the controls, either EDC pre-fixation, the primary antibody, and/ or the secondary antibody incubation was omitted from the detecting procedure. On the cross section of the 1st-internode, tissues including epidermis, cortex and part of the pith were located for photographing to show the distribution of IAA signal. On the longitudinal section of the 2-4th internodes, part of the pith was photographed for confirmation of the IAA signal. IAA signal was compared between different treatments of bulbs.

5.3 Results

5.3.1 Effect of GA_3 injection in overcoming bud deterioration during dry storage of tulip bulbs

Bulbs did not develop bud deterioration and the physical characteristics of buds showed no obvious difference in the state of health between different treatments 4 weeks after bulb treatment (Fig. 5.2A, B, C). Eight weeks after treatment of bulbs, severe bud deterioration was observed in bulbs in the 20 °C treatment (Fig. 5.2E). The outer leaves of the buds in most bulbs became partially dry and the inside flower organs already completely deteriorated (Figs. 5.1, 5.2E; Table 5.2). In contrast, bud deterioration was not discernible in bulbs in the 4 °C treatment and 20 °C with GA_3 treatment at the same stage (Fig. 5.2D, F). Examination on the inner flower buds showed that 73 % success was

achieved in 20 °C with GA_3 treatment in overcoming bud damage after 8 storage weeks (Table 5.2). Twelve weeks after treatment of bulbs, bud deterioration was observed in all bulbs in the 20 °C treatment and the deterioration of buds in 87 % bulbs was degree 3 (Fig. 5.2H; Table 5.2). However, bud deterioration in bulbs in the 20 °C with GA_3 treatment was lighter with symptoms of degree 2 in 53 % bulbs (Fig. 5.2I; Table 5.2). The 20 °C with GA_3 treatment showed 7 % success in overcoming bud deterioration (Table 5.2). Bud deterioration was not observed in bulbs in the 4 °C treatment (Fig. 5.2G).

5.3.2 Immunolocalization of IAA in the 4 °C, 20 °C and 20 °C with GA_3 treatments during dry storage of bulbs.

Controls

The immunolocalization procedure of controls corroborates the specificity of the monoclonal IAA antibody for auxin within the tissues (Moctezuma, 1999). EDC crosslinks the carboxyl group of IAA to structural proteins in the plant tissues, and serves to preserve the antigenicity of IAA to its particular monoclonal IAA antibody (Shi *et al*., 1993; Moctezuma, 1999). The control sections were prepared from bulbs that had been stored at 4 °C for 12 weeks. Without complete procedure, the sections showed no IAA signal but weak background color (Fig. 5.3A-D). With complete procedure, IAA signal (the blue green color) was detected in the epidermis, cortex and pith (Fig. 5.3E, F). IAA signal was slightly more concentrated in the epidermis than in the inside tissues probably due to the small cell size in the epidermis, whole cells of which were covered by the IAA signal (Fig. 5.3E). In the cortex and pith, IAA signal was present in both the parenchyma cells and vascular bundles (VB) (Fig. 5.3E, F).

Immunolocalization of IAA in the 4 ℃, 20 ℃ and 20 ℃ with GA_3 treatments

A weak IAA signal was observed in between the cells (Fig. 5.4B, arrowhead), probably the apoplast, but not inside the cells, probably the symplast, at the beginning of treatment of bulbs (Fig. 5.4A-C). In the VB, the IAA signal seemed to be slightly more concentrated (Fig. 5.4B). Four weeks after treatments of bulbs were started, IAA signal with different strength was observed in three treatments of bulbs (Fig. 5.4). A stronger IAA signal was present inside the cells in the epidermis, cortex and pith in bulbs in the 4 °C treatment than before treatment of bulbs; but discernible change of IAA signal was not observed between the cells (Fig. 5.4D-F). In bulbs in the 20 °C treatment, a weak IAA signal, was detected in between the cells in the epidermis, cortex and pith (Fig. 5.4G-I) and in cells of the sheath surrounding the VB (Fig. 5.4H). In the 20 °C with GA_3 treatment, IAA signal was present both inside the cells in the epidermis, cortex and pith and between the cells (Fig. 5.4J-L). The IAA signal inside the cells was stronger than that in the 20 °C treatment (Fig. 5.4G-I) but weaker than that in the 4 °C treatment (Fig. 5.4D-F).

5.3.3 Flowering of bulbs in 4 °C, 20 °C and 20 °C with GA_3 treatments

Bulbs that had been stored at 20 °C for 4 weeks attained 67 % flowering, which was not achieved when storage period extended to 8 and 12 weeks (Table 5.3). In the 20 °C with GA_3 treatment, 56 % and 22 % flowering were realized in bulbs that had been stored for 4 and 8 weeks respectively but not for 12 weeks. However, all the plants that flowered in both the 20 °C treatment and 20 °C with GA_3 treatment showed abnormal symptoms mostly shorter stems, which were a

little longer in the 20 °C with GA_3 treatment than in the 20 °C treatment (data not shown). The bulbs that had been stored at 4 °C for 4, 8 and 12 weeks attained high flowering and harvestable flower percentages (Table 5.3). Longer periods of storage resulted in earlier flowering in the 20 °C with GA_3 treatment as well as in the 4 °C treatment. The flowering percentage in the 20 °C and 20 °C with GA_3 treatments was lower than the sprouting rate, indicating that the state of health of the bulbs became worse after the bulbs were planted. Bulbs stored at 20 °C for 12 weeks, with or without GA_3 injection, developed bud deterioration in almost all the bulbs (Table 5.2) and resulted in no flowering after planting (Table 5.3), which indicated that dry storage at 20 °C for such a long period had detrimental effect on the bulbs.

5.4 Discussion

The immunohistochemical method for localization of endogenous free IAA in plant tissues in the present study is a reliable immunoassay method (Moctezuma, 1999; Hou and Huang, 2004). In pre-fixation, EDC couples IAA to structural proteins through the carboxyl group and maintains the antigenicity of IAA (Shi *et al*., 1993). Control sections showed the specificity of the monoclonal IAA antibody to endogenous free IAA in the tissues (Fig. 5.3). Without complete procedure, the control sections showed no IAA signal (Fig. 5.3A-D), indicating that the color reaction was dependent on the presence of both the primary and secondary antibodies. This also demonstrated that the background color was very low (Hou and Huang, 2004).

With this immunohistochemical method, IAA was detected in the internodes of shoots before and after treatment of bulbs (Fig. 5.4). IAA signal was distributed mainly between the cells at the beginning of treatment. Moreover, the

strength of IAA signal did not change obviously 4 weeks after treatment of bulbs. It was probably because that the amount of IAA distributed between the cells was low, the change of which was below the detectable level of this immunohistochemical method; or there was no change in IAA level at all in bulbs treated differently. On the other hand, IAA signal inside the cells, which was not detectable at the beginning of bulb treatment (Fig. 5.4A-C), was detected in the 4 °C treatment and 20 °C with GA_3 treatment 4 weeks after treatment of bulbs (Fig. 5.4D-F, J-L). But IAA signal was not detected in the 20 °C treatment at this stage (Fig. 5.4G-I). These results indicated that the IAA was induced by low temperature or by application of GA_3, suggesting that GA_3 had similar effect to low temperature on promoting the activity of IAA inside the cells. The IAA signal, however, was a little stronger in the 4 °C treatment than in the 20 °C with GA_3 treatment (Fig. 5.4 D-F, J-L), indicating that GA_3 was not as effective as the 4 °C treatment in promoting activity of endogenous IAA. Exogenous GAs applied to the plant can promote biosynthesis of IAA in plant tissues (Kuraishi and Muir, 1962, 1964; Jindal and Hemberg, 1976; Law, 1987; Okubo and Uemoto, 1985). Therefore, IAA signal inside parenchyma cells showed that IAA was probably biosynthesized in the cells.

Bud deterioration, which was not discernible at the end of the 4th week, was lighter in the 20 °C with GA_3 treatment (Fig. 5.2F, I; Table 5.2) but severe in the 20 °C treatment 8 or 12 weeks after treatment of bulbs (Fig. 5.2E, H; Table 5.2). These results were consistent with reports of De Munk and Gijzenberg (1977) and Moe (1979) that gibberellins could reduce bud abortion of tulip. Water deficit (Van Kilsdonk *et al*., 2002) and lack of substrate supply to the bud are possible causes of bud abortion, which are the results of impaired natural balance between growth substances (De Munk and Gijzenberg, 1977). The results of the present study indicated that IAA was probably one of the growth

substances whose biosynthesis was impaired during long period of storage of bulbs at 20 °C.

The bulbs that had been treated differently showed different flowering ability (Table 5.3) confirmed that storage of bulbs at low temperature for a proper period is needed for normal flower production (Le Nard and De Hertogh, 1993). Exogenous GA_3 showed an effect on promoting flowering but it was not as effective as the low temperature (Table 5.3). On the other hand, GA_3 treatment extended sprouting ability of bulbs 12 weeks longer and flowering ability 8 weeks longer by overcoming bud deterioration during long time dry storage at 20 °C.

It may be concluded that GA_3 overcomes bud deterioration during dry storage of bulbs at 20 °C, by promoting biosynthesis of endogenous IAA inside the cells of internodes.

Table 5.1 Treatments of bulbs in *Tulipa gesneriana* L. cv. 'Ile de France

Bulb storage	3 weeks before treatment	4, 8, 12 week treatment
Temperature regime	(1) 15 °C	4 °C
	(2) 20 °C	20 °C
	(3) 20 °C	GA_3 before 20 °C

Table 5.2 Bud deterioration in 4 °C, 20 °C and 20 °C with GA_3 treatments after bulbs were stored for 4, 8 and 12 weeks in *Tulipa gesneriana* L. cv. 'Ile de France'

Treatment	Period (week)	Deterioration of flower bud (per 30 bulbs)			
		Degree 0	Degree 1	Degree 2	Degree 3
4 °C	4	30	0	0	0
	8	30	0	0	0
	12	30	0	0	0
20 °C	4	30	0	0	0
	8	3	7	20	0
	12	0	1	3	26
20 °C + GA_3	4	30	0	0	0
	8	25	5	0	0
	12	2	5	16	7

Damage degree **0**, flower bud was healthy without symptoms of deterioration. Damage degree **1**, stamens, filaments and perianth leaves of flower bud shriveled. Damage degree **2**, outer leaves of the bud partially wilted, the top internode wilted, the innermost leaf and all floral organs of flower bud wilted, papery and yellowish brown. Damage degree **3**, all internodes wilted, all leaves wilted, papery and discolored

Table 5.3 Sprouting, days to anthesis, flowering and harvestable flower percentages of bulbs in *Tulip gesneriana* L. cv. 'Ile de France' treated at 4 °C, 20 °C and 20 °C with GA_3 injection

Treatment	Period (week)	Sprouting (%)	Days to anthesis	Flowering (%)	Harvestable flower [b] (%)
4 °C	4	100	75 ± 4 [a]	100	100
	8	100	51 ± 2	100	100
	12	100	34 ± 1	97	93
20 °C	4	100	93 ± 6	67	0
	8	17	-	0	0
	12	0	-	0	0
20 °C + GA_3	4	100	85 ± 7	56	0
	8	89	70 ± 4	22	0
	12	28	-	0	0

Bulbs were planted in a greenhouse (15/ 10 °C, day/ night) on January 1, January 29 and February 26 respectively in year 2006 at the end of the 4th, 8th and 12th week after treatment;

[a] Values ± Standard deviation;

[b] Harvestable flower according to the <Grading standards for the shipment of cut flowers in Japan, 1995>

Fig. 5.1 Grading degrees and symptoms of bud deterioration of bulbs in *Tulipa gesneriana* L. cv. 'Ile de France' stored at 4 °C, 20 °C and 20 °C with GA_3 injection. Damage degree **0**, normal flower bud without deterioration symptoms; Damage degree **1**, stamens, filaments and perianth leaves shriveled; Damage degree **2**, outer leaves of the bud partially wilted, the top internode wilted, the innermost leaf and all floral organs wilted, papery and yellowish brown; Damage degree **3**, all internodes wilted, all leaves wilted, papery and discolored

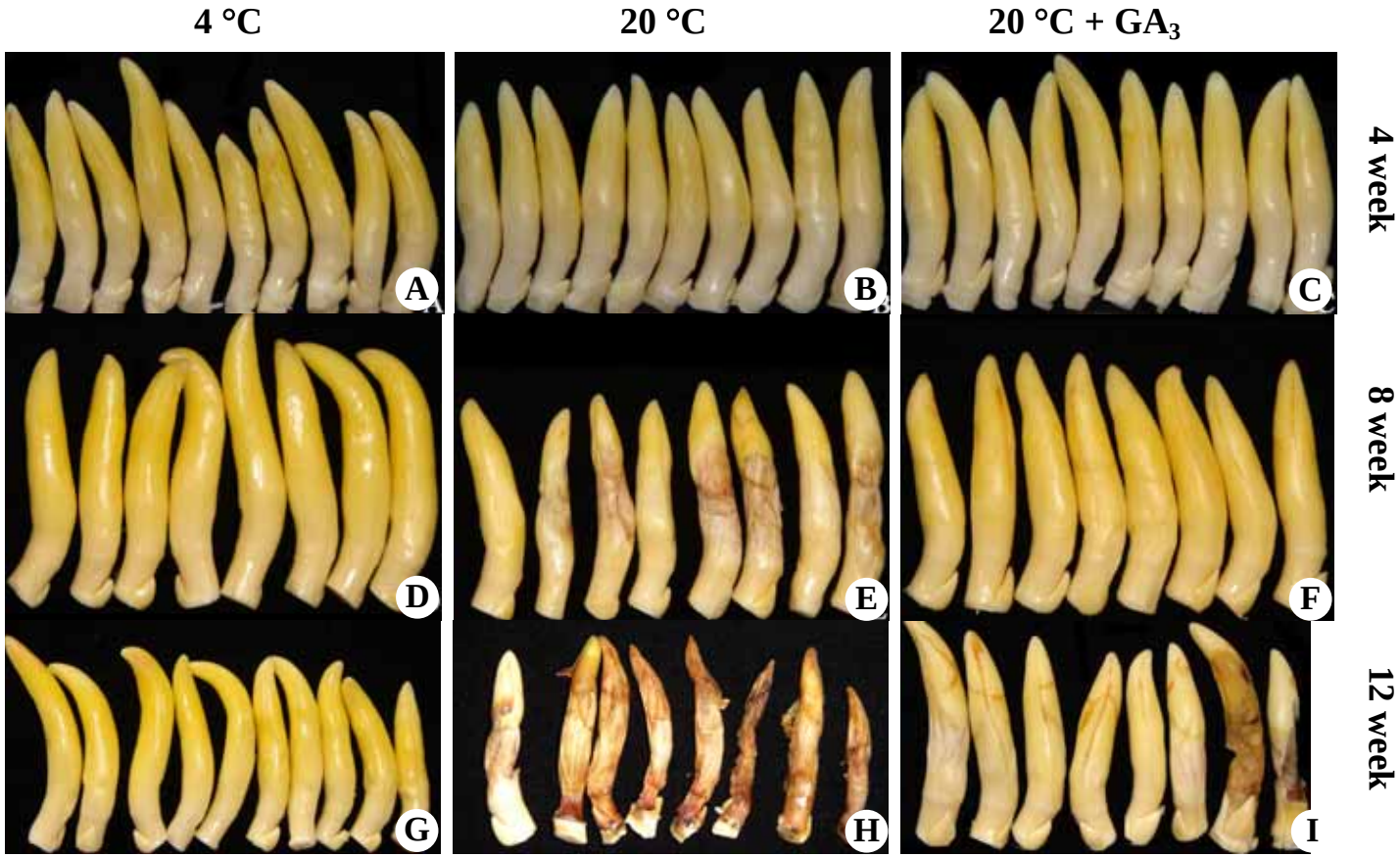


Fig. 5.2 Effect of GA_3 injection in overcoming bud deterioration of bulbs during dry storage at 20 °C in *Tulipa gesneriana* L. cv. 'Ile de France'. **A**, **D**, **G** Buds of bulbs stored at 4 °C for 4, 8, 12 weeks respectively. **B, E, H** Buds of bulbs stored at 20 °C for 4, 8, 12 weeks respectively. **C**, **F**, **I** Buds of bulbs stored at 20 °C for 4, 8, 12 weeks, with GA_3 injection at 0.8 mg ml^{-1} at the beginning of treatment

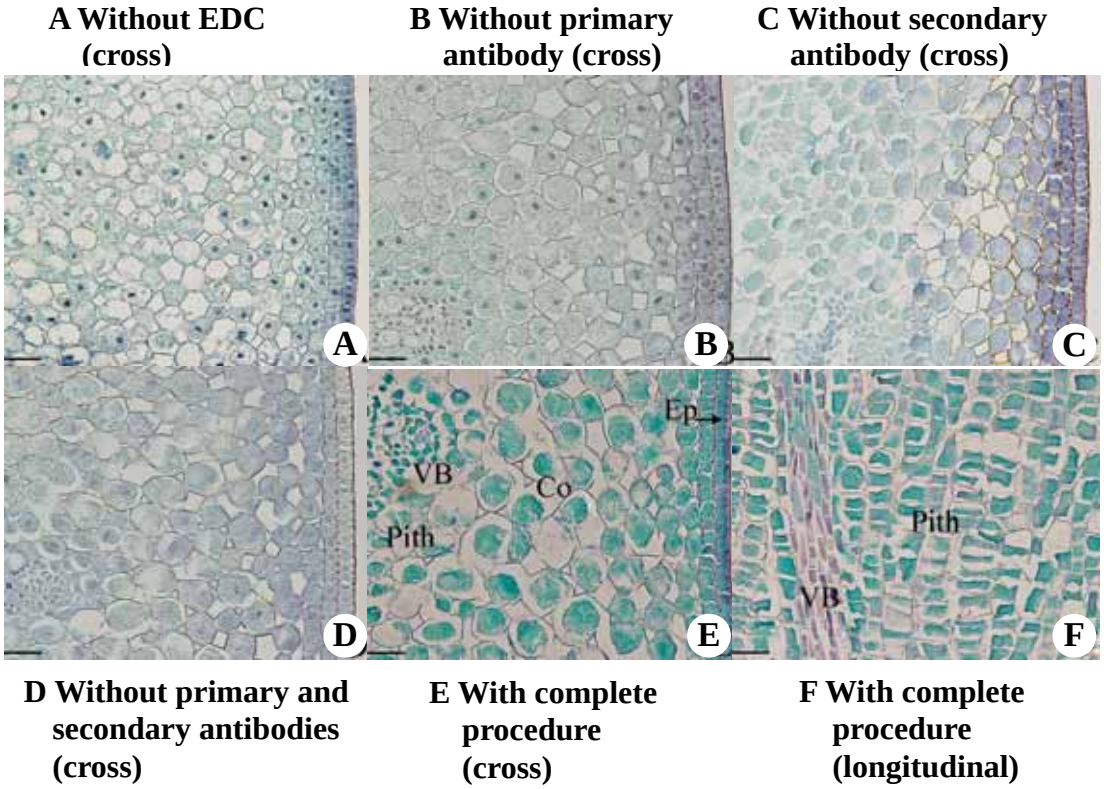


Fig. 5.3 Controls for the IAA immunolocalization technique. The blue-green color on section **E** and section **F** was the IAA signal. **A-E** Cross sections of the 1st-internode showing IAA signal in epidermis, cortex and pith; **F**, longitudinal section of the 2-4th internodes, showing IAA signal. **A** Without EDC; **B** without primary antibody; **C** without secondary antibody; **D** without both primary and secondary antibodies; **E**, **F** with complete IAA immunolocalization procedure. All control sections were prepared from bulbs that had been stored at 4 °C for 12 weeks. **Ep** = epidermis; **Co** = cortex; **VB** = vascular bundle. Scale bar = 50 μm

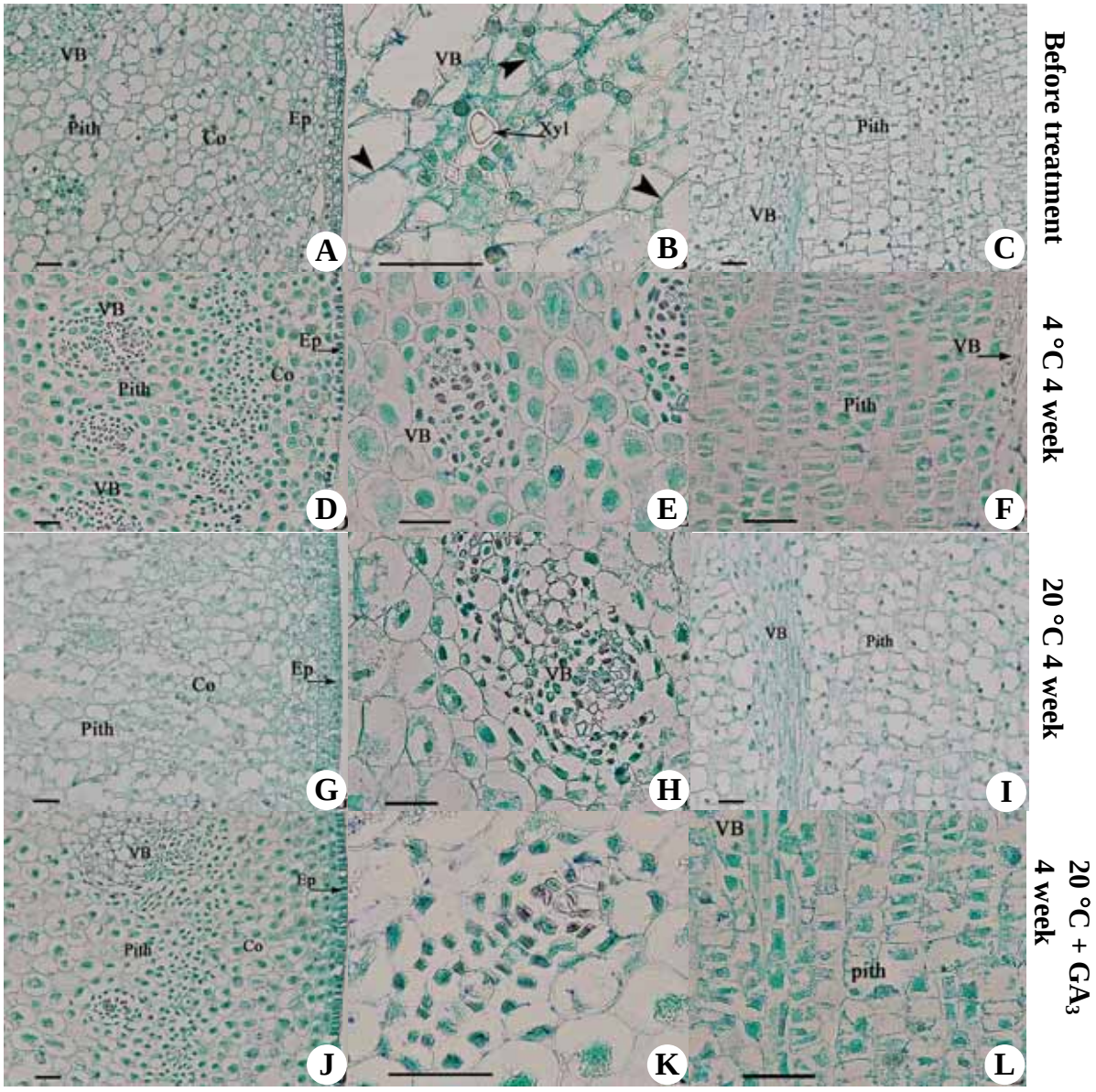


Fig. 5.4 Immunolocalization of endogenous free IAA in the internodes of bulbs treated at 4 °C, 20 °C and 20 °C with GA_3 injection in *Tulipa gesneriana* L. cv. 'Ile de France'. The blue-green color on the sections was the IAA signal. **Ep**, epidermis; **Co**, cortex; **VB**, vascular bundle; **Xyl**, xylem. Scale bar = 50 μm

Chapter 6

Effect of low temperature on endogenous IAA level in different organs of shoots during storage of tulip bulbs

6.1 Introduction

After completion of flower initiation, tulip bulbs require a period of 12-16 weeks of low temperature for floral stalk elongation and flowering (De Hertogh, 1974). Several studies show that IAA plays a key role in the elongation growth of the stem and in the development of flower in tulip (Gabryszewska and Saniewski, 1983; Saniewski and Okubo, 1997; 1998a, b; Saniewski, *et al*., 2005).

Elongation of the last internode is promoted by auxins. In addition, different floral organs show different effects in controlling floral stalk elongation (Op den Kelder *et al*., 1971). This suggests that endogenous auxins are probably produced at different concentrations in different floral organs in the plant of tulip.

However, there is no direct evidence that IAA is produced in the bulbs during storage period. In addition, the distribution of IAA in different organs of the bulb as affected by cold treatment remains unknown. For a thorough understanding of the role of IAA in shoot elongation as affected by low temperature, this study was started aiming at localizing IAA in different organs of shoots during storage of tulip bulbs at low temperature by an immunohistochemical method.

6.2 Materials and methods

6.2.1 Plant material and treatments of bulbs

Tulip (*Tulipa gesneriana* L., cv. 'Ile de France') bulbs, 11 cm in circumference, were stored at 20 °C until mid-November. The bulbs were then stored at 4 °C after pre-stored at 15 °C for 3 weeks. The bulbs stored at 20 °C for the same period were used as control. Ten bulbs were randomly collected from 4 °C or 20 °C bulbs for IAA detection at the beginning and the end of the 4th, 8th and 12th week of storage.

6.2.2 Immunolocalization of IAA in different parts of the shoot of bulbs

Bulb scales were removed and the shoot was cut into the 1st, 2-4th internodes, leaves and flower. The sections of the 1st and 2-4th internodes were cut longitudinally into two parts and one part, 0.5 cm long, was sampled; and 0.5 cm long sections of the outermost leaf, 1st leaf from the basal end, and flower from the basal end, were sampled.

Sections collected from different organs of the shoot were immediately pre-fixed at vacuum for 2 h in a 2 % (w/v) aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, Fluka, Switzerland), then post-fixed for 24 h at 4 °C in a solution containing 4 % paraformaldehyde and 2.5 % glutaraldehyde. After dehydrated the samples were infiltrated in an ethanol-polyester wax graded series, embedded in a polyester wax and sliced to 10 μm. Two ribbons, each with 5 slices, were fixed onto a glass slide after spread with distilled water at 25 °C on a hot plate.

Dried sections were deparaffinized and hydrated in an ethanol-water graded

series, and then processed according to Moctezuma (1999) and Hou and Huang (2004, 2005) with the following modifications: (1) a 1:20 dilution of the primary IAA antibody (1 mg ml^{-1}) in PBS/ BSA solution was prepared, and 100 μl was added to each slide (Agdia, Elkhart, IN, USA), and then the sections were incubated at 20 °C for 5 h in a humidity saturated chamber; (2) 100 μl of the secondary antibody, 1:100 (mg ml^{-1}) dilution of the anti-mouse IgG-alkaline phosphatase-conjugate (1 mg ml^{-1}, Promega, USA), was added and the sections were incubated at 20 °C for 12 h in a humid chamber; (3) after the blue-green color of IAA signal appeared and became steady on the slides, the sections were rinsed in water, dehydrated and mounted with a cover glass using xylene-based mounting medium (Eukitt, O. Kindler, Germany), dried at 40 °C and photographed.

A set of controls were included to verify the effectiveness of the immunolocalization technique as used by Moctezuma (1999) and Hou and Huang (2005). Controls were prepared using cross sections of the first internode by omitting EDC pre-fixation, the primary antibody and/ or the secondary antibody respectively from the procedure.

6.3 Results

6.3.1 Controls for the IAA immunolocalization technique

The immunolocalization procedure of controls showed the specificity of the monoclonal IAA antibody for auxin within the tissues (Moctezuma, 1999). In the four controls, EDC prefixation, the primary monoclonal antibody, the secondary antibody, or both the primary and secondary antibodies were each omitted respectively from the procedure and the blue-green color of IAA signal

was not detected (Fig. 6.1A-D). With complete procedure, IAA signal, shown as the blue green color, was detected (Fig. 6.2A).

6.3.2 Distribution of IAA in different parts of the shoot in bulbs stored at 4 °C for 12 weeks

IAA was localized in the internodes (Fig. 6.2A, B), 1st leaf (Fig. 6.2E), petals (Fig. 6.2D, F), anther (Fig. 6.2G, I, J) and pistil (Fig. 6.2H). In all the organs of the shoot, IAA signal was detected in the cells and between the cells (Fig. 6.2B, F, I, J). In the internodes, IAA was detected at the epidermis, cortex and pith (Fig. 6.2A). In the anther, IAA was mainly detected in the pollen sacs and vascular bundles at the connective of the anther (Fig. 6.2G). In pollen sacs, the tapetum showed strong IAA signal (Fig. 6.2J). In the vascular bundles at the connective, IAA signal was mainly detected between the cells (Fig. 6.2I).

6.3.3 Distribution of IAA in different organs of the shoot of 4 °C and 20 °C bulbs stored for 0, 4 and 8 weeks

IAA signal staining was detected in the leaf, petals and anthers but hardly in the internode at the beginning of storage (Fig. 6.3A, E, I, M). The IAA signal in the pollen sacs of anthers was weaker than in the leaf and petals (Fig. 6.3E, I, M). The distribution of IAA in the internode (Fig. 6.3C, D), leaf (Fig. 6.3G, H) and petals (Fig. 6.3K, L) in 4 °C bulbs did not show apparent changes with storage period extended. However, the IAA signal staining in anthers was stronger in bulbs stored at 4 °C for 4 or 8 weeks than the IAA signal detected at the beginning of storage (Fig. 6.3O, P). In bulbs stored at 20 °C for 4 weeks, IAA was detected in the leaf (Fig. 6.3F) and petals (Fig. 6.3J) but not in the internode

(Fig. 6.3B) and anthers (Fig. 6.3N). Sample of bulbs stored at 20 °C for 8 weeks was not available for IAA detection, because bud deterioration occurred due to the long period of storage at 20 °C.

6.4 Discussion

A period of low temperature is vital to induce stem elongation of tulip bulbs (De Hertogh, 1974; Le Nard and De Hertogh, 1993). It is supposed that auxins are produced in bulbs during storage at cold. Results of the present study showed that IAA was produced in bulbs during storage at 4 °C, as changes in IAA signal were detected in the internodes and anthers (Fig. 6.3A, C, D, M, O, P). The anther is a big part in the flower bud. Changes in IAA level in the anthers would impose much effect on the elongation of the stem. Distribution of IAA in the leaves and petals did not show difference between 4 °C bulbs and 20 °C bulbs (Fig. 6.3). It is probably because that the difference in the concentration of IAA between the 4 °C and 20 °C bulbs was lower than the detectable level of this immunohistochemical method.

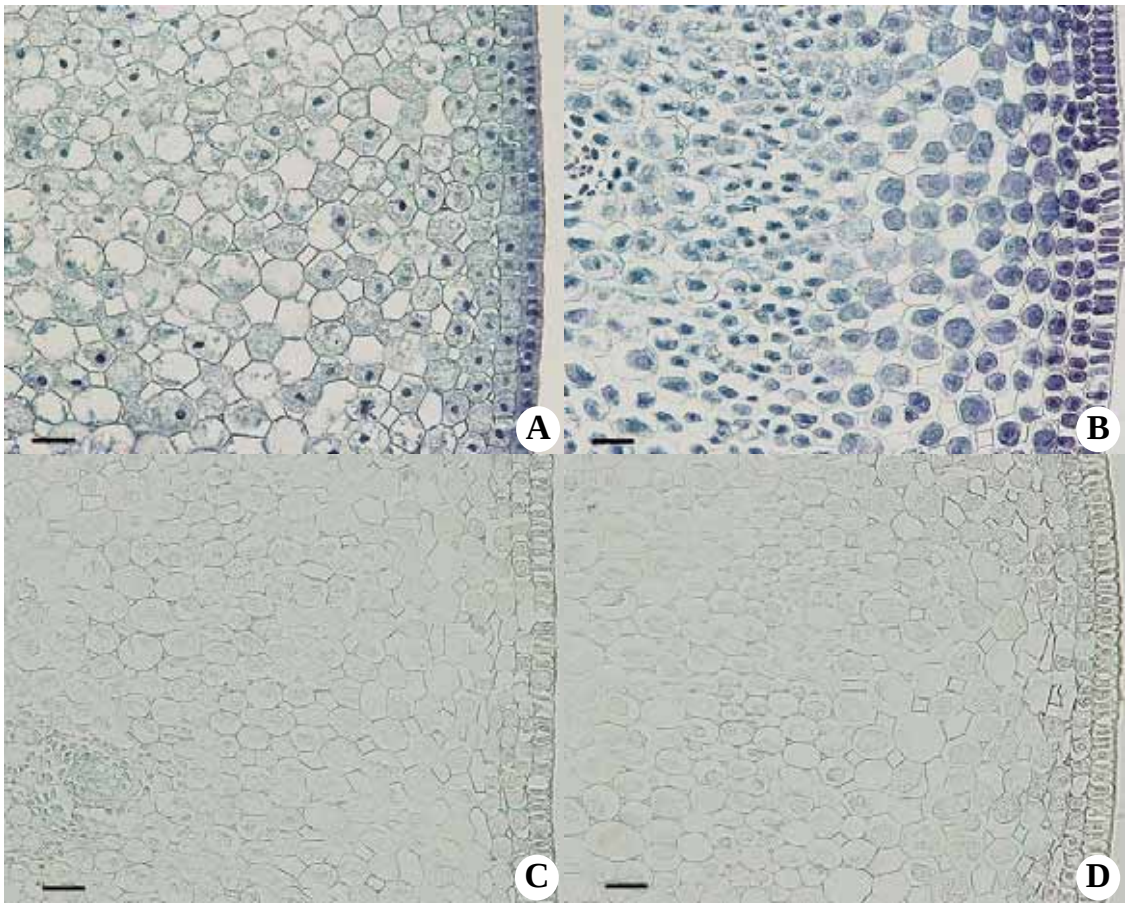
The IAA signal was localized between the cells of different organs of the shoot in bulbs stored at 4 °C. This result provides direct proof for the long-time assumption that IAA is produced in upper organs and transported to the lower parts to promote elongation. IAA is once localized in the nucleus, vacuoles, mitochondria, some dictyosomes and dictyosome-derived vesicles in caps of primary roots of *Z. mays* cv. Kys and it is suggested that dictysomes and vesicles constitute a pathway for IAA movement in and secretion from root cap cells (Shi *et al*., 1993). In the present study, the IAA signal in the cells in 4 °C bulbs covers the nucleus and the vacuoles (Fig. 6.2A-C, E, F). This indicates that IAA is probably transported between cells through some un-identified substances

surrounding the nucleus.

Tulip bulbs had been stored at 20 °C for more than 3 months before the experiment was started in the present study. The long time storage at 20 °C resulted in bud deterioration after bulbs were stored continuously at 20 °C for another 8-12 weeks. Detection of IAA in bulbs stored at 20 °C for 8-12 weeks was not successfully performed, because organs of the shoot became withered and could not be fixed in solutions. Without low temperature, most tulip bulbs develop bud abortion when forced at warm conditions (Le Nard and De Hertogh, 1993). In addition, the deterioration of bud generally starts from the anthers (De Munk, 1973) because without low temperature, the anthers lose water easily and the metabolism of hormones in the anthers might be impaired by loss of water. IAA was not detected in anthers in bulbs stored at 20 °C for 4 weeks indicated that metabolism of IAA was probably impaired. Therefore, storage of bulbs at low temperature is necessary for maintaining normal metabolisms in the anthers and in bulbs. Moreover, the IAA detected in pollen sacs probably plays an important role in the development of the microspores in the locules of anthers.

A Without EDC pre-fixation (cross)

B Without primary antibody (cross)



C Without secondary antibody (cross)

D Without primary and secondary antibodies (cross)

Fig. 6.1 Controls for the IAA immunolocalization technique. All sections were prepared from bulbs that had been stored at 4 °C for 12 weeks. Scale bar = 50 μm

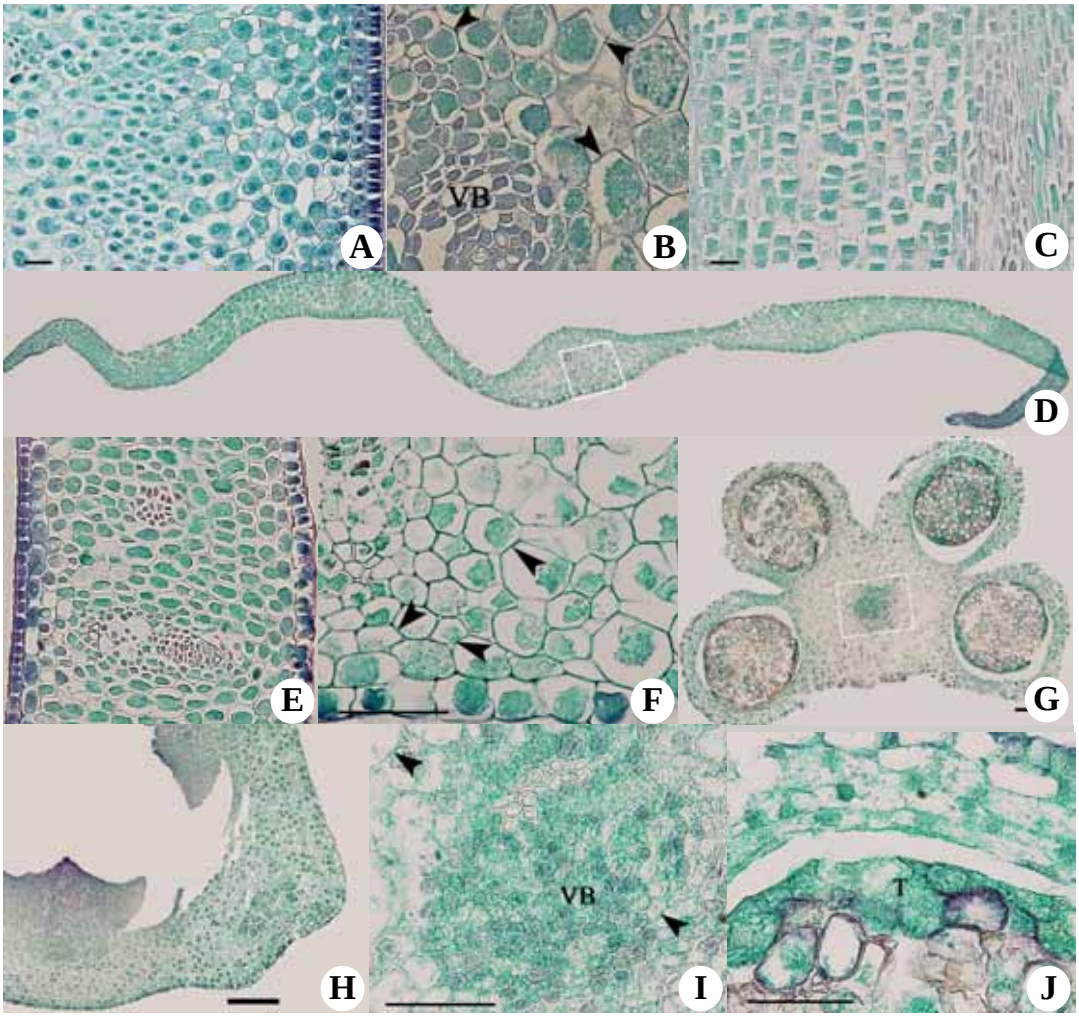


Fig. 6.2 Spatial pattern of IAA distribution in various plant organs of shoots of bulbs stored at 4 °C for 12 weeks in *Tulipa gesneriana* L., cv. 'Ile de France'. **A** 1st internode. **B** Local enlargement of **A**, showing IAA signal in vascular bundles and parenchyma cells at the pith. **C** 4th internode. **D** Petal. **E** Leaf. **F** Local enlargement of **D**, showing IAA signal in and between cells near the vascular bundles in the middle of a petal. **G** Anther. **H** Pistil. **I** Local enlargement of **G**, showing IAA signal in vascular bundles at the connective of the anther. **J** Local enlargement of **G**, showing IAA signal at the tapetum of the anther (**T**, tapetum). Scale bar = 50 μm

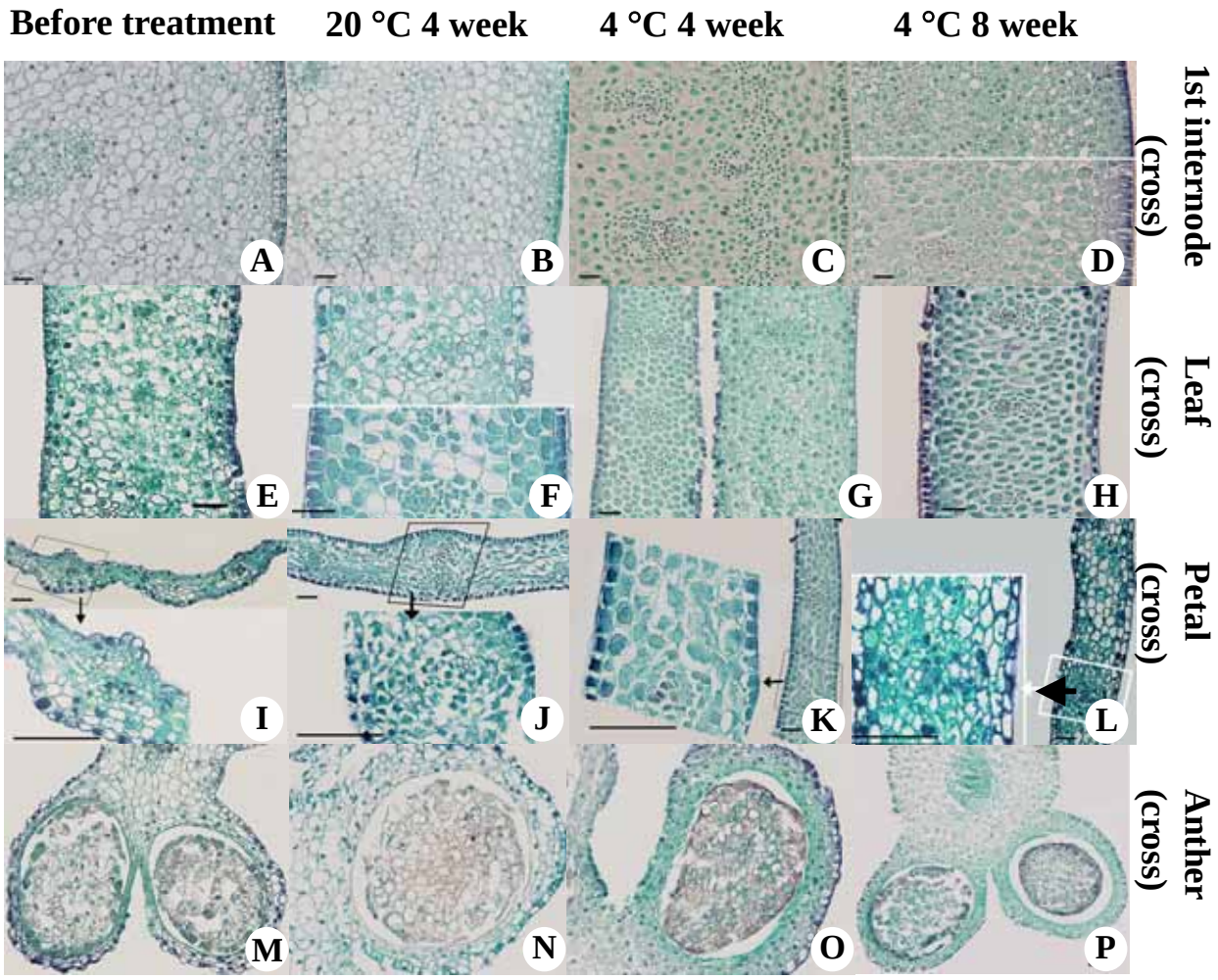


Fig. 6.3 Temporal pattern of IAA distribution in different organs of the shoot of bulbs stored at 4 °C in *Tulipa gesneriana* L., cv. 'Ile de France'. The blue-green color on the sections was IAA signal. Scale bar = 50 μm

Chapter 7

Diffusible IAA from different organs of tulip plant during rapid elongation of the flower stalk

7.1 Introduction

A better understanding of the factors controlling stem elongation of tulip plants is important in commercial production of both potted plants and cut flowers. Growth of the first (lowest) and top internodes of tulips is affected by different control systems; the length of the first internode is markedly affected by the storage period at low temperature, but the length of the top internode is much less affected by the cold duration (Hanks and Rees, 1977). Removal of the flower or floral organs inhibited elongation of the last internode (Op den kelder *et al*., 1971; Hanks and Rees, 1977). Growth of the stem can be restored by treatment of IAA in lanolin paste to the cut surface of the top internode (Op den kelder *et al*., 1971; Saniewski and De Munk, 1981; Saniewski and Okubo, 1997, 1998a, b; Saniewski *et al*., 2005). Therefore, it is suggested that IAA plays a key role in the elongation of the stem and development of the flower in tulip (Op den kelder *et al*., 1971; Saniewski and De Munk, 1981; Gabryszewska and Saniewski, 1983; Saniewski and Okubo, 1997, 1998a, b; Saniewski *et al*., 2005). Moreover, the auxins might be produced in the flower and transported downward to internodes of the stem to promote growth. There is evidence that only basipetally transported IAA or its metabolites account for the induction of elongation of tulip stalk (Saniewski *et al*., 1979; Gabryszewska and Saniewski, 1983; Banasik and Saniewski, 1985). However, the putative native IAA responsible for the elongation of the floral stalk has not yet been chemically

identified in tulip (Le Nard and De Hertogh, 1993).

It is reported that, in *Narcissus,* the shoot possesses two different sites of auxin production, the scape and the apical region represented by the flower bud, the flower or the fruit (Edelbluth and Kaldewey, 1976). The floral stalk of tulip plant, which might function similarly as an auxin production site, has not yet received much attention. Since the availability of unambiguous evidence for IAA in different organs of tulip plant is essential to provide a firm basis for assessing the role of auxins in the elongation of the floral stalk, it is necessary to chemically identify the putative IAA and to confirm the sites of auxin production in the plant of tulips. Compared to extractable hormones, the diffusible hormones may be more related to the physiological phenomena such as stem elongation (Sjut and Bangerth, 1984). Thus, the present study was started with an aim to examine the diffusible IAA levels from different organs of tulip plant and to confirm possible sites of IAA production during the period of rapid elongation of the flower stalk.

7.2 Materials and methods

7.2.1 Plant materials and collection of samples

Bulbs of *Tulipa gesneriana* L., cv. 'Ile de France', 11 cm in circumference, were obtained from a commercial source in Niigata, Japan in 2005. The bulbs, which had reached 'Stage G', were stored well-ventilated at 4 °C until they were planted on 18 November 2005 in plastic planters (44 cm × 25 cm × 40 cm). The planters were placed in an experimental field. A soil mix (leaf mold: compost: red clay = 1: 1: 1) was used. No water was given to the bulbs until March of year 2006, for the climate in autumn and winter in Niigata was very wet. The stage of flowering was defined as the time when the flower buds were fully

colored. The petals of the perianth opened one day after the full coloration of the buds. Flowers were harvested three days after flowering.

The stage of rapid elongation of the floral stalk was defined as the period during which the growth of the stalk increased sharply, which was observed after the floral stalk started growth for 26 days after planting and the plant was about 22 cm in height in the present experiment. The first samples of plant organs were collected on the day when a striking increase in growth of the flower stalk was observed, followed by another four samplings performed when the color was visible on the buds, when the buds became fully colored, when the petals of the perianth opened and when the flowers were harvested, −10 (10 days before flowering), −5 (5 days before flowering), 0 (the flowering day), 1 and 3 days after flowering (DAF), respectively. The stamens were collected three times until flowering, because after flowering the anthers became dehiscent.

In one collection, 40 plants with an even stem height were collected. After the length of the internodes measured, the plant was separated to the leaves, stem and flower. Three leaves on a plant were labeled from the base as the 1st, 2nd and 3rd leaf (Fig. 7.1). The stem was cut at the nodes. Four internodes were labeled acropetally as the 1st, 2nd, 3rd and 4th internode (Fig. 7.1). The flower was divided into the petals, stamens and pistil. Three leaves, four internodes and three types of floral organs of each plant were sampled separately. The collection of diffusible IAA referred to Kojima *et al*. (2002) with some modifications. Briefly, after the basal ends of different organs were cleaned in distilled water for 5 min, plant organs were incubated under continuous white fluorescent light of 750 W m^{-2} at 25 °C for 5 h with the basal ends immersed in distilled water with 50 mg l^{-1} ascorbic acid. During the incubation, 0.5 cm long section from the basal end of each plant organ was cut away at intervals of 1.5 h and the remaining part was returned to incubation after cleaned again with

distilled water for 5 min. The distilled water for incubation with diffusates from plant organs was collected for IAA analysis. The samples were stored at –20 °C until analyzed.

Preliminary experiment showed that samples of the leaves or floral organs, collected from 40 plants respectively, did not contain detectable amount of IAA on the available GC/MS system. Thus, a fluorescence detector, with high sensitivity and selectivity for IAA (Akiyama *et al*., 1983), equipped with an HPLC, was employed for IAA analysis for all types of organs. The chemical identity of the diffusible auxin was analysed on the available GC/MS system (Hitachi M-9000, GC/ 3DQMS system) using a sample mix made of internodes and floral organs.

7.2.2 Identification and analysis of the diffusates from different organs with a liquid chromatography (LC)-fluorometric detector

Identification of diffusible IAA was performed by comparing retention time of the peaks of a sample mix, made of internodes and flower organs, with that of authentic IAA. Diffusates from leaves, internode and floral organs, equivalent to ten, one and one plants respectively, were used for one determination. The samples were extracted and purified according to Kojima (1995) with some modifications. Briefly, after IPA was added as an internal standard, the pH of the samples was adjusted to 2.8 and the aqueous samples were partitioned three times against diethyl ether and evaporated at vacuum to dryness. The samples obtained were dissolved in 50% methanol and fractionated with an HPLC system equipped with a fluorescence detector (L-7480, Hitachi, Japan). The HPLC columns (a series connection of two Inertsil ODS-3 columns, 6 mm × 250 mm, particle Ø5 μm, GL science, Tokyo) were isocratically eluted with 50% methanol solution, to which 20 mM acetic acid was added, at a flow rate of 0.5

ml min^{-1}. IAA content was determined according to Akiyama *et al*. (1983) and Kojima *et al*. (1994), calculated from the ratio of peak area of IAA to that of IPA by the following equation:

IAA content = $IPA_{added} \times 1.24 \times P_{IAA}/ P_{IPA}$, where P_{IAA} is the peak area of IAA and P_{IPA} is that of IPA on a same chromatogram.

7.2.3 Confirmation of chemical identity of IAA with gas chromatography (GC)-mass spectrometry

The chemical identity of the diffusible auxin collected from different plant organs was analyzed and confirmed on a gas chromatography mass spectrometer (GC/3DQMS system, G-7000M, M-9000, Hitachi, Japan) using a sample mix, composed of internodes and flower organs. The extraction and purification of the sample mix, were performed in the same way as in the analyses with the LC-fluorometric detector. But instead of IPA, $^{13}C_6$-IAA was added as the internal standard. The sample mix was fractionated on an ODS-3 column (6 mm × 250 mm, particle Ø5 μm, GL science), monitored with a UV-detector (UV-8010, Tosoh, Japan) at 280 nm, at a flow rate of 1.0 ml min^{-1}. The eluate was collected corresponding to retention time of authentic IAA, dried, methylated and analyzed on a GC/3DQMS system. The most abundant ion, m/z 130, and the molecular ion, m/z 189, of IAA-Me, and m/z 136 and m/z 195 of $^{13}C_6$-IAA-Me, were monitored.

7.3 Results

7.3.1 Identification of IAA in the diffusates from different organs with gas chromatography (GC)-mass spectrometry

The mass spectra of standard IAA-Me (with ^{13}C-IAA-Me) are shown in Fig. 7.2A. The chromatogram of the extract of a sample mix of internodes and flower organs gave a peak with the same retention time as that of authentic IAA. The mass spectra of the material contained in the peak were identical to the spectra of the authentic IAA-Me (with ^{13}C-IAA-Me), having characteristic peaks at m/z 130 and m/z 136, and at m/z 189 and m/z 195 (Fig. 7.2B).

7.3.2 Growth of leaves, internodes and floral organs during rapid elongation of the flower stalk

During rapid elongation of the floral stalk, the 1-3 leaves increased 1.10 g, 0.63 g and 0.26 g respectively from −10 to −5 DAF (Fig. 7.3A). The petals and the pistil of the flower kept high weight increasing till 3 DAF (Fig. 7.3B). The stamens of the flower increased weight only a little before anther dehiscence one day after flowering (Fig. 7.3B).

The patterns of increase in fresh weight and of increase in extension growth of internodes during rapid elongation period were quite similar (Fig. 7.3C, D). The increase in weight of each internode was 2.0-2.8 g during 13 days of rapid elongation of the flower stalk (Fig. 7.3C). The average elongation of the floral stalk (with flower) was 26.3 cm during the 13 days and the growth rate was 2.0 cm per day (Fig. 7.3D). Before the rapid elongation period, average growth rate was only 0.9 cm per day and the elongation was 21.9 cm during 26 days (data not shown). Elongation of the 1-2 internodes slowed down as the floral stalk

extended. The 3-4 internodes grew rapidly until 1 DAF, after which the 4th internode continued rapid elongation, but growth of the 3rd internode slowed down (Fig. 7.3D). Consequently, the average elongation of the 1-4 internodes during 13 days of rapid elongation was 2.4 cm, 5.8 cm, 7.3 cm and 9.8 cm respectively (Fig. 7.3D). Rapidest elongation was in the 4th internode.

7.3.3 Diffusible IAA from leaves, internodes and floral organs during rapid elongation of the flower stalk

The diffusible IAA exported from three leaves respectively remained at a low level (Fig. 7.4A). The amount of IAA from the 1st leaf decreased more than ten-fold during the rapid elongation period. The amount of IAA in the 1st leaf was higher than that in the 2-3 leaves before flowering, but lower after flowering. The amount of diffusible IAA from the 2-3 leaves increased as the stem elongated (Fig. 7.4A). On a basis of fresh weight, the 3rd leaf exported higher level of IAA than the 1-2 leaves (Fig. 7.4D).

The amount of diffusible IAA from the 1st and 2nd internodes changed in a similar way (Fig. 7.4B, E). The amount of IAA increased strikingly till −5 DAF, then declined until 1 DAF, and increased again till 3 DAF (Fig. 7.4B, E). The IAA amount from the 1st internode was higher before flowering but lower after flowering than the amount of IAA from the 2nd internode (Fig. 7.4B). However, on a fresh weight basis, IAA concentration from the 2nd internode was higher than that from the 1st internode, except at the beginning of the period (Fig. 7.4E). The amount of diffusible IAA from the 3rd and 4th internodes also showed a similar changing pattern. The amount of diffusible IAA increased until 0 DAF, declined one day later, increased again till 3 DAF (Fig. 7.4B, E). The amount of IAA increased markedly during the five days before flowering (Fig. 7.4B). On a basis of fresh weight, the concentration of diffusible IAA from the 3rd internode

remained higher than that from the 4th internode at every sampling day, except on 0 DAF (Fig. 7.4E).

The amount of diffusible IAA from petals of the flower increased as the stem extended (Fig. 7.4C). The amount of IAA from the stamens firstly increased and then decreased before 0 DAF (Fig. 7.4C). The IAA amount from the pistil remained somewhat stable until 0 DAF, followed by an increase and thereafter a decrease (Fig. 7.4C). There was no much difference in the amount of diffusible IAA between the petals and pistil (Fig. 7.4C), whereas on a basis of fresh weight, IAA from the pistil was approximately 10-fold of that from the petals at every sampling day during the rapid elongation period (Fig. 7.4F).

There was striking differences in the amount of diffusible IAA from different plant organs. Generally, the amount of IAA from the internodes was more than that from the floral organs, and far more than that from the leaves (Fig. 7.4A-C). However, on the bases of fresh weight, the concentration of IAA from the pistil was the highest on −10, −5, 1 DAF, followed by that from the 3-4 internodes, 1-2 internodes, the petals and stamens, and the leaves (Fig. 7.4D-F).

7.4 Discussion

Development of the 2-4th internodes, with an acropetal increase in elongation, accounts most for extension growth of the floral stalk during the period of rapid elongation of tulip plant, for the 1st internode had almost reached its full length at the beginning of this stage (Fig. 7.3D). The 2-4th internodes each had a sharp growth but appeared during different periods: from −10 to −5 DAF for the 2nd, from −5 to 0 DAF for the 3rd, and during the whole period for the 4th internode (Fig. 7.3D). The levels of diffusible IAA from the 1st and 2nd internodes increased greatly from −10 to −5 DAF and the high IAA level from

the 2nd internode coincides with the sharp elongation of the 2nd internode. The IAA levels from the 3rd and 4th internodes showed a marked increase from −5 to 0 DAF, which is coincident with the rapid growth of the 3-4 internodes (Figs. 7.3D, 7.4B).

On a basis of fresh weight, the 2nd internode exported higher concentration of IAA than did the 1st internode after −10 DAF, which might account for faster elongation of the 2nd internode (Figs. 7.3D, 7.4E). The 4th internode kept faster growth than the 3rd internode till 3 DAF (Fig. 7.3D). However, less amount and lower concentration of IAA, except on 0 DAF, was exported from the 4th internode than from the 3rd internode during the rapid elongation period (Figs. 7.3D, 7.4B, E). Since only basipetally transported IAA is responsible for the induction of tulip stalk elongation (Saniewski *et al*., 1979; Banasik and Saniewski, 1985; Gabryszewska and Saniewski, 1983), the elongation of the 4th internode is undisputedly under the control of the flower, as suggested previously (Op den kelder *et al*., 1971; Hanks and Rees, 1977; Saniewski and De Munk, 1981). However, the amount of IAA provided by a whole flower, including all floral organs, was less than that by each internode, except on −10 DAF (Fig. 7.4B, C). In addition, diffusible IAA from the internodes reached its peak level at different time than the diffusible IAA from the flower during the rapid elongation period. Thus, although the petals and pistil of the flower diffused more amount of IAA than the leaves (Fig. 7.4A, C, D, F), the flower is not the major site of IAA production. Thus, the high level of IAA from the internodes probably did not mainly originate from the flower.

Among all types of plant organs the leaves exported the lowest level of IAA during the rapid elongation period (Fig. 7.4), which may indicate that the leaves are not the major source of auxins during the rapid elongation period. This result, to some extent, provides a direct proof for reports of Banasik and Saniewski

(1985) that excision of leaves, which was conducted when the shoot was 4-7 cm long, caused initially weaker elongation of internodes but finally the plants developed long stalks, similar to those of intact plants; and for report of De Munk (1979) that removal of the leaves initially retards the growth in *Fritillaria*, but the ultimate stem length will be the same.

It was suggested that the leaves and flower are the production sites of auxins controlling elongation of the floral stalk of tulip (Op den kelder *et al*., 1971; Hanks and Rees, 1977; De Munk, 1979; Saniewski and De Munk, 1981). However, these studies had been done by removal of the flower, floral organs or leaves with lanolin paste applied on the cut surface. The surgery, removal of the flower or plant organs, causes irreversible damage to tulip plant. Although covering the cut surfaces with lanolin paste reduces the damage (Hanks and Rees, 1977), it can not recover the physical harm completely. Studies showed that removal of the leaves of tulips often results in desiccation of the flower bud and stem elongation is completely inhibited (De Munk, 1979). It is well known that wounding is one of the stress factors that can induce increased ethylene production by most plant tissue (Yang and Pratt, 1978; Kawa *et al*., 1993). In tulips, it is actually observed that wounding (slicing) of the pistil induces ethylene greatly (unpublished data) (Wegrzynowicz and Saniewski, 1992). In addition, ethylene inhibits stem elongation of tulip cut flower (Nichols and Kofranek, 1982; Suh and Lee, 1997). Therefore, the reduction in elongation of the internodes by decapitation or removal of leaves or floral organs (Op den Kelder *et al*., 1971; Hanks and Rees, 1977; Saniewski and De Munk, 1981) is probably caused by not only the loss of the flower or leaves, both putative IAA sources, but also ethylene that produced after the cut, which might affect the transport of auxin (Abeles, 1973; Nichols and Kofranek, 1982) and normal metabolism of auxin in the internodes.

Investigation on the comparative net biosynthesis of IAA from tryptophan

in cell-free enzyme extracts of different parts of pea (*Pisum sativum* L. cv. Alaska) seedlings and immature pea seeds showed that the regions of most active auxin production are the terminal bud, young stem below the terminal bud, and young leaves; whereas lesser amounts of net auxin biosynthesis are found in enzyme extracts prepared from older stems and leaves and from root tips (Moore, 1989). In the present experiment, the flower stalk exuded most amount of IAA (Fig. 7.4). The level of diffusible IAA exported from the 1-3 internodes, on the bases of fresh weight, follows the order of that the 3rd > the 2nd > the 1st internode. This suggests that upper younger internodes produced IAA more actively than did the lower older ones. The 4th internode exported higher amount of IAA than did the flower during the overall period, except on 1 DAF (Fig. 7.4B, C). This suggests that the top internode was a more important source of auxin than the flower in controlling the extension of the flower stalk.

Different sampling periods may cause the differences between our results and previous ones of other researchers. In the present experiment, plant organs were collected during the period when the floral stalk elongated rapidly. At the beginning of the rapid elongation period, the average growth of the stalk changed from 0.9 cm per day to 2.0 cm per day. Moreover, the 1st internode has almost elongated to its full length and the leaves have already expanded to their full size (Fig. 7.3A, D). Previous reports of other researchers showed that the experiment periods were before the rapid elongation of the flower stalk (Hanks and Rees, 1977; Saniewski and De Munk, 1981). Since the 1st leaf exported IAA decreasingly during the rapid growth period (Fig. 7.4A, D), it is possible that before this period, the 1st leaf exudes higher level of IAA, which might be transported basipetally to the 1st internode to promote elongation. At the beginning of the rapid elongation stage, the flower exported higher level of IAA than the 4th internode (Fig. 7.4B, C). Thus, it is possible that before this stage, the flower exudes higher level of IAA than each internode, acting as the major

site of auxin production.

In conclusion, it is suggested that the top internode is probably the major source of auxins account for the rapid elongation of the flower stalk of tulip.

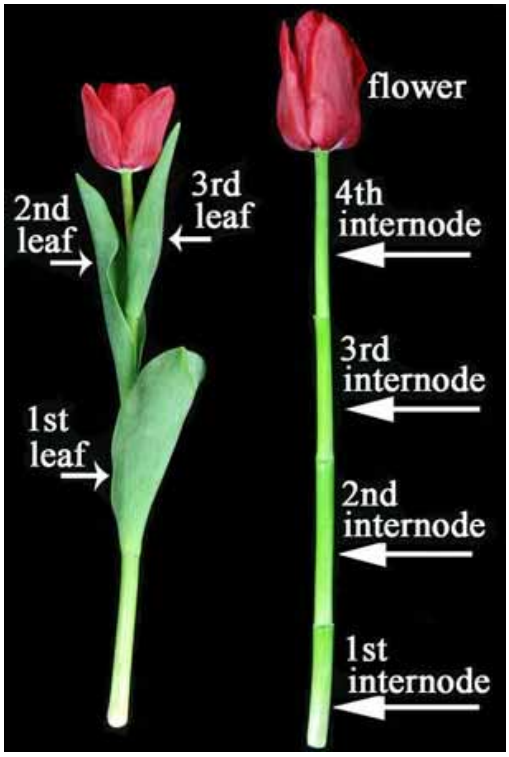


Fig. 7.1 Regions-of-interest in the flower stalk in *Tulipa gesneriana* L., cv. 'Ile de France', which were labeled and collected for diffusible IAA analysis

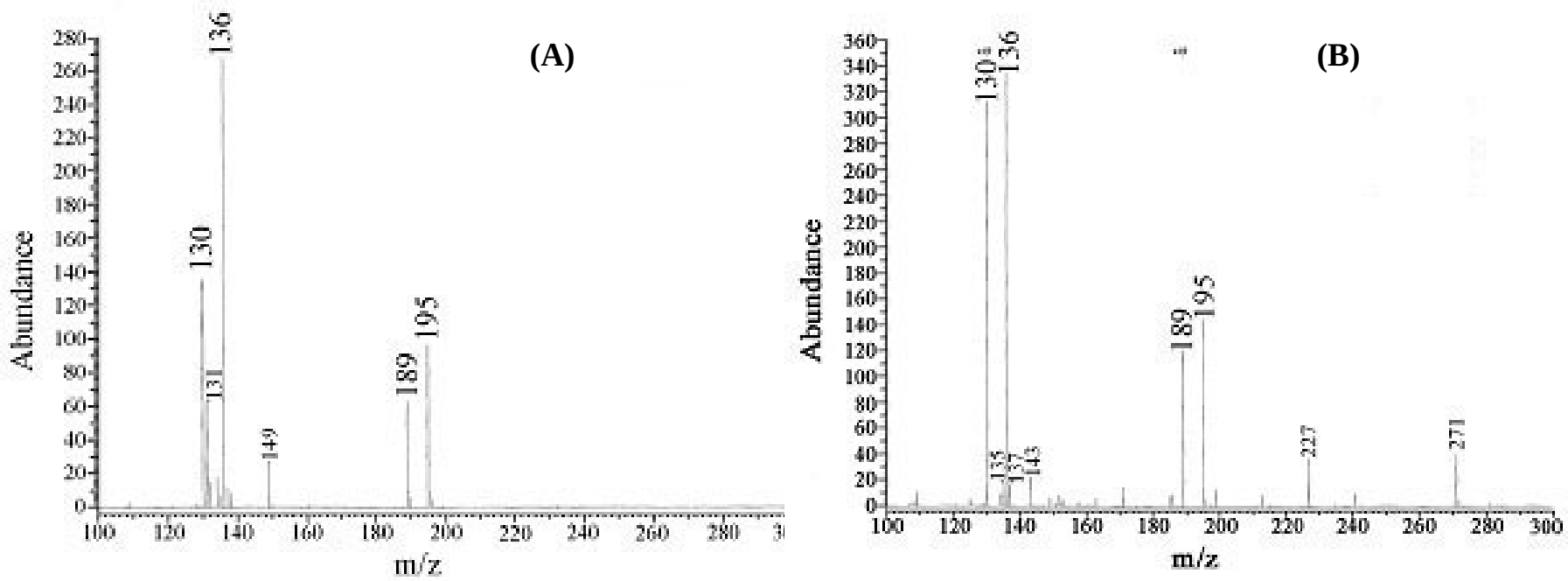


Fig. 7.2 Gas chromatograms and mass spectra of standard methyl indole acetic acid (IAA-Me and $^{13}C_6$-IAA-Me) and of the extract from a sample mix of internodes and flower organs in *Tulipa gesneriana* L., cv. 'Ile de France' after methylation. (**A**) Mass spectra of authentic IAA-Me and ^{13}C-IAA-Me, m/z 130 and m/z 189 of IAA-Me, m/z 136 and m/z 195 of $^{13}C_6$-IAA-Me. (**B**) Mass spectra of IAA-Me from the sample mix and ^{13}C-IAA-Me, m/z 130 and m/z 189 of IAA-Me from the sample mix, m/z 136 and m/z 195 of ^{13}C-IAA-Me

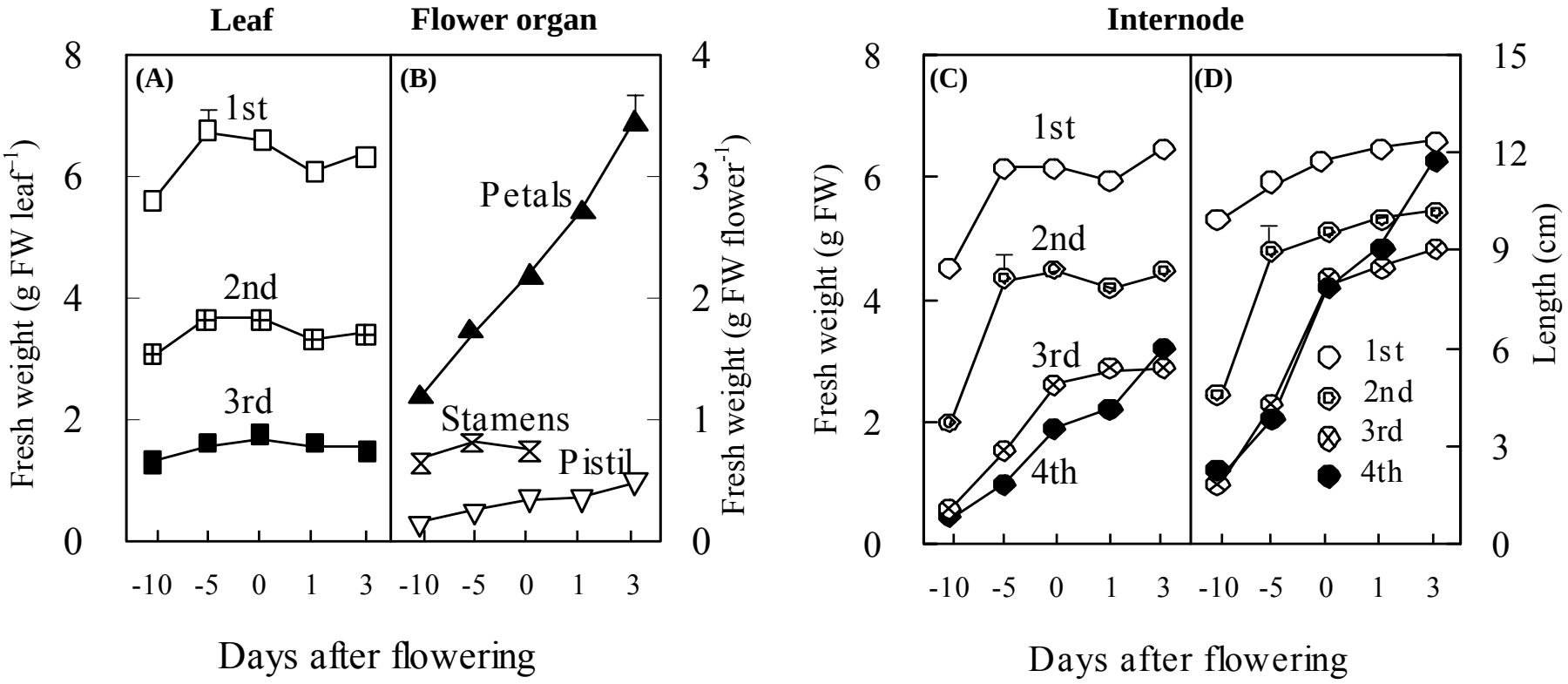


Fig. 7.3 Increase in fresh weight of the leaves (**A**), floral organs (**B**) and internodes (**C**), and increase in the length of internodes (**D**) during rapid elongation of the flower stalk in *Tulipa gesneriana* L., cv. 'Ile de France'. Data are means of 3 determinations, with 1-10 organs used for one analysis. Vertical bars indicate SE, where vertical bars are not shown the limits are within the dimensions of the symbols

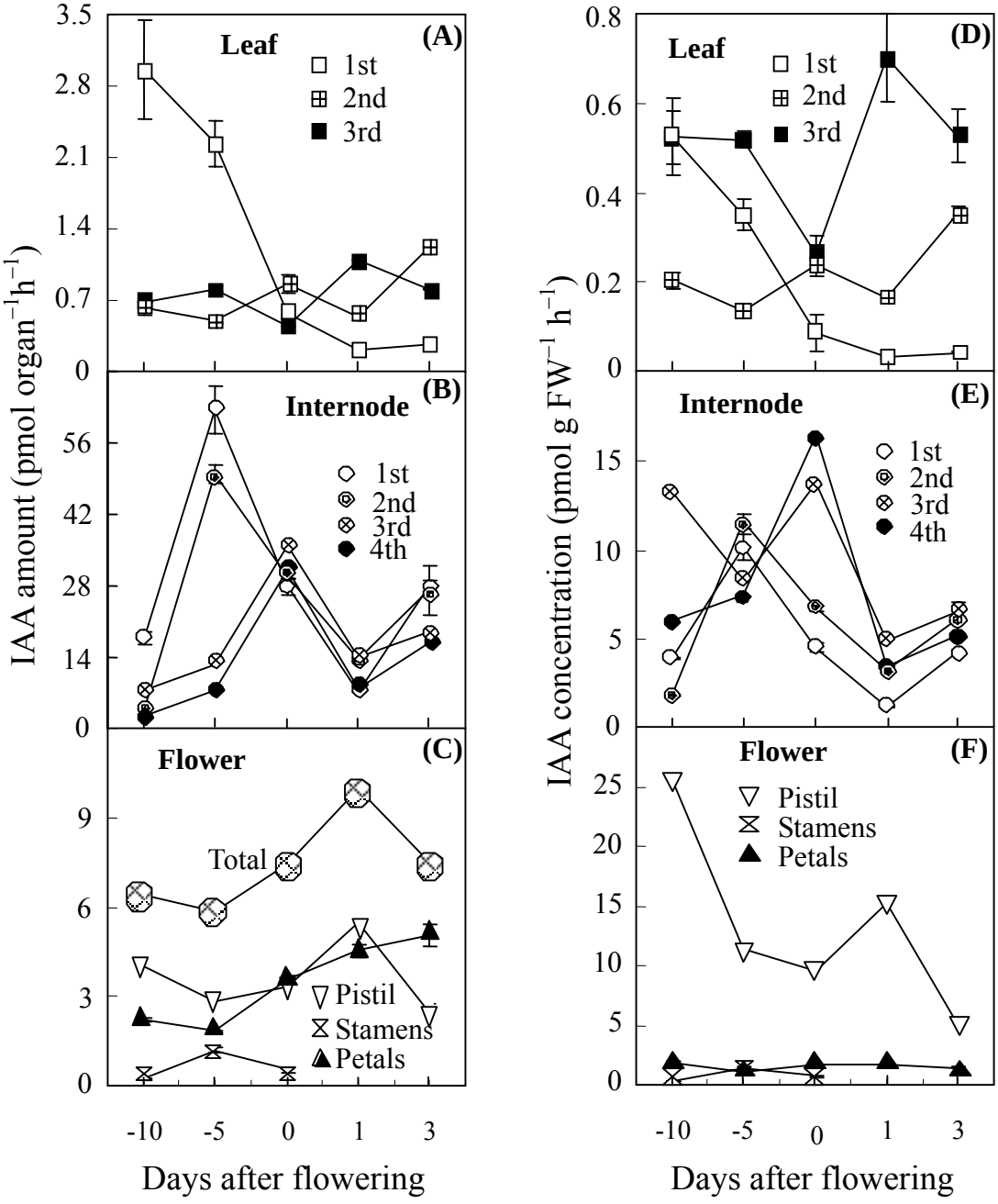


Fig. 7.4 Diffusible IAA from the leaves, internodes and floral organs during rapid elongation of the flower stalk in *Tulipa gesneriana* L., cv. 'Ile de France', detected with a fluorescence detector. **A**, **B**, **C**, the amount of IAA diffused per hour from one organ of the leaves, internodes and flower organs respectively. **D**, **E**, **F**, the concentration, on the bases of fresh weight, of IAA diffused from the leaves, internodes and flower organs respectively per hour. Data are means of three determinations (± SE)

Chapter 8

General discussion

Introduction

Many species and hybrids in the genera *Lilium* and *Tulipa* are widely used in the floral industry as cut flowers and potted ornamentals and in home gardens (Miller, 1993; Beattie and White, 1993; Le Nard and De Hertogh, 1993). Although techniques for controlling flowering are available for many species, the mechanisms directly affecting the processes of scape elongation and flowering have not been elucidated. This is true even in the tulip that has been subjected to extensive research (Le Nard and De Hertogh, 2002). Breaking dormancy of bulbs in *Lilium rubellum* and *Tulipa gesneriana* cultivars by low temperature is usually adopted to induce rapid shoot emergence and flowering, especially in the greenhouse production. Researchers have spent decades trying to discover the mechanisms that control flowering in plants. Much descriptive information has accumulated, but the fundamental factors remain unknown (Beattie and White, 1993). Therefore, basic research on these processes as well as studies on the effects of external factors on physiological processes is absolutely necessary for the commercial use of lily and tulip species (Le Nard and De Hertogh, 2002).

Breaking dormancy in plants is associated with the ABA concentration in many bulbous crops (Djilianov *et al*., 1994; Kim *et al*., 1994; Nagar, 1995; Yamazaki *et al*., 1995, 1999a, b). However, in *Lilium rubellum* the mechanism affecting dormancy breaking is not well understood. In *Tulipa*, although substantial progress has been made regarding endogenous hormones, such as

auxin, GA, cytokinin etc, on their roles in dormancy breaking, shoot elongation and flowering, precise data on the chemical identifies of hormones other than ethylene and ABA are still insufficient in tulip (Le Nard and De Hertogh, 1993). Research in details is still needed for a thorough understanding of the roles of hormones in dormancy breaking and plant development.

Therefore, in 2003 a study was started aiming at elucidating the roles of hormones in bulb dormancy breaking and plant development as affected by low temperature in *Lilium rubellum* and *Tulipa gesneriana*.

The research on effects of cold treatment on changes in endogenous hormone level and plant development in *Lilium rubellum* and *Tulipa gesneriana* comprised several aspects. Changes in ABA concentration in bulb scales in *Lilium rubellum* and in different organs of bulbs in *Tulipa gesneriana* were studied (Chapter 2, 3), development of microspores in anthers as affected by low temperatures for storage of bulbs was studied during the periods of bulb storage and after bulbs were planted in a greenhouse (Chapter 4), effect of long time storage of tulip bulbs at 20 °C room temperature, contrary manipulation to breaking dormancy, on the viability of shoots was investigated (Chapter 5), changes in auxin (IAA) level in shoot of tulip bulbs during cold storage were detected with an immunohistochemical method (Chapter 6), and diffusible auxin from different organs of tulip plants was examined at the stage of rapid elongation of the floral stalk after planting (Chapter 7).

Dormancy breaking of bulbs in *Lilium rubellum* and *Tulipa gesneriana* with low temperature and the role of ABA

To elucidate the role of ABA in bulb dormancy breaking, it was necessary to examine the effect of low temperature on dormancy breaking of bulbs. Dormancy breaking of bulbs was studied on the shoot emergence and flowering

ability in lily and on the stem elongation in tulip (Chapter 2, 3). Shoot emergence and flowering of *Lilium rubellum* bulbs stored for 14 weeks at 4 °C occurred more synchronously, and the time span from the first to last flower in the plants was shorter than that of bulbs stored for 10 weeks at 4 °C. These results showed that dormancy of *Lilium rubellum* bulbs was broken by 14 weeks of storage at 4 °C (Chapter 2).

The stem of both 4 °C and 20 °C tulip bulbs elongated a little rapidly during the 5-6th storage weeks and then the rate of elongation slowed down. It seems that stem elongation of tulip bulbs was initiated after 4 weeks of storage at 4 °C or at 20 °C. Elongation of shoots of 4 °C bulbs increased sharply after the bulbs were planted at 15 °C/ 10 °C (day/ night), whereas shoots of 20 °C bulbs did not show apparent increase in elongation after planting. During the whole storage period the stem of 4 °C tulip bulbs elongated faster than that of 20 °C bulbs. Results obtained show that 4 °C bulbs were released from dormancy (Chapter 3).

ABA concentration in the scales of *Lilium rubellum* bulbs decreased as storage duration extended, and it declined to a constant low level after bulbs had been stored for 10 weeks at 4 °C. This result indicates that the decrease in the endogenous ABA concentration during bulb storage is related to dormancy-release of *Lilium rubellum* bulbs (Chapter 2).

The ABA concentration in the basal plate and innermost scale of tulip bulbs was higher than in other layers of scales. It was suggested that basal plate is one of the probable ABA biosynthetic sites (Aung and De Hertogh, 1979). Since the basal plate and innermost scale are connected directly to the shoot, changes in ABA level in the basal plate and innermost scale would influence more directly dormant condition of the shoot. The concentration of ABA in these two organs decreased sharply to a low level after bulbs stored at 4 °C for 4 weeks and

declined further after another 4 weeks. The sharp increase in the rate of elongation of 4 °C stems after planting shows that decrease in ABA concentration was related to dormancy breaking of 4 °C bulbs. However, storage at 4 °C did not result in more reduction in ABA concentration in the scales than storage at 20 °C. The ABA concentration in 20 °C bulbs declined to a low level too at the end of the storage period. However, the stems of shoots in 20 °C did not show a sharp increase in elongation after planting. This result shows that 20 °C bulbs were not completely released from dormancy. Dormancy of tulips is not deep (Rees, 1992). It is probably easily affected by external factors other than storage temperatures. Water content in 20 °C bulb scales decreased 18 % during storage period, which might have damaged normal metabolic activity of ABA (Chapter 3). Therefore, the marked decrease in ABA level in 20 °C bulbs probably was due to different mechanism.

Microspore development during dormancy breaking of bulbs with low temperature and effect of long time storage of bulbs at 20 °C on the viability of shoots in tulip

It seems that active metabolism had not been initiated in anthers until the bulbs were transferred to low temperature (Chapter 4). The developmental process of microspores in the anthers was not affected by different storage temperatures during the storage of bulbs. Microspores of both 4 °C and 20 °C bulbs reached tetrad stage at the end of storage. However, difference in the development of microspores between the 4 °C and 20 °C bulbs was observed after both bulbs were planted in a warm greenhouse (15-18 °C/ 10 °C, day/ night). Four microspores of the tetrads of bulbs stored at 4 °C separated from each other, developed into vacuolated microspores, engorging pollen, and finally

into mature pollen grains before flowering, whereas the microspores of the tetrads of bulbs stored at 20 °C did not separate from each other after bulbs were planted and finally these tetrads attached together resulting in unshaped pollen grains without viability (Chapter 4). These results indicate that low temperature is vital for normal development of microspores in tulip.

Tulip bulbs developed bud deterioration, when storage period at 20 °C extended further longer than usual. Symptoms of deterioration initially appeared on the stamens of the flower bud (De Munk, 1973). Injection of GA_3 into bulbs at the beginning of bulb storage decreased the damage. Since IAA was detected in bulbs with GA_3 treatment or in bulbs with cold storage (Chapter 5), it is suggested that GA_3 reduces bud deterioration in 20 °C storage, probably by promoting the production of endogenous IAA in the shoot.

IAA was immunolocalized in tulip anthers, especially at the tapetum of pollen sacs, in bulbs during storage at 4 °C (Chapter 4, Chapter 6). The IAA in the anther might affect the division of the tetrad microspores. It is reported that decreased auxin content in anther affects the mitosis of pollen grains by blocking the auxin flow in anther filaments in *Arabidopsis thaliana* (Feng *et al*., 2006). In addition in *Arabidopsis thaliana*, maturing pollen grains accumulate high concentrations of free auxin. It was suggested that during early stages of anther development, tapetum cells produce free-IAA at high concentrations. In addition, the tapetum cells probably supply free-IAA to the developing pollen grains (Aloni *et al*., 2006). Without cold treatment, IAA could not be produced in tulip anthers during storage of bulbs, which would result in defective pollen grains at later stages. However, there is no direct evidence that this abnormal development of microspores would result in bud abortion in tulips. Further study is still needed.

Effect of low temperature on changes in IAA level in different parts of the shoot during storage period and the period of rapid elongation of the floral stalk in tulip

With an immunohistochemical method, IAA was detected in different parts of shoots in tulip bulbs stored at 4 °C for 12 weeks. The IAA signal, shown as the blue-green color on the sections, was obviously darker in 4 °C bulbs than in 20 °C bulbs after bulbs were stored for as short as 4 weeks. It indicated that low temperature induced the production of IAA in the shoot organs, especially in the internodes and anthers of the flower (Chapter 6). Although quantification analysis could not be achieved with the immunoassay, results obtained showed that it is an effective method for IAA detection in tulip bulbs, moreover, small number of bulbs sufficed one detection.

IAA-like activity was once detected in bulbs in *Tulipa* (Ito *et al*., 1960; Syrtanova *et al*., 1973). However, appreciable auxin changes were not found even in *T. alberti* bulbs that had received eight weeks of low temperature at 4 °C. In our preliminary experiment, IAA was chemically identified in different parts of the shoot in tulip with gas chromatography-mass spectrometry at the beginning of storage. However, due to the facts that concentration of IAA is low in different parts of the shoot and the shoot in a bulb is small, extraction of the hormone from different shoot organs was difficult. In addition, a large number of bulbs were used. Thus, further quantification analyses were not performed. A combination of quantification and immunologic localization probably could provide a useful way of analyzing endogenous IAA. An effective extraction method for quantification analysis has to be used first.

The average stalk elongation was 26.3 cm during the 13 days of rapid elongation of the flower stalk that started shortly before flowering, and the rate

of elongation was 2.0 cm per day (Chapter 7). IAA was undisputedly identified in the dissusates collected from different shoot organs. The marked increase in length in the 2nd internode, observed during −10 to −5 DAF, is parallel to the sharp increase in its diffusible IAA concentration. The large increase in length in 3-4 internodes, observed during −5 to 0 DAF, are parallel to the increase in the diffusible IAA level. Although the 1st internode did not elongate much during the rapid elongation period, its fresh weight increased markedly during −10 to −5 DAF, which is parallel to the increase in its diffusible IAA level. The diffusible IAA concentration in the 1-3 internodes, on the base of fresh weight, shows the order of the 3rd > 2nd > 1st. This suggests that upper younger internodes produced IAA more actively than did the lower older ones. The 4th internode maintained faster elongation than the 3rd internode till 3 DAF, whereas less amount and lower concentration of IAA, except on 0 DAF, was exported from the 4th internode. Since IAA can only be basipetally transported, the flower might act as the IAA source. However, the 4th internode exported higher amount of IAA than did the flower during almost the whole period of rapid elongation of the flower stalk, except on 1 DAF (Fig. 7.4B, C). This suggests that the top internode was a more important source of auxin than the flower in controlling the extension of the flower stalk.

The level of diffusible IAA shows difference between organs. It follows the order of that the internodes > floral organs > leaves. This is different from reports of other researchers (Op den kelder *et al*., 1971; Hanks and Rees, 1977; De Munk, 1979; Saniewski and De Munk, 1981). These results further provided a proof for the assumption that the top internode is important in controlling the rapid elongation of floral stalk (Chapter 7).

Auxin is not the only hormone that affects elongation of tulip flower stalk. Under natural light, elongation of the last internode is paralleled by increase in

non-polar free gibberellin (Okubo and Uemoto, 1986). Other hormones might also be involved in the floral stalk elongation.

In conclusion, decreases in the concentration of ABA in bulb scales are related to dormancy release of *Lilium rubellum* and *Tulip gesneriana* cv. 'Ile de France' bulbs during dormancy breaking of bulbs with low temperature. Auxin is produced in the shoot during the cold treatment period, which leads to shoot emergence, floral stalk elongation and finally the flowering. Other hormones, such as GAs and cytokinins might also be involved in shoot elongation and flower development through changes in concentration or by interaction with other hormones during the period of storage at cold or during growth of shoots of bulbs in greenhouses. Further studies are still needed for a thorough understanding of the role of hormones in the flower development.

Summary

Dormancy breaking of bulbs in *Lilium rubellum* and *Tulipa gesneriana* with low temperature and the role of ABA

Concentration of endogenous abscisic acid (ABA) in *Lilium rubellum* bulb scales decreased as storage duration increased, and the concentration of ABA was at a constant low level after being stored at 4 °C for 10 weeks. Synchronous shoot emergence and flowering obtained in bulbs stored at 4 °C for 14 weeks, indicate that decrease in the endogenous ABA concentration during bulb storage is related to dormancy breaking of bulbs in *Lilium rubellum*. In tulip, concentration of ABA in basal plate (BP) and innermost scale (S-4) of bulbs stored at 4 °C decreased during 8 weeks of storage, whereas there was no steady decrease in concentration of ABA in outer scales (S-1, S-2, S-3). The shoot of bulbs stored at 4 °C for 8 weeks elongated sharply after planting, which indicates that dormancy of tulip bulbs was broken by storage at 4 °C for 8 weeks. Therefore, the decrease in concentration of ABA in BP and S-4 of bulbs is related to dormancy breaking of bulbs in tulip.

Microspore development during dormancy breaking of bulbs with low temperature and effect of long time storage at 20 °C on the viability of shoots in tulip bulbs

During dormancy breaking of tulip bulbs by low temperature, microspore mother cells (MMCs) in anthers developed into tetrad microspores, which continuously developed into viable mature pollens after bulbs were planted in a greenhouse. However, without low temperature, viable mature pollens could not be obtained because the tetrad microspores formed during storage period failed to separate from each other after planting of bulbs. Dormant tulip bulbs

developed bud deterioration when stored at room temperature (20 °C) for a longer time than usual. The symptoms of bud deterioration firstly appeared on anthers. Injection of GA_3 solution into bulbs overcame bud deterioration and induced IAA production in the shoot. An increase in IAA level was found in anthers and basal internode of bulbs stored at 4 °C, whereas the IAA in basal internode and anthers was below a detectable level after continuous storage at 20 °C. Since both IAA and ABA decreased in bulbs after storage at 20 °C, normal metabolism of hormones might be damaged during storage at 20 °C. Moreover, the balance in concentration between different hormones in shoots might be negatively affected. Loss of water is probably one vital factor accounting for the above failures in development and hormone metabolism.

Effect of low temperature on changes in IAA level in different parts of shoots during storage period and during the period of rapid elongation of the floral stalk in tulip

IAA was detected in different organs of the shoot in bulbs stored at 4 °C during storage period and during the period of rapid elongation of the flower stalk. With an immunohistochemical method, IAA was localized in internodes, 1st leaf and flower organs in bulbs stored at 4 °C for 12 weeks. In addition, temporal examination of IAA showed that the IAA signal staining was detected in different organs in bulbs during storage at 4 °C. The IAA signal staining was weak in internodes and anthers at the beginning of storage but became stronger after 4 weeks of storage at 4 °C. However, after 4 weeks of storage at 20 °C, the IAA signal was still weak in internodes and became weaker in anthers. The IAA signal staining was also detected in leaves and petals at the beginning of storage. However, the IAA signal did not show an obvious increase in strength after 4, 8 or 12 weeks of storage at 4 °C or a decrease in strength after 4 weeks of storage

at 20 °C. Detection of IAA signal in leaves and anthers was not successfully performed in bulbs stored at 20 °C for 8 and 12 weeks, because shoots were damaged in bulbs during storage at 20 °C for a long period.

With dormancy of tulip bulbs broken, the flower stalk underwent a rapid elongation, which markedly increased shortly before flowering. Diffusible IAA was detected in various parts of shoots. The amount of diffusible IAA from different organs was internodes > flower organs > leaves during the period of rapid elongation of the flower stalk. Moreover, the amount of IAA from the internodes and flowers reached its peak at different time. Results obtained indicate that the top internode is probably the major site of auxin production accounting for the rapid elongation of the flower stalk.

References

Abeles F.B. 1973. *Ethylene in plant biology*. New York, London: Academic Press.

Adkins S.W. and Bellairs S.M. 1995. *Seed dormancy mechanisms in Australian native species*. In: Bellairs, S.M., Marris, J.M. (Eds.), Proc Workshop on Native Species Establishment on Mined Lands in Queensland. Australian Center for Minesite Rehabilitation Research, Brisbane and Chamber of Mines and Energy of WA, Perth, pp. 51-71.

Adkins S.W., Bellairs S.M. and Loch D.S. 2002. Seed dormancy mechanisms in warm season grass species. *Euphytica* 126:13-20.

Aguettaz P., Paffen A., Delvallée I., Van der Linde P. and De Klerk G.J. 1990. The development of dormancy in bulblets of *Lilium speciosum* generated *in vitro* Ⅰ. The effects of culture conditions. *Plant Cell, Tissue and Organ Cultulre* 22:167-172.

Akiyama M., Sakurai N. and Kuraishi S. 1983. A simplified method for the quantitative determination of indoleacetic acid by high performance liquid chromatography with a fluorometric detector. *Plant & Cell Physiology* 24(8):1431-1439.

Algera L. 1936. Concerning the influence of temperature treatment on the carbohydrate metabolism, the respiration and the morphological development of the tulip. Proc. Kon. Akad. van Wet., Amsterdam, 39:1-29.

Ali-Rachedi S., Bouinot D., Wagner M-H., Bonnet M., Sotta B., Grappin P. and Jullien M. 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta*

219:479-488.

Aloni R., Schwalm K., Langhans M. and Ullrich C.I. 2003. Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in *Arabidopsis*. *Planta* 216:841-853.

Aloni R., Aloni E., Langhans M. and Ullrich C.I. 2006. Role of auxin in regulating Arabidopsis flower development. *Planta* 223:315-328.

Arnold W.N. 1968. The selection of sucrose as the translocate of higher plants. *Journal of Theoretical Biology* 21(1):13-20.

Aung L.H., De Hertogh A.A. and Staby G.D. 1969. Gibberelin-like substances in bulb species. *Canadian Journal of Botany* 47:1817-1819.

Aung L.H. and De Hertogh A.A. 1979. Temperature regulation of growth and endogenous abscisic acid-like content of *Tulipa gesneriana* L. *Plant Physiology* 63:1111-1116.

Banasik L. and Saniewski M. 1985. The effect of different auxins on tulip stalk elongation. *Acta Horticulturae* 167:193-204.

Beattie D.J. and White J.W. 1993. *Lilium*-hybrids and species. Chapter 28. In : De Hertogh, A., Le Nard, M. (Eds.), The physiology of flower bulbs. Elsevier, Amsterdam, pp. 423-454.

Beck R. 1984. The ‘hows’ and ‘whys’ of hybrid lilies; establishing a multicolored lily program for cuts or pots. *Florist’s Review,* 175:22,24,27.

Bewley J.D. 1997. Seed germination and dormancy. *The Plant Cell* 9:1055–1066.

Bhargava R. 1997. Changes in abscisic acid and gibberellic acids contents during the release of potato seed dormancy. *Biologia Plantarum* 39(1):41-45.

Corbineau F., Bianco J., Garello G. and Côme D. 2002. Breakage of *Pseudotsuga menziesii* seed dormancy by cold treatment as related to

changes in seed ABA sensitivity and ABA levels. *Physiologia Plantarum* 114:313-319.

De Hertogh A.A. 1974. Principles for forcing tulips, hyacinths, daffodils, Easter lilies and Dutch irises. *Scientia Horticulturae* 2:313-355.

Delvallée I., Paffen A. and De Klerk G.J. 1990. The development of dormancy in bulblets of *Lilium speciosum* generated *in vitro*. II. The effect of temperature. *Physiologia Plantarum* 80:431-436.

De Munk W.J. 1973. Flower-bud blasting in tulips caused by ethylene. *European Journal of Plant Pathology* 79:41-53.

De Munk W.J. and Hoogeterp P. 1975. Flower-bud blasting in tulips. *Acta Horticulturae* 47:149-156

De Munk W.J. and Gijzenberg J. 1977. Flower-bud blasting in tulip plants mediated by the hormonal status of the plant. *Scientia Horticulturae* 7:255-268

De Munk W.J. 1979. Influence of plant growth regulators on the development of bulbous plants with special reference to organ relationships. *Acta Horticulturae* 91:207-219.

Dewitte W. and Van Onckelen H. 2001. Probing the distribution of plant hormones by immunocychemistry. *Plant Growth Regulation* 33:67-74.

Djilianov D., Gerrits M.M., Ivanova A., Van Onckelen H.A. and De Klerk G.J.M. 1994. ABA content and sensitivity during the development of dormancy in lily bulblets regenerated *in vitro*. *Physiologia Plantarum* 91:639-644.

Dole J.M. 2003. Research approaches for determining cold requirements for forcing and flowering of geophytes. *HortScience* 38:341-346.

Edelbluth E. and Kaldewey H. 1976. Auxin in scapes, flower buds, flowers, and fruits of Daffodil (*Narcissus pseudonarcissus* L.). *Planta* (Berl.) 131:285-291.

Feng X.-L., Ni W.-M., Elge S., Mueller-Roeber B., Xu Z.-H. and Xue H.-W.

2006. Auxin flow in anther filaments is critical for pollen grain development through regulating pollen mitosis. *Plant Molecular Biology* 61:215-226.

Franssen J.M. and Voskens P.G.J.M. 1992. Methods to determine abscisic acid and indole-3-acetic acid, and determinations of these hormones in tulip cv. Apeldoorn bulbs as related to the cold-treatment. *Acta Horticulturae* 325:267-276.

Gabryszewska E. and Saniewski M. 1983. Auxin control of tulip stalk elongation in vitro. *Scientia Horticulturae* 19:153-159.

Geng X.M., Ii-Nagasuga K., Okubo H. and Saniewski M. 2005. Effects of TIBA on growth and flowering of non pre-cooled tulip bulbs. *Acta Horticulturae* 673:207-214

Gorin N. and Heidema F.T. 1985. Starch content of freeze-dried anthers and α-amylase activity of their extracts as criteria that dry-stored bulbs (*Tulipa gesneriana*, L.) cultivar 'Apeldoorn' have been exposed to 5 C. *Scientia Horticulturae* 26:183-189.

Hanks G.R. and Rees A.R. 1977. Stem elongation in tulip and narcissus: the influence of floral organs and growth regulators. *New Phytologist* 78:579-591.

Hobson G.E. and Davies J.N. 1977. Mitochondrial activity and carbohydrate levels in tulip bulbs in relation to cold treatment. *Journal of Experimental Botany* 28:559-568.

Hobson G.E. and Davies J.N. 1978. Influence of the extent and duration of cold treatment on the flowering behaviour, composition and metabolic activity of tulip bulbs. *Scientia Horticulturae* 8:279-287

Hou Z.X. and Huang W.D. 2004. Immunohistochemical localization of IAA in developing strawberry fruit. *Journal of Horticultural Science and Biotechnology* 79:693-698.

Hou Z.X. and Huang W.D. 2005. Immunohistochemical localization of IAA and ABP1 in strawberry shoot apexes during floral induction. *Planta* 222:678-687.

Ikeda Y. 1971. Studies on *Lilium rubellum* Baker. 2. Regarding variation and utilization as horticultural variety (in Japanese with English summary). *Bulletin of Niigata Horticulture Experimental Station* 6:42-70.

Ikeda Y. 1997. A system for year-round flowering of *Lilium rubellum* Baker by manipulating chilling temperatures, storage duration, and planting time (in Japanese with English summary). *Journal of the Japanese Society for Horticultural Science* 66:371-377.

Ito H., Kato T. and Toyoda T. 1960. Metabolism of tulip bulbs as related to thermoperiodicity. *Japanese Horticultural Association (Japan*), 29:323-330 (in Japanese with English Summary).

Jacobsen J., Pearce D.W., Poole A.T., Pharis R.P. and Mander L.N. 2002. Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Physiologia Plantarum* 115:428-441.

Jindal K.K. and Hemberg T. 1976. Influence of gibberellic acid on growth and endogenous auxin level in epicotyl and hypocotyl tissues of normal and dwarf bean plants. *Physiologia Plantarum* 38(2):78-82

Junttila O. 1988. To be or not to be dormant: some comments on the new dormancy nomenclature. HortScience 23(5):805-806.

Kamerbeek G.A., Beijersbergen J.C.M. and Schenk P.K. 1972. Dormancy in bulbs and corms. *Proceedings of the 18th International Horticultural Congress* 5:233-239.

Karssen C.M. and Lacka E. 1986. A revision of the hormone balance theory of seed dormancy: Studies on gibberellin and/or abscisic acid-deficient mutants of *Arabidopsis thaliana*. In: Bopp M. (ed.), Plant Growth Substances 1985. Springer-Verlag, Berlin Heidelberg, pp. 315-323.

Kawa L., Le Nard M. and De Hertogh A.A. 1993. The effects of scale wounding of tulip bulbs on ethylene evolution, plant growth and flowering. *Scientia Horticulturae* 53:347-359.

Kerk N.M. and Feldman L.J. 1995. A biochemical model for the initiation and maintenance of the quiescent center: implications for organization of root meristems. *Development* 121:2825-2833.

Khan A.A. 1975. Primary, preventive and permissive roles of hormones in plant system. Botanical Review 4:391-420.

Kim K.S., Davelaar E. and De Klerk G.J. 1994. Abscisic acid controls dormancy development and bulb formation in lily plantlets regenerated in vitro. *Physiologia Plantarum* 90:59-64.

Kojima K., Sakurai N. and Tsurusaki K. 1994. IAA distribution within tomato flower and fruit. *HortScience* 29(10): 1200.

Kojima K. 1995. Simultaneous measurement of ABA, IAA and GAs in citrus – role of ABA in relation to sink ability. *Japan Agricultural Research Quarterly* 29:179-185.

Kojima K. 1996. Changes of abscisic acid, indole-3-acetic acid and gibberellin-like substances in the flowers and developing fruitlets of citrus cultivar 'Hyuganatsu'. *Scientia Horticulturae* 65:263-272.

Kojima K., Ohtake E. and Yu Zh.-Y. 2002. Distribution and transport of IAA in tomato plants. *Plant Growth Regulation* 37:249-254.

Kuraishi S. and Muir R.M. 1962. Increase in diffusible auxin after treatment with gibberellin. *Science* 137(3532):760-761

Kuraishi S. and Muir R.M. 1964. The relationship of gibberellin and auxin in plant growth. *Plant and Cell Physiology* 5(1):61-69

Lang G.A., Early J.D., Arroyave N.J., Darnell R.L., Martin G.C. and Stutte G.W. 1985. Dormancy: toward a reduced, universal terminology. *HortScience* 20:809-812.

Langens-Gerrits M.M., Nashimoto S., Croes A.F. and De Klerk G.J. 2001. Development of dormancy in different lily genotypes regenerated *in vitro*. *Plant Growth Regulation* 34:215-222.

Langens-Gerrits M.M., Miller W.B.M., Croes A.F. and De Klerk G.-J. 2003. Effect of low temperature on dormancy breaking and growth after planting in lily bulblets regenerated *in vitro*. *Plant Growth Regulation* 40:267-275.

Larson R.A. 1992. Introduction to floriculture. 2nd ed. Academic Press, Inc. pp. 195-212.

Law D.M. 1987. Gibberellin-enhanced indole-3-acetic acid biosynthesis: D-Tryptophan as the precursor of indole-3-acetic acid. *Physiologia Plantarum* 70:626-632

Le Nard M. and De Hertogh A.A. 1993. *Tulipa*. Chapter 35. In: De Hertogh, A. and Le Nard, M. (Eds.), The physiology of flower bulbs. Elsevier, Amsterdam, pp.617-682.

Le Nard M. and De Hertogh A.A. 2002. Research needs for flower bulbs (geophytes). *Acta Horticulturae* 570:121-127.

Lersten N.R. 2004. *Pollen development: Details of stages*. Flowering plant embryology. Blackwell Publishing, Iowa, USA, pp.36-64.

Lopez-Carbonell M., Alegre L. and van Onckelen H. 1994. Effects of water stress on cellular ultrastructure and on concentrations of endogenous abscisic acid and indole-3-acetic acid in Fatsia japonica leaves. *Plant Growth Regulation* 14:29-35

Matsuo T. and Mizuno T. 1974. Changes in the amounts of two kinds of reserve glucose containing polysaccharides during germination of the Easter lily bulb. *Plant and Cell Physiology* 15:555-558.

Miller W.B. and Langhans R.W. 1990. Low temperature alters carbohydrate metabolism in Easter lily bulbs. *HortScience* 25:463-465.

Miller W.B. 1993. *Lilium Longiflorum*. Chapter 27. In: De Hertogh A. and Le

Nard M. (Eds.), The physiology of flower bulbs. Elsevier, Amsterdam, pp. 391-422.

Moctezuma E. 1999. Changes in auxin patterns in developing gynophores of the peanut plant (*Arachis hypogaea* L.) *Annals of Botany* 83:235-242.

Moctezuma E. and Feldman L.J. 1999. Auxin redistributes upwards in graviresponding gynophores of the peanut plant. *Planta* 209:180-186.

Moe R. 1979. Hormonal control of flower blasting in tulips. *Acta Horticulturae* 91:221-228

Moore T.C. 1989. *Biochemistry and physiology of plant hormones*. New York, USA: Springer-Verlag.

Nagar P.K. 1995. Changes in abscisic acid, phenols and indoleacetic acid in bulbs of tuberose (*Polianthes tuberosa* L.) during dormancy and sprouting. *Scientia Horticulturae* 63:77-82.

Nichols R. and Kofranek A.M. 1982. Reversal of ethylene inhibition of tulip stem elongation by silver thiosulphate. *Scientia Horticulturae* 17:71-79.

Niimi Y., Endo Y. and Arisaka E. 1988. Effects of chilling- and GA_3-treatments on breaking dormancy in *Lilium rubellum* Baker bulblets cultured *in vitro* (in Japanese with English summary). *Journal of the Japanese Society for Horticultural Science* 57:250-257.

Niimi Y. and Oda M. 1989. Time of initiation and development of flowerbuds in *Lilium rubellum* Baker. *Scientia Horticulturae* 39:341-348.

Nowak J., Saniewski M. and Rudnicki R.M. 1974. Studies on the physiology of hyacinth bulbs (*Hyacinthus orientalis*). I. Sugar content and metabolic activities in bulbs exposed to low temperatures. *Journal of Horticultural Science* 49:383-390.

Ofir M. and Kigel J. 1998. Abscisic acid involvement in the induction of summer-dormancy in *Poa bulbosa*, a grass geophyte. *Physiologia Plantarum* 102:163-170.

Ohkawa K. 1977. Studies on the physiology and control of flowering in *Lilium speciosum rubrum*. *Special Bulletin of the Kanagawa Horticulture Experimental Station*. pp.1-73.

Ohkawa K. 1989. Time of flower bud differentiation in lilies native to Japan (in Japanese, with English summary). *Journal of the Japanese Society for Horticultural Science* 57:655-661.

Okubo H. and Uemoto S. 1985. Changes in endogenous gibberellin and auxin activities during first internode elongation in tulip flower stalk. *Plant and Cell Physiology* 26(4):709-719.

Okubo H. and Uemoto S. 1986. Changes in endogenous gibberellins during internode elongation of tulip flower stalk as affected by light conditions. *Journal of the Japanese Society for Horticultural Science* 55(2):215-220.

Op den Kelder P., Benschop M. and De Hertogh A.A. 1971. Factors affecting floral stalk elongation of flowering tulips. *Journal of the American Society for Horticultural Science* 96(5):603-605.

Rebers M., Vermeer E., Knegt E. and Van der Plas L.H.W. 1996. Gibberellin levels are not a suitable indicator for properly cold-treated tulip bulbs. *HortScience* 31(5):837-838.

Rees A.R. 1992. *Ornamental bulbs*, corms and tubers. Chapter 5: Physiology. C.A.B International Wallingford, Oxon OX10 8DE UK. pp.61-92.

Saniewski M., Banasik L., Antoszewski R. and Lukaszewska A. 1979. Polarity of IAA-1-^{14}C translocation in tulips as related to shoot growth. *Acta Horticulturae* 91:229-234.

Saniewski M. and De Munk W.J. 1981. Hormonal control of shoot elongation in tulips. *Scientia Horticulturae* 15:363-372.

Saniewski M. and Okubo H. 1997. Auxin induces stem elongation in nonprecooled and precooled derooted and rooted tulip bulbs. *Journal of the Faculty of Agriculture, Kyushu University* 42(1-2):53-61.

Saniewski M. and Okubo H. 1998a. Effects of 2, 3, 5-triiodobenzoic acid (TIBA) on stem growth induced by indole-3-acetic acid (IAA) and naphthylacetic acid (NAA) in precooled rooted tulip bulbs. *Journal of the Faculty of Agriculture, Kyushu University* 43(1-2):11-23.

Saniewski M. and Okubo H. 1998b. Inhibitory effect of naphthylphthalamic acid (NPA) on stem growth induced by auxin in precooled tulip bulbs. *Journal of the Faculty of Agriculture, Kyushu University* 43(1-2):59-66.

Saniewski M., Kawa-Miszczak L., Wegrzynowicz-Lesiak E. and Okubo H. 1999. Gibberellin induces shoot growth and flowering in nonprecooled derooted bulbs of tulip (*Tulipa gesneriana* L.). *Journal of the Faculty of Agriculture, Kyushu University* 43(3-4):411-418

Saniewski M., Okubo H., Miyamoto K. and Ueda J. 2005. Auxin induces growth of stem excised from growing shoot of cooled tulip bulbs. *Journal of the Faculty of Agriculture, Kyushu University* 50(2):481-488.

Shi L., Miller I. and Moore R. 1993. Immunocytochemical localization of indole-3-acetic acid in primary roots of *Zea mays*. *Plant Cell and Environment* 16(8):967-973.

Shin K.S., Chakrabarty D. and Paek K.Y. 2002. Sprouting rate, change of carbohydrate contents and related enzymes during cold treatment of lily bulblets regenerated in vitro. *Scientia Horticulturae* 96:195-204.

Sjut V. and Bangerth F. 1984. Induced parthenocarpy – a way of manipulating levels of endogenous hormones in tomato fruits (*Lycopersicon esculentum* Mill.) 2. diffusible hormones. *Plant Growth Regulation* 2:49-56.

Stieglitz H. 1977. Role of β-1,3 gluconase in postmeiotic microspore release. *Developmental Biology* 57:87-97.

Suge H. and Osada A. 1966. Inhibitory effects of growth retardants on the induction of flowering in winter wheat. *Plant and Cell Physiology* 7:617-630.

Suh J.K. and Lee A.K. 1997. Effect of ethephon pretreatment on the stem elongation of cut tulip flowers. *Journal of the Korean Society for Horticultural Science* 38(5):588-591.

Suzuki T., Matsuura T., Kawakami N. and Noda K. 2000. Accumulation and leakage of abscisic acid during embryo development and seed dormancy in wheat. *Plant Growth Regulation* 30:253-260.

Syrtanova G.A., Turetskaya R.K.H. and Rakhimbaev I. 1973. Natural auxins and inhibitors in dormant and growing tulip bulbs, *Tulipa alberti*: *Tulipa ostrowskiana*. *Soviet Plant Physiology* 20:965-967.

Terry P.H., Aung L.H. and De Hertogh A.A. 1982. Identification of abscisic acid in *Tulipa gesneriana* L. by gas-liquid chromatography with electron capture and combined gas-liquid chromatography and mass spectrometry. *Plant Physiology* 70:1574-1576.

Thomas C., Bronner R., Molinier J., Prinsen E., Van Onckelen H. and Hahne G. 2002. Immuno-cytochemical localization of indole-3-acetic acid during induction of somatic embryogenesis in cultured sunflower embryos. *Planta* 215:577-583.

Upreti K.K., Murti G.S.R. and Bhatt R.M. 1997/98. Response of French bean cultivars to water deficits: changes in endogenous hormones, proline and chlorophyll. *Biologia Plantarum* 40(3):381-388

Van Kilsdonk M.G., Nicolay K., Franssen J.M. and Kollöffel C. 2002. Bud abortion in tulip bulbs studied by magnetic resonance imaging. *Journal of Experimental Botany* 53(374):1603-1611

Walker-Simmons M. 1987. ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. *Plant Physiology* 84:61-66.

Wang S.Y. and Roberts A.N. 1970. Physiology of dormancy in *Lilium longiflorum* 'Ace' Thunb. *Journal of the American Society for*

Horticultural Science 95:554-558.

Wang M., Heimovaara-Dijkstra S. and Van Duijn B. 1995. Modulation of germination of embryos isolated from dormant and non-dormant barley grains by manipulation of endogenous abscisic acid. *Planta* 195:586-592.

Wegrzynowicz E. and Saniewski M. 1992. Distribution of ethylene production and ethylene-forming enzyme activity in different stages of growth and development of tulips. *Acta Horticulturae* 325:285-290.

Xu R.Y., Niimi Y., Han D.-S. and Kuwayama S. 2005. Effects of cold treatment on anthesis of tulip with special reference to pollen development. *Acta Horticulturae* 673:383-388.

Yamazaki H., Nishijima T. and Koshioka M. 1995. Changes in abscisic acid content and water status in bulbs of *Allium wakegi* Araki throughout the year. *Journal of the Japanese Society for Horticultural Science* 64:589-598.

Yamazaki H., Nishijima T., Yamato Y., Koshioka M. and Miura M. 1999a. Involvement of abscisic acid (ABA) in bulb dormancy of *Allium wakegi* Araki. I. Endogenous levels of ABA in relation to bulb dormancy and effects of exogenous ABA and fluridone. *Plant Growth Regulation* 29:189–194.

Yamazaki H., Nishijima T., Yamato Y., Hamano M., Koshioka M. and Miura M. 1999b. Involvement of abscisic acid in bulb dormancy of *Allium wakegi* Araki. II. A comparison between dormant and nondormant cultivars. *Plant Growth Regulation* 29:195–200.

Yamazaki H., Nishijima T., Koshioka M. and Miura H. 2002. Gibberellins do not act against abscisic acid in the regulation of bulb dormancy of *Allium wakegi* Araki. *Plant Growth Regulation* 36:223-229.

Yang S.F. and Pratt H.K. 1978. The physiology of ethylene in wounded plant tissues. In: Kahl G, ed. *Biochemistry of wounded plant tissues*. Walter de

Gruyter & Co., Berlin, pp.595-622.

Related publications

Xu R.Y., Niimi Y., Han D.-S., Kuwayama S. 2005. Effects of cold treatment on anthesis of tulip with special reference to pollen development. Acta Horticulturae 673:383-388.

Xu R.-Y., Niimi Y., Han D.-S. 2006. Changes in endogenous abscisic acid and soluble sugars levels during dormancy-release in bulbs of *Lilium rubellum*. Scientia Horticulturae 111:68-72.

Xu R.-Y., Niimi Y., Kojima K. 2007. Exogenous GA_3 overcomes bud deterioration in tulip (*Tulipa gesneriana* L.) bulbs during dry storage by promoting endogenous IAA activity in the internodes. Plant Growth Regulation, in press.

Xu R.-Y. Niimi Y., Han D.-S. Immunohistochemical localization of indole-3-acetic acid in tulip shoot during low temperature treatment of bulbs. (Submitted to Acta Horticulturae, August, 2006).

Xu R.-Y., Niimi Y., Ohta Y., Kojima K. Changes of diffusible indole-3-acetic acid from various parts of tulip plant during rapid elongation of the flower stalk. (Submitted in February, 2007).

Xu R.-Y., Niimi Y. Cold treatment affects microspore development and induces IAA production in pollen sacs in tulip (Submitted in January, 2007)

要約

低温処理による *Lilium rubellum* Baker (ヒメサユリ)とチューリップ球根の休眠打破と ABA の役割

L. rubellum の内生 ABA 濃度は鱗茎の貯蔵期間が長くなると共に減少し、10 週間後にはほぼ一定となった。そして 4℃14 週間処理した鱗茎の出芽および開花はほぼ一斉に起こり、これは鱗茎貯蔵中の ABA の減少が *L. rubellum* 鱗茎の休眠解除と関連していることを示唆している。一方、チューリップでは鱗茎の底盤部および外側から最も内側にある第 4 番目鱗片の ABA 濃度は 4℃、8 週間貯蔵する間に減少したが、外側から 1 から 3 番目までの鱗片の ABA 濃度の減少はほとんど観察されなかった。そして 4℃、8 週間貯蔵した鱗茎はガラス室に植え付け後急速に花茎を伸長したことから、チューリップ鱗茎の休眠は、4℃8 週間の貯蔵で休眠が破れること、また、底盤部および最も内側の鱗片がチューリップ鱗茎の休眠解除に関係していることがわかった。

低温によるチューリップ鱗茎の休眠打破過程中の小胞子発達および 20℃長期貯蔵がシュートの健全性に及ぼす影響

低温処理によりチューリップ鱗茎の休眠を打破する過程で、葯中の花粉母細胞は四分小胞子に発達し、鱗茎がガラス室で栽培される過程で稔性花粉となった。しかし低温処理を受けなかった場合、稔性花粉は形成されず、その原因は貯蔵中に形成された四分子小胞子がガラス室で鱗茎を栽培したあともそれぞれの花粉に分離・独立できないためであることがわかった。

休眠中の鱗茎を 20℃で長期間にわたり貯蔵すると鱗茎内の茎（葉、蕾を含む）に外観上の傷害が起こた。この最初の兆候は葯に現れた。鱗茎にGA_3溶液を注入するとこの傷害は軽減され、茎にIAA生産が起こた。4℃で貯蔵した鱗茎ではIAA含量増加は葯と最基部節間で見られたが、一方 20℃で貯蔵した鱗茎の最基部ではほとんど検出され得ないような量であり、葯では全く検出されなかった。20℃貯蔵の鱗茎ではIAAとABAはともに減少したことから、20℃貯蔵中に正常なホルモン代謝が阻害され、茎内の種々のホルモン濃度のバランスが崩れ、負の影響を生じたかもしれない。また 20℃乾燥貯蔵中の水分含量の低下が鱗茎内の種々の器官の発達を阻害したり、あるいはホルモン代謝に影響した可能性もある。

低温が貯蔵中の鱗茎内シュートおよび花茎伸長中の IAA レベルの変化に及ぼす影響

IAA は 4℃貯蔵中の鱗茎シュートの種々の器官および急速な伸長を起こす花茎から検出された。免疫組織化学的方法で、IAA が 4℃、12 週間貯蔵した鱗茎の各節間、第 1 葉および花器官に分布していることがわかった。4℃で貯蔵中に異なる時期に種々の器官で IAA の検出を行ったが、貯蔵開始時では節間や葯では IAA シグナル（染色強度）は弱かった。この IAA シグナルは節間や葯では４℃貯蔵 4 週間後には強くなったが、20℃4 週間貯蔵した鱗茎の節間では IAA シグナルは貯蔵開始時とほぼ同様で変化はなく、弱かったが、葯では IAA シグナルは貯蔵開始時よりさらに弱くなった。貯蔵開始時の鱗茎内の葉および花被でも IAA シグナルは検出された。４℃で 4，8 及び 12 週間貯蔵後にこれらの器官の IAA シグナルを検出したが、その強度は貯蔵開始時と差がなかった。一方、20℃で 4 週間貯蔵した鱗

茎の葉および花被でも IAA シグナルは検出され、4 週間貯蔵した鱗茎から採取した葉および花被のシグナル強度は貯蔵開始時と差がなかった。しかし、8 週間および 12 週間 20℃貯蔵した鱗茎ではシュートが傷害を起こしたため調査できなかった。

休眠が解除されたと思われる鱗茎をガラス室および圃場で栽培すると花茎伸長は急速に進み、それは開花直前が最も顕著であった。この花茎伸長が急速に進む時期の拡散性 IAA 量は茎＞花器官＞葉の順であったことや各節間や花器官からの拡散性 IAA 量のピーク時期がそれぞれ異なったことから、花直下の節間（第 4 節間）が急速な花茎伸長に関わる拡散性オーキシン生産の主要な場所はである可能性が示唆された。

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