The Physiology and Control of Re-greening in Spathes of *Zantedeschia*

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Abstract

Spathe re-greening is a primary determinant limiting postharvest quality of *Zantedeschia* Spreng. as a cut flower, pot- or landscape-plant. A treatment that can be utilised by growers to delay re-greening offers potential to improve the postharvest quality and provide a marketing advantage. To achieve this, and develop an understanding of the physiological mechanism of re-greening, this project investigated the changes in colour, levels and types of pigment, and differentiation of plastids in spathe tissue durng re-greening; and how this process was controlled by various factors including fructification, light and various plant hormones (e.g. cytokinin and gibberellin).

In the hybrid 'Best Gold', spathe re-greening was initiated within three days after horticultural harvest-maturity and, within two weeks the whole abaxial surface of the spathe had re-greened. During this period, the adaxial surface did not re-green and remained yellow in colour. The change in colour of the abaxial surface primarily resulted from the accumulation of chlorophyll within the subepidermal layers, as reflected by a strong correlation between the colour coordinate hue angle (H°) and total chlorophyll content in that surface (r = 0.98). Monitoring H° can therefore, be used to evaluate the degree of re-greening for 'Best Gold' without chlorophyll analysis. The content of carotenoid (in particular lutein which was predominant) was comparatively steady during re-greening. From an ultrastructural perspective, spathe re-greening was characterized by redifferentiation of chloroplasts from chromoplasts, as compared with *de novo* synthesis of chloroplasts from proplastids. The redifferentiation of chloroplasts involved thylakoid reformation through multiple

mechanisms. In addition to *de novo* synthesis of thylakoid by invaginations of the inner-envelope membrane, it is likely that the thylakoids were either derived from primary thylakoids or plastoglobuli present in mature chromoplasts.

The occurrence of re-greening in the spathe of both *Zantedeschia aethiopica* and 'Best Gold' following the removal of the spadix prior to pollination, contradicted the hypothesis that re-greening was induced by fructification (Pais and Neves, 1982-1983). Further to this, the occurrence of re-greening in the spathe of 'Best Gold' with a spadix naturally devoid of female flowers, and the re-greening of pigmented leaves devoid of any true flower parts, also contradicted this hypothesis. The current findings therefore, indicate that fructification is not necessarily a prerequisite for induction of re-greening.

In absence of light, no chlorophyll accumulated in spathe tissue of 'Best Gold', but the initial redifferentiation of chloroplasts from chromoplasts, as characterized by the formation of double-membrane lamella, was noted. Without light however, redifferentiation of chloroplasts was not completed. These suggest spathe re-greening requires light for the process to complete, but the onset of re-greening can be induced in darkness. The application of 6-benzylaminopurine (BAP) stimulated re-greening in spathe tissue by enhancing accumulation of carotenoid and chlorophyll, and also stacking of grana. But the response to BAP was dependent on the presence/absence of light, the stage of re-greening, and which surface, i.e. abaxial or adaxial. In contrast, the application of gibberellin (GA_3) retarded formation of double-membrane lamella, and thus delayed the onset of re-greening. Hence, a synergistic effect of BAP and GA_3 in delaying the onset of re-greening was likely to be a result of co-regulation between

BAP-stimulated accumulation of carotenoid and GA_3-stimulated retardation of chloroplast redifferentiation.

By integrating both light and hormonal factors, several methods were tested on the actual horticultural commodity, i.e. flowers (peduncle, spathe and spadix) of 'Best Gold', so as to evaluate their efficacy in delaying re-greening. Pulsing flowers in darkness at 5 ºC for 24-h in a solution containing both GA_3 and BAP, was the most effective treatment in postponing re-greening, resulting in a seven-day delay in re-greening of the spathe.

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List of abbreviations

ABA	Abscisic acid
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
C*	Chroma
CPPU	N_1-(2-chloro-4-pyridyl)-N_3-phenylurea
cv.	Cultivar
DW	Dry weight
GA_n	$Gibberellin_n$ – denotes the number
H°	Hue angle
HPLC	High performance liquid chromatography
IAA	Indolylacetic acid
L*	Lightness
LSD	Least significant difference
NAA	1-naphthaleneacetic acid
POR	Protochlorophyllide reductase
RO	Reverse osmosis
SAS	SAS system for statistical analysis
SE	Standard error
TEM	Transmision electron microscopy
TLC	Thin layer chromatography
Type-1	Flattened, double-membrane lamella
Type-2	Swollen membrane thylakoids
Type-3	Vacuole-like, single-membrane-bound bodies

Type-4	Clusters of membrane fragments
10^{-2} M	1×10^{-2} M
10^{-4} M	1×10^{-4} M
10^{-5} M	1×10^{-5} M
10^{-6} M	1×10^{-6} M
10^{-8} M	1×10^{-8} M

Chapter 1 **General Introduction**

1.1 ***Zantedeschia***

Zantedeschia Spreng. (calla lily) is a popular ornamental crop in the international flower market. It has been cultivated as a cut flower, pot plant or garden ornamental throughout the world and contributes to horticultural revenue for many countries including America, Holland, and New Zealand (Funnell, 1993; Singh, 1996). *Zantedeschia* is reported to be ranked within the top 20 cut flowers at Dutch flower auctions, with more than 70 million stems being sold per year (Flower Council of Holland, 2005). In addition, *Zantedeschia* is New Zealand's second highest earner of export revenue for cut flowers, worth $NZ 9 million in 2009 for both tubers and cut flowers (Statistics New Zealand, 2010).

The popularity of *Zantedeschia* in the international flower market is partially because of its distinct and stylish inflorescence and its wide selection of colours (Singh, 1996). The inflorescence consists of a coloured spathe (i.e. modified leaf) on a fleshy peduncle, with the spathe subtending a spadix that contains true female and male flowers (Figure 1.1). In the horticultural context, this combination of spathe, spadix and peduncle is what is harvested and marketed as a cut flower and, for the purposes of this thesis, unless defined otherwise, will be referred to as the "flower". Extensive interspecific breeding has resulted in a wide array of colours of spathe in *Zantedeschia*, including white, red, orange, pink, cream, yellow, purple and dark purple.

The genus *Zantedeschia* belongs to the *Araceae* family and comprises eight species, which are all endemic to Southern Africa, i.e.; *Z. aethiopica* (L.) Spreng., *Z. albomaculata* (Hook.) Basil, *Z. elliottiana* (Watson) Engl., *Z. jucunda* Letty, *Z. rehmannii* Engl., *Z. pentlandii* (Watson) Wittm., *Z. odorata* P. L. Perry and *Z. valida* Letty (Singh et al., 1996b). Based on both morphological features and their cyclic periodicity, *Zantedeschia* can be divided into two informal groups (Funnell, 1993; Letty, 1973; Singh et al., 1996b):

- Winter-flowering group - this group contains *Z. aethiopica* and *Z. odorata*. They are perennial plants with a rhizome or tuber underground. Leaves of *Z. aethiopica* are evergreen while leaves of *Z. odorata* die down in summer. They flower from late winter to late spring. Female and male flowers are interspersed over the lower part of the spadix, which is subtended by a coloured spathe (Figure 1.1 B & D). The colour of the spathe is either white or infrequently pink. During fruiting, the upper part of the spathe withers while the lower part turns green and embraces the enlarging fruits (Figure 1.1 F). The fruits turn orange and soft at maturity.
- Summer-flowering group (also known as Aestivae) - the second group consists of the remaining six species. They are perennial plants with a tuber underground. These six species flower in summer and their foliage dies down in late autumn to spring. Female and male flowers are not interspersed on the spadix, but are separated with male flowers at the top (Figure 1.1 A & C). The colour of the spathe typically ranges from white, pink, maroon, to yellow. Different from the winter-flowering group, during fruiting the spathe does not wither; instead the whole spathe turns

green in colour and completely embraces the fruits (Figure 1.1 E). The fruits remain green and firm at maturity.

Zantedeschia and its hybrids may be grown in temperate to sub-tropical climates (Funnell, 1993). *Z. aethiopica* in the winter-flowering group can continue to grow and flower if temperatures are not below 12 ºC or above 28 ºC. In contrast, the species in the summer-flowering group are more restricted to their natural habitats, i.e. cool-temperature climate with summer rainfall (Letty, 1973). As a result, these species encounter a seasonal dormancy, even if provided with conditions that usually promote growth (Funnell et al., 1988). *Zantedeschia* is usually propagated by using seeds, division of underground organs, or tissue culture. If grown from seeds, the plants of *Zantedeschia* will naturally flower in three years, between 45 to 150 days if grown from two-year-old tubers, and two years if grown from tissue cultured plantlets (Funnell, 1993; Tjia, 1989).

The majority of *Zantedeschia* hybrids cultivated commercially are developed from the summer-flowering group (Funnell, 1993). 'Best Gold' (Figure 1.1 A, C & E) is an example of these hybrids bred locally by Mr. Jim Wilson, Palmerston North, New Zealand. Significant factors that contributed to selecting of 'Best Gold' as the model plant for the current study included the well established commercial crop, and easy access to large quantities locally.

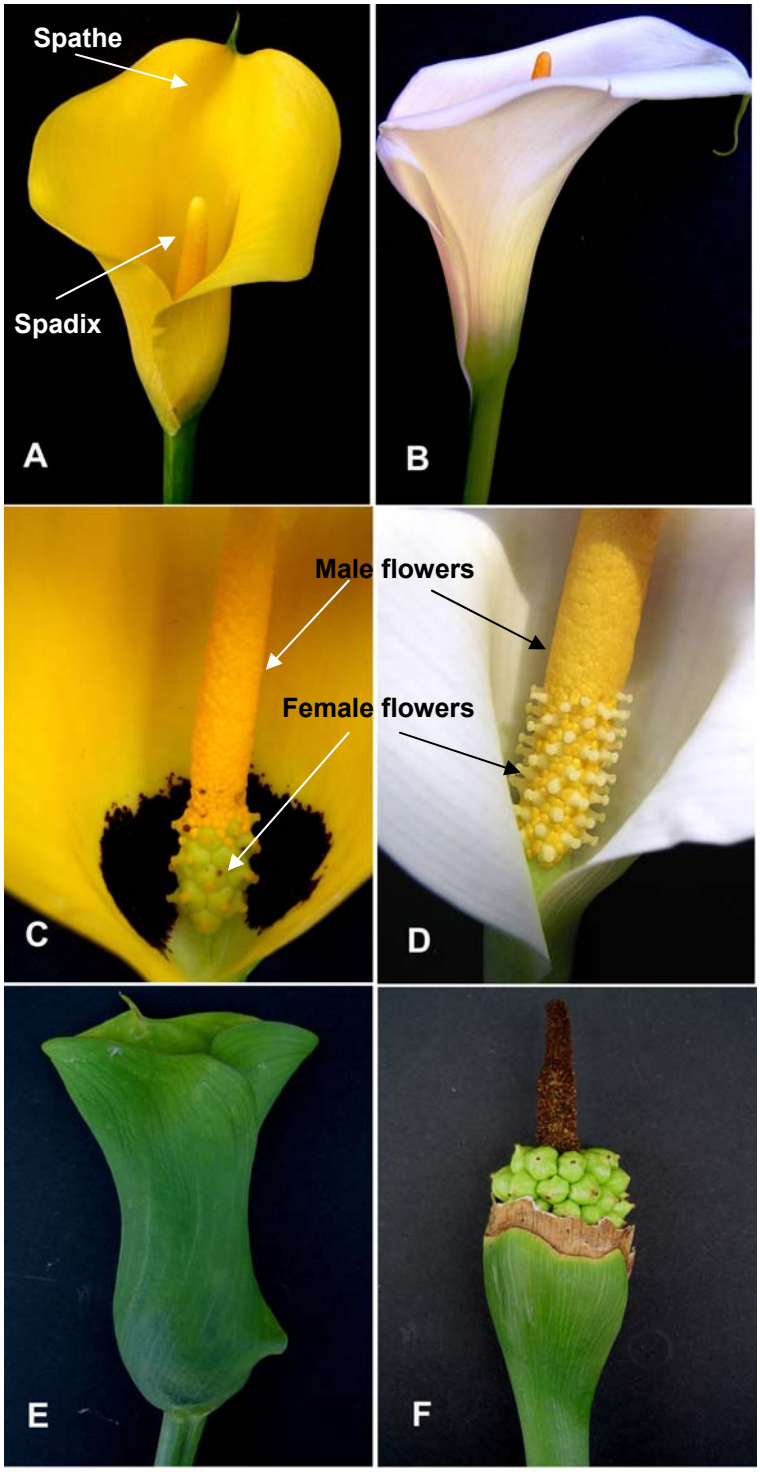

Figure 1.1 Inflorescence of *Zantedeschia* 'Best Gold' (left A, C & E) and *Zantedeschia aethiopica* (B, D, & F). A & B: a leaf-like spathe encloses a spadix; C & D, female and male flowers congregate on the spadix; E: fully re-greened spathe of 'Best Gold' embraces enlarging fruits (not visible); F: fully re-greened spathe of *Z. aethiopica* with upper part of spathe withered while the lower part embraces the enlarging fruits.

1.2 Spathe re-greening of *Zantedeschia*

The development of the spathe of *Zantedeschia* can be described as passing through five broad stages: green bud, yellow-green bud, horticultural harvest-maturity, re-greened and enrolled spathe (Figure 1.2). When the green bud emerges from its enveloping leaf sheath, the spathe is tightly folded and is green in colour. Within 7-10 days, this green colour is lost as the spathe gradually unfolds, spadix matures, and expression of the true colour on both the abaxial and adaxial surfaces becomes evident. Soon after the spathe is fully open and coloured and reaches horticultural harvest-maturity (approximately 2-3 days in 'Best Gold'), it starts to turn green again, first on the abaxial surface and some weeks later on the adaxial surface. Within two weeks after horticultural harvest-maturity, the whole abaxial surface of the spathe or lower part of the spathe (as in *Z. aethiopica*) turns green and, in another two weeks, the re-greened spathe envelops the developing fruit. To the naked eye the fully re-greened spathe is similar in colour to the green colour of a mature leaf. Within this thesis, this process of regaining of green colouration on the spathe is defined as re-greening. The re-greened spathe possesses photosynthetic capability (Tavares et al., 1998), hence the process of re-greening not only offers physical protection for the fruit, but also provides a prospective source of energy for their development.

Although re-greening can be regarded as being of potential biological importance for fruit development and maturation, in horticultural terms it is the primary factor limiting the ornamental quality of *Zantedeschia* flowers. Colour is one of the key qualities that determines the preference of consumers when purchasing a cut flower (Yue and Behe, 2010). In this context, in particular for the species and

hybrids from the summer-flowering group, the rapid appearance of green on the spathe decreases the saturation of its true colour and, therefore, makes it less acceptable to the consumer. In addition to this, for some hybrids, the development of green colouration is extensive, completely overlaying the true colour of the spathe, which reduces the display quality of *Zantedeschia* in a flower arrangement or bouquet. Tjia and Funnell (1986) reported that typically, the vase life of *Z. elliottiana* was 7-8 days. If not for the problem of re-greening, the vase life of *Z. elliottiana* could be significantly extended.

Spathe re-greening is wide-spread in the species and hybrids of *Zantedeschia*, particularly in the summer-flowering group. This is shown by the fact that among 61 commercial species and hybrids, 92% of them were reported to present the characteristic of re-greening (Funnell, 1993). Hence, a technology that can maintain the true colour of the spathe by postponing the onset of re-greening would help improve the quality of the flower and increase its commercial value. For the growers of *Zantedeschia* in New Zealand, developing and utilising such technology is even more necessary, as *Zantedeschia* flowers grown in New Zealand are typically shipped over long distances before they reach the retailer in overseas markets. The shipping time is approximately a week, therefore risk reduction in quality of flowers due to the extended time. It is this potential horticultural application, and commercial benefit, that has motivated this research into the re-greening process of *Zantedeschia*.

Figure 1.2 Left to right, within approximately a five-week timeframe: five progressive developmental stages of the spathe in *Zantedeschia* 'Best Gold' from immature green bud through to fully re-greened and enrolled spathe.

1.3 Re-greening in organs of other plant species

Re-greening has been reported to occur in various organs of plants for a number of species. Re-greening of floral organs (e.g. petal, sepal, or bract) has been shown in *Phalaenopsis* Blume species (Tran et al., 1995), *Helleborus niger* L. (Salopek-Sondi et al., 2002), *Nuphar luteum* L. (Gronegress, 1974), *Orontium aquaticum* L. (Casadoro et al., 1982), *Heliconia aurantiaca* L. and *Spathiphyllum wallisii* Regel. (Weidner et al., 1985). Similar to the spathes of *Zantedeschia*, these floral organs of other species undergo the same characteristic colour changes during flower development. These organs are usually green when they are very young, develop full colour during anthesis, and gradually re-green again after pollination and during subsequent fruit formation.

The re-greening extends the longevity of the floral organs and, in doing so, potentially ensures the successful development and maturation of seeds (Herrera, 2005; Salopek-Sondi et al., 2000). Moreover, this colour change also provides signals to pollinators that the flowers are no longer fertile, which in turn increases the efficiency of pollination for other flowers. These floral organs therefore, fulfil two roles:

- functioning as an attractant to pollinators during anthesis;
- as a potential source of energy for the developing seeds and fruits during re-greening.

In other plant species the transition between these two roles of the floral organs has been associated with the occurrence of fructification (Salopek-Sondi et al., 2000; Tran et al., 1995). While discussed further in Sections 1.4 and 1.5, the precise biochemical and molecular signals linking fructification and re-greening, remain largely unknown. For the purposes of providing a starting point for the research into spathe re-greening

of *Zantedeschia* presented in this thesis, it was assumed that what was known about re-greening occurring in floral organs of other plant species was likely to be most influential.

In some species a yellowing leaf or cotyledon can be induced to re-green before the point where the tissue commits to senescence (van Doorn, 2005). This kind of re-greening has been examined in *Nicotiana rustica* L. (Zavaleta-Mancera et al., 1999b), *Linum usitatissimum* L. (Greening et al., 1982), *Glycine max* (L.) Merr. (Skadsen and Cherry, 1983) and *Vicia faba* L. (Dyer and Osborne, 1971). In these plants, when the lower leaves or cotyledons turn yellow, they can be induced to re-green by the removal of all the younger leaves or by spraying the leaf or cotyledon with ammonium nitrate. This indicates that the yellowing resulted in part from internal competition for nitrogen, and was somehow reversible. In addition, this re-greening could only be induced when the roots were attached or when cytokinin was applied to the detached yellowing leaf or cotyledon. Since root tips were proposed as one of the sites for cytokinin synthesis, the cytokinin originating from the roots was suggested to be a trigger for the re-greening in the yellowing leaf or cotyledon after the removal of younger leaves (Colbert and Beever, 1981). As discussed further in Sections 1.4 and 1.5, re-greening in yellowing leaves or cotyledons has been extensively studied due to its connection with the process of leaf senescence. At the outset of the research presented in this thesis, it was unclear if re-greening in leaves, cotyledons and floral organs, e.g., spathe of *Zantedeschia*, share a similar biochemical and molecular mechanism. But it was believed that the current knowledge of re-greening in leaves or cotyledons would provide some insight for research into spathe re-greening in *Zantedeschia*.

Re-greening has been observed on the peel of ripe fruits of *Citrus sinensis* (L.) Osbeck when left on the tree till the following spring or summer (Huff, 1983; Thomson et al., 1967), and also on the harvested fruits of *Cucumis sativus* L. (Prebeg et al., 2008), *Citrus grandis* Merr. (Saks et al., 1988) and *Cucurbita pepo* L. (Devide and Ljubesic, 1974), when exposed to light. The skin of these fruit is green at an immature stage, turns yellow when mature and, gradually develops a green colour again after maturity. Re-greening in fruits like these has not been extensively studied and, to date, the evolutionary advantage that might be gained from re-greening remains unclear.

In summary, re-greening of plant tissue is a unique but not rare process occurring naturally during plant development. It is present naturally or artificially in various organs of plants including leaves, cotyledons, floral organs and fruits. Despite its occurrence in different parts of plants, re-greening of these tissue shares many similarities from the perspective of the physiological mechanism including regaining of green colouration, accumulation of chlorophyll, reformation of chloroplasts (discussed further in Section 1.4.4), and potentially serving as an alternative source of photosynthate (Prebeg et al., 2008; Salopek-Sondi et al., 2000; Zavaleta-Mancera et al., 1999b). However, re-greening in these various plant organs might differ in the mechanism of how this process is induced and proceeds. For example, re-greening in sepals of *H. niger* was believed to be induced (at least in part) by fructification (Salopek-Sondi et al., 2000; Tarkowski et al., 2006). Re-greening in peel of fruits in *C. sinensis* on the other hand, was reported to be induced by a decrease in the content of sucrose within the fruits (Huff, 1984).

Compared with other developmental processes within the plant, e.g. greening and senescence (refer Section 1.4), re-greening has attracted less attention by researchers. As a result, the evolutionary advantage of re-greening, and how it is induced and controlled, is still largely unknown. The research on spathe re-greening of 'Best Gold' presented in this thesis has attempted to fill some gaps in the scientific knowledge of re-greening *per se*, meanwhile aiming to also address the horticultural problem caused by re-greening in *Zantedeschia* (refer Section 1.2).

1.4 Greening, de-greening, re-greening and senescence

At a simplistic level: greening, de-greening, re-greening and senescence are four developmental processes occurring in plants that each involves the gain or loss of chlorophyll, chloroplasts and photosynthetic activity. In a normal life span, plants or plant tissues (e.g. leaves) undergo greening, followed by de-greening (e.g. with ripening in fruits and anthesis in flowers) and, subsequently, senescence. In some plant species (refer list in Section 1.3), an extra process, i.e. re-greening, occurs between de-greening and senescence. These four processes are characterized by extensive metabolic activities, e.g. protein turnover, pigment biosynthesis and degradation, and ultrastructural variations (Biswal et al., 2003; Sarafis, 1998; Smart, 1994; Thomas et al., 2003; Vothknecht and Westhoff, 2001; Waters and Pyke, 2004; Zhou et al., 2005). As discussed in detail in the following sections, in plants the processes of greening, de-greening, re-greening and senescence in plants are linked, which is reflected in a dynamic interrelationship between the different forms of plastids (Figure 1.3).

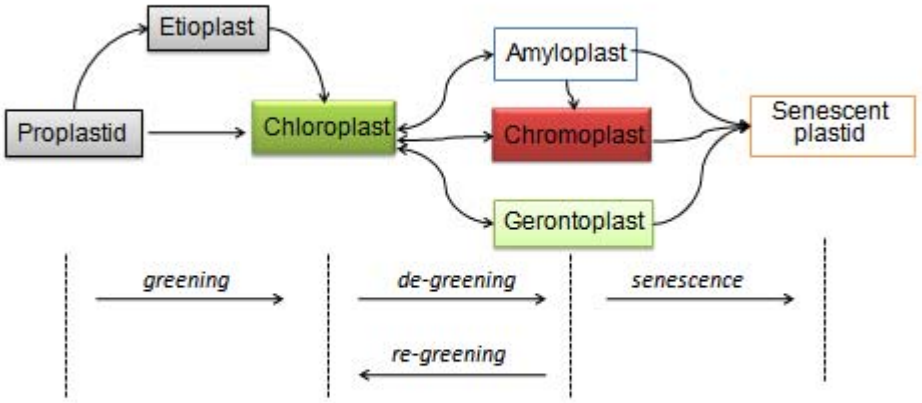

Figure 1.3 Interrelationship between different forms of plastids in higher plants and their association with some developmental processes including: greening, de-greening, re-greening and senescence. Note that this diagram is not comprehensive, as only the forms of plastids that relate to the current research have been included.

1.4.1 **Plastids**

Plastids are typical organelles found in the cells of a plant. There are various forms of plastids, which each fulfil different roles in different types of cells. These plastids can interconvert between different types accompanying the progress of plant development (Figure 1.3). A brief summary of the basic types of plastids and their functions relevant to greening, de-greening, re-greening and senescence is presented below. Some key components of the different plastids are highlighted, so as to identify distinguishing features of possible use when monitoring plastids in the spathe of 'Best Gold' during re-greening:

- Proplastids are the precursors of all plastids most commonly present in meristematic regions. They can further develop into more complex forms of plastids, e.g. chloroplast and chromoplast (Figure 1.3). They normally contain a trace of thylakoid membrane and, sometimes, starch. Some studies have shown that proplastids can be formed through incorporation of cytoplasmic structures (Osafune et al., 1990), but the origin of proplastids largely remains a mystery;
- Etioplasts are partially differentiated chloroplasts, derived from proplastids prior to exposure to light. They are characterised by a crystalline prolamellar body, which is a complex comprising the enzymes and precursors for chlorophyll biosynthesis (Boddi et al., 1989). These plastids undergo rapid transformation to functional chloroplasts upon illumination (Figure 1.3);
- Chloroplasts are green-coloured plastids found in green tissue of plants, and are responsible for photosynthesis. They are characterised by a comprehensive network of thylakoid system with granal stacks interlinked

by stroma thylakoids. The thylakoid system is surrounded by the stroma. As a reservoir of excess lipids, plastoglobuli are frequently present in chloroplasts, though their size and number are small. Within the chloroplast, chlorophyll, and its accessory pigments, i.e., carotenoids, are integrated into the bilayer of membranes and bound with proteins to form light-harvesting antennae and photosystem I and II (Young, 1993b). The circular, plastid DNA, is attached to the thylakoid membranes. Chloroplasts can generally convert into other forms of plastids, e.g. chromoplasts and amyloplasts and *vice versa* (Figure 1.3, Waters and Pyke, 2004);

- Chromoplasts are coloured plastids, and accumulate pigments that are from the carotenoid family. They provide the red, orange and yellow colours seen in flowers, fruits and roots. The exact evolutionary function of chromoplasts is unclear, but they are commonly believed to act as attractants to insects and animals. The carotenoids are stored in various forms in chromoplasts, e.g., concentrated in plastoglobuli with lipids, distributed as crystals in the stroma, or appearing as microfibrils. A trace of thylakoid membranes is often evident in chromoplasts. Chromoplasts can be developed from proplastids, chloroplasts and amyloplasts (Figure 1.3). Some evidence has also shown chromoplasts can redifferentiate to form chloroplasts (Gronegress, 1974; Mayfield and Huff, 1986; Prebeg et al., 2008);
- Amyloplasts are plastids that store excess photosynthate in a form of starch. In doing so, they provide long-term storage of energy in the plant, and are present in meristems, seeds, roots and fruits. Amyloplasts are

usually fully occupied by large starch granules. They may find their origin either directly from proplastids or dedifferentiation from chloroplasts (Figure 1.3, Tavares et al., 1998);

- Gerontoplasts are partially degraded chloroplasts that appear in yellowing leaves (Biswal et al., 2003). It is difficult to precisely define the structures of gerontoplasts. They are not dead organelles, but plastids with the task of salvaging material for use in the plant elsewhere (Thomas, 1997). To some extent, gerontoplasts are morphologically comparable to chromoplasts, but the former can only develop from mature chloroplasts and do not divide (Biswal et al., 2003). Gerontoplasts retain the capacity for recovery of thylakoid systems and chlorophyll, which have been lost to a degree, and conversion back to chloroplasts (Figure 1.3) before senescence occurs (van Doorn, 2005).

A significant amount of our knowledge and understanding about plastids has come from microscopy studies, in particular observing thin sections of plant tissue using a transmission electron microscope (TEM). The size for some key sub-components in the plastids, e.g. plastoglobuli and grana in chloroplasts of a re-greened spathe in *Z. elliotiana*, ranges from 0.1 to 0.5 μm in length (Gronegress, 1974). For this reason, TEM with a maximum resolution of 0.1 nm can be used to reveal the details for some key sub-components in the plastids. In contrast, a conventional light microscope with a highest resolution 0.2 μm is limited in its capability when studying the plastids at the ultrastructural level. Thus, in order to understand the process of re-greening of spathe tissue at the ultrastructural level, TEM was identified as a critical research tool for this project.

1.4.2 **Greening: formation of functional chloroplasts**

Greening, defined as a process of gaining green colouration in plant tissue, occurs at the early stage of plant development, e.g. upon germination of seed and emergence of a leaf or flower bud. The attainment of fully functional chloroplasts (i.e. completion of greening) enables a seedling or piece of plant tissue to establish an independent energy source prior to the depletion of any existing energy reserves. The independent energy source is achieved by the formation of photosynthetically competent chloroplasts from proplastids (Figure 1.3). From a morphological aspect, this plastid conversion is characterized by the constitution of a thylakoid system including the *de novo* synthesis of thylakoid membranes and pigment-protein complexes (Waters and Pyke, 2004). The thylakoid membranes initially appear as long double-membrane lamellar, and quickly compact to form granal stacks, which are connected by stroma thylakoids. It is believed that the origin of thylakoid membranes is from the invaginations of the inner-envelope membranes of the plastids, at least during the early stage of differentiation, being a continuum between inner-envelope membrane and thylakoid membranes (Vothknecht and Westhoff, 2001). However, this continuum has rarely been detected toward the later stage of differentiation, i.e. the maturation of the chloroplast. It is, therefore, unclear whether there is an alternative site for continuous synthesis of thylakoid membrane within the plastids, or whether some transportation agents (e.g. vesicles) are produced to take care of the traffic between the inner envelope membrane and thylakoid system.

If seedlings geminate in darkness, the proplastids develop into etioplasts that contain a prolamellar body (Figure 1.3). As such, etioplasts represent a temporarily blocked stage along the path of development to chloroplasts (Waters and Pyke, 2004).

At this stage, they are primed to dismantle their prolamellar body to constitute a thylakoid system upon illumination.

Thylakoid biogenesis during greening is coordinated with a rapid accumulation of both chlorophyll and carotenoids. These pigments are essential components of photosynthetic apparatus in chloroplasts. Proplastids are devoid of any pigments. Thus, all the pigments that accumulate during the conversion of proplastids to chloroplasts are via *de novo* biosynthesis. Upon illumination, the chlorophyll can be detected within 24 hours in cotyledons of *Lupinus luteus* L. (Makeev et al., 1995) or *C. sativus* (Cohen et al., 1988). The biosynthesis of chlorophyll is performed entirely within the chloroplast, but the precise locations of this biosynthesis remain elusive. Upon illumination, a relocation of chlorophyll synthetase activity from the prolamellar body to developing thylakoids indicates that newly-formed thylakoids might be a possible site for chlorophyll biosynthesis during greening (Lindsten et al., 1993). Integrating this result, chlorophyll-forming centres, that are situated near to or on the surface of thylakoid membranes, have been proposed by Sundqvist and Dahlin (1997). Similar to chlorophylls, carotenoids rapidly accumulate during the construction of thylakoid systems (Biswal et al., 2003). They are synthesized within plastids, but it is also unclear where the exact sites are for carotenoid biosynthesis. Some evidence indicates that the thylakoid membranes and stroma of chloroplasts are possible sites for the biosynthesis of carotenoids (Camara et al., 1982; Lopez et al., 2008).

During greening pigments such as chlorophyll *a*, chlorophyll *b*, carotenes and xanthophylls bind to specific apoproteins to construct light harvesting complexes in thylakoid systems (Sundqvist and Dahlin, 1997). In a mature chloroplast, the light

harvesting complexes consist of a reaction centre surrounded by antennae that harvest and transfer light energy to the reaction centre. Generally, β-carotene and chlorophyll *a* are located at the reaction centre as its only pigments, while chlorophyll *a*, chlorophyll *b* and the xanthophylls (neoxanthin, violaxanthin and lutein) are embedded in the surrounding antenna (Young, 1993a). Numerous lines of evidence indicate that the presence of chlorophyll and carotenoids is essential for constructing and stabilizing the thylakoid systems (Axelsson et al., 1982; Dahlin and Franzen, 1997; Dahlin and Timko, 1994; Park et al., 2002; Sundqvist and Dahlin, 1997).

The preceding knowledge with regard to greening, provides some insights into the type of research associated with the process of re-greening, as greening and re-greening both involve formation of a functional chloroplast. Hence, for the research into the re-greening of spathe tissue in *Zantedeschia* reported in this thesis, thylakoid formation and pigment biosynthesis during the reformation of chloroplasts are likely to be important factors to be monitored.

1.4.3 **De-greening: conversion of chloroplasts to other forms of plastids**

De-greening is defined as a process of losing green colouration and chlorophyll pigmentation in plant tissue prior to senescence. It occurs in flower tissue during anthesis, ripening of fruit or the yellowing of leaves. It is normally accompanied by changes in pigmentation of flowers or fruits, enabling them to be attractive to pollinators or animals for pollination or seed dispersal.

Due to its horticultural relevance, de-greening in flowers and fruits has been extensively studied, e.g. in the fruit of *Lycopersicum esculentum* L. (Cheung et al., 1993; Harris and Spurr, 1969; Kahlau and Bock, 2008), *C. sinensis* (Rodrigo et al., 2004; Thomson, 1966), *Capsicum annuum* L. (Hornero-Mendez and Minguez-Mosquera, 2002; Spurr and Harris, 1968), *Narcissus pseudonarcissus* L. (Salim et al., 1996), and *Tagetes erecta* L. (Moehs et al., 2001). As part of this existing research, the ultrastructural, biochemical and molecular mechanism of the transition from chloroplast to chromoplast has been determined. At the ultrastructural-level, de-greening is not a true reversal of greening, as it is characterized by the plastid differentiation from chloroplasts to amyloplasts, chromoplasts or gerontoplasts, but not to proplastids (Figure 1.3). This plastid transition mainly involves a breakdown of thylakoid systems and formation of plastoglobuli. The first sign of thylakoid degradation is the appearance of electron dense regions in the granal and intergranal thylakoid, which are thought to be a consequence of deposition of lycopene, an initial product in the pathway of carotenoid biosynthesis (Harris and Spurr, 1969). Later the intergranal regions swell, followed by degradation of the grana. In mature chromoplasts, some stroma thylakoids or thylakoid plexi are often observed, indicating an incomplete loss of thylakoids. A concomitant increase in size and number of plastoglobuli with the loss of thylakoids, has led to a suggestion that the degenerating thylakoids might contribute to the formation of plastoglobuli (Brehelin and Kessler, 2008). Plastoglobuli are, therefore, believed to serve as a reservoir for lipids.

Along with the thylakoid turnover during de-greening of fruits and flowers, loss of chlorophyll and accumulation of carotenoid take place simultaneously. Analysis in the fruits of the *green flesh* mutant of *L. esculentum*, indicated that the

loss of chlorophyll is not a prerequisite for extensive accumulation of carotenoids (Cheung et al., 1993). The loss of chlorophyll and accumulation of carotenoids were controlled separately via distinct nuclear genes. The types of carotenoids accumulated during de-greening differ greatly both among species and, even within a species. For example, the fruits of *C. grandis* at the green stage primarily contained chloroplast carotenoids including β-carotene and xanthophylls (Gross et al., 1983). During the de-greening stage, these chloroplast carotenoids gradually decreased while typical chromoplast carotenoids, e.g. ζ-carotene and cryptoxanthin appeared. In contrast, the pathway of chlorophyll breakdown is comparatively conserved between species, and has been extensively reviewed elsewhere (Hörtensteiner, 1999; Hörtensteiner, 2006; Thomas, 1997). Because chlorophyll breakdown is not the focus of this thesis, it will not be reviewed further here.

De-greening in leaves occurs concomitantly with the formation of gerontoplasts from chloroplasts (Biswal et al., 2003). Although this plastid differentiation results in a decline in photosynthesis and a partial degradation of thylakoids, pigments and some proteins, the gerontoplasts are not a dead plastid (Biswal et al., 2003). They possess genetic material that controls the active functions including mobilization of nutrients and synthesis of protein. Furthermore, gerontoplasts retain the capability of reversion back to chloroplasts if provided with the right conditions, e.g. low light level (Zavaleta-Mancera et al., 1999b). Hence, the formation of gerontoplasts in a de-greening leaf takes place before senescence (van Doorn, 2005).

1.4.4 **Re-greening: reformation of functional chloroplasts**

Compared with greening, de-greening and senescence, re-greening has attracted less attention from researchers. From the structural point of view, re-greening of plant tissue is all about the redifferentiation of functional chloroplasts (Figure 1.3). The majority of studies have shown no evidence of *de novo* synthesis of chloroplasts from proplastids, but a direct formation of chloroplasts from amyloplasts (Tavares et al., 1998), chromoplasts (Gronegress, 1974; Mayfield and Huff, 1986; Prebeg et al., 2008), or gerontoplasts (Dyer and Osborne, 1971; Zavaleta-Mancera et al., 1999b). As such, to some extent, re-greening can be viewed as a reversal of de-greening (Figure 1.3). Upon re-greening, a progression of intermediate stages of plastids with mixed characteristics of chloroplast, chromoplast or amyloplasts can often be observed. Hence, in the current study, microscopic examination of plant tissue and viewing this mixture of ultrastructural organelles should be able to be used to determine the occurrence and progress of re-greening in the spathe of 'Best Gold'.

Redifferentiation of chloroplasts during re-greening involves reconstitution of the thylakoid system. Although prior to re-greening the thylakoid system has largely vanished from the plastids, some thylakoid remnants can frequently be observed in the stroma (Gronegress, 1974; Thomson et al., 1967). The persistence of remnants of thylakoid in mature plastids like chromoplasts is considered to be indicative of their potential for redifferentiation to chloroplasts. Whatley (1978) suggested that the thylakoid system in chloroplasts is in a state of dynamic equilibrium. As such, when the dedifferentiation of thylakoids surpasses redifferentiation, chloroplasts develop into chromoplasts or gerontoplasts. Similarly, redifferentiation of thylakoids can begin again before dedifferentiation has resulted in total loss of thylakoids. This

capacity for reversal to chloroplasts is determined by the rate of thylakoid turnover. This explains why the plastids capable of re-greening usually contain thylakoid remnants. As noted previously (refer Section 1.4.2), in the current research, focussing on the dynamic changes in the thylakoid system within plastids could prove useful to determine the status of re-greening in the spathe of 'Best Gold'.

As re-greening is initiated, the plastids undergo redifferentiation of chloroplasts in a manner resembling that noted for greening of proplastids or etioplasts, but are distinguished by some differences:

- Firstly, re-greening proceeds much more slowly than greening. For example, re-greening of fruits in *C. sinensis* occurs over a period of three to four months, whereas greening of a young leaf takes place in less than a week (Thomson et al., 1967).
- Secondly, for re-greening, the origin of the newly formed thylakoids was not only from the inner envelope membrane, but also from plastoglobuli, which retain close continuity with thylakoids and decline in size and number along with the accumulation of thylakoids (Mayfield and Huff, 1986; Prebeg et al., 2008; Salopek-Sondi et al., 2002).
- Thirdly, unlike those mature chloroplasts developed from proplastids during greening, the redifferentiated chloroplasts usually contain large plastoglobuli (Gronegress, 1974).
- Finally, plastid division is rather rare in these redifferentiated chloroplasts, particularly those from gerontoplasts (Zavaleta-Mancera et al., 1999b). This is supported by the fact that there was no increase

in chloroplast number in re-greened leaves. However, it is unclear whether the redifferentiated chloroplasts from chromoplasts or amyloplasts are capable of plastid division.

In spite of characterizing the process of re-greening at the ultrastructural level, its characterization in terms of pigment turnover has not received much consideration. It has been reported that re-greening is accompanied by the re-accumulation of chlorophyll (Pais and Neves, 1982-1983; Zavaleta-Mancera et al., 1999a), but how chlorophyll biosynthesis is recovered has not been accurately defined. In addition, how chlorophyll biosynthesis is coordinated with the dynamic changes of other pigments, e.g. carotenoids, is unknown. Some plastids like chromoplasts contain carotenoids and some of them (e.g. β-carotene and lutein), are also a key component of the reformed thylakoid systems. Thus, one of the main questions concerning carotenoid accumulation is whether there is a relocation of carotenoid from existing storage reservoirs (e.g. plastoglobuli) to redifferentiated thylakoid membranes, or an initial breakdown of chromoplast carotenoids and then *de novo* synthesis of chloroplast carotenoids. The study reported in this thesis was aimed to provide some insights into answering some of these questions via examination of ultrastructure and pigment content at different stages of spathe re-greening in 'Best Gold'.

Spathe re-greening in *Zantedeschia* has not been extensively studied. The current knowledge is mainly based on the pioneering work from a group of researchers in Portugal: Pais and Neves (1982-1983), Tavares et al. (1998) and Lino-Neto et al. (1999). They reported that spathes of *Z. aethiopica* undergo senescence soon after maturation, but fructification (the process leading to the formation of fruits)

inhibited this senescence and resulted in spathe re-greening. The maturation (de-greening) of the spathe is associated with the formation of amyloplasts from chloroplasts, while re-greening is the result of redifferentiation of chloroplasts from amyloplasts (Figure 1.3). All existing knowledge is based on the research utilising *Z. aethiopica*, which belongs to the winter-flowering group of *Zantedeschia* (refer Section 1.1). Other than it is known to occur (Funnell and Downs, 1987), little detailed knowledge is available on re-greening of spathes in *Zantedeschia* from the summer-flowering group. It is unclear if re-greening of *Zantedeschia* from the winter- or summer-group follows a similar mechanism. Therefore, a direct comparison between *Z. aethiopica* and a hybrid from the summer-flowering group, i.e. 'Best Gold', would help to answer this question. Chapter 4 of this thesis, attempts to address this topic.

1.4.5 **Senescence: dismantling of plastids**

Senescence is a terminal stage of the life span of plant tissue and, as noted above, it is a different process from greening, de-greening and re-greening. It has been defined as a process that leads to the programmed death of individual cells, organ and whole plants (van Doorn and Woltering, 2004). Senescence involves a highly regulated series of biochemical and physiological changes including complete dismantling of cell structures (e.g. plastids), loss of pigments, degradation of proteins, nucleic acids and membranes, and the recycling of components and nutrients to other cells or organs. These changes have been extensively reviewed elsewhere (Eason, 2006; Smart, 1994; Thimann, 1980; Thomas et al., 2003; Zhou et al., 2005). In this thesis, in order to distinguish it from de-greening, senescence is defined as a process leading to irreversible death of cells, organs and/or whole plants. It is accompanied by

the plastid differentiation from gerontoplasts, chromoplasts and amyloplasts to senescent plastids (Figure 1.3). This process however, was not proposed as the centre of attention in this thesis, but is noted here so as to highlight the difference of this process to re-greening.

1.5 Induction and control of re-greening

As a process of plant development, re-greening will have some level of control at the genetic level. Despite the importance and uniqueness of re-greening in plants, how re-greening is induced and controlled at the genetic level remains unresolved. The limited studies on spathe re-greening of *Z. aethiopica* at this level have revealed that the transcription of the genes that encode some proteins of the chloroplast, e.g., RUBISCO (ribulose-1, 5-bisphosphate carboxylas/oxygenase) arrested at the white-spathe stage and recovered during spathe re-greening (Piques et al., 1999). This was accompanied by the *de novo* synthesis of RUBISCO in the spathe during re-greening (Tavares et al., 1999). In addition, re-greening of a yellowing leaf of *N. rustica* was also associated with a reappearance of chlorophyll-biosynthesis enzymes, e.g. protochlorophyllide oxidoreductase (POR), and chlorophyll-binding proteins (Zavaleta-Mancera et al., 1999a). Therefore, re-greening is believed to involve the re-activation of some chloroplast-related genes and re-accumulation of the chloroplast proteins. This raises some interesting questions:

- What are the signals/factors which induce these genetic activities of re-greening?
- Why can only some species respond to these re-greening signals/factors and re-green in their normal life span?

- Are there genes that pre-determine the capability of plant tissue to re-green at a certain stage of development?

The focus of the current study was on environmental and physiological factors likely to be involved in influencing spathe re-greening of *Zantedeschia*, which may also have potential for horticultural application for postponing re-greening. This study was not designed to address these questions through molecular analysis, although molecular analysis would clearly be the next step required to understand regulation of the re-greening process once the causal factors are identified.

As discussed in detail in the following sections, based upon the existing knowledge of re-greening and its comparable process, i.e. greening, it is hypothesized that re-greening can be affected by various factors. These factors include: fructification, plant hormones, sugar levels, light, and other chemical treatments.

1.5.1 **Fructification**

Re-greening in floral organs is associated with fructification in many species including: *Phalaenopisis* (Tran et al., 1995), *Z. aethiopica* (Pais and Neves, 1982-1983), *N. luteum* (Gronegress, 1974) and *H. niger* (Salopek-Sondi et al., 2000). In all these species, unpollinated floral organs were reported to senesce soon after their formation, whereas those which were pollinated and preceded to fruit development, turned green, developed photosynthetic capability, and had the timing of their natural senescence delayed. Hence, fructification appears to trigger re-greening of floral organs in some species. The underlying mechanism of this signalling pathway is, however, largely unknown. Some evidence has shown that fructification was

associated with increased levels of endogenous plant hormones including cytokinins, gibberellins or ethylene (Ayele et al., 2010; Jones and Woodson, 1997; Tarkowski et al., 2006). The concept of fructification in inducing spathe re-greening has not previously been tested in the summer-flowering group of *Zantedeschia*. Therefore, in Chapter 4 of this thesis, the influence of fructification on spathe re-greening of 'Best Gold' was examined.

1.5.2 **Cytokinins**

Previous research on spathe re-greening of *Z. aethiopica* has reported that the effect of fructification on re-greening can be substituted by the application of cytokinins. Some cytokinins, e.g. 6-(o-hydroxybenzylamino)-purine and its riboside 6-(o- hydroxybenzylamino)-9-β-D-ribofuranosylpurine, were isolated from the fruits of *Z. aethiopica* (Chavesdasneves and Pais, 1980). Following the removal of the spadix, application of synthetic cytokinins, that were considered equivalent to the ones isolated from the fruits, resulted in spathe re-greening; otherwise the spathe senesced (Pais and Neves, 1982-1983). These authors then proposed that spathe re-greening of *Z. aethiopica* was induced by the accumulation of these cytokinins in the spathe, being translocated from developing fruits on the spadix. To date however, no evidence has actually illustrated this transportation of the cytokinins, or any signalling by this endogenous cytokinin, that leads to the re-greening of the spathe. Hence, it is unclear whether fructification stimulates the re-greening of *Zantedeschia* directly or through a cytokinin-signalling pathway.

Re-greening of yellowing leaves or cotyledons also has an association with cytokinins. After decapitation, an accumulation of root-borne cytokinins in the one

remaining yellowing leaf or cotyledon has been shown to be a direct cause of the re-greening in *C. pepo* L., *L. esculentum*, *Phaseolus vulgaris* L., and *N. rustica* (Colbert and Beever, 1981; Staden and Carmi, 1982; Venkatarayappa et al., 1984). The role of cytokinins in re-greening has been confirmed by both increased levels of endogenous cytokinin in re-greening tissue and, promotion of re-greening by the exogenous application of cytokinins (Ananieva et al., 2004; Zavaleta-Mancera et al., 1999a). In addition, cytokinins are believed to be able to directly stimulate the expression of chloroplast-associated nuclear and plastid genes, and also promote the synthesis of proteins required for construction of photosynthetic apparatus and pigment biosynthesis during greening (Kusnetsov et al., 1998; Kusnetsov et al., 1994; Thomas et al., 1997; Yaronskaya et al., 2006; Zubo et al., 2008). Hence, accumulation of endogenous cytokinins, both prior to and during re-greening, appears to be one of the signals that triggers and promotes re-greening. No research has been published clarifying the role of cytokinin in stimulating re-greening of the spathe in the summer-flowering group of *Zantedeschia*. Therefore, in Chapters 5 and 6 of this thesis, the influence of exogenous cytokinin on spathe re-greening of 'Best Gold' was investigated.

1.5.3 **Gibberellins**

Gibberellins are also believed to be able to influence re-greening in some species. A study of sepals of *H. niger* has shown that re-greening can be stimulated by an application of exogenous gibberellin (GA_3) on an unfertilized flower (Salopek-Sondi et al., 2002). On the other hand, re-greening of a fertilized flower can be inhibited by an application of paclobutrazol, an inhibitor of gibberellin biosynthesis (Salopek-Sondi et al., 2002). This role of gibberellin in the induction of re-greening

has further been supported by analysing the endogenous gibberellin profile in the sepal of *H. niger* prior to, and during, re-greening (Ayele et al., 2010). It was found that the level of the endogenous gibberellin in the sepal was highest prior to re-greening. In sepals that did not re-green, gibberellin levels were very low. Based on these studies, it is plausible to suggest that accumulation of gibberellin in sepals of *H. niger* is associated with the induction of re-greening. To our knowledge, there is no report so far on the influence of gibberellin on spathe re-greening of *Zantedeschia*. Therefore, in Chapters 5 and 6 of this thesis, this topic was addressed by investigating the effect of an application of exogenous gibberellin on spathe colour, pigment accumulation and, plastid variation, during re-greening.

1.5.4 **Other plant hormones**

The influence of ethylene on re-greening of plant tissue has not been extensively studied. Ethylene is commercially used to promote de-greening in citrus, as ethylene stimulates both chlorophyll degradation and carotenoid accumulation in peel of the fruits (Goldschmidt et al., 1993; Rodrigo and Zacarias, 2007). On the other hand, as a reversed process of de-greening, re-greening in fruits of *C. sinensis* has been reported to be inhibited to some extent by application of ethephon, a plant growth regulator that is converted into ethylene by plant metabolism (El-Zeftawi, 1978). Ethylene however, has been shown to have no influence on change in colour of the spathe during re-greening of *Zantedeschia* hybrids, and no ethylene was detected from these hybrids during postharvest evaluation (Funnell and Downs, 1987). These results indicate that the spathe at the stage of re-greening was not sensitive to ethylene. However, it is unclear whether ethylene was present at some time prior to re-greening, which might induce re-greening. Further study is therefore, required to clarify the role

of ethylene in the induction and progression of re-greening. Given the general lack of response of *Zantedeschia* hybrids to ethylene (Funnell and Downs, 1987), in this thesis, ethylene was not included in the investigation.

Few investigations of the effect of auxin and abscisic acid (ABA) on re-greening of plant tissues have been undertaken. Application of auxin has been shown to increase the content of chlorophyll, but did not affect chloroplast differentiation in detached leaf discs of *C. sativus* (Kovacs et al., 2007). Additionally, the formation of chloroplasts during the re-greening of sepals in *H. niger* was not affected by auxin (Salopek-Sondi et al., 2002). To date, there is no evidence that ABA influences re-greening in plant tissue, although some research indicated that ABA repressed greening of cotyledons in *L. luteus* (Kusnetsov et al., 1998; Kusnetsov et al., 1994). Given the limited information available, and the timeframe for a PhD thesis, the influence of ABA on re-greening was not investigated in this thesis. However, the influence of auxin on re-greening of 'Best Gold' was examined further in Chapter 5.

1.5.5 **Sugars**

Re-greening in peel of *C. sinensis* is affected by various factors including gibberellins (Coggins, 1962) and concentrations of nitrogen and sucrose in the fruit (Huff, 1983). Huff (1984) showed that a reduction in soluble sucrose concentration in fruits was a major factor that stimulated the re-greening of *C. sinensis* in spring and summer. Furthermore, a recent study on leaves of *L. esculentum* revealed that accumulation of glucose in leaves led to a decrease in the content of both carotenoid and chlorophyll (Mortain-Bertrand et al., 2008). The decrease in the content of carotenoid was associated with a repression of genes encoding enzymes of carotenoid

biosynthesis. Given this evidence, it was hypothesised that an increase in sugar content within plant tissue might be a potential way to postpone re-greening in spathe tissue of *Zantedeschia*. This hypothesis was tested in Chapter 5 of this thesis.

1.5.6 **Light**

Light is known to be an external signal that induces the process of greening in plant tissue. To date, molecular analysis of the process of greening has revealed that the signalling pathway of light is initiated at the perception of light by the phytochromes (Quail, 2002; Smith, 2000). With the energy from light, these phytochromes undergo photoconversion to an active form. They were associated with the expression of the genes in the nucleus that encode proteins for chloroplast formation and chlorophyll biosynthesis (Kusnetsov et al., 1996; Kusnetsov et al., 1994). In addition, light can be directly absorbed by pigments, and their precursors, to initiate a cascade series of metabolic activities. For example, in the chlorophyll biosynthesis pathway of angiosperms, the reduction of protochlorophyllide to chlorophyllide was the sole light-dependent step (Armstrong and Apel, 1998; Armstrong et al., 1995; Vonwettstein et al., 1995). The light was absorbed by protochlorophyllide itself, which then drove the transfer of electrons and activated the enzyme to catalyze the reduction of protochlorophyllide (Griffiths, 1991). These studies on the effects of light are all based on investigations focussed on greening and, to date, knowledge of how light is involved in signalling for re-greening has not been reported.

Similar to greening, re-greening also involves a light-dependent process, i.e., the formation of a functional chloroplast. It is thus hypothesised that light, as an

external signal, is required for induction and manipulation of re-greening. This is partially supported by the fact that the re-greening of harvested fruits in *C. grandis* was induced by storing the fruits under daylight (Saks et al., 1988). Likewise, the re-greening of fruits in *C. sinensis* also required the presence of light, as the chloroplast formation and chlorophyll biosynthesis in the rind of the fruits was unable to proceed without it (El-Zeftawi, 1978). Further studies at the ultrastructural, biochemical and molecular levels are, however, required to reveal to what degree re-greening is regulated by light. Similarly, if this information is applicable as a model to be tested with re-greening in spathe tissue of *Zantedeschia*, a series of questions immediately emerge:

- Is the onset of chloroplast redifferentiation directly induced by light, or is it only the maturation of the functional chloroplast which requires the presence of light?
- Is light interacting with other signals (e.g. cytokinin) in induction and manipulation of re-greening?

Such questions represent challenges, but need addressing before progress in understanding the process of spathe re-greening in *Zantedeschia* can be made. Hence, in Chapter 6 of this thesis some of these questions were addressed by investigating the influence of light and dark treatments on spathe re-greening of 'Best Gold'.

1.5.7 **Other chemical treatments**

A range of exogenous chlorophyll inhibitors have been shown to have the ability to alter the rate of greening or re-greening in a range of horticultural commodities (Anstis and Northcote, 1973; Hsu et al., 1989). A number of these

inhibitors are herbicides, such as cycocel and amino triazole (Wolf, 1977). To be commercially acceptable, any final treatments to slow re-greening in spathes of *Zantedeschia* should ideally contain compounds with low mammalian toxicity. In addition, given the timeframe for a PhD thesis, it was decided that before evaluating such chemical treatments, plant hormones and other naturally occurring compounds would be evaluated. Hence this review limits itself to acknowledging the existence of such compounds only.

1.6 Measurement of re-greening and human perception

1.6.1 What is colour?

It is a well established fact that the perceived colour of an object results from a selective absorption of certain wavelengths of light by pigment particles in the object (Hunter and Harold, 1987). Hence, when a beam of light strikes the surface of a non-metallic object, the majority of it penetrates the surface and encounters the pigment particles. The light is then partially absorbed by the pigments while the rest of the light spectrum is reflected back from the pigments to the surface of the object, resulting in the colour we perceive. In addition, the colour of an object is also influenced by the topography of the surface. For an object with a surface coated with cuticle waxes (e.g. apple), a portion of the incident light is reflected without penetrating the surface. This reflected light gives rise to the glossy appearance of the objects. The glossy appearance makes the colour appear more vibrant and intense. Conversely, for an object with a rough surface (e.g. epidermal hairs of a kiwifruit), the incident light is scattered in numerous directions. Colourless scattered light is added to the coloured light that is reflected from the pigment particles in the object, and thus

reduces the saturation of the colour. Hence, to determine the factors contributing to the final colour we perceive, it is essential to understand the pigment profiles and surface topography of the object.

Given the fact that the surface of spathes of *Zantedeschia* is smooth, it is reasonable to suggest that their colour primarily results from an interaction between light and the pigments in the tissue of the spathe. Analysis of pigment profiles of *Zantedeschia* hybrids from the summer-flowering group has revealed that anthocyanins were the main pigments responsible for the red and pink colour, carotenoids were associated with the yellow colour, and an overlay of carotenoids by anthocyanin gave rise to the orange colour (Lewis et al., 2003). The anthocyanins were typically located in the epidermal cells, while the carotenoids were located in the mesophyll layers. Hence in the experimental crop used in this thesis, i.e. *Zantedeschia*, it is recognised that pigments can be distributed within different cell layers of spathe tissue. Re-greening of the spathe in *Zantedeschia* has been associated with an accumulation of chlorophyll (Gronegress, 1974; Tavares et al., 1998). However, it is unclear whether this re-greening is determined solely by the accumulation of chlorophylls masking the existing pigments, or by the simultaneous disappearance of existing pigments and appearance of chlorophylls and, whether any such changes differentially occur in different cell layers. Using 'Best Gold' as a model plant, Chapter 2 of this thesis attempts to address the questions of:

- whether re-greening is determined solely by the accumulation of chlorophyll masking the existing pigments, or by the simultaneous disappearance of existing pigments and appearance of chlorophyll and,

- how the pigments change in terms of content and distribution within the cell layers, as re-greening proceeds.

1.6.2 How do our eyes perceive re-greening?

Human perception of colour is a process in which the reflected light from an object is perceived by eyes and is processed by the brain. Human eyes contain three types of colour receptors in the retina that sense red, green and blue light, respectively. Light at any visible wavelength (400 nm to 700 nm) can stimulate one or more of these receptors, which pass signals to the brain via nerve fibres. The brain then interprets the colour by combining the data of the excited types together with the quantity of receptors. This eye-brain mechanism is highly sensitive, and it can distinguish about 10 million different colours (Hunter and Harold, 1987). In addition, within the range of visible wavelengths, human eyes are most sensitive to light around 550 nm, i.e., what we generally see as green light. As a result of this greater sensitivity, the green colour is more readily seen by human eyes than any other colour. Hence, it is reasonable to suggest that the progressive appearance of green colouration on the spathe of *Zantedeschia* is readily detected by human eyes. Further to this however, for *Zantedeschia* hybrids with various colours, the ability of the human eye to detect re-greening in the spathe is likely to differ. For example, re-greening on a deep-purple coloured hybrid (e.g. 'Hot Chocolate') is likely to be less discernible to the human eye than that occurring with 'Best Gold', a yellow-gold coloured hybrid of *Zantedeschia*. Hence in the research reported in this thesis, use of the hybrid 'Best Gold' is considered to be a good choice for re-greening research, as such changes in colour should be more readily perceived than in other hybrids.

As introduced in Section 1.2, colour is the most noticeable attribute of appearance in fresh produce that consumers rely on when they make their purchases (Yue and Behe, 2010). *Zantedeschia* flowers with a re-greening spathe are perceived as having low quality (Funnell and Downs, 1987), the saturation of the existing colour of the spathe is reduced, and the appearance of green may also be associated with over-mature flowers. Re-greening on the spathe therefore, reduces the marketability of *Zantedeschia*. This again reiterates the market-driven need for development of a method to postpone re-greening in this flower crop; a motivating factor of the research presented in this thesis.

1.6.3 **Colour measurement**

As human perception of colour is subjective, and is influenced by many factors including lighting conditions and angle of observation, an objective colour measurement is required to assess colour. Tristimulus colorimeters have been designed to reproduce the way human eyes see colours and express these colours numerically, based on international standards, e.g. Commission International de I'Eclariage (CIE) system (Hunter and Harold, 1987). Colorimeters contain three filters that absorb light at the same wavelengths as the human eye (Francis, 1980). The reflected light from an object passes through the filters and is received by photocells to produce electric current that can be quantified. The colorimeters use the same light source and illumination method for each measurement. For this reason, the perception of colour by the colorimeters will not be influenced by the external lighting conditions and, therefore, provide accurate and consistent data. Moreover, colorimeters are portable, quick to operate and are suitable for repeated colour measurement for horticultural crops both in the field or laboratory. They have been commonly used to describe the colour of horticultural crops, including *Zantedeschia*

(Funnell and Downs, 1987; Helyes et al., 2006; Iglesias et al., 2001; Peppi et al., 2006) and, therefore, in this study, a tristimulus colorimeter was chosen to quantify the colour change in the spathe of 'Best Gold' during re-greening.

A variety of numerical scales have been used to describe colour. The scale most frequently used in quantifying the colour of horticultural crops is CIELAB (Maftoonazad and Ramaswamy, 2006). With this scaling system any given colour is allocated as a point in a three-dimensional space (Figure 1.4). Lightness (L*) represents the z-axis of the space, and describes the brightness of a subject from 0% (absolute black) through 50% (grey) to 100% (absolute white). The x-axis of the space is the coordinate of a*. Its positive values indicate colours in the red spectrum, and negative values refer to green spectrum. The y-axis of the colour space is the coordinate of b*. Its positive values refer to yellow, and negative values to blue. Chroma (C*) represents the saturation of the colour, and ranges from 0% at its centre, which is completely unsaturated, to 100% at the edge of the circle representing maximum saturation. Hue angle (H°) refers to a tint of colour and is represented in a circular axis using units of degrees, ranging from 0° red, 90° yellow, 180° bluish-green, and 270° blue. C* and H° are derived from the values of a* and b* based on equations [1.1] and [1.2]. Since these colour scales can be readily obtained from a tristimulus colorimeter, they are often used to describe colour for horticultural crops including *Zantedeschia* hybrids (Funnell and Downs, 1987). However, it has not previously been reported how relevant and sensitive any change in these colour-scale values reflect any change in pigment content in the spathe of *Zantedeschia*. Hence, Chapter 2 of this thesis was designed to answer this question.

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad [1.1]$$

$$H^{\circ} = \tan^{-1}(a^*/b^*) \qquad [1.2]$$

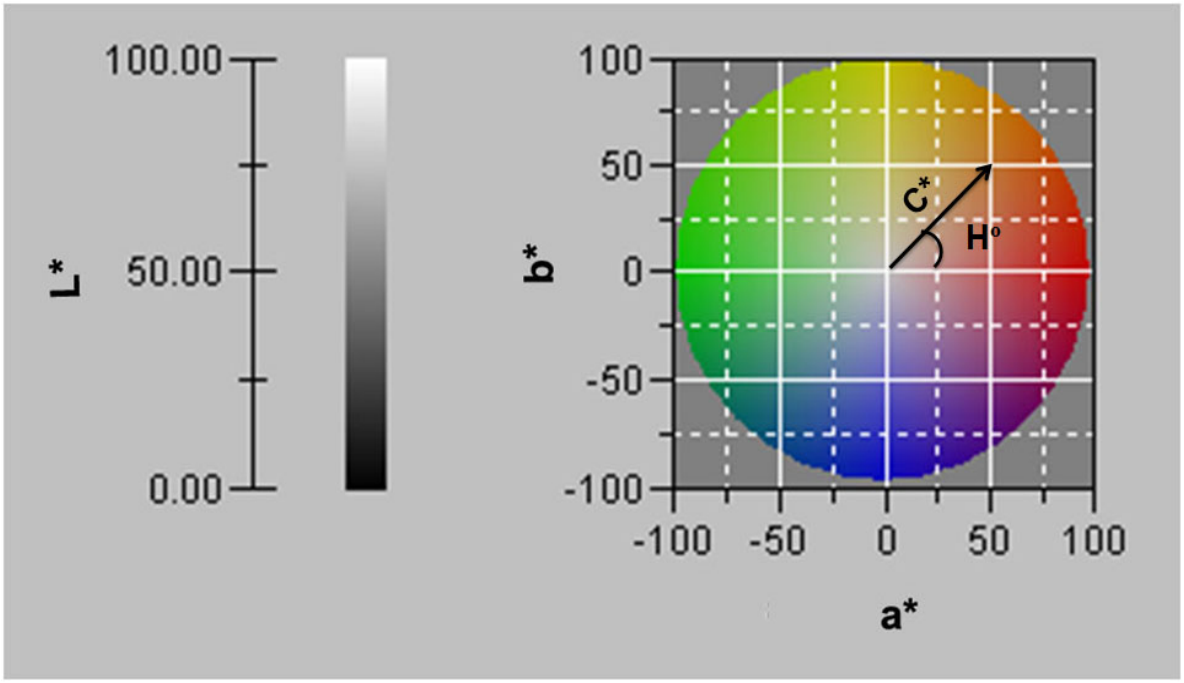

Figure 1.4 CIE LAB colour space. Adapted from SpectraMagic™ NX (Konica Minolta, Japan). L*: lightness; C*: chroma; H°: hue angle.

1.6.4 **Pigment analysis**

As noted in the preceding sections, re-greening of plant tissue has been associated with chlorophyll accumulation. Hence, monitoring the change in the composition and content of pigments in the spathe through pigment analysis is viewed as a potential research tool to measure the progress of re-greening. Pigment analysis usually requires the extraction of pigments from plant tissue by solvents. As pigments differ in their polarity, solvents chosen for extraction of pigment must be suitable for the types of pigments present in plant tissue. For example, for chlorophyll and carotenoid, acetone is commonly used for the extraction, while anthocyanin is mainly extracted with a mixture of methanol and water (Andersen and Francis, 2004; Schiedt and Liaaen-Jensen, 1995). The stability of pigments in solvent solution is detrimentally affected by oxygen, acidity, light and high temperature. For this reason, precautions should be undertaken during pigment extraction to avoid the artefacts caused by these factors. For instance, addition of an antioxidant to the solvent, operation of extraction under low light condition, storage of pigment solution in the dark and at low temperature, and addition of $NaHCO_3$ or $CaCO_3$ to neutralize plant tissue containing acids, are all recommended protocols for the extraction of pigments, including carotenoids (Britton, 1985).

To identify and quantify pigments, a number of methods are available including spectroscopic and chromatographic methods, which have been described in detail in the literature (Andersen and Francis, 2004; Gilmore and Yamamoto, 1991; Schiedt and Liaaen-Jensen, 1995; Wellburn, 1994). The choice of method is usually determined by the type of information needed and availability of facilities, funding and time. As part of the decision making process of which methods to use in the

current study, the most used methods of identification and quantification of pigments are briefly summarized as follows:

- Spectroscopic method

The method is a comparatively rapid and simple way to primarily identify and quantify the key pigments present in a tissue. An absorbance spectrum can be attained for an extract containing a mixture of pigments using a spectrophotometer. Comparison of this spectrum with those of pigment standards available in the literature (Britton, 1995) provides preliminary identification of key pigments present in the extract. Based on the equations that have been developed and published in the literature (Wellburn, 1994), concentration of pigments can also be estimated. When there are many types of pigments present in the extract, and some of them share similar spectra (this is often the case for tissue containing carotenoids), this method is limited in its ability to provide precise identification and quantification of individual pigments (Schoefs, 2002). The spectroscopic method is, therefore, suitable for a crude identification and quantification of pigment groups present in plant tissue.

- Chromatographic methods

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) are two of the most common chromatographic methods used for separation, identification and quantification for individual pigments present in plant tissue (Britton, 1985; Chen et al., 2004; Hart and Scott, 1995). TLC is a cheap and rapid way to isolate pigments, but is less accurate in identification of pigments and, is also limited in its ability to separate some pigment isomers, e.g., lutein and zeaxanthin or α- and β-carotene. On the other hand, compared with TLC, HPLC is a

relatively expensive, but accurate and sensitive method for separation, identification and quantification of individual pigments. Some pigment isomers (e.g. lutein and zeaxanthin) can be separated by using HPLC (Gilmore and Yamamoto, 1991). In addition to this, supplementation of HPLC with a photodiode array or a mass spectrometer has increased the sensitivity and accuracy of HPLC in identification of individual pigments (Fraser et al., 2000; Stintzing et al., 2002).

In summary, both the spectroscopic and chromatographic methods present their own strengths in pigment analysis, but none of them by itself is entirely suitable for an exhaustive separation, identification and quantification of a complicated mixture of pigments. Hence, a combination of these methods to suit the need of the research is considered ideal to achieve pigment analysis. For example, the spectroscopic method can be used for a preliminary screening of key pigment groups present in plant tissue, TLC for rapid identification of individual pigments, and then HPLC for a more accurate qualification and quantification of the individual pigments. This strategy of pigment analysis was applied in the research presented here, especially Chapters 2 and 6.

1.7 Thesis goals and objectives

The research presented in this thesis is part of an ongoing programme with the broad research goals of:

1. providing *Zantedeschia* growers with methods of postponing spathe re-greening and,
2. understanding the physiological mechanism of re-greening.

Within these goals, based on the preceding literature review, the following were the primary research objectives addressed in this thesis:

1. to determine the relationship between the changes in colour of the spathe of 'Best Gold' and the changes in chlorophyll or carotenoid content (Chapter 2);
2. to describe the pattern of re-greening in terms of changes in colour, content, types and distribution of pigments, and differentiation of plastids (Chapters 2 & 3);
3. to determine whether fructification is associated with spathe re-greening in representative selections of both the winter-flowering group of *Zantedeschia*, i.e. *Z. aethiopica* and the summer-flowering group, i.e., 'Best Gold' (Chapter 4);
4. to screen a range of chemicals including plant hormones and sucrose for their potential in postponing re-greening in spathes of 'Best Gold' (Chapters 5 and 6);
5. to examine the role of light in combination with hormonal treatments in influencing the initiation and progression of spathe re-greening of 'Best Gold' (Chapter 6).

Chapter 2 **Relationship between changes in colour and pigment content during spathe re-greening of *Zantedeschia* 'Best Gold'**

2.1 Introduction

The colour of the spathe in *Zantedeschia* was mainly influenced by the quantity of pigments and their distribution in cell layers (Lewis et al., 2003). Carotenoids were the key pigments contributing to the yellow colour in *Zantedeschia* hybrids (like 'Best Gold') and were located throughout the subepidermis and mesophyll of the spathe tissue (Lewis et al., 2003). As introduced in Chapter 1, spathes of 'Best Gold' undergo re-greening after horticultural harvest-maturity. The abaxial surface of the spathe is the external facing and, therefore, most visible surface that the viewer uses to assess the quality of flowers, and it re-greens more quickly than the adaxial surface (inner spathe surface; refer Chapter 3). Re-greening in the spathe of *Zantedeschia* has been previously associated with the increase in content of total chlorophyll (Gronegress, 1974; Pais and Neves, 1982-1983; Tavares et al., 1998). At the commencement of the current study however, it was unclear how the pigment composition and distribution in the tissue of the spathe varied during re-greening, and how this change influenced the change in the colour of the spathe, particularly on the abaxial surface.

To enable objective comparisons, colour is often expressed in numeric scales. The most used scale in measuring plant colour was set by the Commission Internationale de L'Eclairage (CIE LAB), including the colour coordinates of lightness (L*), a* (red-green), b* (yellow-blue), chroma (C*), and hue angle (H°; refer Chapter 1). These coordinates can readily be attained from a tristimulus colorimeter. L*, C*, H° and their equivalents have been successfully used to describe changes in colour for many horticultural commodities, e.g. fruits of *Lycopersicum esculentum* L. (Helyes et al., 2006), *Vitis vinifera* L. (Peppi et al., 2006), *Citrus unshiu* (Mak.) Marc. (Iglesias et al., 2001) and spathes of *Zantedeschia* hybrids (Funnell and Downs, 1987). The de-greening in the fruits of *C. unshiu* from green to orange was quantified through a colour index, wherein the breakpoint in colour from green to orange corresponded to a H° of 90°. Opposite to the de-greening of *C. unshiu*, spathe re-greening of 'Best Gold' is represented by a colour change from yellow to green. Therefore, in the research reported here, a H° of 90° is used as a breakpoint for the colour change for spathes of 'Best Gold' from the visible yellow to green. In other words, a H° greater than 90° is required so that humans perceive the visible colour as green.

Because colour measurement through a tristimulus colorimeter is easy to obtain, frequent attempts have been made to use the colour coordinates to predict pigment content, instead of destructive and tedious extraction and quantification for pigments. A good correlation between pigment content and the colour coordinates of the CIE LAB, has been reported in a range of horticultural commodities, e.g. fruits of *Cucurbita moschata* Poir. (Itle and Kabelka, 2009), *L. esculentum* (Thiagu et al., 1993) and *Rubus idaeus* L. (Moore, 1997). However, a number of reports in the literature

illustrated that for some commodities, pigment content did not correlate well with the colour coordinates, e.g. in fruits of *Actinidia deliciosa* C.F. Liang and A.R. Ferguson (Lawes, 1989), and *Malus*×*domestica* Borkh. (Lancaster et al., 1994; Singha et al., 1991). Lancaster and Lister (1997) suggested that correlations may only be evident when the plant tissue under investigation comprises a limited number of pigment groups. However, when more than two pigment groups predominate, these correlations are less significant, as under this situation, each set of L*, C* and H° values can be derived from many different quantitative combinations of pigments. Hence, the correlations between pigment content and colour coordinates appear to be tissue-dependent. This highlights the need for an examination of the correlation for individual plant tissue type before any prediction can be made for pigment content using colour coordinates.

The colour coordinates attained from a tristimulus colorimeter have previously been used to describe re-greening in spathes of *Zantedeschia* hybrids (Funnell and Downs, 1987), but have not been related to actual pigment content in the spathe. Hence, to use L*, C* and H° more confidently for evaluation of spathe re-greening in 'Best Gold', in this chapter, an examination of relationship between these colour coordinates and changes in chlorophyll and carotenoid content in spathes of 'Best Gold' was undertaken. In doing so, the broader aim was to find an accurate, rapid and resource-efficient way to compare, in subsequent chapters, the efficacy of various postharvest treatments on spathe re-greening.

2.2 Materials and Methods

2.2.1 Plant materials and handling

Plants of *Zantedeschia* 'Best Gold' were grown either in a greenhouse at the Plant Growth Unit, Massey University or outdoors at a local commercial property (Palmerston North, New Zealand; 40°20'S). To increase flower production in the greenhouse, tubers were sprayed with a solution of 100 ppm GA_3, and allowed to dry before planting. Tubers were planted in 3-litre plastic pots containing a 50:30:20 bark: peat: pumice mixture (v:v:v), plus 2.0 kg/m^3 each of agricultural lime and dolomite, 1.0 kg/m^3 gypsum, and 3.0 kg/m^3 Osmocote® 16N-3.5P-10K (Grace-Sierra International, Netherlands). The plants were irrigated using capillary matting, which was kept moist at all times through automated drippers three times per day for 5 minutes (7 minutes in summer). To minimize buildup of soluble salts, the pots were also overhead watered by hand once per week. The greenhouse was heated to 15 °C, ventilated at 20 °C, and received natural sunlight and photoperiod (9 to 15-h).

Flowers (spathe plus spadix and peduncle) with a similar horticultural harvest-maturity (i.e. just before pollen shed, spadix turned corn-yellow, spathe fully coloured (Funnell and Downs, 1987)) were used for monitoring colour change after harvest in a vase life room. The room was controlled under recommended environmental conditions for postharvest evaluation (Reid and Kofranek, 1980), i.e., temperature 20 ± 1 ºC, relative humidity 60 to 70%, 12-h (0600 HR – 1800 HR) photoperiod and light intensity 25 μmol·m^{-2}·s^{-1} at bench height provided by cool-white fluorescent tubes.

As described in Chapter 3, discs excised from the spathe of 'Best Gold' followed a similar pattern of re-greening as that occurring in the detached entire spathe. Hence in the current study, discs of spathe tissue were used. Once harvested, two discs per stem were excised from either side of the midrib at the basal area of spathes using a cork borer (diameter 14 mm). In sampling discs, any green tissue at the basal area was avoided. The discs were placed on filter paper soaked in RO water with the adaxial surface facing down in closed petri dishes. The filter paper was kept moist throughout the experiment.

2.2.2 **Experimental design and data analysis**

The experiment was a completely randomized design. A total of 336 discs from 168 spathes were collected and allocated at random in batches of 16 to petri dishes. Each petri dish was treated as an experimental unit. The discs were randomly sampled for colour measurement and pigment analysis at two- to three-day intervals, within the 14 day period after harvest. Hence, there were seven sampling dates in total. At each date of sampling there were 3 replicates, each comprising 16 discs.

Data were tested initially to ensure they met the requirement for ANOVA using the general linear procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Means were separated by using the unrestricted LSD procedure. Pearson's correlation coefficients were determined by using Minitab 15 (Minitab Inc., Pennsylvania, USA) and linear regression was conducted using SigmaPlot (Systat Software Inc., Richmond, Califonia, USA)

2.2.3 **Colour measurement**

L*, C* and H° of both the abaxial and adaxial surfaces of discs were measured using a tristimulus colorimeter CM-2600d/2500d (Konica Minolta, Japan). The discs were then dissected with a scalpel into abaxial and adaxial layers via cutting through the middle parenchyma tissue. The replicate samples for each surface were dehydrated using a Hetosicc freeze dryer (Heto Ltd., Denmark) and stored at -20 °C pending pigment analysis.

2.2.4 **Pigment analysis**

Freeze-dried tissue was weighed and ground, and extracted overnight in 2 ml 80% acetone at 4 ºC in darkness. The samples were centrifuged and the supernatant removed to a 15 ml tube wrapped in aluminium foil. A further 1 ml 80% acetone was added to the pellet and the procedure was repeated three to four times as above, until the tissue was colourless. The supernatant was combined and the final volume was adjusted to 5 ml. Absorbance of the extract was measured at wavelengths of 470, 647, and 663 nm using a spectrometer (Hitachi U-2000; Hitachi, Japan). Wellburn's (1994) equation was used to calculate the content of chlorophyll *a*, chlorophyll *b*, total chlorophyll and total carotenoid. All pigments were expressed in units of both $\mu g \cdot g^{-1}$ dry weight (DW) and $\mu g \cdot cm^{-2}$.

2.2.5 **Histology**

Discs of the spathe of 'Best Gold' were sampled at: day 0 (horticultural harvest-maturity), day 7 (early re-greening) and day 14 (re-greened) for histological examination. Using a scalpel, thin cross-sections from the basal area of spathe tissue

were taken, discriminating between the abaxial and adaxial surfaces by trimming at an angle. In sampling discs, any green tissue at basal area was avoided. The sections were vacuum-infiltrated in Reverse Osmosis (RO) water to remove air bubbles. They were then placed on a glass slide with a drop of RO water and a cover slip was applied. To determine the distribution of pigmentation across the cross-section, each slide was examined under a compound light microscope (Zeiss, Germany) with a Leica DFC 320 digital camera attached (Leica Microsystems, Wetzlar, Germany). The slide was also viewed under a Leica model TCS SP5 DM6000B confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) for examination of the distribution of chlorophyll autofluorescence across the section.

2.3 Results

2.3.1 Change in pigment distribution

At horticultural harvest-maturity (day 0) histological examination of the spathe tissue showed carotenoid-containing chromoplasts were mainly concentrated in subepidermal layers (three to four cell layers beneath the epidermis), of both abaxial and adaxial surfaces, with some scattered in the mesophyll cells (Figure 2.1 A). The confocal image revealed that there was no autofluorescence of chlorophyll from either the abaxial or adaxial surfaces at this stage of horticultural harvest-maturity (Figure 2.1 C&E). By day 7, newly-synthesized chloroplasts appeared at the subepidermis of the abaxial, but not the adaxial surface. This was further confirmed by the autofluorescence of chlorophyll (shown as red colouration) within the subepidermis of the abaxial surface, but not from the adaxial surface (Figure 2.1 D&F). An enlarged image of the abaxial surface (Figure 2.1 F) showed that the red coloured fluorescence

was from chloroplasts. Along the same layer in the subepidermis, the distribution of the chloroplasts was relatively uniform between the individual adjacent cells by day 7. The carotenoid-containing chromoplasts in both the abaxial and adaxial surfaces were less evident by day 7. By day 14, the subepidermis of the abaxial surface had accumulated even more chloroplasts than were evident at day 7 (data not shown).

2.3.2 Change in colour

Within the 14 day period of observation, the abaxial but not adaxial surface of discs re-greened. During this period, the colour of the abaxial surface progressed from golden-yellow through yellow-green to green. This change in colour was quantitatively described as an increase in H° from 85° to 96°, and a decrease in both L* (76% to 61%) and C* (70% to 48%; Figure 2.2). During the first week, the rate of increase of H° (1.29°/day) was 3.4 times higher than that evident in the second week (0.29°/day). By day 5, the H° of the abaxial surface was already greater than 90°, being evident as visible green colouration on the discs. For the adaxial surface, within the 14-day period the colour varied from vivid yellow to dull yellow, and was quantified by an increase in H° (86° to 90°) and a decrease in both L* (78% to 71%) and C* (66% to 39%; Figure 2.2). Compared to the abaxial surface, changes in magnitude of H° and L* of the adaxial surfaces, progressed within a narrower range of values, but within a wider range for values of C*.

2.3.3 Change in pigment content

During the 14-day period of observation, re-greening on the abaxial surface was accompanied by an approximate 10-fold increase in the content of both

chlorophyll *a* and chlorophyll *b*, from 143 to 1300 $\mu g \cdot g^{-1}$ DW and, from 43 to 475 $\mu g \cdot g^{-1}$ DW, respectively (Figure 2.2). At the same time, the total carotenoid content of this surface decreased by 20% from 1127 to 895 $\mu g \cdot g^{-1}$ DW.

For the adaxial surface however, over this 14-day period, less than 60 $\mu g \cdot g^{-1}$ DW of either chlorophyll *a* or *b* was detected (Figure 2.2). The initial content of total carotenoid of the adaxial surface was 841 $\mu g \cdot g^{-1}$ DW, which was lower than that of the abaxial surface (Figure 2.2). Over the 14-day period, the adaxial surface showed a slight decrease in total carotenoid from 841 to 730 $\mu g \cdot g^{-1}$ DW.

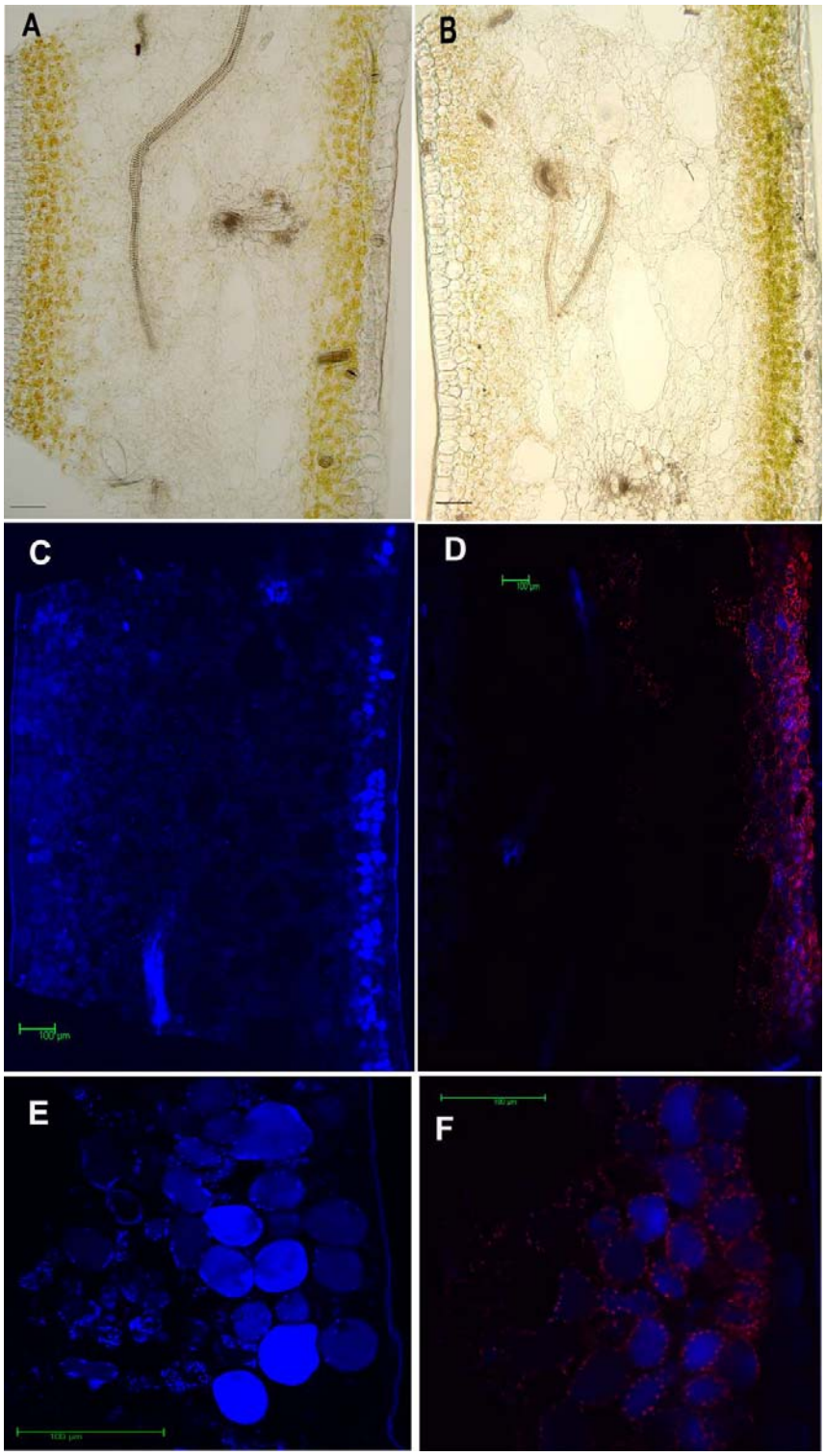

Figure 2.1 Pigment distribution on both abaxial (right side of section) and adaxial (left side of section) surfaces of the spathe of *Zantedeschia* 'Best Gold' at, A (day 0) and B (day 7) under bright-field of light microscope; C (day 0) and D (day 7) under confocal microscope. Red colour indicates chlorophyll autofluoresence. E (day 0) and F (day 7) are enlargements of the abaxial surface. Scale bars = 100 µm.

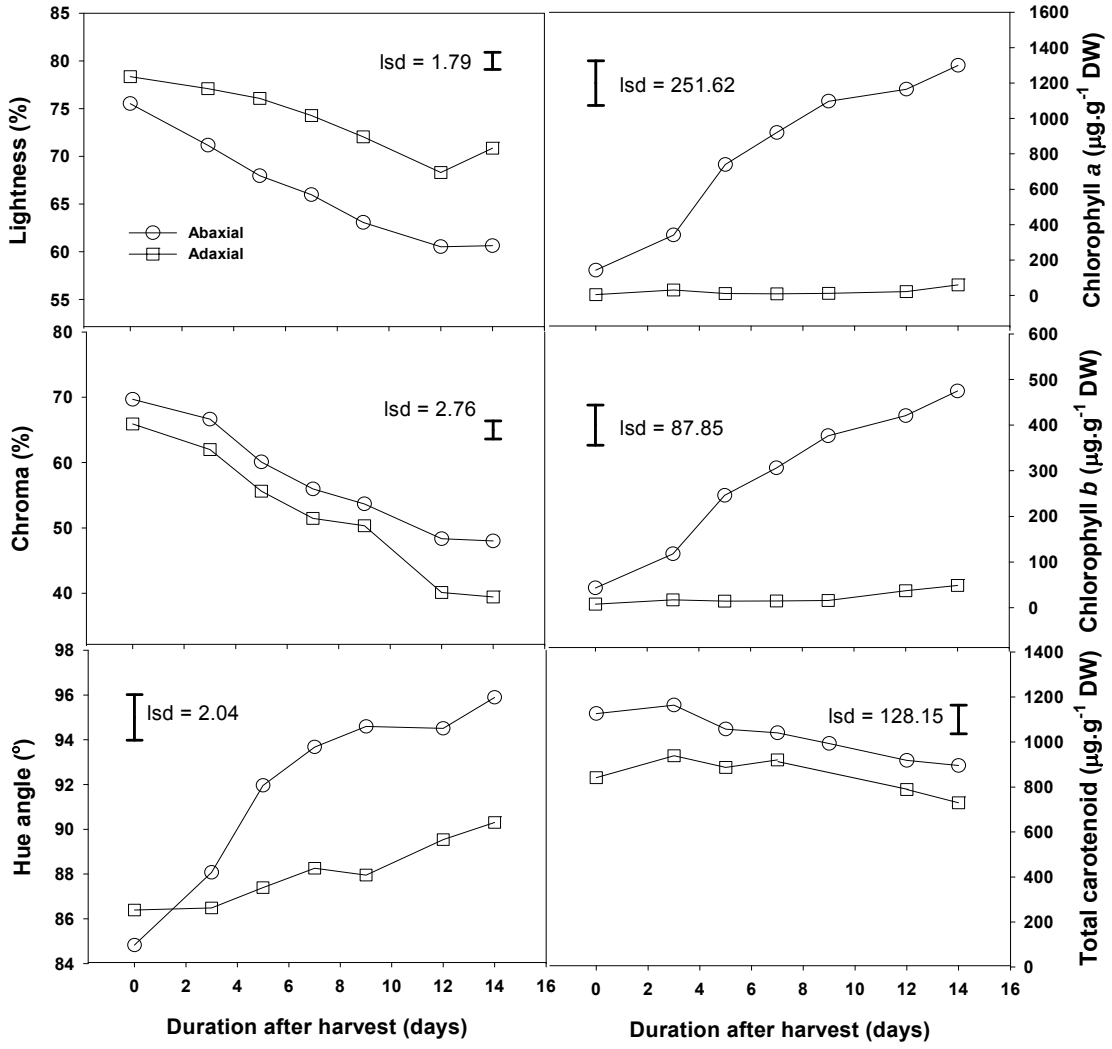

Figure 2.2 Colour coordinates (lightness, chroma, and hue angle) and content of pigments (chlorophyll *a*, chlorophyll *b* and total carotenoid) of spathe tissue of *Zantedeschia* 'Best Gold' during 14 days after horticultural harvest-maturity. The line bars represent the LSD at $P < 0.05$. n=3.

2.3.4 Correlations between lightness, chroma and hue angle with pigment content

2.3.4.1 Chlorophyll

For the abaxial surface, the content of chlorophyll *a*, *b* or total chlorophyll, on both a dry weight (μg·g^{-1} DW) and an area (μg·cm^{-2}) basis, was negatively correlated with L* and C*, while positively correlated with H$^{\circ}$ (Figure 2.3; Figure 2.4). For these parameters the absolute value of Pearson's correlation coefficients, i.e. $|r|$, ranged between 0.89 and 0.98 ($P < 0.001$; Table 2.1).

The correlations between the content of chlorophyll *a*, *b* or total chlorophyll, with H$^{\circ}$, were between 1% to 9% higher than the correlations with L* and C* (Table 2.1). The highest correlation was between H$^{\circ}$ and the content of chlorophyll *a* or total chlorophyll on a per area (μg·cm^{-2}) basis, with a *r* value of 0.98.

The correlation between H$^{\circ}$ and the content of chlorophyll *a*, *b* or total chlorophyll, was slightly (1%) higher on a per unit area than per dry weight basis. Because of this, a linear regression was performed for the correlation pair between H$^{\circ}$ and the pigment content per unit area. There was a quadratic relationship between the content of chlorophyll *a*, *b* or total chlorophyll (μg·cm^{-2}) with H$^{\circ}$ accounting for 97% or 98% of variation ($P < 0.001$; Figure 2.5).

For the adaxial surface, there was a correlation between the content of chlorophyll *b*, but not chlorophyll *a*, with L*, C* and H$^{\circ}$ (Figure 2.3; Figure 2.4; Table 2.1). As the quantity of both chlorophyll *a* and *b* in the adaxial surface was negligible

(< 60 μg·g^{-1} DW) compared with that in the abaxial surface, and the adaxial surface is not the principal surface limiting quality of flowers, regression analysis was not performed for data from this surface.

2.3.4.2 Total carotenoid

For the abaxial surface, the content of total carotenoid, expressed on both the basis of per unit area and per dry weight, was positively correlated with L* and C*, and negatively with H° (Figure 2.3; Figure 2.4), with values of $|r|$ ranging from 0.66 to 0.9 ($P \leq 0.001$; Table 2.1).

The correlations between L*, C* and H° with the content of total carotenoid, were between 10% to 15% higher on a per unit area basis than on a per dry weight basis (Table 2.1). The highest correlation was between the total carotenoid content (μg·cm^{-2}) with C* ($r = 0.9$; $P < 0.001$). A simple linear regression best described this relationship, accounting for 81% of variation ($P < 0.0001$; Figure 2.5).

For the adaxial surface, the correlations were significant between L*, C* and H° with the content of total carotenoid on an area basis, but not on a per dry weight basis (Figure 2.3; Figure 2.4; Table 2.1). The highest correlation for this surface was also between the content of total carotenoid and C*. A simple linear regression best described for this correlation, accounting for 71% of variation ($P < 0.0001$; Figure 2.6).

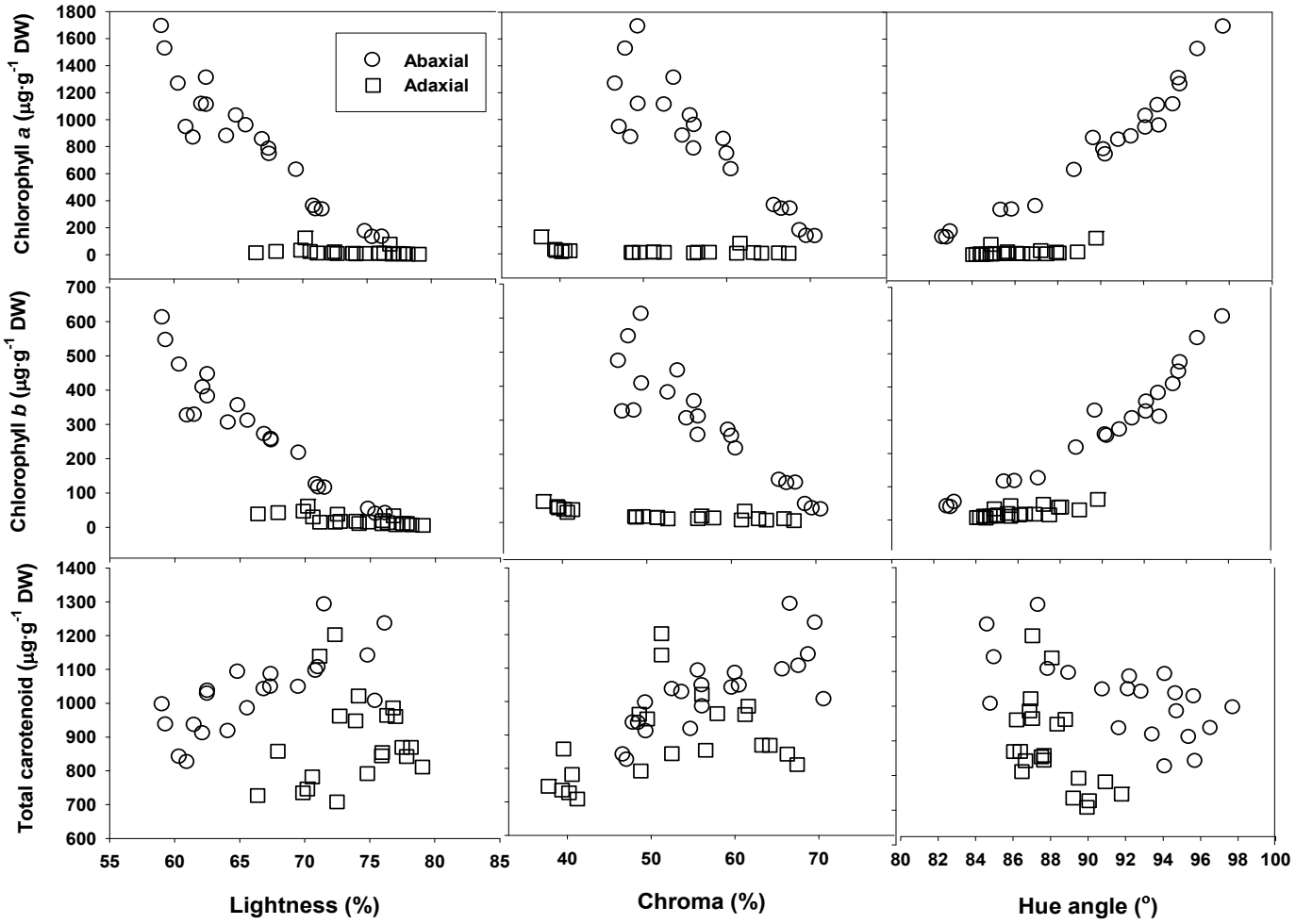

Figure 2.3 Content of chlorophyll *a*, chlorophyll *b*, or total carotenoid (µg·g^{-1} DW) versus lightness, chroma, and hue angle of both abaxial and adaxial surfaces of the spathe of *Zantedeschia* 'Best Gold' over a 14-day period from horticultural harvest-maturity.

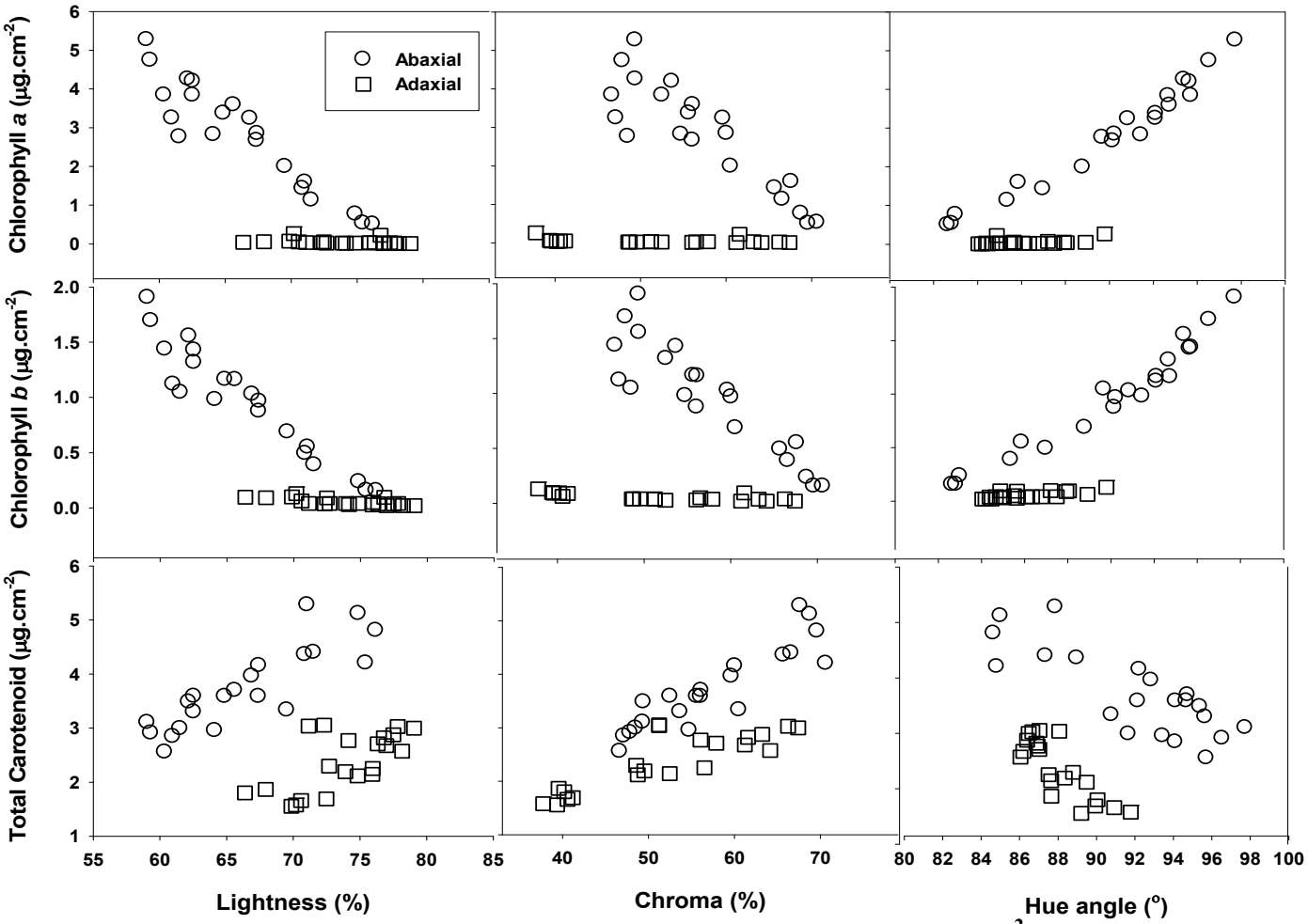

Figure 2.4 Content of chlorophyll *a*, chlorophyll *b*, or total carotenoid (µg·cm^{-2}) versus lightness, chroma, and hue angle of both abaxial and adaxial surfaces of spathes of *Zantedeschia* 'Best Gold' over a 14-day period from horticultural harvest-maturity.

Table 2.1 Pearson correlation coefficient (r), and probability level, between lightness (L*), chroma (C*), or hue angle (Hº) and chlorophyll a, chlorophyll b, total chlorophyll or total carotenoid, for both the abaxial and adaxial surfaces of *Zantedeschia* 'Best Gold', on both the basis of per unit dry weight (µg·g^{-1} Dry Weight) and per unit area (µg·cm^{-2}).

Abaxial surface												
	Per Dry Weight (µg·g^{-1})						Per Area (µg·cm^{-2})					
	L*		C*		Hº		L*		C*		Hº	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Chlorophyll *a*	-0.95	<.001	-0.9	<.001	0.97	<.001	-0.94	<.001	-0.89	<.001	0.98	<.001
Chlorophyll *b*	-0.95	<.001	-0.91	<.001	0.96	<.001	-0.95	<.001	-0.9	<.001	0.97	<.001
Total chlorophylls	-0.95	<.001	-0.90	<.001	0.97	<.001	-0.94	<.001	-0.89	<.001	0.98	<.001
Total carotenoids	0.74	<.001	0.8	<.001	-0.66	0.001	0.86	<.001	0.9	<.001	-0.81	<.001

Adaxial surface												
	Per Dry Weight (µg·g^{-1})						Per Area (µg·cm^{-2})					
	L*		C*		Hº		L*		C*		Hº	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Chlorophyll *a*	-0.3	0.195	-0.38	0.091	0.51	0.019	-0.22	0.345	-0.28	0.215	0.416	0.061
Chlorophyll *b*	0.74	<.001	-0.79	<.001	0.74	<.001	-0.68	0.001	-0.71	<.001	0.684	0.001
Total chlorophylls	-0.47	0.03	-0.55	0.009	0.62	0.003	-0.39	0.082	-0.45	0.043	0.533	0.013
Total carotenoids	0.17	0.454	0.35	0.117	-0.52	0.016	0.657	0.001	0.852	<.001	-0.82	<.001

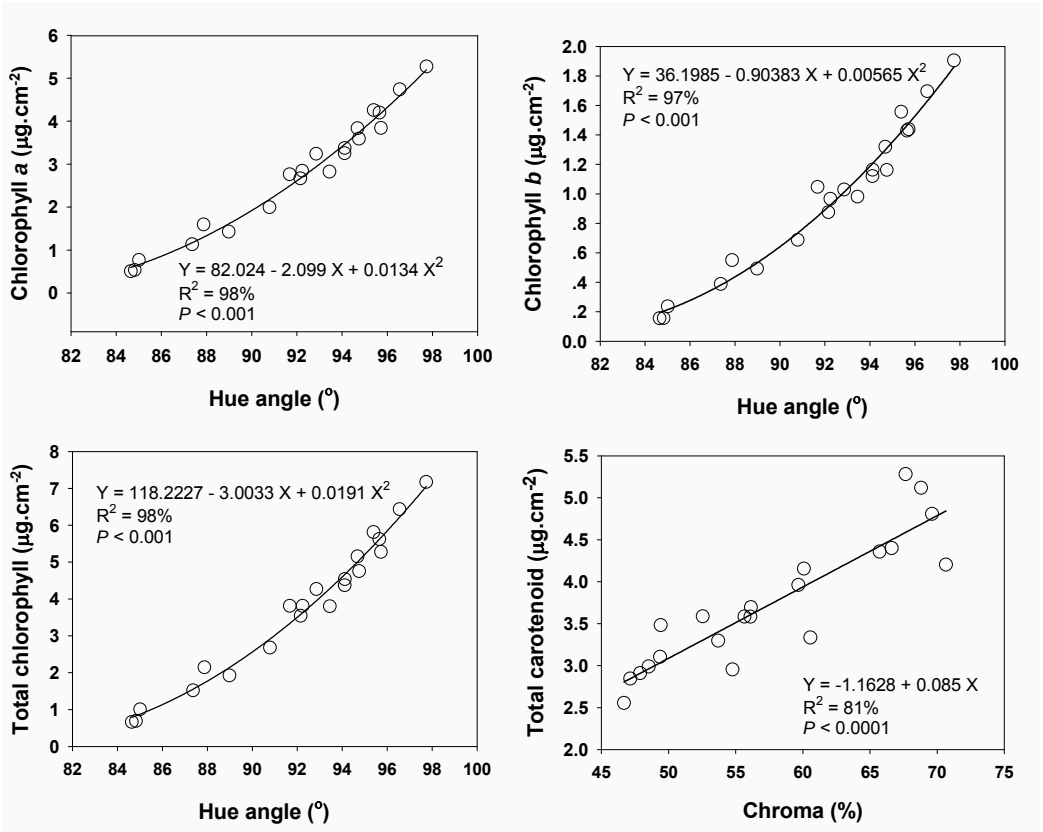

Figure 2.5 Relationships between the content of chlorophyll *a*, chlorophyll *b* or total carotenoid with, hue angle or chroma, for the abaxial surface of the spathe of *Zantedeschia* 'Best Gold' over a 14-day period from horticultural harvest-maturity.

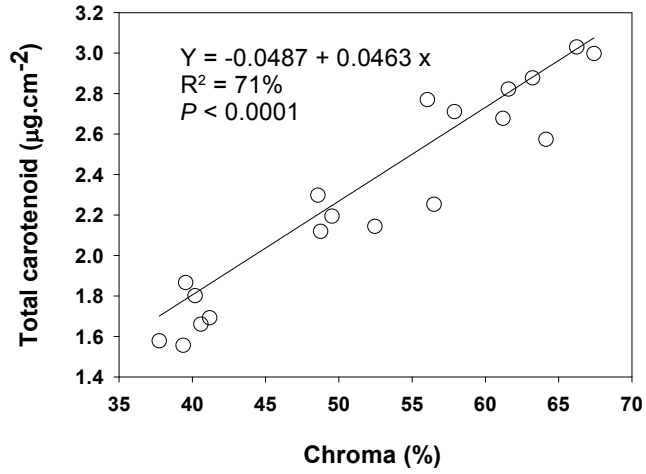

Figure 2.6 Relationship between the content of total carotenoid with chroma for the adaxial surface of the spathe of *Zantedeschia* 'Best Gold' over a 14-day period from horticultural harvest-maturity.

2.4 **Discussion**

In order to develop an accurate, rapid and resource-efficient way to compare the efficacy of various postharvest treatments on spathe re-greening, a primary aim of this experiment was to determine what if any relationship existed between changes in L*, C* and H° and changes in chlorophyll or carotenoid content. In the current experiment, re-greening on the abaxial surface of the spathe of 'Best Gold' resulted from a 10-fold accumulation of chlorophyll *a* and *b* and a less than 1-fold decrease in the content of total carotenoid (Figure 2.2). The change in content of these pigments was shown to be highly correlated with the changes in value of L*, C* or H°, with values of $|r|$ ranging from 0.66 to 0.98 (Table 2.1). These results are in agreement with those reported for leaves of *Petroselinum sativum* L. and fruits of *L. esculentum*, in which there was a significant correlation between the colour coordinates (particularly H°) and pigment content (Berset and Caniaux, 1983; Thiagu et al., 1993). These horticultural commodities contained a relatively simple pigment composition (i.e. one or two pigment groups) similar to the situation that occurred in 'Best Gold'. Thus significant correlations may in part be explained by the change in colour primarily resulting from changes in one of the predominant pigments, e.g. chlorophyll in the case of 'Best Gold'. Conversely, in some fruits, e.g. *Malus*×*domestica*, where colour was determined by more than two pigment groups including chlorophyll, carotenoid and anthocyanin, the change in colour during fruit ripening resulted from significant and simultaneous changes in all these pigments (Lancaster et al., 1994; Singha et al., 1991). As a consequence, no or only a poor correlation was found between the values of the colour coordinates and pigment content with these fruits. Additionally, in the current research, the predominant pigment, i.e. chlorophyll, was concentrated in the subepidermis of the spathe (Figure 2.1), and not spread throughout the mesophyll

layers. As such, the accumulation of chlorophylls during re-greening can be readily discerned by a tristimulus colorimeter. Hence, the simplicity in dynamics of both the pigment composition and distribution in spathes of 'Best Gold' during re-greening, likely contribute to the highly significant correlations between content of chlorophyll and carotenoid with colour coordinates achieved in this study.

Among the three colour coordinates, the change in H° showed the strongest correlation with the accumulation of chlorophyll during the re-greening of 'Best Gold', with a *r* value of 0.98. Hence, measuring the change in H° can be used to infer a change in chlorophyll content and, therefore, evaluate the progress of re-greening for future research in spathe re-greening of 'Best Gold'. However, to use this method with confidence, its limitations need to be acknowledged.

One of the limitations for this method is that its sensitivity is not consistent at different stages of re-greening. As shown in the current experiment, within the range of data examined, the relationship between H° and chlorophyll content was not linear but curvilinear (Figure 2.5). For example, an increase in H° by 7 units from 85° to 92° during the early stage of re-greening (i.e. crossing the break-point for the colour change from yellow to green) correlated with a change in total chlorophyll content of 2.6 $\mu g \cdot cm^{-2}$ (Figure 2.5). In contrast, a similar increase in H° from 92° to 99°, during the later stage of re-greening, was associated with a greater change in chlorophyll content of 4.5 $\mu g \cdot cm^{-2}$ (Figure 2.5). This suggests that H° reflects the change of chlorophyll content more sensitively during the early stages of re-greening, where chlorophyll content is relatively low, than at later stages when there is a higher total chlorophyll content in tissues. In other words, a subtle increase in chlorophyll content

during the early stages of re-greening can result in a rapid change in H°. In the later stages of re-greening, i.e. beyond 7 days in this study, increases in the content of chlorophyll did not result in as large a change in H°, but mainly contributed to a reduction in the lightness of the colour. Hence, H° is likely to be a less sensitive indicator for the change in chlorophyll content during this later stage of re-greening. This reduced sensitivity of H° in reflecting the change in pigment content, after it reaches a saturated level (i.e. curvilinear instead of linear relationship between H° and pigment content), has also been reported with fruits of *Malus*×*domestica* (Dixon, 1993; Iglesias et al., 2008), *L. esculentum* (Thiagu et al., 1993) and, *V. vinifera* (Peppi et al., 2006). This inconsistency in sensitivity of H° in inferring changes in chlorophyll content at different stages of spathe re-greening however, is not perceived as reducing the validity of this method when applied in the future research on re-greening in spathe tissue of 'Best Gold'. This is because it is the initial stage of re-greening, reflected by the values of H° around 90°, which are most relevant to the research interest of this thesis. Therefore, in the subsequent chapters of this thesis, after horticultural harvest-maturity, an increase in H° can be used to infer an accumulation in chlorophyll. In addition, values of H° greater than 90° can be used to indicate that the content of chlorophyll has reached the level that humans perceive green colouration (Iglesias et al., 2001).

As a further limitation of the methodology, since the relationship between chlorophyll content and H° was developed based on the data from the abaxial surface of the spathe of 'Best Gold', measuring the change in H° can not be used to infer the change in chlorophyll content (if any) on the adaxial surface. In the current experiment no chlorophyll accumulation was detected on the adaxial surface of the

spathe, and yet a slight increase in H° from 86° to 90° was observed within the 14-day period of observation (Figure 2.2). This change in H° on the adaxial surface might partially result from a decrease in the content of total carotenoid, as evident by a significant correlation between H° and the content of total carotenoid for this surface (Table 2.1). Therefore, the method developed in the current experiment is only suitable for evaluating re-greening on the abaxial surface.

Compared with other experiments, (refer Chapter 5) the range of H° values examined in this study (i.e. 85° to 98°) was comparatively narrow. Due to variation in environmental conditions (e.g. light intensity), flowers harvested from different seasons are likely to vary in their initial colour (Ben-Tal and King, 1997; MacKay et al., 1987) and pigment content, hence presenting a wider range of H° values than those encountered in the current experiment. As a result, the quadratic relationship between H° and chlorophyll content derived from the current experiment must be considered limited for an accurate estimation of chlorophyll content across a wider range of H° values. To solve this limitation, repeating the current experiment under different growing environment conditions is required. Since resources did not permit this to occur, with regard to the subsequent experiments presented in this thesis, application of the correlations found will be limited to only inferring changes in chlorophyll content have occurred.

Carotenoids remained as a key pigment group in the spathe of 'Best Gold' during the 14-day period of monitoring re-greening. By day 7, they still accounted for about 46% of total pigment content in the abaxial surface (Figure 2.2). Compared with total chlorophyll however, during re-greening the change in the content of total

carotenoid appears to contribute less to the colour change of the spathe. This is partially due to the fact that within the layers of the subepidermis where chlorophyll and carotenoids coexist, the newly-synthesized chlorophyll appears to mask the visibility of the carotenoids. This kind of masking effect is common in green leaves, e.g. *Spinacia oleracea* L. and fruits, e.g. *A. deliciosa* and *Mangifera indica* L. (Alkema and Seager, 1982; Ketsa et al., 1999; Kidmose et al., 2001; McGhie and Ainge, 2001).

The change in the content of total carotenoid on both abaxial and adaxial surfaces during re-greening of the spathe of 'Best Gold' was more closely reflected by the change in C* than in H° (Table 2.1). This is also in agreement with the correlation reported for total carotenoid in fruits of *C. moschata* (Itle and Kabelka, 2009). In the current experiment however, the two surfaces differ in magnitude of the parameters describing the linear relationship between total carotenoid content and C* (Figure 2.5; Figure 2.6). Hence, a given value of C* cannot be inferred to predict the same content of total carotenoid for both surfaces. This is because the presence of chlorophyll on the abaxial surface also partially contributes to the saturation of colour viewed. The pigment composition, therefore, likely determines the relationship between the change in pigment content and colour. In other words, the method developed in this study to evaluate spathe re-greening of 'Best Gold' cannot simply be used for other hybrids of *Zantedeschia* that contain a different pigment composition from that of 'Best Gold'.

2.5 **Conclusion**

Particularly during the initial stages of spathe re-greening, changes in chlorophyll content can be inferred by the change in H° of the abaxial surface. An increase of H° to a value greater than 90° therefore, can indicate that spathe re-greening and an accumulation of chlorophyll have progressed to where it is visible to the human eye. Hence, in the later chapters of this thesis, L*, C* and particularly H°, can be used with confidence to evaluate the onset and degree of re-greening, so as to compare the influence of various postharvest treatments on chlorophyll accumulation.

Chapter 3 **Pattern of re-greening in the spathe of *Zantedeschia* 'Best Gold'**

3.1 Introduction

Zantedeschia is a worldwide, commercial, cut flower and pot plant (Funnell, 1993) and is New Zealand's second largest export flower (Statistics New Zealand, 2010). The leaf-like floral structure (spathe) of *Zantedeschia* re-greens after it fully opens and reaches commercial maturity. This re-greening is a primary determinant limiting the postharvest quality of *Zantedeschia*. Efforts have been undertaken to understand the mechanism of spathe re-greening in *Zantedeschia* and to investigate potential ways of postponing it (Funnell and Downs, 1987; Pais and Neves, 1982-1983; Pais, 1981).

The current knowledge of spathe re-greening in *Zantedeschia* is mainly based on the pioneering work from a group of researchers in Portugal: Pais and Neves (1982-1983), Tavares et al. (1998) and Lino-Neto et al. (1999). They reported that the spathe of *Z. aethiopica* underwent senescence soon after its maturation, but fructification inhibited this senescence, and resulted in spathe re-greening. The earlier maturation of the spathe is associated with the formation of amyloplasts from chloroplasts (de-greening), while re-greening after maturation is the result of redifferentiation of chloroplasts from amyloplasts. Along with the reformation of chloroplasts, chlorophyll accumulates. All this existing knowledge is based on the research utilising *Z. aethiopica*, which belongs to the winter-flowering group of

Zantedeschia (refer Section 1.1). Other than it is known to occur (Funnell, 1993; Funnell and Downs, 1987; Gronegress, 1974), little quantitative detail is available to describe the pattern of re-greening in the summer-flowering species or hybrids, e.g. 'Best Gold', in terms of change in colour, pigment and plastid differentiation.

As noted in Section 1.4, re-greening of plant tissue resembles the process of greening, wherein both involve formation of functional chloroplasts. The process of greening is coordinated with a rapid accumulation of both chlorophyll and carotenoid (Schoefs et al., 1998; von Lintig et al., 1997), as these pigments are essential components of photosynthetic apparatus in chloroplasts (Sundqvist and Dahlin, 1997; Young and Britton, 1993). Likewise, re-greening has previously been associated with the increase in chlorophyll content, but has not been linked to the accumulation of carotenoids. In many situations, prior to re-greening, the carotenoids are already present in the plastids, e.g. chromoplasts in the peel of *Citrus sinensis* (L.) Osbeck. fruits (Mayfield and Huff, 1986) and gerontoplasts in yellowing leaves of *Nicotiana rustica* L. (Zavaleta-Mancera et al., 1999a). In such cases, as chlorophylls increase and chloroplasts reform during re-greening of plant tissue, it is unclear whether these existing carotenoids persist, reform or degrade and, whether there are changes in the types of carotenoids present. As described by Lewis et al. (2003), carotenoids including lutein and β-carotene were responsible for the yellow colour of spathes of *Zantedeschia* hybrids. Similarly, in Chapter 2 of this thesis, spathe tissue of 'Best Gold' at horticultural harvest-maturity (i.e., prior to re-greening), mainly contained carotenoids, and the re-greening was associated with a slight decline in total carotenoid. To date, however, the changes in individual pigments (e.g., lutein and β-carotene) that occur during re-greening of spathe tissue, have not previously been

reported. Therefore, in the current study, the change in pigment content, including chlorophyll and individual carotenoids, in association with chloroplast formation during re-greening, was evaluated.

A protocol of using segments of pericarp tissue from citrus fruit has previously been developed for investigating the re-greening in *C. senesis* (Mayfield and Huff, 1986). With advantages of easier handling and reducing the amount of experimental material required, in the current study, an attempt was made to develop a similar protocol using discs of tissue excised from the spathe of *Zantedeschia* flowers for research into re-greening.

The objectives of this study were therefore, to develop a protocol to use discs of spathe tissue to investigate re-greening of 'Best Gold' and to describe the pattern of re-greening in terms of changes in colour, pigment and plastid differentiation, and any associations between such changes.

3.2 Materials and methods

3.2.1 Plant materials and handling

The plant materials used in this study were flowers (spathe plus spadix and peduncle) of 'Best Gold'. They were produced under the same conditions and harvested at the same maturity, as described in Chapter 2 (refer section 2.2.1). Flowers with a similar horticultural harvest-maturity (i.e. just before pollen shed; spadix turned corn yellow; spathe fully coloured) were obtained for colour monitoring in a vase life room. The room was controlled under recommended environmental

conditions for postharvest evaluation (Reid and Kofranek, 1980), i.e., temperature 20 ± 1 ºC, relative humidity 60 to 70%, 12-h (0600 HR – 1800 HR) photoperiod and light intensity 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height provided by cool-white fluorescent tubes. Once harvested, flowers were held in individual vases containing reverse osmosis (RO) water throughout their postharvest evaluation. If discs were used, they were excised from either side of the midrib, at the positions specified in the individual experiment, by using a cork borer (diameter 14 mm). The discs were placed on filter paper soaked in RO water, with the adaxial surface facing down in closed petri dishes. The filter paper was kept moist with RO water throughout each experiment.

3.2.2 **Disc versus spathe**

To determine if the pattern of re-greening of discs of spathe tissue differed to that for detached entire spathes, the changes in colour of both were monitored. Lightness (L*), chroma (C*) and hue angle (H°) of the abaxial surface of both discs, and detached entire spathes, were recorded every two to three days, over a 14-day period after horticultural harvest-maturity, using a tristimulus colorimeter CM-2600d/2500d (Konica Minolta, Japan). Two discs were sampled from the same position (central position on either side of mid-rib) on the spathe where the readings were taken on detached entire spathes. There were four individual-flower replicates per treatment and, two discs or measurement points, per replicate.

3.2.3 **Pattern of re-greening**

To examine if the pattern of re-greening differed at various positions of the spathe, the change in colour of the abaxial and adaxial surfaces of the discs excised at

symmetrical positions on both the left and right sides of the mid rib, from each of the basal, central and upper positions of spathe was monitored (Figure 3.1). Values of L*, C*, and H° of the discs were recorded every two to three days for a period of 14 days. There were four individual-flower replicates per position.

Based on the results presented in Chapter 2, the change in H° can be used to describe the change in chlorophyll content in the abaxial surface (but not the adaxial surface), particularly during the initial stage of spathe re-greening. For the abaxial surface, a value of H° greater than 90° was indicative of a level of accumulation of chlorophyll (i.e. re-greening) visible to the human eye (Iglesias et al., 2001). Hence, in the experiments presented here, particular attention was paid to changes in H°.

3.2.4 **Pigment analysis**

3.2.4.1 General

To determine the pigment profile in spathe tissue of 'Best Gold', samples were collected from entire spathes and discs at day 0 (horticultural harvest-maturity), day 7 (re-greening spathe), and day 14 (re-greened spathe) for pigment analysis. There were four individual-flower replicates per sampling date, and four discs per replicate were excised from the central position of the spathe. The discs were then dissected with a scalpel into abaxial and adaxial layers, via cutting through the middle parenchyma tissue. The replicate samples for each surface were dehydrated using a Hetosicc freeze dryer (Heto Ltd., Denmark) and stored at -20 $^{\circ}$C pending pigment analysis. For the purposes of comparison, tissue was similarly sampled from a mature leaf of 'Best Gold' for pigment analysis.

3.2.4.2 Pigment extraction and purification

Pigment extraction and purification was conducted following the methodology of Lewis et al. (2003) with some modification. The freeze-dried tissue was weighed before being ground and extracted in 1 ml of acetone: methanol (7:3; v:v) in 0.1% (w/v) Butylated hydroxytoluene (BHT), 10 mg $CaCO_3$, and 0.5 ml internal standard β-Apo-8'-carotenal (10 μg/ml; Fluka, Switzerland). The samples were vortexed, soaked for more than 10 min, and then centrifuged at 16,000 rpm for 5 min at 4°C. The supernatant was removed to a clean 15 ml tube wrapped in aluminium foil. A further 1 ml acetone: methanol (7:3; v:v) was added to the pellet and the procedure repeated three to four times, as above, until the tissue was colourless. The supernatant was combined and dried under a stream of O_2-free N_2. The extract was then resuspended in 1 ml ethyl acetate and transferred to a 2 ml micro-centrifuge tube. The micro-centrifuge tube was sealed with parafilm and kept at -20 °C pending pigment identification and quantification.

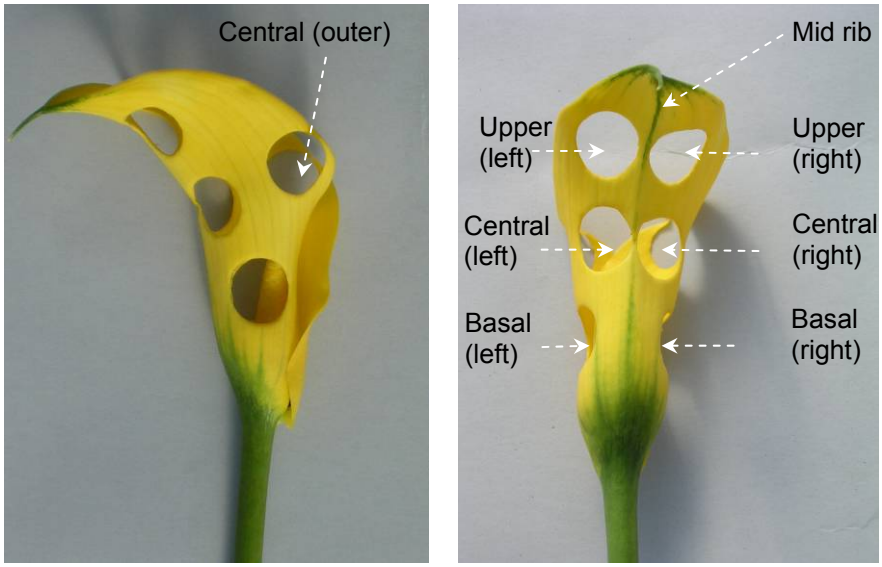

Figure 3.1 Positions on the spathe of 'Best Gold' where discs were sampled.

3.2.4.3 Pigment identification

The pigments were identified by comparison of both their spectroscopic and chromatographic properties, with those of authentic standards, when available, or by matching the observed versus published spectral data and retention time under identical chromatographic conditions. The leaves of parsley and spinach, which are rich in chlorophyll and lutein, were also used as a reference for co-chromatography with the pigments present.

Thin layer chromatography (TLC) was used for primary pigment isolation and identification. The pigment extract (5 µl) was loaded on a 25 cm X 25 cm silica gel 60 F254 thin layer plate (Merck, Germany). Based on pilot trials, a mixture of solvents, i.e., hexane: diethyl ether: acetone (6:3:3; v:v:v) was selected as a moving phase for TLC. The retardation factor (RF) was recorded. The colour bands were then removed from the plate and re-dissolved in acetone, ethanol or chloroform, for the measurement of spectral absorption using a spectrometer (Hitachi U-2000; Hitachi, Japan). These pigment extracts were further purified by high performance liquid chromatography (HPLC). The absorption spectra of individual peaks were recorded through the 'online scan' function of a Dionex Ultimate 3000 VWD-3000 detector.

3.2.4.4 Pigment quantification

The pigments (chlorophyll and carotenoid) were separately quantified by HPLC using a Dionex DX500 chromatography system, including GP40 gradient pump and UltiMate 3000 variable wavelength detector. The column was PerkinElmer

BROWNLEE SPHERI-5 ODS (5 μm, 220 X 4.6 mm). The peaks were integrated at three visible wavelengths, 415 nm, 440 nm and 660 nm.

Elution (1 ml/min) was performed using a solvent system comprising solvent A (Acetonitrile: Methanol: Tris-HCL buffer; 72:8:3; v:v:v) and B (Methanol: Ethyl acetate; 68:32; v:v). All solvents were HPLC grade (Merck, Germany). The solvents were filtered through 0.2 μm nylon, and degassed using ultrasound. Based on pilot trials, a step gradient was developed for the pigment analysis by using four solvent bottles (Table 3.1).

Table 3.1. Step gradient for HPLC analysis. Solvent A (Acetonitrile: Methanol: Tris-HCL buffer; 72:8:3; v:v:v); Solvent B (Methanol: Ethyl acetate; 68:32; v:v).

	Time interval (min)						
	0-10	10-15	15-20	20-30	30-32	32-34	34-44
A:B (v:v)	95:5	65:35	30:70	0:100	30:70	65:35	95:5

An aliquot of the original pigment extract (60 μl) was dried and re-dissolved in ethyl acetate (30 μl) together with the HPLC starting solvent (95% A + 5% B, 270 μl). The extract was filtered through a 0.22 μm nylon syringe filter (Phenomenex, USA) before being injected into the HPLC system.

The external standards used to quantify the concentration of the pigments in samples included: β-carotene (Sigma), lutein and chlorophyll *a*. The standards of lutein and chlorophyll *a* were prepared from leaves of *Petroselinum crispum* Mill. using a methodology adopted from Chen at al. (2004).

3.2.5 **Histology**

To examine changes in plastid ultrastructure during re-greening via light microscopy and transmission electron microscopy (TEM), tissue samples were collected from both the entire spathe and discs at day 0 (horticultural harvest-maturity), day 7, and day 14. There were two individual-flower replicates per sampling date, and four discs per replicate excised from the central position of the spathe. For the purposes of comparison, tissue from an immature spathe at green-bud stage and a mature leaf were also collected for histological observation.

Samples were fixed, postfixed, dehydrated and then embedded in resin following the methodology of Gronegress (1974) with some modification. More than ten sub-samples (about 1 mm^3) were randomly excised from the four discs for each treatment. Using a scalpel, thin cross-sections were taken, discriminating between the abaxial and adaxial surfaces by trimming at an angle. The sub-samples were immediately fixed and vacuum infiltrated with 2% (w/v) formaldehyde and 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (PH 7.2) for 2 hours at room temperature. After three washes in the same buffer, the sub-samples were postfixed with 1% (w/v) OsO_4 in the same buffer for 1 hour at room temperature. The three buffer washes were repeated. The sub-samples were then dehydrated by using an acetone/water series (25%, 50%, 75%, 95%, 100%) and were kept in each concentration for 10 min and another 100% acetone solution for one hour. The sub-samples were first embedded with an acetone:resin Procure 812 (ProSciTech, Australia) mixture (50:50) on a stirrer over night, changed to 100% resin for another 8 hours, and then mounted in 100% fresh resin at 60 ˚C for 48 hours.

Sections 1 μm in thickness were cut from trimmed resin blocks using a glass knife and Ultramicrotome (Leica, Austria). They were heat-mounted onto a glass slide, stained with 0.05% Toluidine Blue, and were initially examined under a compound light microscope (Zeiss, Germany) with a Leica DFC 320 digital camera attached (Leica Microsystems, Wetzlar, Germany). Digital images of the section were taken and areas of interest were chosen for examination using TEM.

Ultra thin sections (100 nm) were cut using a diamond knife and an Ultramicrotome, and were collected on a copper grid. The sections were stained with saturated uranyl acetate in 50% ethanol for 4 min, followed by lead citrate for another 4 min. Examination of the specimens was conducted using a Philips CM10 transmission electron microscope (Philips, The Netherlands). Ultrastructural analyses were performed on plastids located within both abaxial and adaxial surfaces of spathe tissue (approximately within the first five cell layers of subepidermis). A representative plastid among 100 analysed plastids has been presented in the thesis.

3.2.6 **Data analysis**

Data were tested initially to ensure they met the requirement for ANOVA, using the general linear procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Where significant ($P < 0.05$) treatment effects were detected, means were separated by using the unrestricted LSD procedure.

3.3 Results

3.3.1 Disc versus spathe

As illustrated by changes in the value of H°, re-greening of both the detached entire spathe and discs of spathe tissue followed a similar pattern, with no differences detected at any date of measurement ($P > 0.05$; Figure 3.2). The H° remained constant from harvest for the first three days, and subsequently increased as both the spathe and tissue discs progressively turned from yellow to green. The H° reached the value of 90° by day 5, indicating the time when re-greening was visible. In addition, the progressive change in H° was also consistent between discs, detached and intact entire spathes (data not shown).

3.3.2 Pattern of re-greening

The first signs of re-greening of the spathe started from the basal position on the abaxial surface, and progressed upward as well as outward from the midrib. Differences in the initial rates of increase in H° of the abaxial surface from basal, central, upper and outer positions of the spathe confirmed this pattern of re-greening (Figure 3.3 A & B). From day 2 to 5, when an increase in the H° was first recorded, the H° of the basal discs increased at the rate of 3.4 °/day, which was faster than that from the central position at 2.5 °/day, central-outer position at 0.78 °/day and upper position at 0.88 °/day (Figure 3.3 A & B). By day 5, H° of both the basal and central discs was greater than 90° (i.e. visibly green in colour), whereas H° of the discs from the central-outer and upper positions was less than 90° (i.e. visibly yellow in colour). Discs from symmetrical positions on left and right sides of the mid rib, followed the same pattern in increase of the H° with time after harvest ($P < 0.05$; Figure 3.3 A & B).

Within the 14-day period of observation, re-greening was not observed on the adaxial surface of the discs sampled at all positions of the spathe. At all positions on the spathe, H° of the abaxial surface increased by approximately 17° over the 14 days after harvest (Figure 3.3 A & B). In contrast, over the same time period H° of the adaxial surface increased by 4°, 5°, 8°, and 10° at upper, central, central outer, and basal positions, respectively (Figure 3.3 C & D). By the end of the 14-day period, discs from the upper and central positions of the adaxial surface of the spathe visibly remained yellow in colour, while discs from basal and central outer positions were pale yellow. This is in distinct contrast to the apparent green colour of the abaxial surface of all discs at this time.

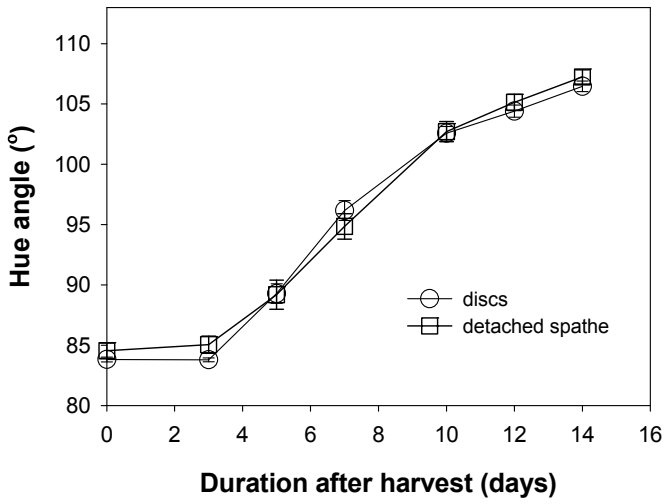

Figure 3.2 Hue angle of detached entire spathe and discs of spathe tissue of *Zantedeschia* 'Best Gold'. Measurements at the same position from the abaxial surface, i.e. central left and right (refer Figure 3.1). Each point is mean ± se of four replicates.

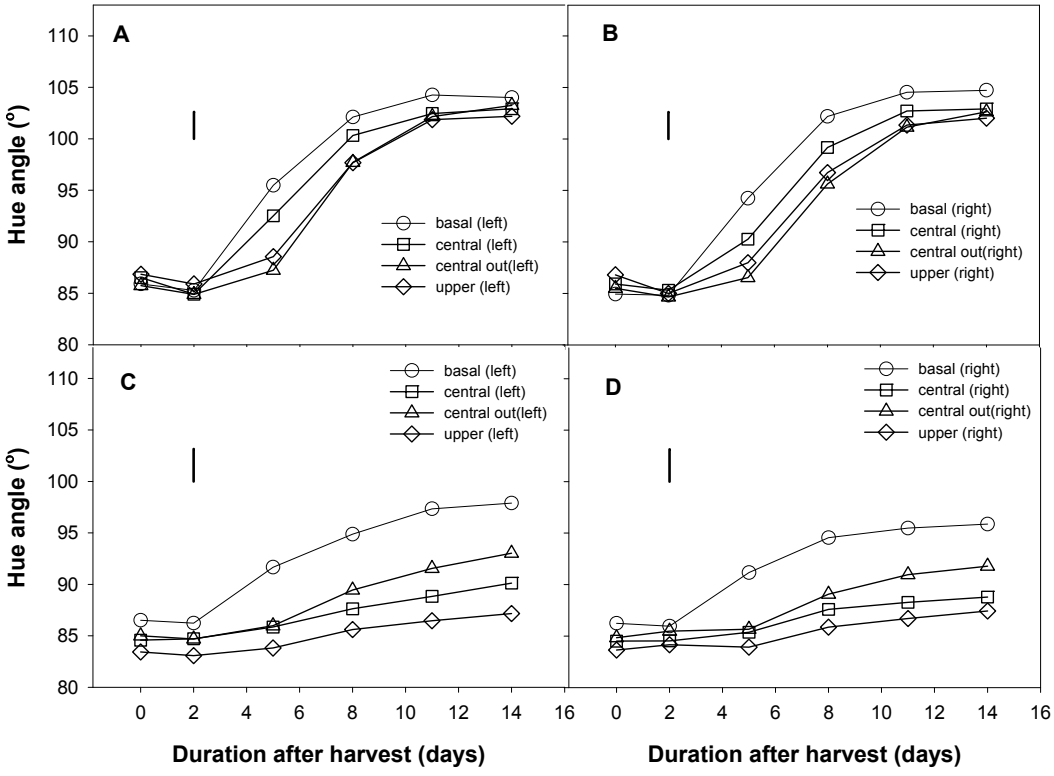

Figure 3.3 Hue angle of both abaxial (A & B) and adaxial (C & D) surfaces of discs of spathe tissue excised from left (A & C) and right (B & D) sides of the midrib at basal, central, central-outer and upper positions of spathe (positions refer Figure 3.1). Vertical bars represent LSD at $P < 0.05$ (n = 4).

3.3.3 **Pigment profile**

The pigments of spathe tissue of 'Best Gold' were initially isolated and identified by TLC. Five obvious yellow coloured bands were separated from both the abaxial and adaxial surface (data not shown) of discs of spathe tissue at three stages, i.e. day 0, 7 and 14, as well as a mature leaf of 'Best Gold' (Figure 3.4). Two pale-yellow bands were present only from the spathe tissue, but not from the leaf (Figure 3.4 question marks). Four green bands were separated from both the leaf and abaxial surface of the spathe at day 14, two from the spathe at day 7, and none at day 0. These green bands were absent from the adaxial surface of the discs (data not shown). In addition, using TLC both the spathe and leaf contained the pigments that matched the standards of β-carotene and lutein, but not zeaxanthin (Figure 3.5). The front band (i.e. RF = 10) from both discs of spathe tissue and the leaf, was thicker than that of the β-carotene standard, indicating the possibility that this band might contain more than one pigment (refer results below). At day 14, the pigments present in the abaxial surface of discs of spathe tissue, and the leaf were similar. The identity of pigments isolated from the detached entire spathe was consistent with those present in the discs of spathe tissue at all measurements (data not shown).

After separation by TLC, the individual bands of pigments were further purified and identified by HPLC. Each band on the TLC corresponded to a single peak in HPLC, except for the front band that was separated into two peaks by HPLC (Figure 3.6). Six carotenoids and two chlorophylls were putatively identified by comparing their retention time, Rf values, and visible absorption spectrum with those of authentic standards, when available, or those from published data (Table 3.2).

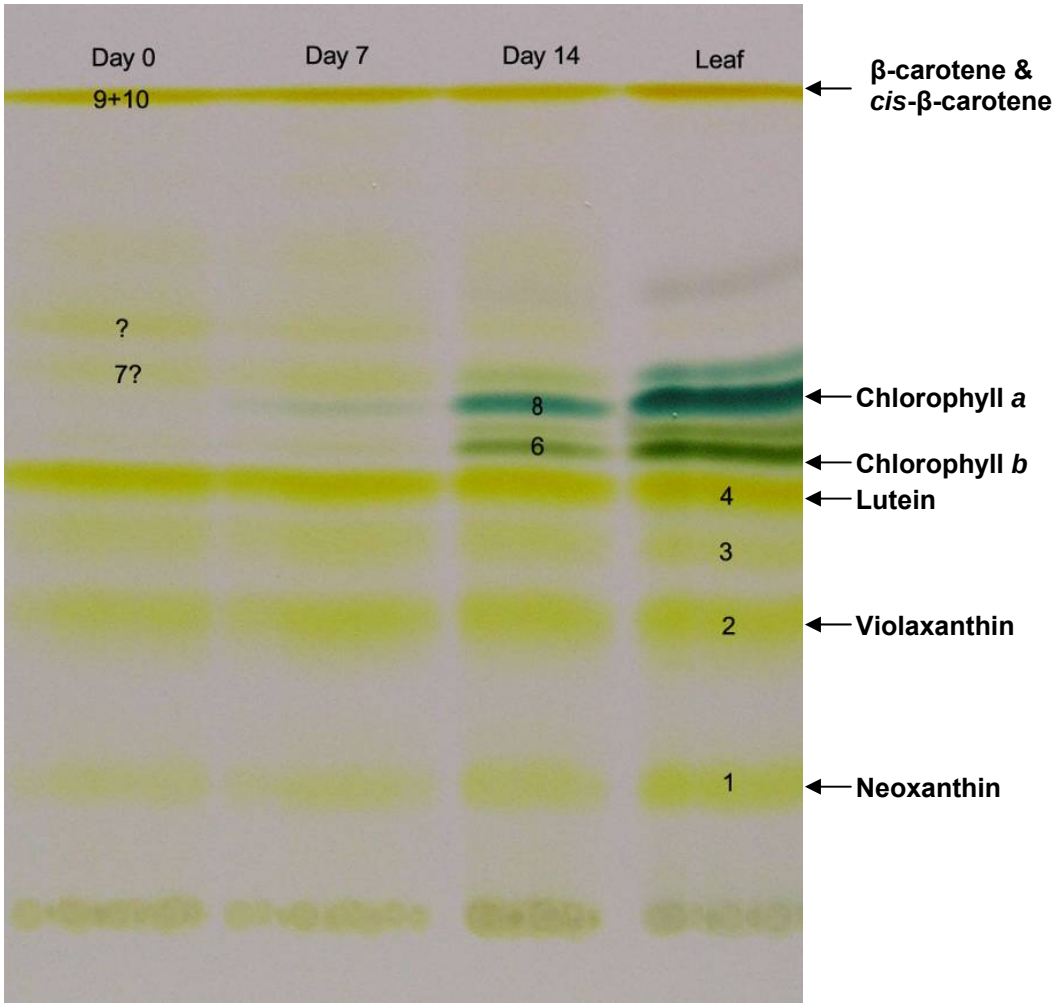

Figure 3.4 Pigments in the abaxial surface of discs of spathe tissue of *Zantedeschia* 'Best Gold' at horticultural harvest-maturity (day 0), day 7 and day 14, as well as the mature leaf of 'Best Gold' were separated by thin layer chromatography. The putatively identified pigments were as listed in Table 3.2.

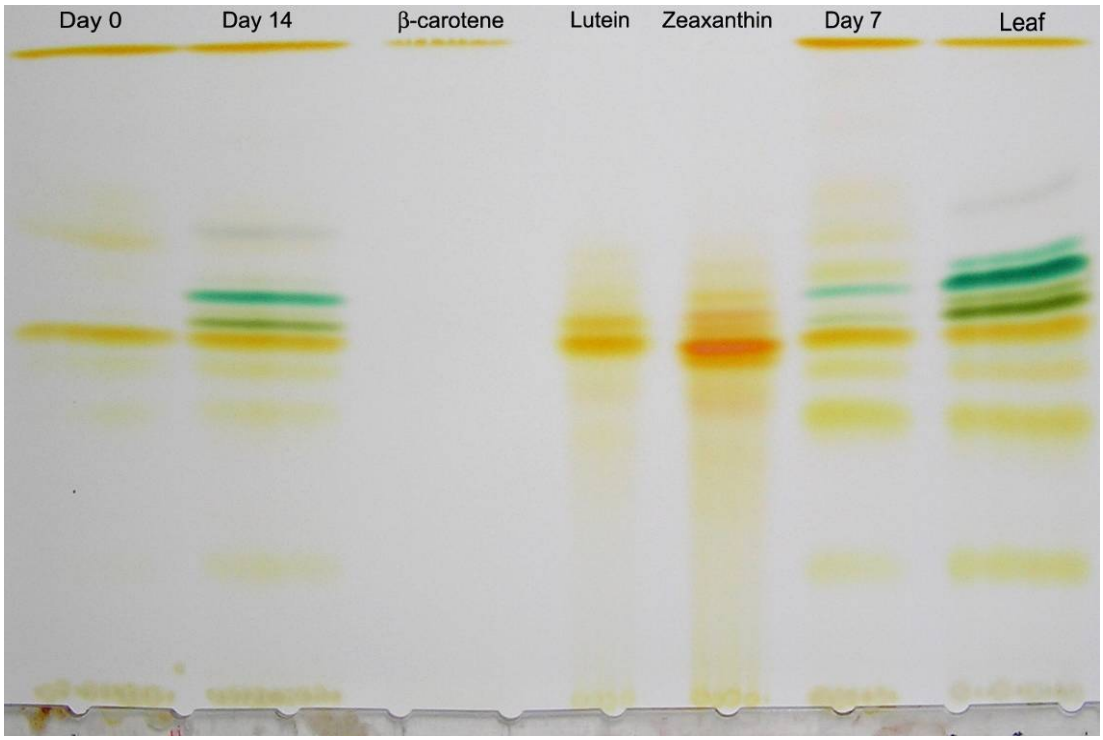

Figure 3.5 Pigments in the abaxial surface of spathe tissue and leaf of *Zantedeschia* 'Best Gold' were co-eluted with standards of β-carotene, lutein and zeaxanthin by thin layer chromatography.

Table 3.2 Elution order and absorption characteristics of pigments from discs of spathe tissue of *Zantedeschia* 'Best Gold' encompassing the 14-day period from horticultural harvest-maturity through re-greening

Peak no.	RT [a] (min)	Pigment	Maxima Ethanol (nm)	Maxima Acetone (nm)	Maxima In-line [b] (nm)	Maxima Ethanol from literature [c]	Maxima Acetone from literature [c]	RF (relative to β-carotene
1	6.05	Neoxanthin	413/438/466	--	414/438/467	415/439/467	416/440/470	2.2
2	7.55	Violaxanthin	416/440/470	--	418/441/470	419/440/470		4.5
3	8.32	unknown		426	412/436/455			5.5
4	13.66	lutein	(418)/442/474	425/445/471	423/445/473	422/445/474		6.0
5	15.59	8-apo-carotenal[c]	--	--	458	--	--	--
6	18.67	Chlorophyll *b*	460/649	453/645	463/650	460/648		6.3
7	19.7	α-Carotene	(421)/445/472	445/475	444/473	423/444/473		7.6
8	20.84	Chlorophyll *a*	432/665 (in chloroform)	411/663	431/663	432/666 (chloroform)	432/663	6.6
9	26.4	β-carotene	--	451/478	450/476	424/450/476	429/452/478	10
10	26.93	*Cis*-β-carotene	--	--	445 /470	420/446/471		10

[a] Retention time of each pigment in HPLC.
[b] The spectra were recorded in the step gradient of solvent A (Acetonitrile: Methanol: Tris-HCL buffer, 72: 8: 3, v/v) and B (Methanol: Ethyl acetate, 68: 32, v/v).
[c] The spectra were as reported by Britton (1995) and Bonora et al. (2000).

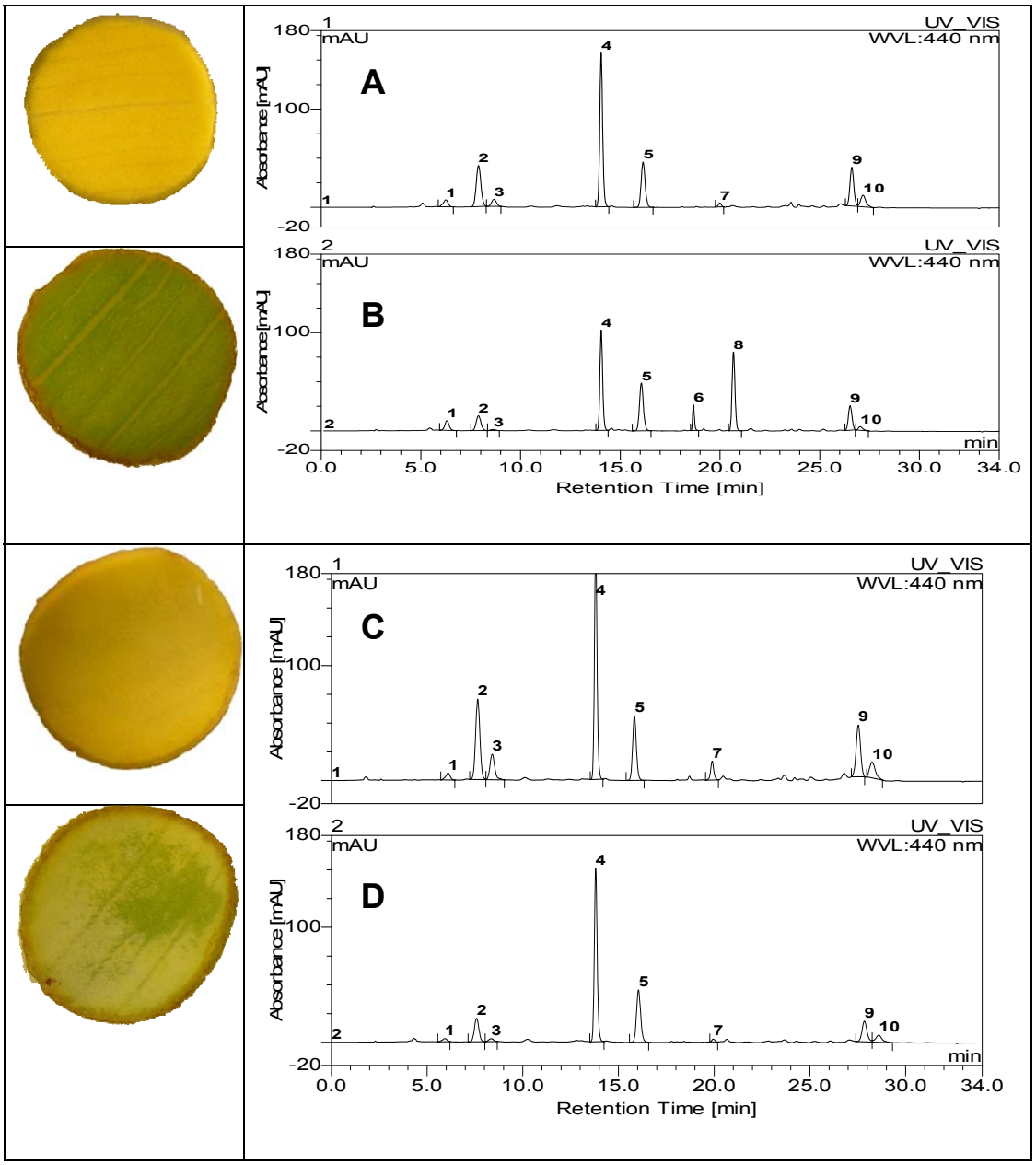

Figure 3.6 HPLC chromatogram traces of pigments (right column) from discs of spathe tissue of *Zantedeschia* 'Best Gold' (left column) on day 0 (A: abaxial; C: adaxial) and day 14 (B: abaxial; D: adaxial). Peaks: 1, neoxanthin; 2, violaxanthin; 3, unknown; 4, lutein; 5, 8-apo-carotenal; 6, chlorophyll *b*; 7, α-carotene; 8, chlorophyll *a*; 9, β-carotene; 10, *cis*-β-carotene.

3.3.4 **Pigment quantity**

Re-greening in the abaxial surface of both discs and detached entire spathes was associated with accumulation of chlorophyll and a slight loss of total carotenoid (Figure 3.7). At horticultural harvest-maturity (day 0), carotenoids were the only pigment group detected in spathe tissue. Lutein was the most abundant carotenoid (47% of the total pigments) followed by violaxanthin, β-carotene and *cis*-β-carotene at 16%, 13% and 7%, respectively. By day 7, in the abaxial surface of discs and entire spathes, there was a rapid increase in the content of both chlorophyll *a* and *b* ($P < 0.05$; Figure 3.7 A & B), which contributed approximate 46% and 13% of total pigments, respectively. From day 7 to day 14, in the discs of spathe tissue, there was minimal increase in the content of both chlorophyll *a* and *b* ($P > 0.05$). This is in contrast with a continuous increase in chlorophyll content found in the entire spathe over this period (Figure 3.7 A & B). As a result, the entire spathe contained higher levels of both chlorophyll *a* and *b* than that of the discs at day 14 ($P < 0.05$).

With regard to carotenoid content, both discs and the entire spathe followed a similar pattern of change with duration from harvest: overall a 13-23% decrease in the content of total carotenoid. With the exception of neoxanthin which increased, all other individual carotenoids showed no significant change (e.g. the most abundant, lutein; Figure 3.7 E) or a decrease in their content over the 14-day period (Figure 3.7 C, D, F, G & H). Particularly for the entire spathe, the content of neoxanthin increased more than 2 fold over this period ($P < 0.05$), following a similar pattern to the change in chlorophyll content (Figure 3.7 A, B & C).

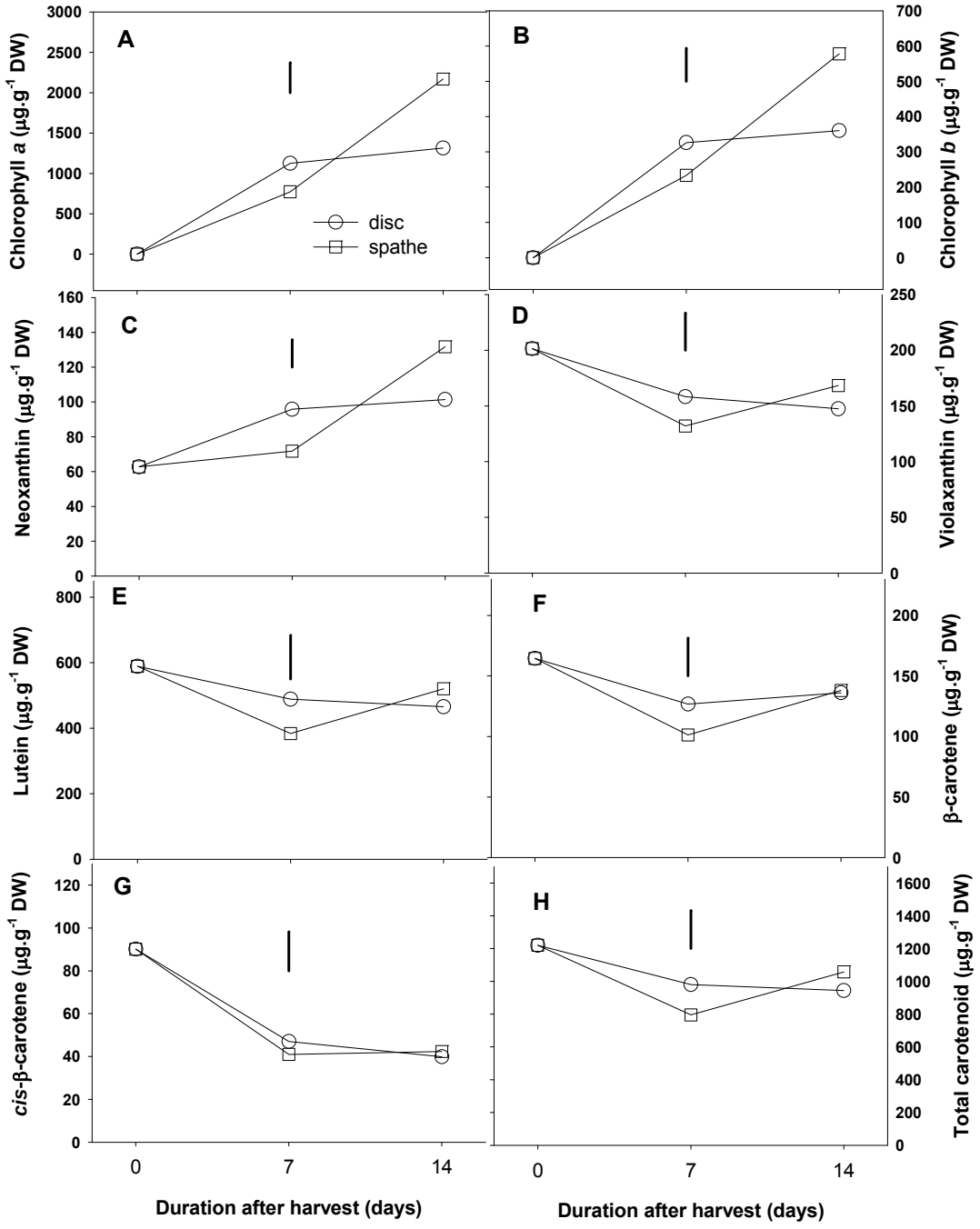

Figure 3.7 Content of pigments present in the abaxial surface of detached entire spathe or discs of spathe tissue of *Zantedeschia* 'Best Gold' during 14 days after horticultural harvest-maturity. Vertical bars represent the LSD at $P < 0.05$, n=4.

For the adaxial surface, over the 14-day period of observation, no chlorophyll was detected and, therefore, there was no substantial change in the relative composition of pigments (data not shown). Throughout this period, lutein remained as the dominant pigment in the adaxial surface with the proportion changing from 40% to 48% of total pigments.

3.3.5 **Plastid redifferentiation**

At the green-bud stage, cells within the subepidermal layers of the abaxial surface contained chloroplasts with well-organized granal regions, a few starch grains and small spherical plastoglobuli (Figure 3.8 A).

At horticultural harvest-maturity (day 0), when the spathe became fully yellow, the number and size of the plastoglobuli was significantly greater in the plastids; a few swollen thylakoid fragments remained; and the chloroplasts developed into chromoplasts (Figure 3.8 B).

As spathe re-greening proceeded in the abaxial surface of the detached entire spathe, by day 7 thylakoid membranes increased and orientated along one axis; the plastoglobuli reduced in number and size and remained in close proximity to the thylakoid membrane throughout the plastid differentiation; starch grains also gradually disappeared (data not shown).

When the entire spathe had fully re-greened by day 14, typical chloroplasts with well stacked grana, interlined by stroma thylakoids, reappeared in the subepidermal layer (Figure 3.8 C). These chloroplasts showed no discernible

difference in structure to those found in a mature leaf of ‘Best Gold’ (data not shown). At this stage of development, an intermediate stage of plastid between chromoplasts and chloroplasts was occasionally observed in the spathe tissue (Figure 3.8 C arrow). This plastid was characterised by large-sized plastoglobuli and newly formed thylakoids along the inner-envelope membrane. Throughout the re-greening, a double-membrane envelope of plastids remained intact and no sign of degradation of this membrane was observed.

The adaxial surface of spathe tissue showed less obvious signs of plastid differentiation during the three stages of spathe development in contrast with the abaxial surface. At the green-bud stage, plastids showed the characteristics of amyloplasts (Figure 3.8 D block arrow), mainly occupied by large starch grains and a few small plastoglobuli. As the spathe turned yellow at horticultural harvest-maturity (day 0), the amyloplasts had developed into chromoplasts, though their shape was different from those evident within the abaxial surface (Figure 3.8 E). By day 14, the plastids in the adaxial surface of the detached entire spathe remained as chromoplasts (Figure 3.8 F), in contrast to those in the abaxial surface that developed into chloroplasts (Figure 3.8 C). There were no well-organized grana apparent at either the green-bud or re-greened stages of spathe in the adaxial surface, but some thylakoid membranes were found.

The plastid differentiation during re-greening of the discs of spathe tissue was similar to that occurred in the detached entire spathe, except that at day 14, the disc tissue contained a less number of well-stacked grana and less electron-dense stroma, than that evident within the entire spathe (data not shown).

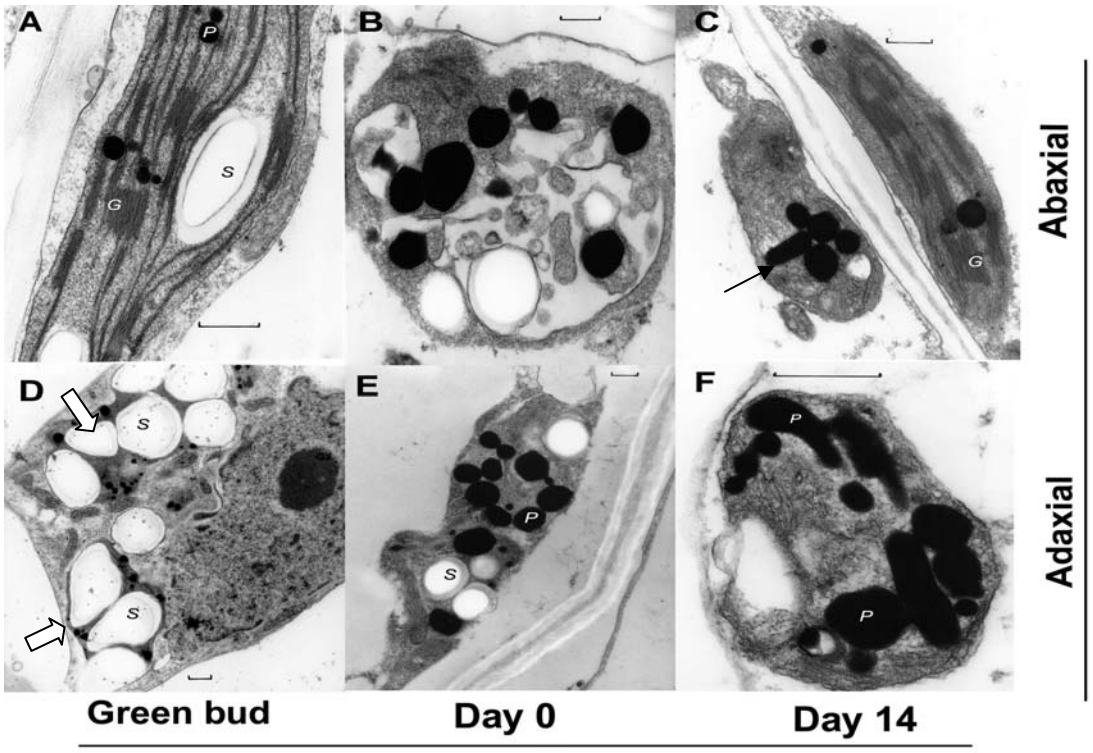

Figure 3.8 Electron micrographs of plastids in subepidermal cell layers of both abaxial and adaxial surfaces at different stages of spathe development in *Zantedeschia* 'Best Gold'. A) Chloroplast present in the abaxial surface of spathe at green-bud stage, showing well-stacked grana and a few plastoglobuli; B) Chromoplast appears in the abaxial surface of the yellow spathe at horticultural harvest-maturity; C) Chloroplast (right) and an intermediate stage of plastid (left) between chloroplast and chromoplast were observed in abaxial surface of the detached entire spathe at day 14; D) Amyloplast present in the adaxial surface of the spathe at green-bud stage; E) Chromoplast found in the adaxial surface of the yellow spathe at horticultural harvest-maturity; F) Plastid remain in chromoplast-form in the adaxial surface of detached entire spathe at day 14. Bar = 1 µm. *G*, grana; *P*, plastoglobuli; *S*, starch grain.

3.4 Discussion

As described by the value of H°, re-greening of discs of spathe tissue closely followed that of the detached entire spathes. These discs were readily able to be maintained in an active state over the 14-day period of observation, confirming discs as a potential model for future investigations into re-greening. The data for pigment content were also consistent between the two tissue sources, except that the entire spathe contained a higher level of chlorophyll than the discs toward the later stage of re-greening (Figure 3.7). It is probable that in comparison with the entire spathe, the discs were exposed to additional stress and the tissue was isolated from a potential supply of substrate/energy from nearby tissue, thereby limiting the extent to which the discs can accumulate chlorophyll. This is in part supported by the ultrastructural observation that at day 14 the chloroplasts present in the disc tissue contained a lower number of well-stacked grana and had less electron-dense stroma, compared to chloroplasts present in the entire spathe. The inconsistency in variation of pigment content and plastid ultrastructure toward the later stage of re-greening between the discs and entire spathe however, is not perceived as reducing the validity of using discs as a model system for future research on re-greening. This is because the initial stage of re-greening, wherein discs and entire spathe showed no discernible difference in the progress of re-greening, is the most relevant timeframe to the research interest of this thesis. Hence, using discs of spathe tissue is appropriate for further investigations into re-greening.

Due to the progressive onset of development of re-greening from the base towards the upper areas of the spathe, discs sampled from different vertical positions

of the spathe varied in their rate of re-greening (Figure 3.3). Additionally, the rates of re-greening were also different between horizontal positions relative to the midrib, but were consistent from symmetrical positions on left and right sides of the mid rib. The combination of both positional effects limits the number of discs with uniform behaviour that can be sampled per spathe. It is therefore recommended that while discs are appropriate to be used for re-greening research, a position-effect needs to be taken into consideration.

Re-greening in spathe tissue of *Z. elliotiana* or *Z. aethiopica* was concomitant with an increase in total chlorophyll, as chloroplasts reformed (Gronegress, 1974; Pais and Neves, 1982-1983). In the current study with 'Best Gold', this finding was confirmed but, in addition, showed that the content of both chlorophyll *a* and chlorophyll *b* increased, while total carotenoid decreased slightly, as re-greening proceeded. At horticultural harvest-maturity, carotenoids were the only pigment group present and the individual carotenoids in the chromoplasts of the spathe were typical of those found in the chloroplasts of mature leaf tissue: mainly lutein, with smaller amounts of other xanthophylls and β-carotene. This is in agreement with that reported previously (Lewis et al., 2003). As re-greening progressed, these carotenoids persisted and, in particular, the predominant lutein remained at a comparatively steady level (Figure 3.7; Figure 3.8). It is not surprising that these carotenoids were retained, as they are essential components for reconstruction of the photosynthetic apparatus in chloroplasts (Siefermann-Harms, 1987; Sundqvist and Dahlin, 1997; Young, 1993a). It is, however, unclear whether:

- carotenoids undertake initial degradation as the chromoplast structures are dismantled (e.g. plastoglobuli) and re-synthesized as the thylakoid system is reconstructed, or,
- a direct relocation of carotenoids from plastoglobuli to the newly formed thylakoids occurs.

In the current study, the thylakoids were found to be closely coupled to plastoglobuli throughout the plastid differentiation from chromoplasts to chloroplasts, and appearance of thylakoids occurred simultaneously with disappearance of plastoglobuli (Figure 3.8). These changes suggest a possibility of relocation of the existing carotenoids to newly formed thylakoids. This hypothesis is further supported by a recent study conducted by Austin et al. (2006) using the techniques of freeze-fracture electron microscopy and electron tomography, wherein they revealed that plastoglobuli remain in a physical continuum with the thylakoid membrane via half-lipid bilayers. Carotenoids and other lipid molecules stored in the plastoglobuli are reported to be in a dynamic equilibrium with those in the thylakoid membranes. Further research using similar microscopy techniques is required to test the application of this hypothesis of relocation of some carotenoid from plastoglobuli to thylakoids during re-greening of spathe tissue in ‘Best Gold’.

A continuous biosynthesis of some carotenoids (e.g. neoxanthin) may also occur during re-greening of spathe tissue in ‘Best Gold’. During re-greening, the content of neoxanthin increased following a similar pattern to that of chlorophyll accumulation, although neoxanthin only contributed a relatively small proportion to total pigments (4% - 5%; Figure 3.7). Neoxanthin is the end product of carotenoid metabolism through the β-carotene-branch. The fact that neoxanthin increased in

content while lutein (end product of α-carotene-branch) remained comparatively stable, implies that the progress of re-greening facilitates the flux of carotenoid biosynthesis through the β-carotene-branch. On the other hand, as chlorophyll and neoxanthin are primary components of light-harvesting antennae in chloroplasts (Peter and Thornber, 1991; Young, 1993b), a continuous increase in content of chlorophyll and neoxanthin during re-greening suggests an active constitution of light-harvesting antennae in photosystems, which eventually leads to formation of a functional chloroplast.

During development of the spathe of 'Best Gold' from green-bud to fully re-greened, plastids in the subepidermal layers of the abaxial surface underwent sequential redifferentiation from chloroplast to chromoplast to chloroplast (Figure 3.8 A, B & C). This is similar to results reported for *Z. aethiopica* (Melo et al., 1995) and *Z. elliotiana* (Gronegress, 1974). In the abaxial surface, intermediate stages of plastids between chromoplast and chloroplast, as well as complete chloroplasts, were observed in the fully re-greened spathe (Figure 3.8 C). This intermediate stage of plastid contained not only a new developing thylakoid system, but also a large number of different shaped plastoglobuli remaining from the chromoplast. In addition, no proplastids were observed in the re-greening tissue and the double-envelope membrane of plastid remained intact throughout the chromoplast-chloroplast transition. These ultrastructural changes suggest that the chloroplasts were directly differentiated from the chromoplasts, not formed *de novo* from proplastids. Similar observations have been previously reported in the re-greening of peel of *C. sinensis* (Mayfield and Huff, 1986), fruits of *Cucurbita pepo* L. (Devide and Ljubesic, 1974), and the spathe of *Z. elliotiana* (Gronegress, 1974).

Despite the fact that the abaxial and adaxial surfaces of spathe tissue contained similar chromoplasts and pigment levels at horticultural harvest-maturity, there was a distinct difference in both the previous and subsequent differentiation of plastids between these two surfaces, as the spathe developed from green-bud to the fully re-greened stage (Figure 3.8). The occurrence of such unique and variable processes of plastid differentiation in the subepidermal layers of the abaxial and adaxial surfaces, which are only about 10 cells apart, has not been reported previously. Re-greening naturally occurs on the adaxial surface of the spathe, but at a much slower rate compared with the abaxial surface. Although the precise mechanism that determines these spatial and temporal variations in the induction and progression of re-greening between these two surfaces of spathe tissue is unknown, it is possible that:

1. the availability of re-greening associated signals (RAS), e.g. the level of endogenous plant hormones, differs between the abaxial and adaxial surfaces;
2. abaxial and adaxial surfaces vary in their sensitivity, e.g., number of receptors (Firn, 1986) to RAS;
3. there may be some transcription factors that specifically control this spatial and temporal variation of re-greening, as is the case in pigmentation of petals in *Antirrhinum majus* L. (Shang et al., 2010).

In Chapters 5 and 6 of this thesis, these spatial and temporal variations in re-greening of the abaxial and adaxial surfaces will be further examined by evaluating their response to exogenous plant hormones.

3.5 Conclusion

Re-greening in spathe tissue of 'Best Gold' was characterized by the regaining of green colouration on the yellow spathe. This change in colour initiated from the basal area of the abaxial surface, and progressed upward as well as outward from the midrib. The regaining of green colouration was associated with accumulation of both chlorophyll *a* and *b*, and retention of carotenoids, in particular lutein, the most prevalent carotenoid in spathe tissue. Concomitant with changes in pigments, redifferentiation of chloroplasts from chromoplasts, but no *de novo* synthesis of chloroplasts from proplastids, took place in the subepidermis of the abaxial surface. Further studies on the redifferentiation of chloroplast in terms of thylakoid reconstruction are still required. Chapter 6 will address this issue by evaluating the response of spathe tissue to various treatments (e.g. light and plant hormones) at more frequent sampling times.

Discs of spathe tissue and the detached entire spathe followed a similar pattern of re-greening indicating that discs are appropriate to be used for re-greening research. Due to the progressive onset of development of re-greening from basal to upper and from inner to outer (relative to midrib), discs sampled from various positions of the spathe varied in their rate of re-greening. Therefore, positional effects need to be taken into account when using discs as a model system to study re-greening.

Chapter 4 **Influence of fructification and cytokinin on spathe re-greening: comparison of winter- and summer-flowering species of *Zantedeschia***

4.1 Introduction

Re-greening in floral organs after anthesis occurs in many plant species (Casadoro et al., 1982; Salopek-Sondi et al., 2002; Tran et al., 1995; Weidner et al., 1985), including all seven species of *Zantedeschia* (Singh et al., 1996a). In winter-flowering species, e.g. *Z. aethiopica*, only the proximal area (basal) of the abaxial surface of the spathe re-greens while the distal area (upper) eventually withers to expose the developing fruit. In summer-flowering species (e.g. *Z. elliotiana* and *Z. rehmannii*), the entire abaxial surface of the spathe re-greens, wrapping the fruit completely inside the re-greened spathe. 'Best Gold', the selection used in the current research, is a hybrid of summer-flowering species. The whole spathe of 'Best Gold' re-greens and in-rolls within two weeks after the spathe is fully open and coloured (Funnell and Downs, 1987).

Once re-greened, floral organs typically achieve photosynthetic capacity (Salopek-Sondi et al., 2000; Tavares et al., 1998; Weidner et al., 1985), and have been demonstrated to serve as an additional source of assimilates for developing seeds and fruits (Herrera, 2005). Considering this need and timing of re-greening (i.e. after pollination), it is plausible that re-greening is associated with the occurrence of

pollination and/or fructification. Spathe re-greening in *Z. aethiopica* has been proposed as being linked to the occurrence of fructification (Lino-Neto et al., 1999; Pais and Neves, 1982-1983). Pais and Neves reported that in the absence of fructification (e.g. removal of the spadix), spathes of *Z. aethiopica* underwent senescence soon after the removal of the spadix. In contrast, if the spadix was substituted by the application of synthetic cytokinins, that were equivalent to those naturally occurring and isolated from the fruits *Z. aethiopica*, the spathe re-greened. The authors then hypothesised that spathe re-greening in *Z. aethiopica* was induced by these naturally occurring cytokinins, following their translocation from the fruits to the spathe. This hypothesis is based on research utilising *Z. aethiopica*, which belongs to the winter-flowering group. However, little information is available on the mechanism of re-greening for the summer-flowering group of *Zantedeschia*, e.g., 'Best Gold'. Whether re-greening in the spathe tissue of 'Best Gold' is related to fructification and/or cytokinins is unknown.

The true female and male flowers mature at different times in *Z. aethiopica*, while they mature at similar times in the summer-flowering species (New, 1964). The female flowers of *Z. aethiopica* are receptive at the bud stage when the spathe begins to unfold, and the female flowers progressively lose their receptivity when the spathe is fully open and all male flowers become mature and dehisce (New, 1964; Singh et al., 1996a). Hence, *Z. aethiopica* is not self fertile. In contrast, for the summer-flowering group of *Zantedeschia*, their female and male flowers mature at similar times (New, 1964). As a result, species within the summer-flowering group have the potential of being self fertile. Unpublished observations of 'Best Gold' similarly show synchronised flowering of male and female flowers and, therefore, the capability of

self-pollination (pers. comm. Dr. Keith Funnell). Hence for *Z. aethiopica*, if spadix removal was to be used to prevent fructification or pollination, or any potential signalling induced by fructification or pollination, the spadix would need to be removed when the spathe begins to unfurl or earlier. In contrast, for 'Best Gold', the spadix would need to be removed one to two days before pollen shed. In light of this information, the objectives of the current study were:

1. to determine whether fructification is associated with spathe re-greening in representative selections of both winter-flowering species of *Zantedeschia*, e.g. *Z. aethiopica*, and summer-flowering species, e.g. 'Best Gold' and,
2. to examine the influence of exogenous cytokinin on spathe re-greening in selections of both species.

4.2 Materials and Methods

4.2.1 Fructification

4.2.1.1 Presence / absence of spadix

Stems (spathe plus spadix and peduncle) of *Z. aethiopica* were obtained from plants grown in the display gardens of Massey University, Palmerston North. Stems were cut from plants either at:

- the unfurling bud-stage, when white colouration was appearing on the edge of the previously green-coloured buds, with the bud starting to unfurl, and with the male and female flowers still tightly wrapped by the spathe; or

- horticultural harvest-maturity, i.e., one to two days before pollen shed; spathe fully open and de-greened.

Stems of 'Best Gold' at horticultural harvest-maturity (i.e. 1-2 days before pollen shed, spadix turned corn-yellow, spathe fully coloured (Funnell and Downs, 1987)), were attained from plants grown in a heated glasshouse (growing conditions as described previously, refer Chapter 2 Section 2.2.1).

The spadix was either left intact or removed immediately after the stem was cut from the plant. For *Z. aethiopica*, due to the difficulty of removing the spadix when the bud is tightly wrapped, the spadix was removed at the unfurling bud-stage.

The four individual stem replicates, for each treatment, were subsequently monitored for changes in colour of the spathe, while held in a vase-life room with reverse osmosis (RO) water supplied in glass jars. The vase-life room was controlled to environmental conditions recommended by Reid and Kofranek (1980): room temperature 20 ± 1 ºC, relative humidity 60 to 70%, 12-h (0600 HR – 1800 HR) photoperiod and light intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height provided by cool-white fluorescent tubes.

Measurement of colour changes of the abaxial surface of the spathe at basal, central and upper positions, were conducted as described in Chapter 2, which comprised recording the colour coordinates of lightness (L*), chroma (C*), and hue angle (H°), using a tristimulus colorimeter CM-2600d/2500d (Konica Minolta, Japan). The measurement of colour was repeated every 2 to 4 days for a period of two weeks. A change in H° can be used to describe the changes in chlorophyll content in the

abaxial surface of 'Best Gold', particularly during the initial stage of spathe re-greening (Chapter 2). While a similar correlation has not previously been established for *Z. aethiopica*, preliminary tests validated that H° could be used to describe re-greening of the spathe. A value of H° greater than 90° has previously been found to be indicative of a level of chlorophyll visible to the human eye in the peel of *Citrus unshiu* (Mak.) Marc. (Iglesias et al., 2001), and this criterion was also applied for the abaxial surface of the spathe in 'Best Gold'. Hence, in this experiment, for both 'Best Gold' and *Z. aethiopica*, the primary data of interest were H°.

4.2.1.2 Presence / absence of female flowers

In some instances the spadix of 'Best Gold' is naturally devoid of the true female flowers, with only male flowers being present. This divergence in morphology was not observed in *Z. aethiopica* grown in the gardens of Massey University and, therefore, in the current experiment only 'Best Gold' was studied for the influence of the presence or absence of female flowers on re-greening. Four stems of 'Best Gold', either containing female flowers or no female flowers, were attained at horticultural harvest-maturity, and were monitored for changes in colour in a vase-life room for two weeks (refer Section 4.2.1.1).

Some leaves of 'Best Gold' naturally contain regions of pigmentation, of the same yellow-gold colour as occurs in the spathe. This divergence in leaf morphology was not observed in *Z. aethiopica* grown in the gardens of Massey University and, therefore, in the current experiment only yellow-pigmented leaves of 'Best Gold' were studied for changes in colour over two weeks after the leaves had unfurled.

4.2.2 **Application of cytokinin**

As described in Chapter 3, discs excised from the spathe of 'Best Gold' followed a similar pattern of re-greening as that occurring in the detached entire spathe. Hence in the current study, discs of spathe tissue were used for evaluating the effect of cytokinin in re-greening for both *Z. aethiopica* and 'Best Gold' sampled at horticultural harvest-maturity. Using a cork borer (diameter 14 mm), discs of spathe tissue were sampled from either side of the midrib at basal, central and upper positions of spathes for both *Z. aethiopica* and 'Best Gold'. The discs were placed on filter paper soaked in treatment solutions with the adaxial surface facing down in closed petri dishes.

Pais and Neves (1982-1983) reported that application of the cytokinin-like compound 6-(o-hydroxybenzylamino)-purine at 10^{-4} M stimulated re-greening in the spathe of *Z. aethiopica* with the spadix removed. 6-benzylaminopurine (BAP) is a synthetic cytokinin that is readily available, close in structure to the compound used in the study conducted by Pais and Neves (1982-1983), and physiologically active in *Zantedeschia* (Subbaraj et al., 2010). Hence, in the current study, either BAP at 10^{-4} M or RO water (the control) was used as treatment solutions.

From the time of harvest the colour of the abaxial surface of discs was monitored, throughout a 14- to 17-day period of observation. Colour measurement of individual discs was discontinued early if tissue senesced, illustrated by visible browning. There were four individual stem replicates per treatment and two discs per position per spathe.

4.2.3 **Pigment analysis**

Discs from the central position of spathe of *Z. aethiopica* and 'Best Gold', sampled either at horticultural harvest-maturity (day 0) or when re-greened (day 14), were analysed for pigment content. There were three stem replicates and four discs per replicate. Pigment extraction and quantification was conducted as described in Chapter 3, which comprised the freeze-dried tissue first being extracted in acetone: methanol (7:3; v:v). The supernatant was combined and dried under a stream of O_2-free N_2. The extract was then resuspended in ethyl acetate and kept at -20 $^{\circ}$C pending pigment identification and quantification by high performance liquid chromatography.

4.2.4 **Statistical analysis**

Data were tested initially to ensure they met the requirement for ANOVA using the general linear procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Where significant ($P < 0.05$) treatment effects were detected, means were separated by using the unrestricted LSD procedure.

4.3 **Results**

4.3.1 **Presence / absence of spadix**

Regardless of whether or not the spadix was removed at horticultural harvest-maturity, re-greening was observed in the spathes of both *Z. aethiopica* and 'Best Gold'. The re-greening of *Z. aethiopica* was most evident at the basal and central areas of the spathe, with the upper area withering within the 14 days of observation **(**Figure 4.1). In contrast, with 'Best Gold', the re-greening spread to the entire abaxial

surface of the spathe. Additionally, as re-greening progressed in the spathes of either *Z. aethiopica* or 'Best Gold', with the spadix present no fructification or enlargement of ovules was observed. The failure to observe fructification continued for eight weeks after commencement of the experiment, a timeframe previously regarded as adequate in *Zantedeschia* if fructification was to occur (Yao, 1992).

For stems of both *Z. aethiopica* and 'Best Gold' harvested at horticultural maturity, across the entire 14-day period, there was no difference between the spadix being removed or attached, in the values of L*, C* or H° measured at either the basal position or central position of spathes ($P > 0.05$; Figure 4.2). At horticultural harvest-maturity, the basal position of the spathe of *Z. aethiopica* was pale green in colour (L*: 76 %; C*: 44 %; H°: 99°), and by 14 days it had progressed to an intense-green colour (L*: 52 %; C*: 40 %; H°: 110°). With 'Best Gold', across the 14-day period of observation, the central position of spathe progressively changed in colour from golden-yellow (L*: 76 %; C*: 77 %; H°: 84°) to green (L*: 56 %; C*: 50 %; H°: 104°).

Re-greening was also observed on the spathes of *Z. aethiopica* where the spadix had been removed at the unfurling bud-stage. The spathe initially de-greened as it unfurled during the first five days after being harvested. At the basal position of the spathe this developmental period was associated with: an increase in values of L* from 55% to 88%, a decrease in C* from 50% to 8%, and a decrease in H° from 106° to 100°, and corresponded to a colour change from green to pale-green. Re-greening of the spathe commenced once the spathe had first de-greened to maximum, corresponding to a decrease in L* from 88% to 48%, an increase in C* 8% to 40%, and H° from 100° to 115°.

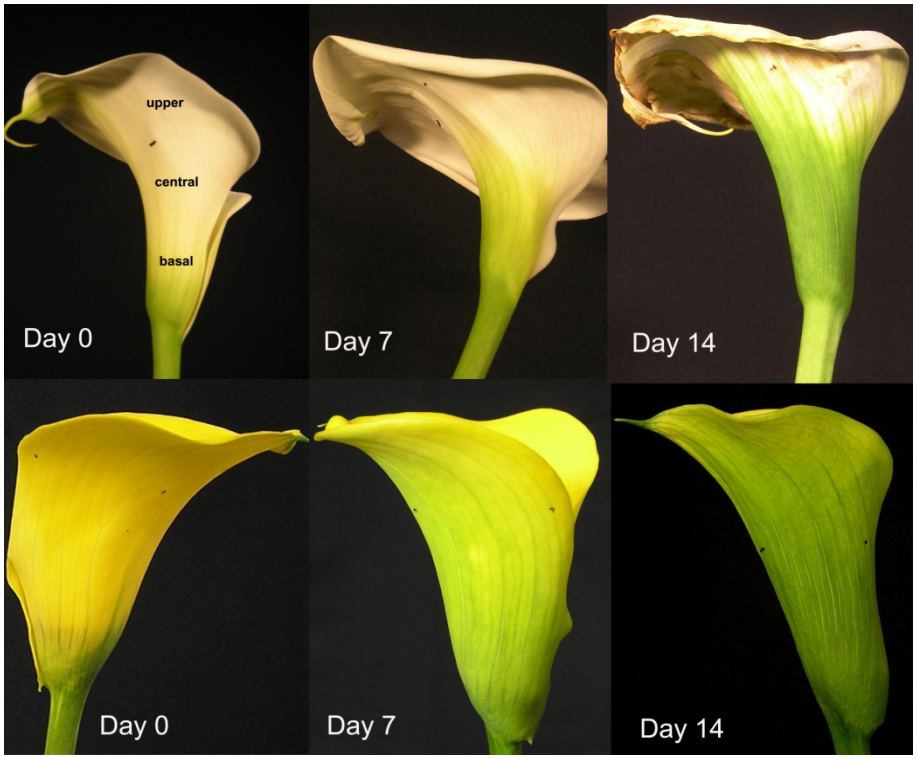

Figure 4.1 Re-greening of the spathe of *Zantedeschia aethiopica* (top) and *Zantedeschia* 'Best Gold' (bottom). Flowers were harvested at horticultural harvest-maturity (day 0), i.e., spathe fully open and prior to pollen release.

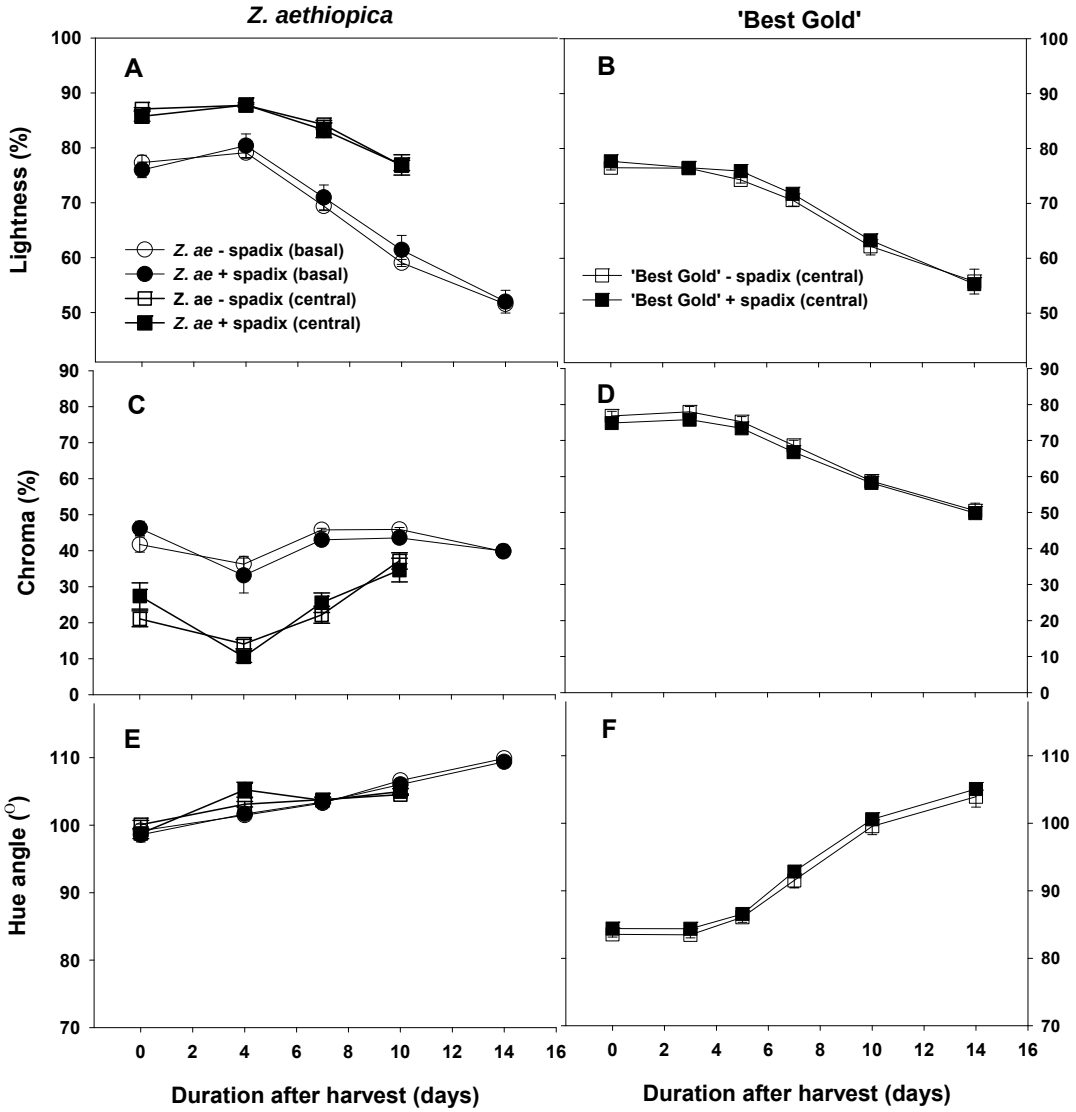

Figure 4.2 Colour coordinates of lightness, chroma, and hue angle measured at the basal and central positions of the spathes of *Zantedeschia aethiopica* (left) or at the central position of *Zantedeschia* 'Best Gold' (right) with either the spadix removed (open symbols) or attached (solid symbols). Flowers were harvested at horticultural harvest-maturity. n=4, vertical bars = Standard error.

4.3.2 Presence / absence of female flowers (‘Best Gold’ only)

The spathe of ‘Best Gold’ re-greened regardless of the presence or absence of female flowers. The spathes began to turn green during the period when pollen was released. At any time of measurement during the period of observation, there was no difference between the spathes with or without female flowers, in the values of L*, C* or H° ($P > 0.05$). Within the 14-day period of observation, the values of L* and C* of all spathes decreased from 78% to 48%, and 76% to 41%, respectively, while H° increased from 84° to 108°.

4.3.3 Re-greening of yellow-pigmented leaves (‘Best Gold’ only)

Within the 14-day period of observation, the abaxial surface of leaves showing yellow pigmentation gradually re-greened, while the adaxial surface did not (Figure 4.3).

4.3.4 Pigment profile

For *Z. aethiopica*, at day 0, lutein was the only pigment detected in the spathe tissue (Table 4.1). As re-greening progressed, concomitant with the accumulation of chlorophyll, other typical chloroplast-carotenoids (e.g., neoxanthin, violaxanthin and β-carotene) appeared. At day 14, chlorophyll *a* and *b* were the predominant pigments, contributing approximately 70% of total pigments. The content of lutein increased 3-fold within the 14-day period.

Figure 4.3 Changes in pigmentation in adaxial and abaxial surfaces of yellow-pigmented leaves of *Zantedeschia* 'Best Gold'.

Table 4.1 Pigment content (µg·g^{-1} dry weight) of spathe tissue in *Zantedeschia aethiopica* or *Zantedeschia* 'Best Gold' at horticultural harvest-maturity (day 0) and when re-greened (day 14). Each value is the mean of three measurements, SE = standard error.

	Z. aethiopica				'Best Gold'			
Pigment	**Day 0**		**Day 14**		**Day 0**		**Day 14**	
		SE		SE		SE		SE
Neoxanthin	0.00		131.78	10.00	83.96	6.46	154.85	11.17
Violaxanthin	0.00		238.34	19.15	235.81	33.73	188.98	14.74
Lutein	100.22	7.14	392.82	34.06	592.40	30.53	589.91	47.57
Chlorophyll b	0.00		483.76	47.22	0.00		604.50	82.56
Chlorophyll a	0.00		1633.26	162.99	0.00		2492.27	376.79
β-carotene	0.00		110.94	4.09	225.60	14.19	214.02	19.39
***cis*- β-carotene**	0.00		44.54	3.06	100.54	8.32	50.96	3.79
Total carotenoid	0.00		992.19	63.60	1336.48	72.81	1258.12	92.55
Total pigment	0.00		3109.21	272.64	1336.48	72.81	4354.89	550.63

For 'Best Gold', unlike *Z. aethiopica*, carotenoids including lutein, violaxanthin and β-carotene were abundant at day 0 (Table 4.1). Their content, particularly for lutein, remained relatively constant over the 14-day period of observation, while both chlorophyll *a* and *b* increased significantly.

4.3.5 **Application of cytokinin**

For *Z. aethiopica*, without BAP treatment (i.e. control), re-greening was observed only on the discs excised from the basal position of the spathe, but by day 10 even these re-greened discs had senesced (i.e. turned brown; Figure 4.4; Figure 4.5 A, C & E). The discs from the central position of the spathe did not re-green and senesced after five days (Figure 4.4; Figure 4.5 A, C & E), while those from the upper position had senesced within three days (data not shown).

With BAP treatment, the discs from all three positions on the spathe of *Z. aethiopica* progressively re-greened over the 17-day period of observation. The BAP-treated discs from the basal position were initially pale green in colour (L*: 77 %; C*: 43 %; H°: 97°) and progressed to become intense-green (L*: 55 %; C*: 44 %; H°: 107°) at 12 days after horticultural harvest-maturity (Figure 4.4; Figure 4.5 A, C & E). With the treatment of BAP, the discs from central and upper positions of the spathe re-greened more slowly and, by day 12, green colouration was visible on these discs (Figure 4.4). This is in distinct contrast with control discs that had not been treated with BAP, wherein they had senesced by day 12 (Figure 4.4).

For 'Best Gold', within the 14-day period of observation, discs sampled from basal, central and upper positions of the spathe progressively re-greened, regardless of

treatment. In the control treatment, the H° of discs from the basal position reached the critical value of 90° (i.e. re-greening deemed visible) by day 2, which was approximately 1 day earlier than that achieved by discs from the central position (Figure 4.5 F) and 2 days earlier than for the upper position (data not shown). Compared with the control treatment, at each position of sampling on the spathe, the treatment with BAP delayed the time when H° reached 90° by approximately 1 day. As the period of observation progressed to day 10, the H° of the BAP-treated discs from the basal position showed no difference to that of the control ($P > 0.05$; Figure 4.5 F).

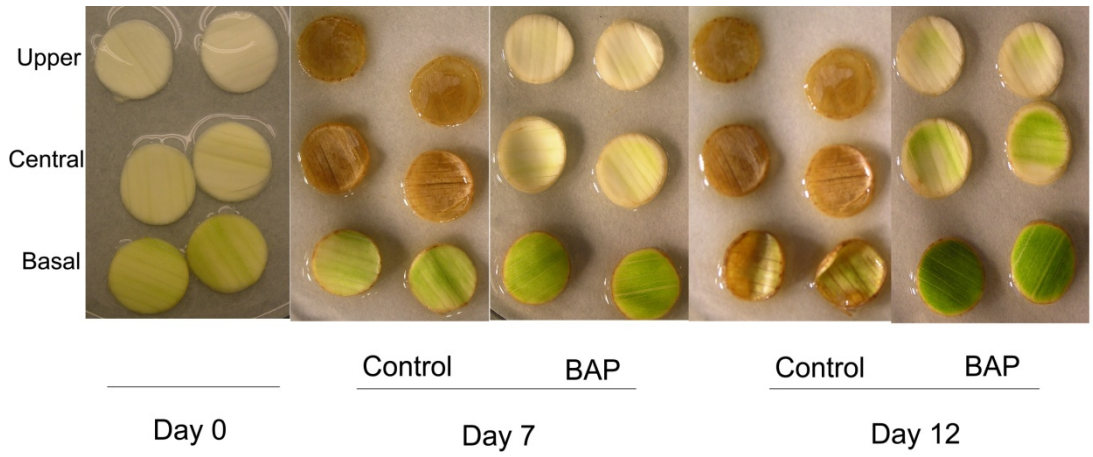

Figure 4.4 Discs of tissue of *Zantedeschia aethiopica* from basal, central and upper positions of the spathe, when treated with either RO water (control) or BAP at 10^{-4} M.

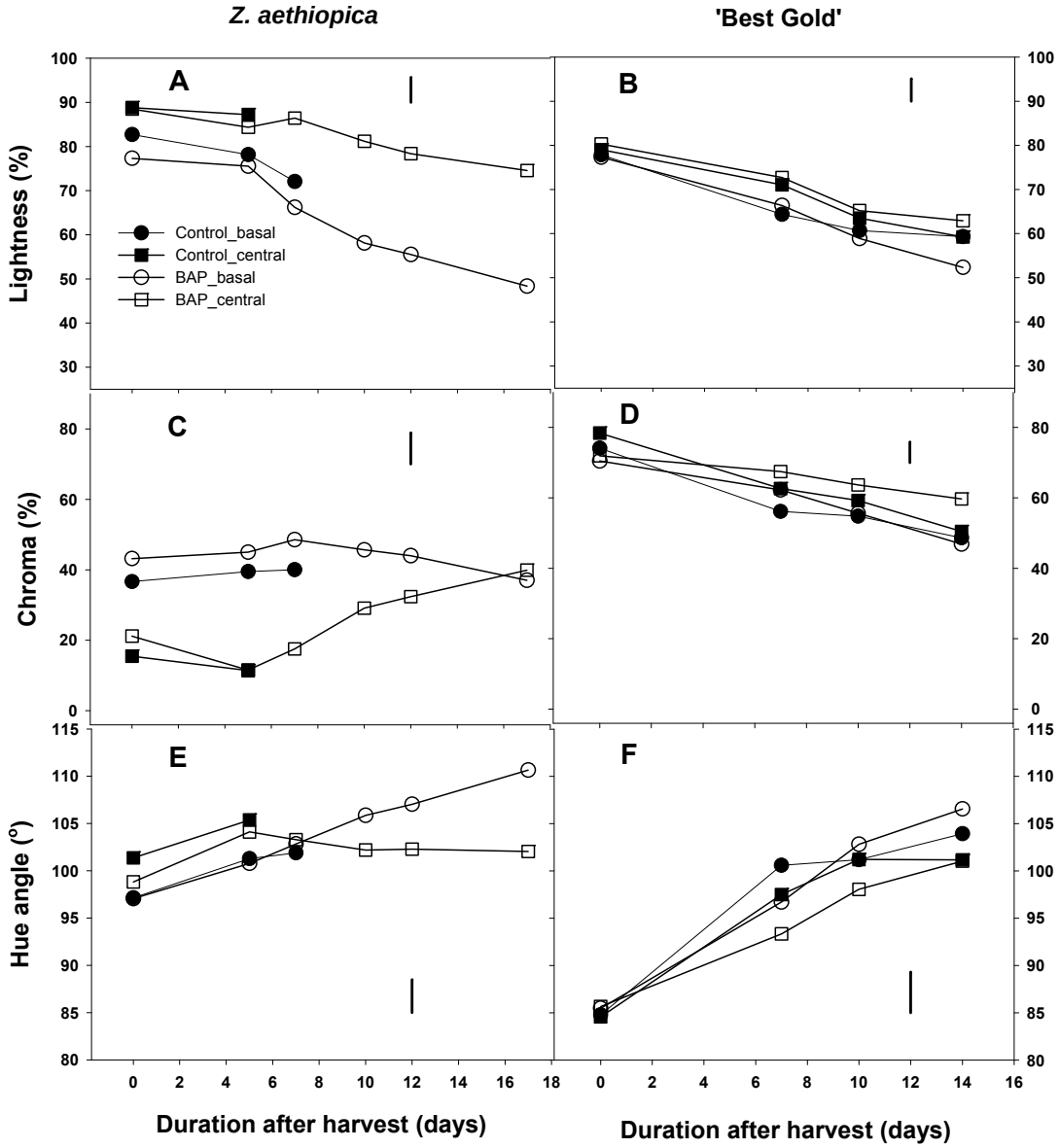

Figure 4.5 Influence of BAP on colour coordinates of lightness, chroma, and hue angle of the abaxial surface of discs of *Zantedeschia aethiopica* (left) and *Zantedeschia* 'Best Gold' (right). Discs were sampled from basal and central positions of the spathe for stems harvested at horticultural harvest-maturity. For *Z. aethiopica*, colour measurement stopped when discs turned brown in the control treatment after day 5 (central) and day 7 (basal). Vertical bars represent LSD at $P < 0.05$ (n = 4).

4.4 Discussion

The hypothesis proposed by Pais and Neves (1982-1983), that spathe re-greening of *Z. aethiopica* is induced by cytokinin-like compounds transferred from the fruits to the spathe, in part conflicts with the current findings. In the current study, while the exogenous application of BAP did result in re-greening in spathe tissue from all positions (Figure 4.4; Figure 4.5), the basal and central positions of the detached spathe in *Z. aethiopica* re-greened, despite removal of the spadix (Figure 4.1; Figure 4.2). This re-greening even occurred when the spadix was removed at the unfurling bud-stage, let alone at horticultural harvest-maturity. By removing the spadix at the unfurling bud-stage when the female flowers were still tightly enveloped by the spathe, pollination and fructification would have been prevented (New, 1964; Singh et al., 1996a). As additional support of the lack of involvement of fructification in re-greening, with the spadix attached, when the spathes of *Z. aethiopica* had re-greened, no fruit set was evident, even when observations continued for eight weeks; a timeframe previously regarded as adequate in *Zantedeschia* if fructification was to occur (Yao, 1992). It is probable that the different environmental conditions where the experiments were conducted, may partially explain the difference in results between the current study and the one reported by Pais and Neves. Pais and Neves conducted their experiment in an outdoor garden in Lisbon, Portugal. The more extreme weather conditions during their experiment, in particular the higher temperatures and lower relative humidity (no data are presented by Pais and Neves), is likely to have created extra desiccation stress for flowers of *Z. aethiopica*. In the current study however, the experiment was performed in an environment-controlled room, in which the temperature, humidity and light intensity were optimally regulated

for postharvest evaluation. These conditions appear to be favourable for spathe re-greening of *Z. aethiopica*, irrespective of whether or not the spadix was removed. Hence, it can be concluded that fructification is not required for the induction of spathe re-greening of *Z. aethiopica*.

The hypothesis that fructification is not a prerequisite for spathe re-greening is further supported by the observations with 'Best Gold'. In the current study, the entire spathe of 'Best Gold' re-greened despite the various approaches to eliminating fructification, i.e.:

1. the natural absence of female flowers on the spadix,
2. manual removal of spadix before female flowers were receptive (i.e., visually recognisable as being before pollen release (New, 1964; Singh et al., 1996a), or
3. the natural absence of a spadix (e.g. in a yellow-pigmented leaf).

In addition, as encountered with discs of spathe tissue, isolation of the discs from nearby tissue did not prevent them from re-greening. Collectively, these responses all support the hypothesis that like *Z. aethiopica*, fructification is not required for induction of spathe re-greening in 'Best Gold'.

The current findings, however, do not completely exclude a potential connection between fructification and spathe re-greening. In this study, the total chlorophyll content found in the fully re-greened spathe of *Z. aethiopica* was approximately 4-fold less than that reported in a fruit-bearing, re-greened spathe of *Z. aethiopica* (Tavares et al., 1998). Hence, it is possible that occurrence of fructification promotes chlorophyll accumulation, and ensures the completion of re-greening in

floral organs, but does not directly trigger the process of re-greening *per se*. Similar conclusions have been suggested with regard to other plant systems which re-green, e.g. sepals of *Helleborus niger* L. (Salopek-Sondi et al., 2002; Tarkowski et al., 2006), wherein re-greening was initiated in the depistillated flower, but did not proceed to completion. However, the mechanism for this potential mode of interconnection between fructification and re-greening is unclear. Further research is, therefore, required to clarify what if any role fructification may have in promoting completion of spathe re-greening of *Zantedeschia*.

If re-greening in spathe tissue of *Zantedeschia* is not triggered by fructification, the question as to how re-greening is induced, remains. Is the onset of re-greening determined by the ontogenetic age of tissue, while the progression and completion of re-greening (i.e. formation of functional chloroplasts) requires the presence of other external (e.g. light) and internal factors (e.g. plant hormones)? To in part address this question, Chapters 5 and 6 of this thesis evaluated some treatments that have been shown to be associated with re-greening in plant tissue, e.g. cytokinins, gibberellin (Ananieva et al., 2004; Ayele et al., 2010), light (Saks et al., 1988) and carbohydrate (Huff, 1984), for their potential influence in changing spathe re-greening of *Zantedeschia*.

Exogenous cytokinins have been shown to stimulate the re-greening of plant tissue in many species, e.g. sepals of *H. niger* (Salopek-Sondi et al., 2002) and leaves of *Nicotiana rustica* L. (Zavaleta-Mancera et al., 1999a). Similarly, in the current study the application of BAP promoted re-greening in discs of spathe tissue that were sampled from *Z. aethiopica* and 'Best Gold'. Although re-greening in the discs

sampled from all three positions of the spathe in 'Best Gold', and from the basal position of the spathe in *Z. aethiopica*, did not require the application of BAP, within the 14 to 17-day period of observation, BAP enhanced the extent by which the tissue re-greened (Figure 4.4; Figure 4.5). In contrast, re-greening in the discs that were excised from the central and upper positions of spathe of *Z. aethiopica* was uniquely stimulated by treatment with BAP because, without BAP, the discs senesced within five days (Figure 4.4; Figure 4.5). This may be a direct response of BAP-stimulation re-greening or an indirect response to a delayed de-greening within the spathe tissue achieved by the application of BAP.

In *Z. aethiopica*, at horticultural harvest-maturity, the discs excised from the basal position of the spathe typically retained a certain amount of green-coloured tissue (Figure 4.4). This is in contrast to the absence of any green colouration in the tissue from the central and upper positions. This green tissue resulted from the retention of some chloroplasts and/or thylakoid remnants (unpublished data), which has the potential to photosynthesize (Tavares et al., 1998). Hence, when isolated from the surrounding spathe tissue, discs from the basal position are likely to survive longer than those from the central and upper positions before re-greening can be induced. From this point of view, the application of BAP might have postponed the senescence in discs from the central and upper positions, so that the tissue retained its integrity, before the initiation of re-greening. On the other hand, in the basal position, the chloroplasts which were retained and/or thylakoid remnants, might contribute more than just functional ultrastructure to the capability of these discs to re-green. Such additional contributions could include the genetic information, e.g. chloroplast RNA (Dyer and Osborne, 1971) or plastid ribosomes (Carde et al., 1988; van Doorn, 2005),

that are required for reconstruction of the chloroplast, which is not completely lost during de-greening of the spathe. It has been demonstrated by Dyer and Osborne (1971), in yellowing leaves of *N. rustica*, that application of cytokinin increased the level of chloroplast RNA, and thus enhanced the re-greening of leaves. Following this logic, in the current study, it is possible that BAP directly stimulated the expression of chloroplast-related genes, which in turn triggered the process of re-greening in the discs from central and upper positions of *Z. aethiopica*. Therefore, while a clear answer to the question of whether BAP is involved in the initiation of re-greening is not yet possible, the application of BAP clearly enhanced the degree of re-greening, and ensured the completion of re-greening in spathe tissue of both *Z. aethiopica* and 'Best Gold'.

It is worth noting that the discs from all three positions of the spathe in 'Best Gold' re-greened within the 14-day period of observation, while the discs from the central and upper positions of the spathe in *Z. aethiopica* failed to re-green, and senesced within five days after harvest. It appears that the spathe tissue of 'Best Gold' is more capable of re-greening than that of *Z. aethiopica*. This difference in their capability of re-greening might be simply related to the fact that the spathe of *Z. aethiopica* is more prone to desiccation than that of 'Best Gold', before re-greening can be initiated (Tjia and Funnell, 1986). Additionally, the difference in the capability of re-greening between *Z. aethiopica* and 'Best Gold' might be partly reflected by the variations at biochemical (e.g. pigment composition) and ultrastructural (e.g. plastid) levels in the spathe tissue between these two species. Numerous lines of evidence indicate that the presence of carotenoids is essential for constructing and stabilizing the thylakoid systems during the formation of chloroplasts (Axelsson et al., 1982;

Bolychevtseva et al., 1995; Dahlin and Timko, 1994). Under these circumstances, in the current study, the abundance of typical chloroplast-carotenoids, including lutein, violaxanthin and β-carotene in spathe tissue of 'Best Gold', might have provided some advantage over *Z. aethiopica* for the initiation of re-greening. The contrasting situation with *Z. aethiopica*, was that only a comparatively small amount of lutein was detected at horticultural harvest-maturity (Table 4.1). Furthermore, from the perspective of ultrastructure at horticultural harvest-maturity, the white spathe of *Z. aethiopica* was characterized by the amyloplasts, with little thylakoid membrane, but large starch granules (Pais and Neves, 1982-1983). In contrast, at horticultural harvest-maturity the yellow spathe of 'Best Gold' was distinguished by the presence of chromoplasts (refer Chapter 3). Irrespective of the area/position of the spathe, chromoplasts contain some thylakoid membranes and a large number of plastoglobuli, which is believed to serve as a storage of lipids that can be utilized to reconstruct the thylakoid system in chloroplasts (Brehelin and Kessler, 2008). Based on the differences on ultrastructure and pigments, it is, therefore, reasonable to suggest that the chromoplasts in the spathe tissue of 'Best Gold' are more primed than the amyloplasts in the spathe tissue of *Z. aethiopica* for a rapid conversion into chloroplasts.

4.5 **Conclusion**

In the current study, fructification was found not to be a prerequisite for the occurrence of re-greening in spathe tissue of both the winter-flowering species, *Z. aethiopica*, and the summer-flowering selection, 'Best Gold'. This disagrees with the hypothesis proposed by Pais and Neves (1982-1983) that spathe re-greening is

induced by cytokinin-like compounds transferred from the fruits to the spathe. Is cytokinin involved in initiation of spathe re-greening in *Zantedeschia*? A clear answer is not yet possible, but the application of BAP clearly enhanced the degree of re-greening and ensured the completion of re-greening in spathe tissue of both *Z. aethiopica* and 'Best Gold'. Given the limited range of concentrations and types of cytokinins tested in the current experiment, screening of various types of cytokinins at a wider range of concentrations is required to further clarify the role of cytokinin in the induction and progression of re-greening. Chapter 5 of this thesis will address this issue.

Chapter 5 **Preliminary screening of plant hormones and sucrose on altering re-greening in *Zantedeschia* 'Best Gold'**

5.1 Introduction

As detailed in Section 1.5, cytokinin promotes re-greening in leaves or floral organs in many species (Ananieva et al., 2004; Tarkowski et al., 2006; Venkatarayappa et al., 1984; Zavaleta-Mancera et al., 1999a), including spathes of *Zantedeschia aethiopica* (L.) Spreng. (Pais and Neves, 1982-1983). For example, application of a synthetic cytokinin, 6-benzylaminopurine (BAP) at 10^{-4} M on a yellowing leaf of *Nicotiana rustica* L. stimulated leaf re-greening (Zavaleta-Mancera et al., 1999a). Similarly, treatment with BAP or zeatin at concentrations of 10^{-5} M to 10^{-2} M induced re-greening of sepals in *Helleborus niger* L. (Salopek-Sondi et al., 2002). Pais and Neves (1982-1983) reported that application of an aromatic cytokinin (6-(o-hydroxybenzylamino)-purine) at concentrations of 10^{-8} M to 10^{-4} M resulted in re-greening in the spathes of *Z. aethiopica* with the spadix removed. So far, no research has been done to clarify the role of cytokinin in altering spathe re-greening of the summer-flowering group of *Zantedeschia*. Before committing to larger and more detailed experiments, attempts have been made here to undertake a preliminary screening of some commonly used and readily available synthetic cytokinins, e.g. BAP, zeatin and N_1-(2-chloro-4-pyridyl)-N_3-phenylurea (CPPU), at concentrations up to 10^{-4} M, for their potential influence on modifying spathe re-greening of *Zantedeschia* 'Best Gold'.

Gibberellin has also been reported to stimulate re-greening in some species, e.g. *H. niger* (Ayele et al., 2010; Salopek-Sondi et al., 2002). Re-greening of sepals of an unfertilized flower in *H. niger* was stimulated by the treatments of gibberellins (GA_3, GA_4 or GA_7) at concentrations of 10^{-5} M to 10^{-3} M, but was inhibited by an application of paclobutrazol, an inhibitor of gibberellin biosynthesis (Salopek-Sondi et al., 2002). Similarly, application of gibberellin was associated with an enhanced re-greening in peel of *Citrus sinensis* (L.) Osbeck. (El-Zeftawi, 1978). Prior to commencing this study, there were no reports on the influence of gibberellin on spathe re-greening of *Zantedeschia*. Hence, in the current chapter, before proceeding to larger and more complex experiments, the effect of an application of exogenous gibberellin (e.g. GA_3), at concentrations up to 10^{-4} M, on the change in spathe colour of 'Best Gold' during re-greening was investigated.

Numerous lines of evidence have suggested a combined effect of cytokinin and gibberellin in many processes of plant development. For instance, a synergistic effect between these two hormones was found in promoting seed germination in *Lactuca sativa* L. (Christine et al., 1976), in enhancing fruit size of *Malus×domestica* (Bangerth and Schröder, 1994), and in preventing leaf yellowing in *Lilium longiflorum* Thunb. (Han, 1997; Whitman et al., 2001). On the other hand, an antagonistic effect of these two hormones has also been shown in increasing the number and weight of grains in barley (Mishra and Gaur, 1985). Hence, depending on the individual situation, the combined effect of cytokinin and gibberellin can not be predicted. Prior to commencing this study, no reports were evident which detailed any effect of the combination of both cytokinin and gibberellin in re-greening of plant tissue. Hence, in the current study, the combination of cytokinin (e.g. BAP) and

gibberellin (e.g. GA_3) on the change in colour of spathes in 'Best Gold' during re-greening was tested.

The influence of auxin on re-greening of plant tissue has been rarely studied. Kovacs et al. (2007) reported that treatment with indolylacetic acid (IAA) enhanced chlorophyll accumulation during the re-greening of the leaf in *Cucumis sativus* L. On the other hand, Salopek-Sondi et al. (2002) showed that application of IAA did not induce any distinct effect on re-greening in the sepal of *H. niger*. Furthermore, Yuan et al. (2001) found that re-greening of the peel of *C. sinensis* was concomitant with a decrease in the level of endogenous IAA in the peel. These contrasting results reflect the possibility that exogenous application of auxin might offer a tool for modifying the re-greening process of spathe tissue, albeit differing plant tissue do not appear to always respond in the same manner. Hence, as a preliminary screening, the potential effect of auxin on the change in colour of spathes in 'Best Gold' during re-greening was evaluated.

Sucrose has been associated with the processes of de-greening and re-greening in the peel of *C. sinensis* (Huff, 1983; Huff, 1984). The de-greening was correlated with the accumulation of sucrose in the peel, while the peel re-greened when the accumulated sucrose disappeared (Huff, 1984). Supplementation of 1.5×10^{-3} M sucrose *in vitro*, to the peel of *C. sinensis*, inhibited the re-greening. The effect of sucrose on the re-greening of spathes in *Zantedeschia* has not been previously reported. Hence, as a preliminary screening, a potential effect of sucrose on the change in colour of spathes in 'Best Gold' during re-greening was tested in the current study.

To test the hypothesis that cytokinin, gibberellin, auxin and sucrose are likely to influence re-greening, in the present study, the effects of several synthetic plant hormones and sucrose on the change in colour of spathes of 'Best Gold' during re-greening were examined. The tested chemicals included: BAP, zeatin, and CPPU, GA_3, 1-naphthaleneacetic acid (NAA), and sucrose. Subsequently, the effect of a combination of BAP and GA_3 on changes in colour of the spathe of 'Best Gold' during re-greening was examined. Through these studies, it was attempted to identify an effective method for postponing spathe re-greening of 'Best Gold'.

5.2 Materials and Methods

5.2.1 Plant materials and handling

The plant materials used in this study were flowers (spathe plus spadix and peduncle) of 'Best Gold'. They were produced under the same conditions and harvested at the same maturity, as described in Chapter 2 (refer section 2.2.1). One disc from either side of the midrib at the central position of spathes was excised by using a cork borer (diameter 14 mm). The discs were placed on filter paper soaked in treatment solutions with the adaxial surface facing down in closed petri dishes. The filter paper was kept moist with treatment solutions throughout the experiment. Each experiment was conducted under controlled environmental conditions recommended by Reid and Kofranek (1980): room temperature 20 ± 1 ºC, relative humidity of 60 to 70%, 12-h (0600 HR – 1800 HR) photoperiod and light intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height provided by cool-white fluorescent tubes.

Measurement of colour changes of the discs was conducted as described in Chapter 2, which comprised recording the colour coordinates of lightness (L*), chroma (C*), and hue angle (H°) of both the abaxial and adaxial surfaces of discs, using a tristimulus colorimeter CM-2600d/2500d (Konica Minolta, Japan). The colour measurement was repeated every 2 to 4 days for a period of two to three weeks, depending on individual experiments. Based on the results presented in Chapter 2, the change in H° can be used to describe the changes in chlorophyll content in the abaxial surface, particularly during the initial stage of spathe re-greening. For the abaxial surface, a value of H° greater than 90º was deemed indicative of a level of accumulation of chlorophyll (i.e. re-greening) visible to the human eye (Iglesias et al., 2001). Hence, for the purposes of a preliminary screening of treatment effects, in this chapter, changes of H° on the abaxial surface were the primary focus of data interpretation. In some instances, values of L* and C* were also included for further interpretation of treatment effects.

5.2.2 Treatments, experimental design and statistical analysis

Experiment 1: effect of cytokinin and gibberellin on the change in spathe colour during re-greening

Based on pilot trials (data not presented), a concentration of BAP at $5x10^{-4}$ M resulted in what appeared to be phytotoxic effects on discs of spathe tissue (i.e. brown colouration and rapid senescence). Hence in the preliminary screening experiments presented in this chapter, concentrations lower than this were evaluated, i.e.:

- BAP (Sigma) at 10^{-4} M, 10^{-6} M, and 10^{-8} M,
- Zeatin (Sigma) at 10^{-4} M, 10^{-6} M, and 10^{-8} M,
- CPPU (SKW 20010, Germany) at 10^{-4} M, 10^{-6} M, and 10^{-8} M,

- GA_3 (Sigma) at 10^{-4} M, 10^{-6} M, and 10^{-8} M,
- Control, i.e. reverse osmosis (RO) water.

Experiment 2: effect of auxin on the change in spathe colour during re-greening

- NAA (Sigma) at 10^{-4} M,
- Control, i.e. RO water

Experiment 3: effect of sucrose on the change in spathe colour during re-greening

- sucrose at 1.5×10^{-3} M,
- Control, i.e. RO water.

Experiment 4: Combination of GA_3 and BAP on the change in spathe colour during re-greening

Based on the results from Experiment 1, treatment solutions comprising combinations of both BAP and/or GA_3 were evaluated for the effect on re-greening. There were five combinations of these two hormones resulting in ratios of GA_3: BAP (v:v) and their equivalent concentrations when mixed together as follows:

- 2:1 (GA_3 1.63×10^{-4} M : BAP 0.67×10^{-4} M),
- 1:1 (GA_3 10^{-4} M : BAP 10^{-4} M),
- 1:0 (GA_3 10^{-4} M),
- 0:1 (BAP 10^{-4} M),
- 1:2 (GA_3 0.67×10^{-4} M : BAP 1.63×10^{-4} M),
- Control (RO water).

Each of the individual experiments listed above was conducted as a split-plot design, with the treatment solution as the main plot, and time (i.e. time from harvest of repeated colour measurement) as the split plot. There were four individual spathes (replicates) for each treatment and two discs (as a sub-sample) per replicate. Data were tested initially to ensure they met the requirement for ANOVA using the general linear procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Where significant ($P < 0.05$) treatment effects were detected, means were separated by using the unrestricted LSD procedure.

5.2.3 **Preparation of treatment solutions**

BAP powder (225 mg) was dissolved in 10 ml warm RO water (about 50° C) and RO water was added to the volume of 100 ml to make up a stock solution of 10^{-2} M. The stock solution was then diluted in RO water to attain the required concentrations.

GA_3 powder (346 mg) was dissolved in a few drops of 50% ammonia and the extra ammonia was evaporated using a rotary evaporator (Rotavapor-R, Buchi Laboratories, Switzerland). The remaining crystal was then re-dissolved in RO water to 100 ml, to make up a stock solution of 10^{-2} M. The stock solution was subsequently diluted in RO water to attain the required concentrations.

NAA powder (186 mg) was dissolved in a few drops of absolute ethanol and RO water was added to 100 ml, to make up a stock solution of 10^{-2} M. The stock solution was diluted to 10^{-4} M for the experiment.

Sucrose (51 mg) was directly dissolved in 100 ml RO water to make up a solution with the required concentration of $1.5x10^{-3}$ M.

5.3 Results

5.3.1 Change in spathe colour in control treatment

During the 21-day period of observation, the colour of the abaxial surface of discs within the control treatment changed from intense golden-yellow through green, and subsequently to yellowish-green (Figure 5.1). The visible expression of re-greening was initiated from the central area of the discs, and progressed outward. The colour changes corresponded with the change in values of H° that consisted of three phases wherein:

1. H° remained constant (84°) for the first two days (golden-yellow),
2. showed a linear increase from 84° to 106° between day 2 and day 12 (yellow to green) reaching 90° (i.e. visibly green) by approximately day 5, and,
3. subsequently decreased from 106° to 97° between day 14 and day 21 (green to yellowish-green; Figure 5.2).

Although the value of H° on day 21 was similar to that on day 7, the values of L* and C* on day 21 were lower than those on day 7 ($P < 0.05$), indicating the colour on day 21 was less saturated and not as bright as on day 7. In addition, within the 21-day period of observation, both the values of L* and C* decreased, from 78% to 50% and from 79% to 41%, respectively (Figure 5.2 A & B).

Within the 21-day period of observation, re-greening was not observed on the adaxial surface of discs in the control treatment. During this period, the colour of discs changed from golden-yellow to nearly white (Figure 5.1), corresponding to an increase in H° from 86° to 90° and a decrease in both L* and C* from 79% to 65% and from 76% to 28%, respectively (data not shown).

5.3.2 Cytokinins and changes in spathe colour

The application of BAP at 10^{-4} M slightly delayed the time when re-greening was visible (i.e., H° reached a value of 90°) on the abaxial surface of the discs (Figure 5.2 C). The H° of the abaxial surface of discs treated with BAP at 10^{-4} M reached the value of 90°, between day 6 and day 7, which is approximately 1-2 days later than occurred in the control treatment. This delay was not observed in discs treated with BAP at either 10^{-6} M or 10^{-8} M (Figure 5.3). Similarly, for discs treated with the other sources of cytokinin (i.e., zeatin and CPPU at all three concentrations), this delay was not evident. Within the first 14-day period, the change in H° for discs in these treatments was similar to that of the control ($P > 0.05$; Figure 5.2 C).

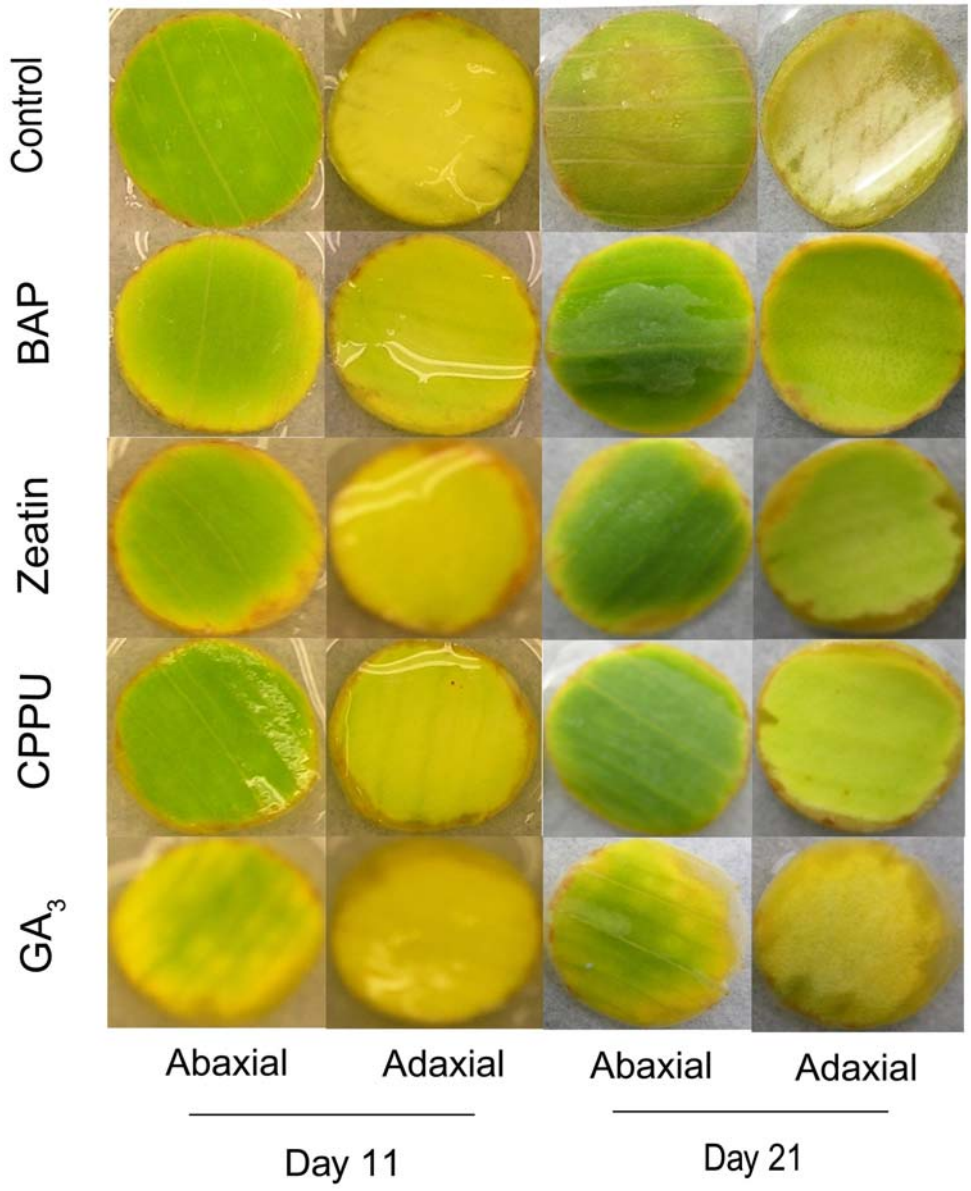

Figure 5.1 Discs of spathe tissue of *Zantedeschia* 'Best Gold' treated with either BAP, zeatin, CPPU, or GA_3 at a concentration of 10^{-4} M after 11 and 21 days. Control discs were held in RO water.

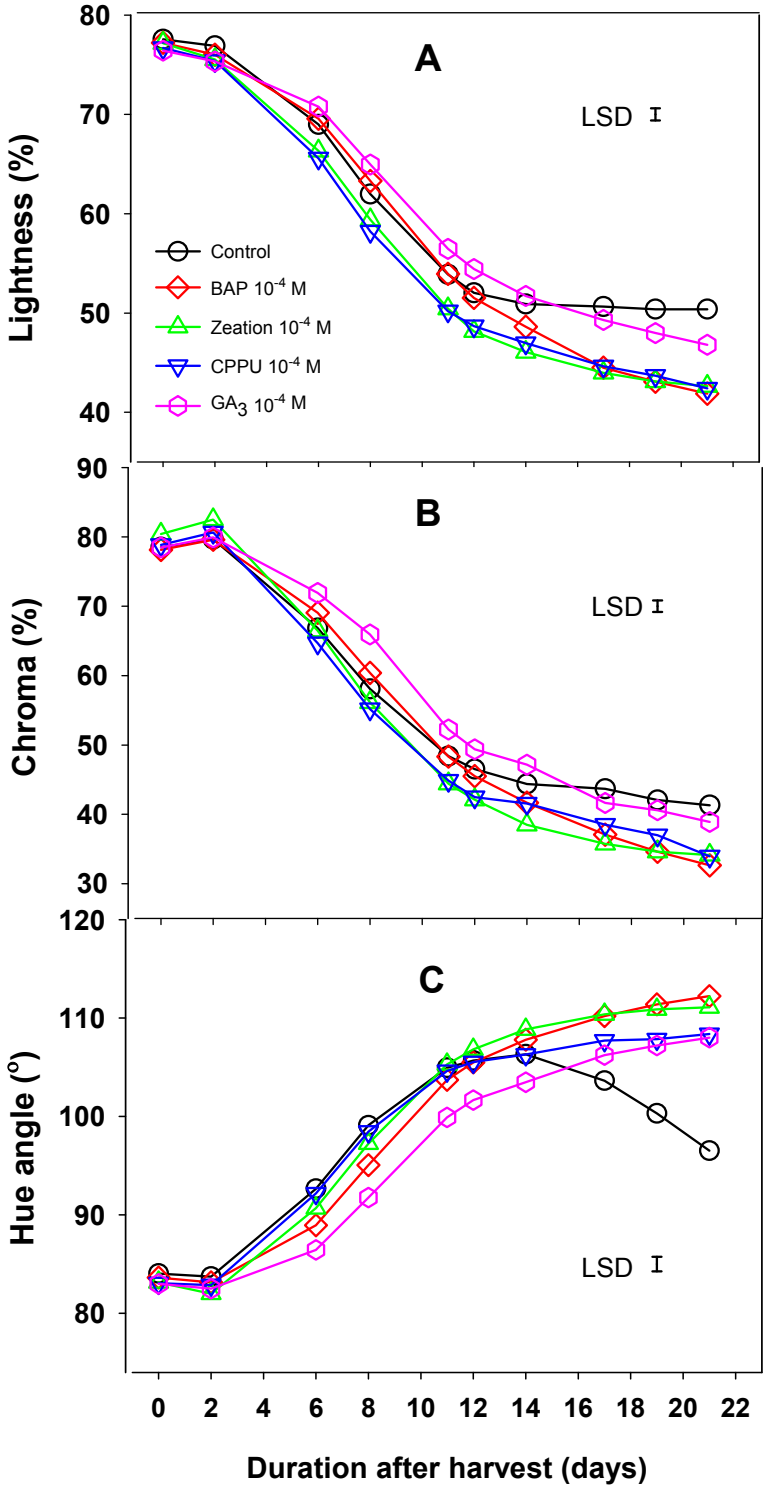

Figure 5.2 Lightness, chroma and hue angle of the abaxial surface of the spathe of *Zantedeschia* 'Best Gold' treated with: BAP, zeatin, CPPU, or GA_3. Only the data pertaining to the control and 10^{-4} M of each compound are presented. Vertical bars represent LSD at $P < 0.05$ (n = 4). Controls were held in RO water.

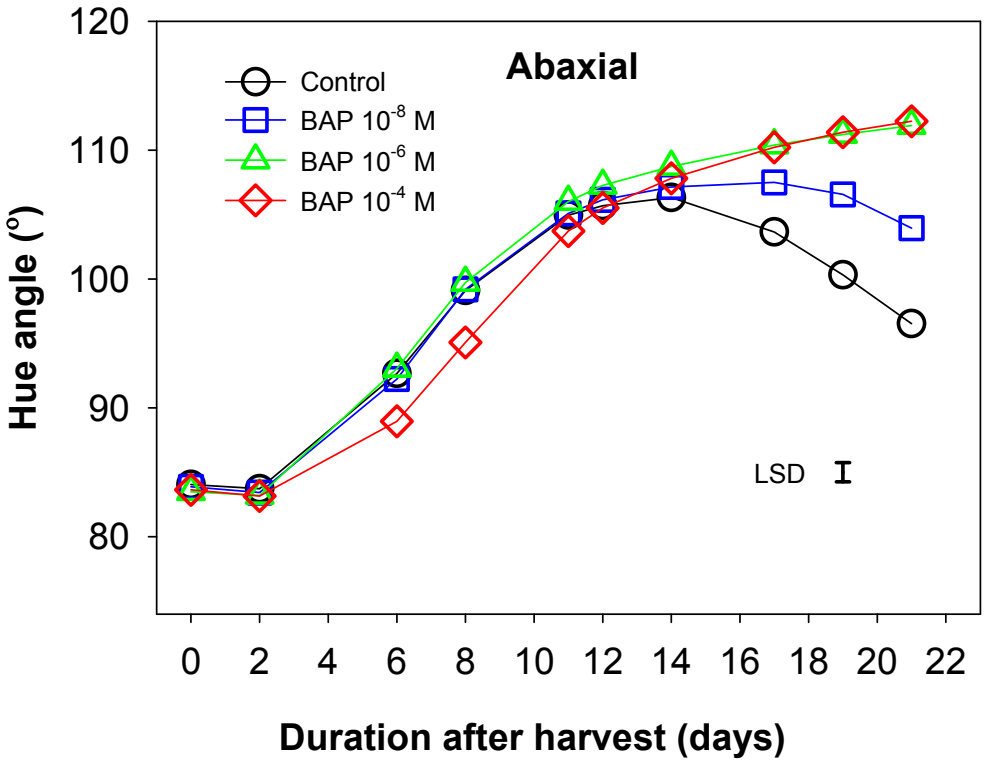

Figure 5.3 Hue angle of the abaxial surface of the spathe of *Zantedeschia* 'Best Gold' treated with BAP at 10^{-8} M, 10^{-6} M or 10^{-4} M. Vertical bar represents LSD (1.49°) at $P < 0.05$ (n = 4). Controls were held in RO water.

During the last week of observation (i.e. day 14 to 21), BAP at both 10^{-4} M and 10^{-6} M stimulated a continuous increase in H° of the abaxial surface (Figure 5.3). This continued increase was in distinct contrast to the decreased value of H° for discs in the control treatment during this same period. Likewise, the application of the other sources of cytokinin (i.e., zeatin and CPPU at 10^{-4} M and 10^{-6} M) also resulted in a continuous increase in the value of H° after day 14, with the magnitude of this change being higher in BAP and zeatin treated discs than that of CPPU ($P < 0.05$; Figure 5.2 C). By day 21, the discs treated with the cytokinins (particularly BAP at 10^{-4} M) were dark-green, whereas the control discs were pale-green (Figure 5.1).

For the adaxial surface, in contrast to no re-greening being observed for the control treatment, re-greening was found in the discs treated with all cytokinins at the concentration of 10^{-4} M (Figure 5.1). Unlike the abaxial surface wherein re-greening was initiated in the central area of discs, in these treatments re-greening of the adaxial surface started from the edge and progressed inward. Within the 21-day period of observation, this colour change corresponded with an increase in H° from 86° to 103°, and a decrease in both L* and C* from 79% to 70% and from 78% to 53%, respectively (data not shown).

5.3.3 GA_3 and changes in spathe colour

Treatment of GA_3 at 10^{-4} M (and 10^{-6} M; data not shown) delayed the time when re-greening was visible (i.e., H° reached a value of 90°) on the abaxial surface of the discs (Figure 5.2 C). In these treatments the H° reached 90° between day 6 and day 8, which was approximately 2-3 days later than the control treatment, and 1 day later than with BAP at 10^{-4} M (Figure 5.2 C). This delay was not found in the discs that

were treated with GA_3 at 10^{-8} M, wherein the colour change of these discs followed that of the control (data not shown). After day 14, the abaxial surface of the discs treated with GA_3 at both 10^{-4} M and 10^{-6} M showed a continuous increase in H°, similar to those evident in the cytokinin-treated discs (Figure 5.2 C).

Throughout the 21-day period of observation, re-greening was not observed on the adaxial surface of the GA_3-treated discs. Instead, by day 21, the adaxial surface of discs under the treatment of GA_3 at 10^{-4} M remained yellow (Figure 5.1). This retention of colour on the adaxial surface was in direct contrast to the control treatment wherein the yellow colour of this surface has been lost by day 21.

5.3.4 NAA and changes in spathe colour

The treatment of NAA at 10^{-4} M did not affect the time when re-greening was visible (i.e., when H° reached a value of 90°) on the abaxial surface of the discs. During the first week of observation, the NAA-treated discs and the control showed a similar value of H°, reaching 90° approximately at day 6 ($P > 0.05$; Appendix II). After day 7, H° of the NAA-treated discs was lower than that of the control ($P > 0.05$) and, by day 15, NAA treated discs had turned light brown, while the control discs remained green.

5.3.5 Sucrose supplementation and changes in spathe colour

Re-greening on the abaxial surface of the sucrose-treated discs, i.e. a H° of 90° or greater, was not noted until approximately six days after it was visible in the control treatment (Figure 5.4). At all periods of measurement after harvest, H° of the

sucrose-treated discs was less, but L* and C* (data not shown) were higher, than that of the control ($P < 0.05$). When the re-greening occurred on the abaxial surface of the sucrose-treated discs, it was mainly concentrated in the central area of the discs, leaving the outer regions yellow in colour, in contrast to an even distribution of green colouration on the abaxial surface of discs in the control treatment (Figure 5.6).

5.3.6 Combination of GA_3 and BAP and changes in spathe colour

Simultaneous application of GA_3 and BAP postponed re-greening by more than ten days. This contrasts to the effect of individual GA_3 or BAP treatments that delayed the time when H° of the abaxial surface reached 90° (i.e. visibly green) by only five or two days, respectively. Among the five combinations of GA_3 and BAP investigated, the longest delay occurred with a 1:1 ratio, resulting in no evident green colouration (i.e. $H^\circ < 90^\circ$) on the abaxial surface throughout the 14-day period of observation (Figure 5.5; Figure 5.6). Up to day 10, there was no difference in the values of H° between the discs treated with GA_3 + BAP at ratios of 2:1, 1:1 or 1:2. By day 14, the H° of the discs treated with GA_3 + BAP at ratios of 2:1 or 1:2 reached 90° (i.e. had became visibly green), which were higher in value than that achieved with GA_3 + BAP at a ratio of 1:1 ($P < 0.05$; Figure 5.5).

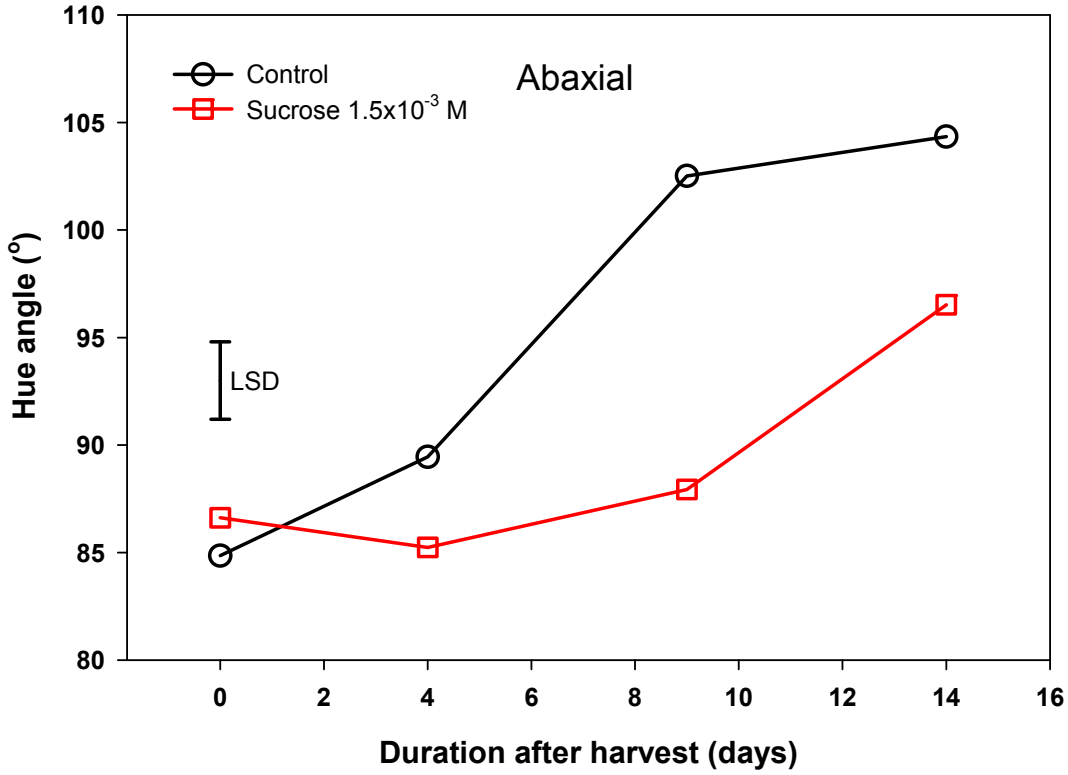

Figure 5.4 Hue angle of the abaxial surface of spathe tissue of *Zantedeschia* 'Best Gold', treated with either RO water (control) or sucrose at 1.5×10^{-3} M. LSD = 3.59° at $P < 0.05$ (n = 4).

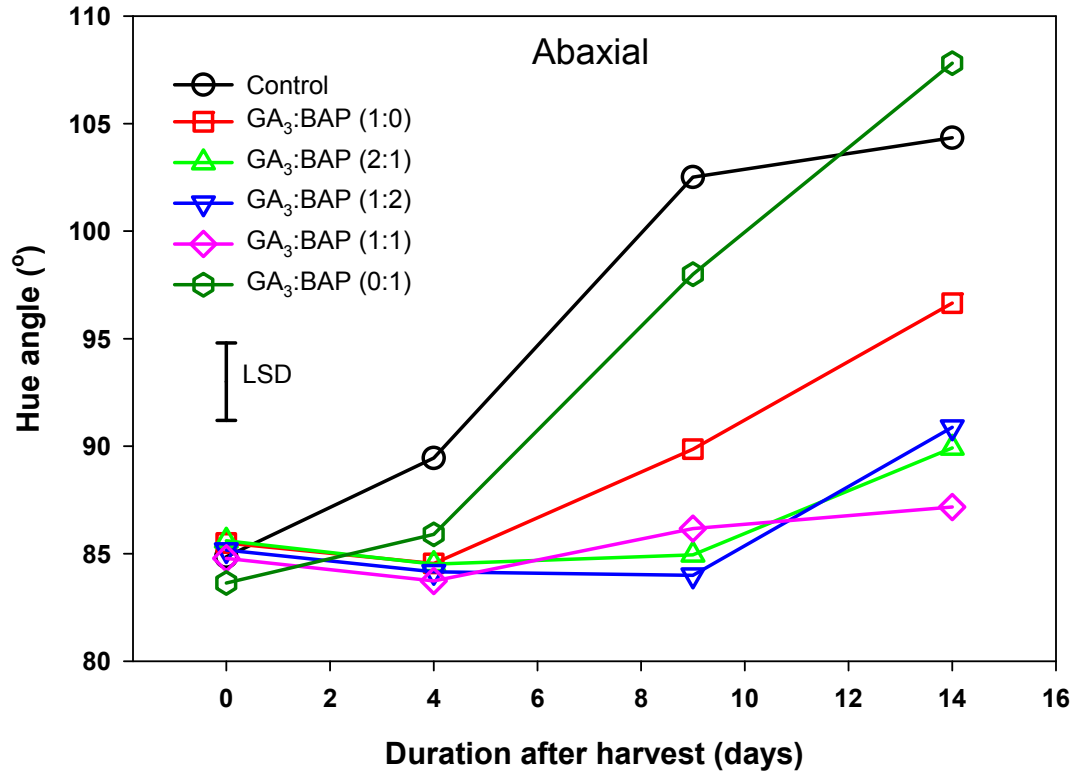

Figure 5.5 Hue angle of the abaxial surface of spathe tissue of *Zantedeschia* 'Best Gold', simultaneously treated with GA_3 and BAP at ratios of GA_3:BAP (v:v) of 1:0, 2:1, 1:2, 1:1 or 0:1. LSD = 3.59° at $P < 0.05$ (n = 4). Controls were held in RO water.

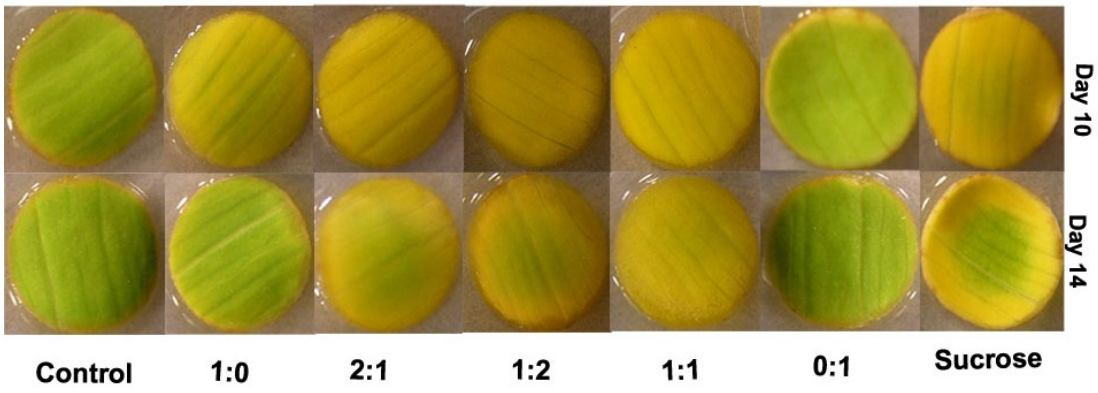

Figure 5.6 The abaxial surface of discs from the spathe of *Zantedeschia* 'Best Gold' treated with sucrose (1.5×10^{-3} M) or combinations of GA_3 and BAP in ratios of 1:0, 2:1, 1:2, 1:1 or 0:1 (v:v). Control discs were held in RO water.

5.4 Discussion

Among all the treatments tested in the current study, simultaneous application of BAP and GA_3 at 10^{-4} M (1:1), to discs of spathe tissue, resulted in the longest delay (more than 10 days) in time until when re-greening became visible (Figure 5.5). When applied alone, both BAP and GA_3 at 10^{-4} M delayed the time when re-greening was visible by 2 and 5 days, respectively (Figure 5.2; Figure 5.5). This delay was enhanced for an additional five days by simultaneous application of BAP and GA_3, indicating a synergistic effect between BAP and GA_3 in postponing the colour change of spathe tissue during re-greening. A synergistic effect of combining cytokinin and gibberellin has previously been reported in preventing leaf yellowing in *L. longiflorum* L. (Han, 2001; Whitman et al., 2001) and *Viola cornuta* L. (Waterland et al., 2010). Chandler (2009) suggested that synergistic effects can arise when components from independent signaling pathways of separate hormones mutually cooperate. As presented in Chapter 2 and Chapter 3, the physical change in colour of the spathe of 'Best Gold' from yellow to green is a result of changes in pigment accumulation and ultrastructure. To explore the hypothesis that this mutual cooperation between the signaling pathways may also be operating in the current system, it would help if the response to BAP and/or GA_3 of other components associated the process of re-greening could be clarified. In Chapter 6, therefore, the effects of the combination of GA_3 and BAP on re-greening will be examined further, at the levels of pigment accumulation and plastid differentiation during re-greening.

The response of spathe tissue to exogenous cytokinins (BAP in particular) appears to be inconsistent at different stages of re-greening. For the abaxial surface,

dependent upon concentration, the application of BAP alone resulted in either no influence or a slight delay in the change of colour from yellow to green during the initial stage of re-greening (i.e. first week of observation; Figure 5.2; Figure 5.3). In contrast, during the last week of observation, when de-greening occurred in discs within the control treatment, cytokinins stimulated continuous re-greening. This positive role of cytokinins in stimulating re-greening during the last week is in agreement with that reported for leaves of *N. rustica* (Zavaleta-Mancera et al., 1999a) and spathes of *Z. aethiopica* (Pais and Neves, 1982-1983). However, the initial insensitive or negative response of spathe tissue in 'Best Gold' to exogenous cytokinin has not previously been reported. As revealed in other model systems, e.g. sepals of *H. niger* (Salopek-Sondi et al., 2002; Tarkowski et al., 2006) and cotyledons of *Cucurbita pepo* L. (Ananieva et al., 2004), re-greening was associated with an increased or sustained level of endogenous cytokinins. It is possible that in the spathe tissue of 'Best Gold' at horticultural harvest-maturity, the level of endogenous cytokinin was sufficient to induce re-greening and ensure its progression. The tissue therefore, did not appear to respond to exogenous cytokinin (e.g. BAP at 10^{-6} M) or provoked a negative response via feedback inhibition (e.g., BAP at 10^{-4} M). On the other hand, it is also reasonable to suggest that during the initial stages of re-greening the role of exogenous cytokinins in influencing re-greening might not be accurately determined simply by monitoring the change in colour of spathe tissue. For example, the treatment of BAP might not directly influence chlorophyll accumulation, but exert an effect on other components of the re-greening process, e.g. the accumulation of thylakoids (Kusnetsov et al., 1994; Wilhelmova and Kutik, 1995; Zavaleta-Mancera et al., 1999b). This initial positive effect of cytokinins, however, is not revealed by simply observing the colour change of the spathe tissue. This highlights the need of

further examination of the response of spathe tissue to cytokinin at biochemical (e.g. pigment) and ultrastructural (plastid differentiation) levels. Therefore, Chapter 6 of the thesis was designed to address the following questions:

1. Does cytokinin influence carotenoid accumulation at the initial stage of re-greening?
2. Does cytokinin prevent chlorophyll accumulation during the initial stage of re-greening?
3. Does cytokinin stimulate chloroplast formation (e.g., thylakoid accumulation) during the initial stage of re-greening?

It is interesting that, for the adaxial surface, re-greening was induced only in the cytokinin-treated discs at 10^{-4} M, regardless of the different types of cytokinin. While this confirms the role of cytokinin in stimulating re-greening in spathe tissue, the reason for why the two surfaces of spathe tissue differ in their response to exogenous cytokinins is unclear. The adaxial surface is clearly not limited in its ability to re-green by the mere absence of structural components (refer Chapter 3). It is possible that the adaxial surface of spathe tissue contains no or low levels of endogenous cytokinins, that results in it not being sufficient to induce and/or sustain re-greening. If so, the adaxial surface is more likely than the abaxial surface to respond to exogenous cytokinins by re-greening. On the other hand, the cells or cellular components in the adaxial surface are potentially not as sensitive to cytokinin as the abaxial surface. More research targeted at analysing endogenous cytokinins and localization of cytokinin receptors in both the abaxial and adaxial surfaces at various stages of re-greening is required to test these hypotheses. The current study however,

was not designed to further address this question through hormonal and molecular analysis.

Application of gibberellins has previously been shown to stimulate re-greening in some species, e.g. sepals of *H. niger* (Salopek-Sondi et al., 2002) and peel of *C. sinensis* (El-Zeftawi, 1978). Ayele et al. (2010) also reported that re-greening in the sepal of *H. niger* was associated with an increased level of the endogenous gibberellins in the sepal. Conversely, in the current study, the application of GA_3 postponed the time when re-greening of spathe tissue was visible, showing a greater delay than the tested cytokinins (Figure 5.2; Figure 5.5). Gibberellin appears to be a retardant of re-greening while also retaining the existing colouration. This role of gibberellin is similar to that found in the process of de-greening in the peel of *C. sinensis* fruits (Fidelibus et al., 2008; Garcia-Luis et al., 1986) and in leaves of *Z. aethiopica* (Skutnik et al., 2001) or *Pelargonium×hortorum* cv. 'Kira' (Rosenwasser et al., 2010), where gibberellin delayed the colour change from green to yellow/orange by preventing chlorophyll breakdown. Gibberellin, however, did not prevent the dismantling of the chloroplast during the de-greening in leaves of 'Kira' (Rosenwasser et al., 2010). The current findings, in light of those reported by other workers, have raised some questions regarding the role of gibberellins in re-greening of spathe tissue, including:

1. Are changes in re-greening associated with changes in gibberellin content, activity and/or sensitivity in the spathe tissue?
2. Does gibberellin retard the visible signs of re-greening by preventing the breakdown of carotenoids?
3. Does gibberellin maintain the integrity of chromoplasts?
4. Does gibberellin inhibit chlorophyll accumulation?

In Chapter 6, by monitoring the change in pigment accumulation and plastid differentiation of spathe tissue in response to the treatment with GA_3, the questions 2-4 are addressed.

Compared with the control treatment, NAA at 10^{-4} M did not result in any notable influence on re-greening of discs, but exerted a detrimental or phytotoxic effect. The findings presented in the current study do not support those reported by Kovacs et al. (2007) wherein the application of an auxin (indole-3-acetic acid) promoted re-greening in leaf discs of *C. sativus*. However, due to the limited range of concentrations and types of auxin tested in the current experiment, it is not appropriate to conclude that auxins provoke no effect on re-greening of spathes in 'Best Gold'. Further screening with a range of auxin types at a wider range of concentrations is recommended for investigation in future research, but the attainment of positive results from the other plant hormones in the current study precluded any further investigations with auxin treatments in this thesis.

Sucrose supplementation accelerated the timing of colour break from green to yellow in the peel of *Citrus* by promoting chlorophyll degradation (Huff, 1983; Huff, 1984; Iglesias et al., 2001). In this study, re-greening (from yellow to green) on the abaxial surface of spathe tissue was retarded by an application of sucrose, showing 1 or 4 days more delay than with the treatment of GA_3 or BAP alone (Figure 5.4). When re-greening occurred on the abaxial surface of the sucrose-treated discs, it was mainly concentrated in the central area of the discs, leaving the outer regions yellow in colour (Figure 5.6). If sucrose is accepted as having an active role in delaying re-greening, this difference between regions of the disc might be explained if the outer regions of

the discs absorbed and consequently metabolised more sucrose than the central area. In doing so re-greening would be further delayed at the edge of the discs than the central area of the discs. The increase of sucrose in the spathe tissue of 'Best Gold' may partially inhibit chlorophyll synthesis. This explanation is supported by the studies with *Daucus carota* L. (Edelman and Hanson, 1971; Pamplin and Chapman, 1975) and leaves of *Lycopersicon esculentum* L. (Mortain-Bertrand et al., 2008), where chlorophyll accumulation was suppressed in the tissue grown in a medium containing sucrose. From a horticultural perspective, sucrose is readily available and is comparatively cheap to purchase. Hence, as a means of delaying re-greening of spathes of 'Best Gold', supplementation of vase solutions with sucrose warrants further investigation for commercial potential. Due to the limited timeframe for a PhD project, sucrose treatment was not investigated further in this study.

5.5 Conclusion

Several synthetic plant hormones and sucrose were screened for their efficacy in postponing re-greening in the spathe of 'Best Gold'. The most effective treatment in delaying re-greening was from the simultaneous application of GA_3 and BAP at 10^{-4} M, i.e. a ratio of 1:1 (v:v), wherein re-greening was delayed for more than ten days. This treatment, therefore, offers good potential for future development into a method that can be used commercially by growers or retailers to postpone re-greening of the spathe in 'Best Gold'. As the current results were based on the response of disc tissue excised from the spathe, further studies are required to test the combination effect of BAP and GA_3 on re-greening of entire flowers (i.e. spathe plus spadix and peduncle), so as to define the optimal concentration and application methods for the commercial

product (e.g. pulsing or as a preservative solution). Chapter 7 of this thesis was designed to address these issues. On the other hand, for the academic interest of further clarifying the role of cytokinin and/or gibberellin in influencing spathe re-greening, an examination of the response of spathe tissue to the treatment of these hormones at pigment and ultrastructural levels is required. Chapter 6 of this thesis will investigate this topic in more detail.

Chapter 6 **Ultrastructural and pigment changes resulting from cytokinin, gibberellin, light, and their combination, during spathe re-greening of *Zantedeschia***

6.1 Introduction

Cytokinins and gibberellins have been previously shown to stimulate re-greening in leaves or floral organs in some species, e.g. *Zantedeschia aethiopica* (L.) Spreng., *Helleborus niger* L. and *Nicotiana rustica* L. (Pais and Neves, 1982-1983; Salopek-Sondi et al., 2002; Zavaleta-Mancera et al., 1999a). Conversely, as reported in Chapter 5, the cytokinin 6-benzylaminopurine (BAP) and gibberellin (GA_3) either exerted no effect or an initial delay to the colour change from yellow to green in the spathe tissue of *Zantedeschia* 'Best Gold'. Further to this, the magnitude of this delay was enhanced by the simultaneous application of BAP and GA_3. As revealed in Chapter 3, re-greening in spathe tissue of *Zantedeschia* 'Best Gold' was characterized by plastid redifferentiation from chromoplasts to chloroplasts, with a concomitant accumulation of chlorophyll and loss of carotenoids. Having identified BAP and GA_3 as affecting the change in colour of the spathe during re-greening, it remained unclear whether the treatment-induced delay in the colour change was associated with:

- a delay in chlorophyll accumulation,
- a retention or accumulation of carotenoid, and/or
- a retardation of chloroplast redifferentiation from chromoplast.

To answer these questions, in the current study, the influence of the treatments of BAP and/or GA_3 on pigment accumulation and plastid differentiation during spathe re-greening of 'Best Gold' was examined.

In angiosperms, chloroplast formation and chlorophyll biosynthesis during leaf greening are light-dependent processes (Armstrong and Apel, 1998; Armstrong et al., 1995; Biswal et al., 2003; Bolle et al., 1996; Vonwettstein et al., 1995). Without the presence of light, the reduction of protochlorophyllide to chlorophyllide, in the biosynthetic pathway of chlorophyll, can not proceed (Armstrong et al., 1995), and proplastids are unable to differentiate into functional chloroplasts during leaf greening (Biswal et al., 2003). Similarly, re-greening in plant tissue also involves chlorophyll biosynthesis and formation of functional chloroplasts (Mayfield and Huff, 1986; Tavares et al., 1998; Zavaleta-Mancera et al., 1999b). It is thus hypothesized that re-greening in spathe tissue of 'Best Gold' is similarly light-dependent, however, it is unclear to what degree re-greening is regulated by light; is the onset of chloroplast redifferentiation directly induced by light, or is it only the maturation of the functional chloroplast which requires the presence of light; is the net gain or loss of pigments (chlorophyll or carotenoid) along with chloroplast redifferentiation also regulated by light?

Chloroplast formation is not only regulated by light, but also influenced by plant hormones (e.g., cytokinins and gibberellins). The application of cytokinins promoted an initial development of chloroplasts in etiolated seedlings of *Arabidopsis thaliana* L. and cotyledons of *Lupinus luteus* L., but it was insufficient to fully override a light requirement for formation of a functional chloroplast (Chory et al.,

1994; Kusnetsov et al., 1994). This effect of cytokinins was enhanced in the presence of light. Furthermore, gibberellin was able to repress chloroplast development in *Pisum sativum* L. (pea) seedlings in darkness, and this repression could be overcome by light (Alabadi et al., 2004; Mathis et al., 1989). All these results suggested that there are some levels of cross talk between light, cytokinin and gibberellin in influencing the formation of chloroplasts.

By monitoring the changes in colour, pigment content and ultrastructure of spathe tissue of 'Best Gold', the objectives of the current study were, to determine:

1. the influence of BAP and GA_3 (applied alone or simultaneously) on changes in content of chlorophyll and carotenoid, and chloroplast redifferentiation during re-greening;
2. the effect of light/darkness on changes in content of chlorophyll and carotenoid, and chloroplast redifferentiation during re-greening;
3. the combination of light and hormone treatments (i.e. BAP and/or GA_3) on changes in content of chlorophyll and carotenoid, and chloroplast redifferentiation during re-greening.

6.2 Materials and Methods

In response to the treatments applied, monitoring the change in pigment content of spathe tissue was conducted in a separate experiment from that focussed upon the histological examination of ultrastructure in spathe tissue. In the following sections (i.e. 6.2.1 and 6.2.2), these two experiments are, therefore, presented separately.

6.2.1 Pigment accumulation

6.2.1.1 Plant materials and handling

The plant materials used in this study were flowers (spathe plus spadix and peduncle) of 'Best Gold'. They were produced under the same conditions and harvested at the same maturity, as described in Chapter 2 (refer section 2.2.1). Using a cork borer (diameter 14 mm), discs were excised from the central position of the spathe, with each experimental unit comprising eight randomly selected discs in a closed petri dish. The discs were placed on filter paper soaked in the relevant treatment solution, with the adaxial surface facing down in the closed petri dishes. The filter paper was kept moist throughout the experiment with the relevant treatment solution.

The experiment was conducted under controlled environmental conditions recommended for postharvest evaluation of vase life (Reid and Kofranek (1980): room temperature 20 ± 1 ºC, relative room humidity of 60 to 70%, 12-h (0600 HR – 1800 HR) photoperiod at a light intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height provided by cool-white fluorescent tubes.

6.2.1.2 Treatments and experimental design

The experiment was conducted as a split-plot design with light or dark treatment as the main plot, and hormone treatment solution and sampling time as sub plots. The four hormone treatment solutions comprised: control (RO water), GA_3 10^{-4} M, BAP 10^{-4} M, or GA_3+BAP with both at 10^{-4} M in the final treatment solution. The four sampling times were: 0, 7, 10 or 14 days after horticultural harvest-maturity.

The dark treatment involved keeping the discs of plant material in a 60 cm × 40 cm plastic box, which was wrapped with aluminium foil, while the light treatment comprised a 12-h (0600 HR – 1800 HR) photoperiod at a light intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height, provided by cool-white fluorescent tubes. To reduce the amount of plant material required for this study, as there was no treatment effect expected at day 0, the dark treatment was not applied at this sampling time. In total, therefore, there were 28 individual treatment combinations, with five experimental replicates (i.e. petri dishes).

6.2.1.3 Colour measurement

Lightness (L*), chroma (C*) and hue angle (H°) of both the abaxial and adaxial surfaces of discs (4 out of 8 discs per sample) were recorded at each sampling time using a tristimulus colorimeter CM-2600d/2500d (Konica Minolta, Japan). The discs were then dissected with a scalpel into abaxial and adaxial layers via cutting through the middle parenchyma tissue. The replicate samples for each surface were immediately stored at -20 $^{\circ}$C, pending dehydration using a Hetosicc freeze dryer (Heto Ltd., Denmark). The freeze-dry samples were stored at -20 $^{\circ}$C pending pigment analysis.

6.2.1.4 Pigment analysis

Pigment extraction and quantification was conducted following the methodology of Lewis et al. (2003) with some modification (details refer Chapter 3 Section 3.2.4), wherein the freeze-dried tissue were extracted in acetone: methanol

(7:3; v:v). The supernatant was combined and dried under a stream of O_2-free N_2. The extract was then resuspended in ethyl acetate and kept at -20 $^{\circ}$C pending pigment identification and quantification by high performance liquid chromatography (HPLC; refer Chapter 3 Section 3.2.4).

6.2.1.5 Data analysis

Data were tested initially to ensure they met the requirement for ANOVA using the general linear procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Where significant ($P < 0.05$) treatment effects were detected, means were separated by using the unrestricted LSD procedure.

6.2.2 Histology

6.2.2.1 Plant materials and experimental design

The plant materials used in this study were as described above for analysis of pigment (Section 6.2.1.1), and in Chapter 2, i.e. spathes of 'Best Gold' attained at horticultural harvest-maturity (refer section 2.2.1). A total of 224 discs were collected and allocated at random in batches of four to a petri dish. Each petri dish was treated as an experimental unit. The treatment and experimental design were the same as described in section 6.2.1.2, with the exception that there were only two replicates per treatment.

6.2.2.2 Colour and histological procedures

L*, C* and H° of both the abaxial and adaxial surfaces of the discs were measured using a tristimulus colorimeter CM-2600d/2500d (Konica Minolta, Japan), before being processed for ultrastructural observation using both light microscopy and transmission electron microscopy (TEM).

The samples were fixed, postfixed, dehydrated and then embedded in resin following the methodology of Gronegress (1974) with some modification (details refer Chapter 3 Section 3.2.5). Examination of the specimens was conducted using a compound light microscope (Zeiss, Germany) with a Leica DFC 320 digital camera attached (Leica Microsystems, Wetzlar, Germany) and a Philips CM10 transmission electron microscope (Philips, The Netherlands). Ultrastructural observation was performed on plastids from within both abaxial and adaxial surfaces of spathe tissue (targeted primarily within the first five cell layers of the subepidermis), at day 0, 7, 10 and 14. To assess treatment effects on chloroplast redifferentiation during re-greening, the ultrastructural analysis at each sampling time primarily comprised qualitative assessment of thylakoid reconstruction and changes in plastoglobuli.

6.3 Results

6.3.1 Colour changes

Colour measurement was used to provide "points of reference" to determine whether the state of spathe tissue at harvest and its pattern of re-greening were consistent between the two experiments of pigment accumulation (i.e. Section 6.2.1) and histology (i.e. Section 6.2.2). The values of colour coordinates (i.e., L*, C* and H°)

were not significantly different in these two experiments, allowing the results from these two experiments to be combined. Hence, only the data of colour coordinates relating to the experiment of pigment accumulation are presented here.

In the presence of light the abaxial surface of discs, treated with GA_3+BAP, showed a delay of approximately five days as to the time when re-greening was visible (i.e., H° reached the value of 90°), compared with the control treatment (Figure 6.1 E). The discs treated with GA_3 alone showed a similar, but smaller delay in colour change (by approximately three days). BAP alone however, did not affect the time when re-greening was visible. Compared to the control, the BAP-treated discs exhibited no difference ($P > 0.05$) in the values of L*, C* and H° throughout the 14-day period, except that at day 14, H° of the BAP-treated discs was higher than that of the control, resulting in discs appearing more green in colour than the control ($P < 0.05$; Figure 6.1 A, C & E). By day 14, the abaxial surface of all discs had re-greened, with those from the BAP treatment appearing the most intense green colour.

With exception of BAP-treated discs, in the presence of light re-greening was not observed on the adaxial surface within the 14-day period of observation. The treatment of GA_3+BAP or GA_3 alone retained the yellow colouration of this adaxial surface for about 10 days, followed by a reduction in the value of C* and an increase in H° (Figure 6.1 D & F). Throughout the 14-day period, there was no obvious change in the values of L* for the adaxial surface in all treatments, apart from a decrease from day 10 to 14 in the control (Figure 6.1 B). On day 14, BAP-treated discs had a higher value for H° than any other treatment (Figure 6.1 F).

In the absence of light, re-greening was not observed on either the abaxial nor adaxial surfaces of the discs, regardless of hormone treatment. Both surfaces remained yellow throughout the 14-day period of observation. Consequently, values of H° of both surfaces of the discs in the dark treatment varied within a narrower range (84° to 88°; Figure 6.2 E & F), as compared with the discs kept in the light (i.e. 84° to 108°; Figure 6.1 E & F). The values of L* for the two surfaces from the treatments with GA_3 and/or BAP in darkness were constant within the 14-day period, while values for the control discs decreased after day 10 ($P < 0.05$; Figure 6.2 A & B). For both surfaces, values of C* showed a decrease over the 14-day period, with the least change occurring in GA_3+BAP-treated discs, and the largest change in the control discs (Figure 6.2 E & F).

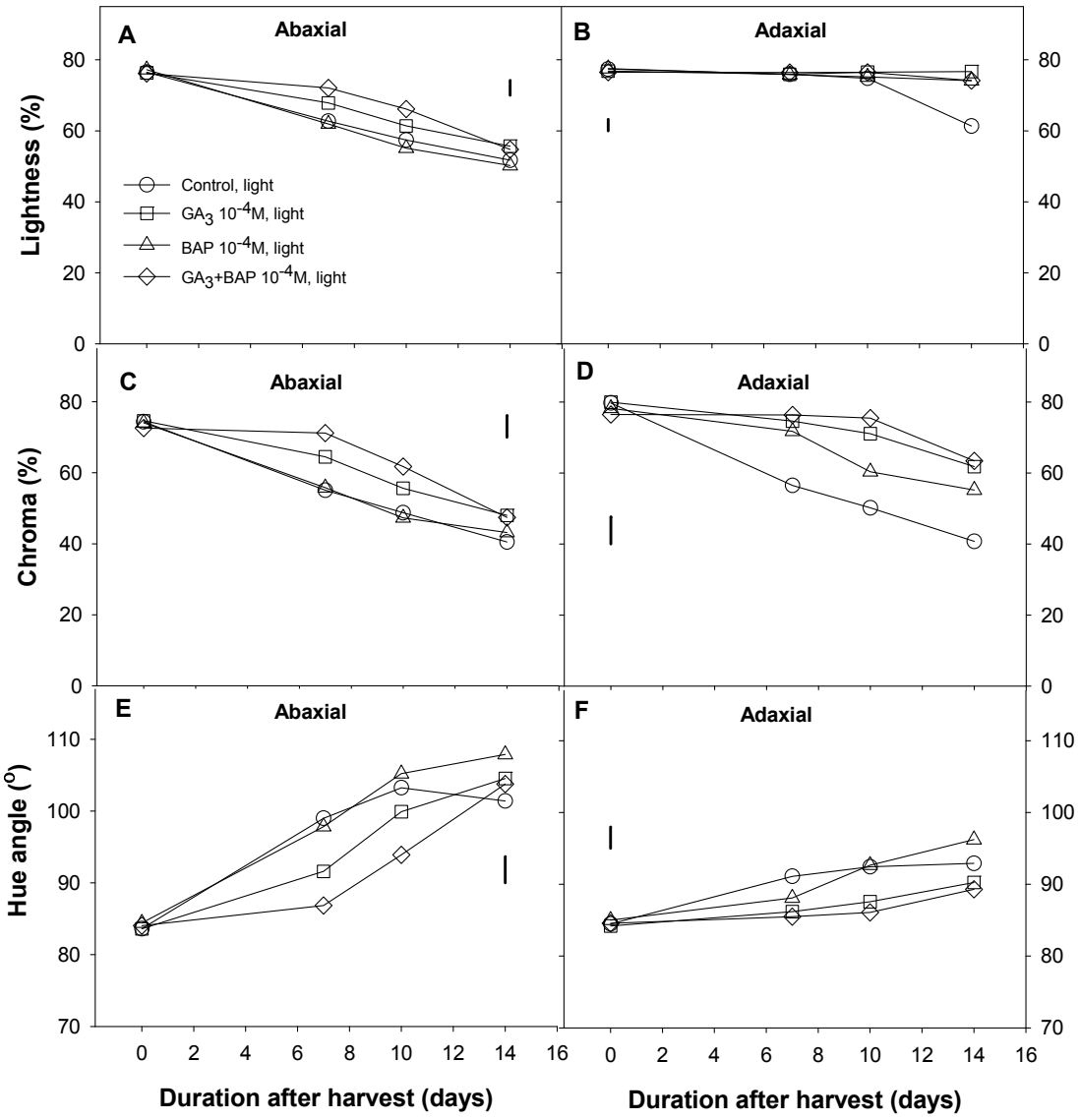

Figure 6.1 Colour coordinates of lightness (A & B), chroma (C & D) and hue angle (E & F) of both abaxial (A, C & E) and adaxial (B, D & F) surfaces of *Zantedeschia* 'Best Gold' spathe tissue, treated with BAP, GA_3 or GA_3+BAP in light. Controls were held in RO water. Each point is the mean of 5 replicates. Vertical line bars are the LSD at $P < 0.05$.

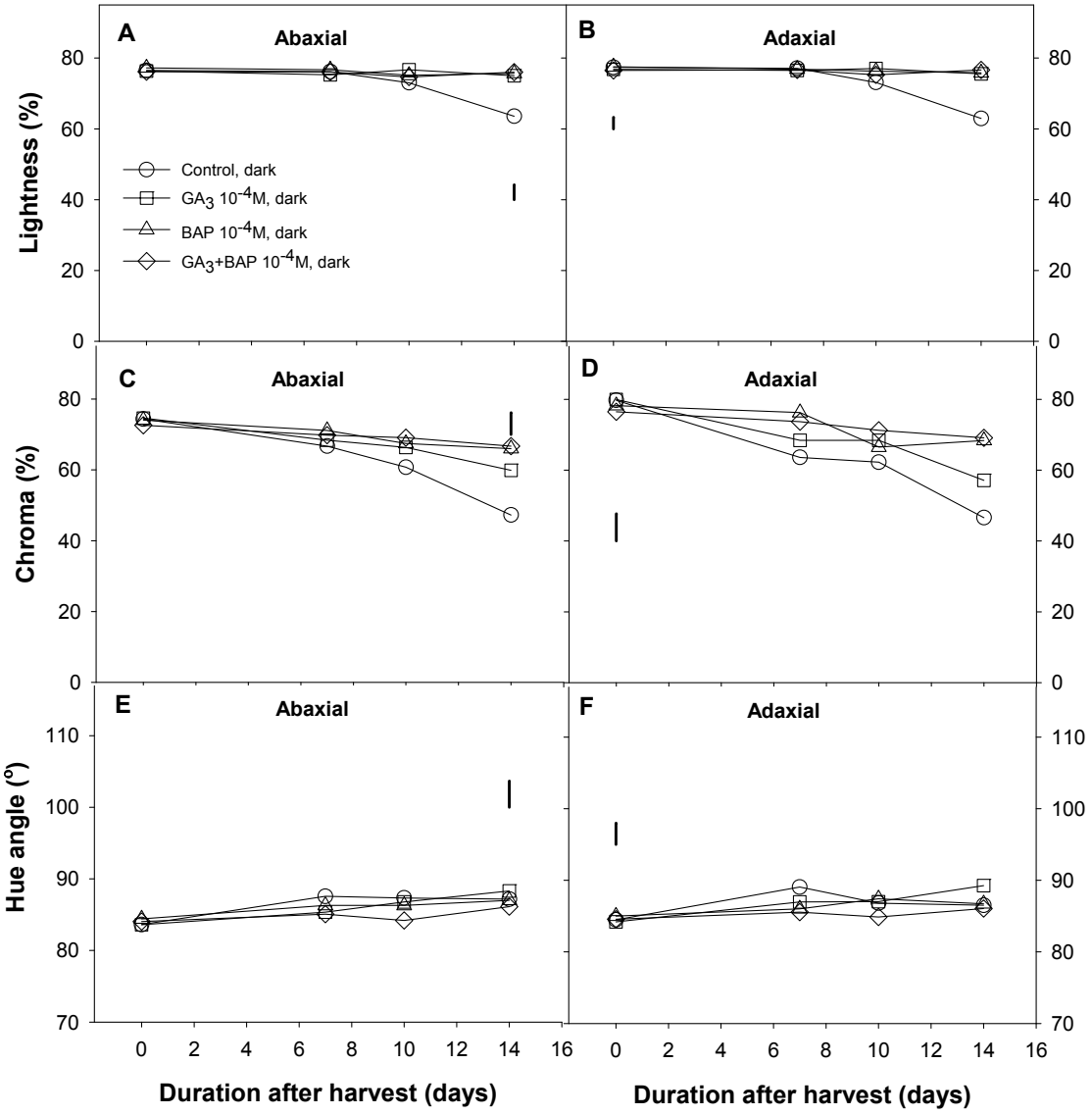

Figure 6.2 Colour coordinates of lightness (A & B), chroma (C & D) and hue angle (E & F) of both abaxial (A, C & E) and adaxial (B, D & F) surfaces of *Zantedeschia* 'Best Gold' spathe tissue, treated with BAP, GA_3 or GA_3+BAP in darkness. Controls were held in RO water. Each point is the mean of 5 replicates. Vertical line bars are the LSD at $P < 0.05$.

6.3.2 Changes of pigment content in light

6.3.2.1 Abaxial surface

In the presence of light, over the 14-day period of observation an increase in the content of both chlorophyll *a* (from 0 to 2492 $\mu g \cdot g^{-1}$ dry weight (DW)) and chlorophyll *b* (from 0 to 605 $\mu g \cdot g^{-1}$ DW) was found in the abaxial surface of discs within the control treatment ($P < 0.05$; Figure 6.3 A & B). When treated with BAP in the light, the abaxial surface showed no difference in the content of both chlorophyll *a* and *b* from that found in control at both day 7 and day 10 ($P > 0.05$). By day 14 however, discs treated with BAP showed a 1.3- to 1.5-fold higher content of both chlorophyll *a* and *b* than that of the control ($P < 0.05$).

Compared to the control, the GA_3-treated discs exhibited a delay in the accumulation of chlorophyll in the abaxial surface when under light. On both day 7 and day 10, the contents of both chlorophyll *a* and *b* in the GA_3-treated discs were about 1.2- to 1.3-fold less than that of the control (Figure 6.3 A & B). By day 14, however, the content of both chlorophyll *a* and *b* in the GA_3-treated discs exceeded that of the control.

When treated with BAP+GA_3, the abaxial surface of the discs showed a 2- to 3-fold lower content of both chlorophyll *a* and *b* than that of the control, at both day 7 and 10 (Figure 6.3 A & B). By day 14 however, their chlorophyll content was similar to that for the control, but was less than that from those treated with GA_3 or BAP ($P < 0.05$).

In the presence of light, the content of total carotenoid in the abaxial surface of control discs showed a slight decrease from 1336 to 1199 $\mu g \cdot g^{-1}$ DW over the 14-day period of observation ($P > 0.05$; Figure 6.3 H). In these discs, except for an increase in the content of the neoxanthin from 84 to 155 $\mu g \cdot g^{-1}$ DW ($P < 0.05$), all other carotenoids either showed a decrease or no discernible change in their content (Figure 6.3 C, D, E, F & G). The most abundant carotenoid, i.e. lutein, had no significant change, varying between 562 and 590 $\mu g \cdot g^{-1}$ DW throughout the 14-day period ($P > 0.05$).

When treated with BAP in light, the abaxial surface of the discs showed an increase in the content of all individual carotenoids by 1.3- to 1.7-fold within the first week of observation ($P < 0.05$; Figure 6.3 C, D, E, F & G). At all times after harvest, a higher content of all carotenoids was found in the BAP-treated discs than that occurring in the control ($P < 0.05$). Compared with other treatments, the abaxial surface of the BAP-treated discs had the highest content of neoxanthin at all times after harvest. The abaxial surface of the GA_3-treated discs showed a similar but less marked change in the content of carotenoids to those treated with BAP (Figure 6.3 C, D, E, F, G & H). An increase in the content of carotenoids (particularly lutein; $P < 0.05$) was also noted in the first week of observation (Figure 6.3 F & G). Compared to other treatments, a 1.2- to 2.2-fold higher content of β-carotene and *cis*-β-carotene were noted in the abaxial surface of the GA_3+BAP-treated discs at day 7 and day 10 ($P < 0.05$; Figure 6.3 F & G). No difference in the content of violaxanthin and lutein was found between the BAP-treated and GA_3+BAP-treated discs over the 14-day period of observation ($P > 0.05$; Figure 6.3 D & E). The progressive increase in the

content of neoxanthin was comparable between the GA_3+BAP-treated and discs from the control treatment (Figure 6.3 C).

6.3.2.2 Adaxial surface

For the adaxial surface, in the presence of light, chlorophyll was not detected except for those treated with BAP, which increased in chlorophyll *a* over the 14-day period of observation from 0 to 371 $\mu g \cdot g^{-1}$ DW ($P < 0.05$; Figure 6.4 A). The magnitude of increase, however, was notably less than that found in the abaxial surface of the BAP-treated discs (from 0 to 3345 $\mu g \cdot g^{-1}$ DW; Figure 6.3 A). There was no chlorophyll *b* detected on the adaxial surface in any treatment.

For the adaxial surface, the content of total carotenoid at day 0 (i.e. 1369 $\mu g \cdot g^{-1}$ DW; Figure 6.4 H) was similar to that of the abaxial surface (Figure 6.3 H). In the presence of light the content of the total carotenoid in the adaxial surface decreased over the 14-day period of observation by 30%, which was greater than that encountered in the abaxial surface (i.e. 10%). For the individual carotenoids, neoxanthin and lutein, in the adaxial surface, the contents were maintained at similar levels over the 14-day period, while other carotenoids showed a decrease ($P < 0.05$; Figure 6.4). The content of α-carotene was insignificant compared with other carotenoids, but was still detectable on the adaxial surface by day 14. In contrast, for the abaxial surface, α-carotene was only detected at day 0.

When treated with BAP and/or GA_3 in light, no increase in the content of carotenoids was noted on the adaxial surface of the discs during the first week, unlike that occurring on the abaxial surface ($P > 0.05$; Figure 6.4). The treatment with

hormones, particularly BAP+GA_3 however, retained the carotenoid content in the adaxial surface over the 14-day period of observation, in contrast with a decrease in the content of all carotenoids evident in the control treatment.

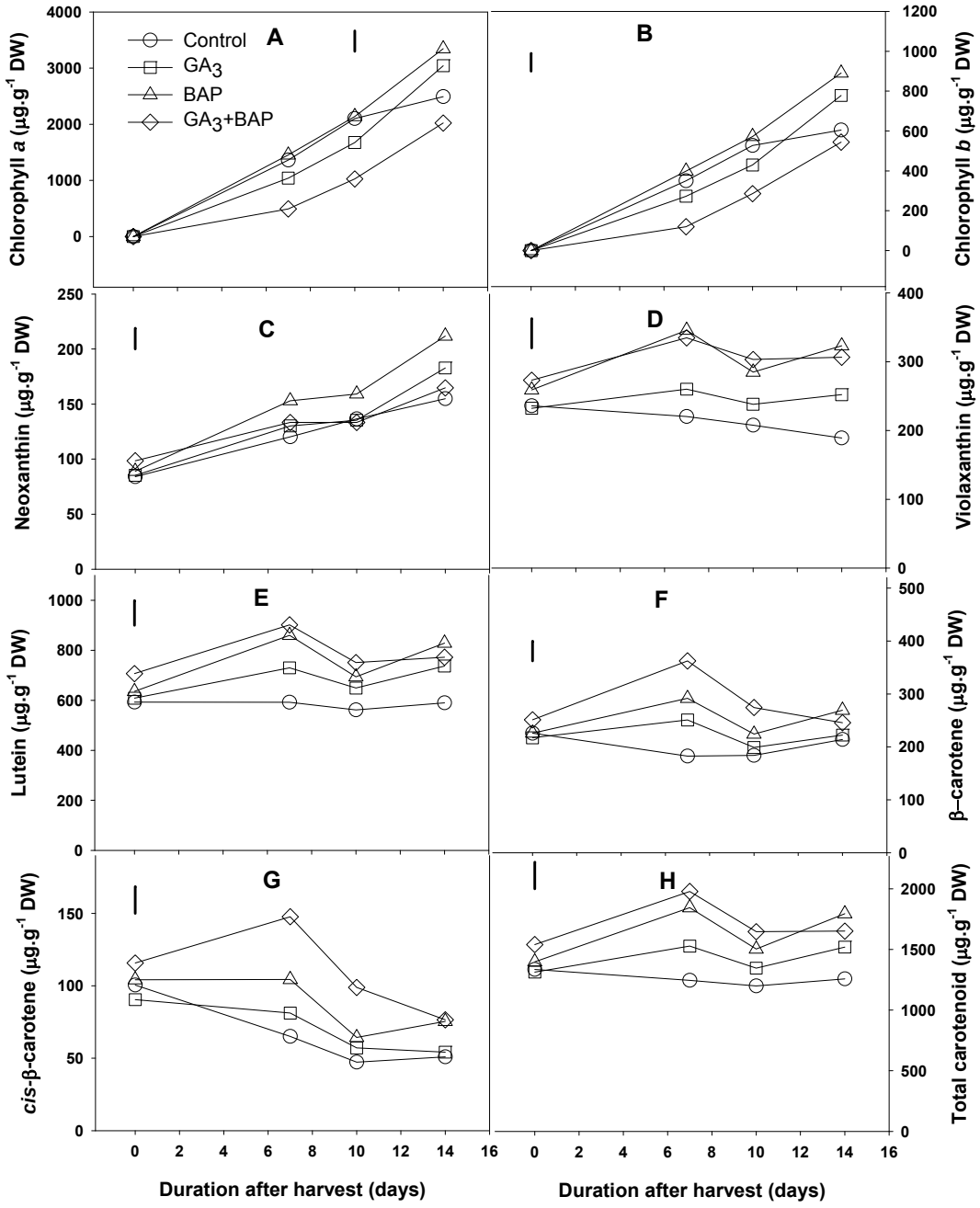

Figure 6.3 Content of chlorophylls and carotenoids in the ABAXIAL surface of the spathe of *Zantedeschia* 'Best Gold', treated with BAP, GA_3 or GA_3+BAP in light over 14 days after horticultural harvest-maturity. Controls were kept in RO water. Each point is the mean of five replicates. Vertical line bars represent the LSD at $P < 0.05$.

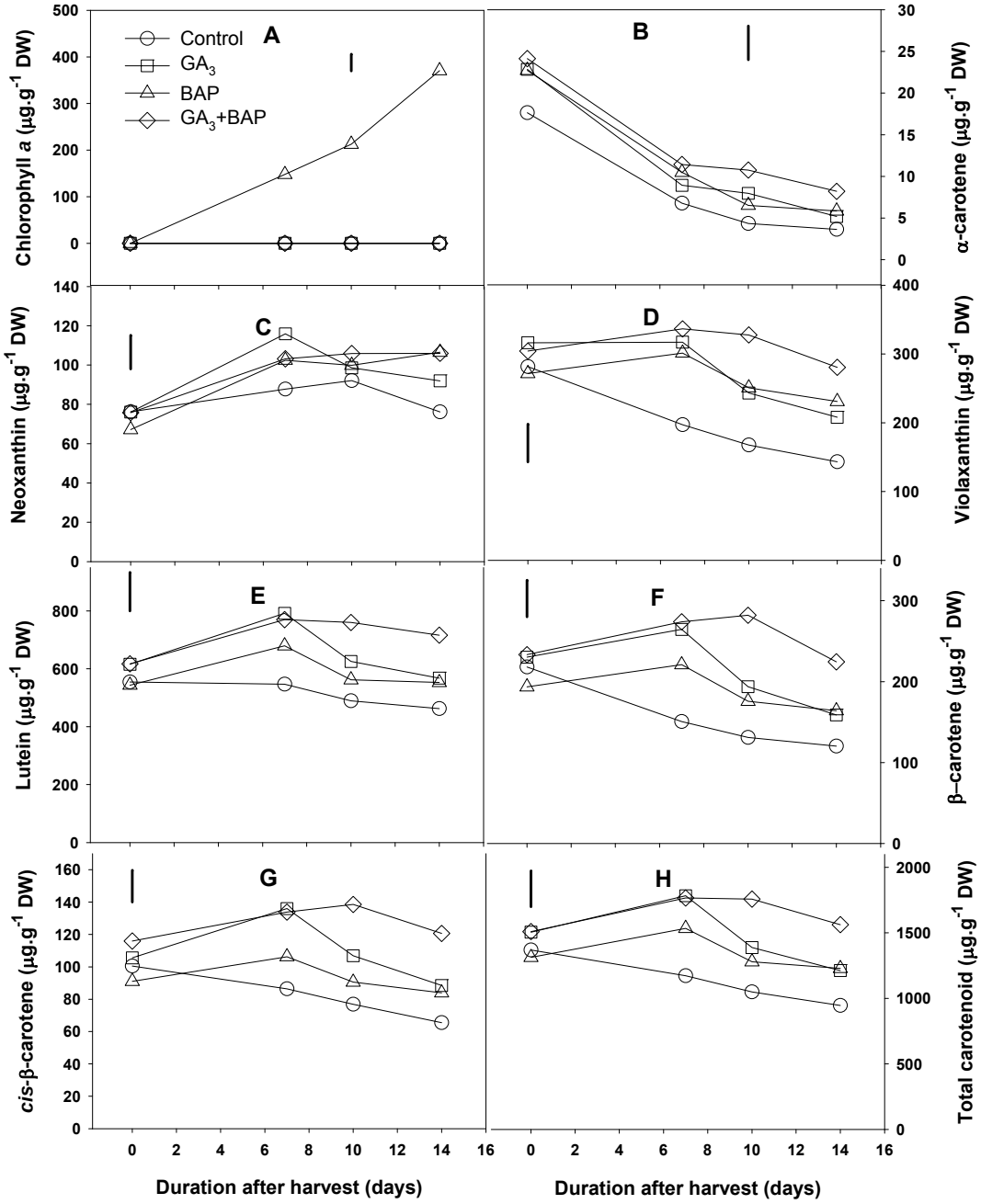

Figure 6.4 Content of chlorophyll *a* and carotenoids in the ADAXIAL surface of the spathe of *Zantedeschia* 'Best Gold', treated with BAP, GA_3 or GA_3+BAP in light over 14 days after horticultural harvest-maturity. Controls were kept in RO water. Each point is the mean of five replicates. Vertical line bars represent the LSD at $P < 0.05$.

6.3.3 Changes of pigment content in darkness

6.3.3.1 Abaxial surface

In the absence of light, chlorophyll was not detected in the abaxial surface of the discs, regardless of the hormone treatment applied. In contrast, carotenoids were detected in this surface of the discs throughout the 14-day period of darkness. During this period, the content of total carotenoid declined from 1336 to 770 $\mu g \cdot g^{-1}$ DW ($P < 0.05$; Figure 6.5 F), with this primarily being the result of a decrease in the content of the three major carotenoids, i.e., lutein, β-carotene and violaxanthin by 41%, 45% and 57% of initial values, respectively (Figure 6.5 B, C & D). In the absence of light, the treatment of BAP or BAP+GA_3 retained the content of carotenoids in the abaxial surface, showing a higher content of individual carotenoids than that of the control at all sampling times subsequent to harvest ($P < 0.05$; Figure 6.5). Compared with the discs held in the light, in darkness the abaxial surface of the discs contained lower levels of individual carotenoids (except for *cis*-β-carotene) at all sampling times subsequent to harvest, regardless of hormone treatment ($P < 0.05$; Figure 6.3; Figure 6.5).

6.3.3.2 Adaxial surface

For the adaxial surface of the discs kept in darkness, no chlorophyll was detected, regardless of hormone treatment. The change in the content of carotenoids for the adaxial surface was similar to that determined in light (data not shown).

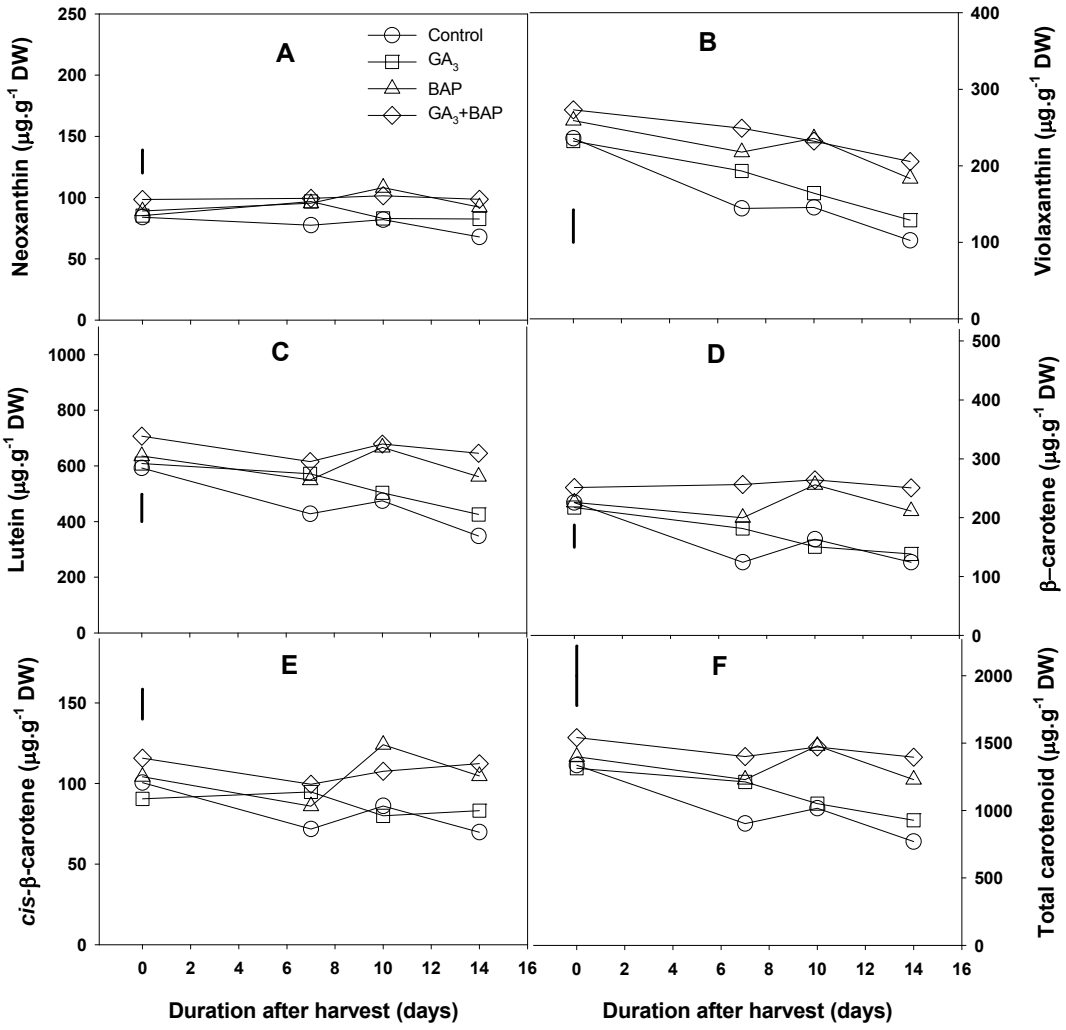

Figure 6.5 Content of carotenoids on the ABAXIAL surface of spathes of *Zantedeschia* 'Best Gold', treated with BAP and/or GA_3 in darkness over 14 days after horticultural harvest-maturity. Controls were kept in RO water. Each point is the mean of five replicates. Vertical line bars are the LSD at $P < 0.05$.

6.3.4 Plastid differentiation in light

6.3.4.1 Control treatment

At horticultural harvest-maturity (day 0), the subepidermal cells in both the abaxial and adaxial surfaces of the spathe were characterized by the presence of rounded-, oval- or shoe-shaped chromoplasts (Figure 6.6). These chromoplasts were surrounded by double-membrane envelopes, and were mainly occupied by large, irregularly shaped, and high electron-dense, plastoglobuli. Infrequently in some chromoplasts, starch granules were noted. At this stage of maturity, the chromoplasts also contained primary-thylakoids which typically appeared to be either the remnants of the thylakoid system remaining from chloroplasts present at the green-bud stage, or newly formed thylakoids. Four types of these primary-thylakoids were evident:

type-1, flattened, double-membrane lamellae (membrane sheets), that flowed around the plastoglobuli or were positioned along the inner-envelope membrane (Figure 6.6 B & D, solid arrow);

type-2, swollen membrane thylakoids, that appeared to be in a state of flux with the plastoglobuli, as evident by either being clustered around the plastoglobuli or frequently attached to the inner-envelope membrane (Figure 6.6 A, B & D, dashed arrow);

type-3, vacuole-like, single-membrane-bound bodies, that sometimes were irregularly shaped or roundish, occurring in various sizes, which were embedded in the stroma and showed close connection with the plastoglobuli and inner-envelope membrane (Figure 6.6 B & C, arrow head);

type-4, clusters of membrane fragments, that appear to be remnants of granal thylakoids. These were often observed to be in the close vicinity of either the inner-envelope membrane or type-3 thylakoids (Figure 6.6 B; Figure 6.10, block arrow).

As re-greening progressed in the presence of light, chromoplasts in the subepidermal cell layers of the abaxial surface showed signs of redifferentiation to chloroplasts. For the control treatment at day 7, the cells were distinguished by plastids at various intermediate stages between chromoplast and chloroplast. The initial stage of the chloroplast redifferentiation was characterized by an increase in double-membrane thylakoids (type-1), forming along the inner-envelope membrane (Figure 6.7 A, solid arrow). As the chromoplast lengthened and developed into a chloroplast, these thylakoids gradually stacked together and were arranged in parallel. Compared with day 0, the number and size of the plastoglobuli had declined at day 7, as the thylakoid membranes increased (data not presented). During plastid redifferentiation, the plastoglobuli remained in close proximity to the thylakoids. At this stage of plastid development, type-3 thylakoids were observed only occasionally.

As the abaxial surface of discs continued to re-green, the plastids became lengthened and differentiated into a more advanced stage of chloroplast. At day 10, more type-1 thylakoids accumulated in parallel, and were stacked to form grana (Figure 6.7 B). The grana were also visibly interconnected by the stroma thylakoids. At this stage of development, the high electron-dense plastoglobuli still persisted, and were arranged in clusters.

By day 14, when the abaxial surface had re-greened even further, most of the plastids had developed into well-organized, lense-shaped chloroplasts (Figure 6.7 C). At this time these chloroplasts (approximately 12 μm^2 in size) were bigger than the chromoplasts evident at day 0 (approximately 5 μm^2). The grana were well stacked and had more layers than those in day 10. They were interconnected by stroma thylakoids to form a comprehensive membrane system. Large starch granules were frequently observed at this latter stage of assessment. The plastoglobuli were small and round-shaped and, rather than clustered together, they were scattered within the plastids. These plastoglobuli remained in close proximity to the new thylakoids. The stroma was less dense in comparison with the chloroplasts at day 10. The double membranes of the plastids remained intact as chromoplasts differentiated into mature chloroplasts.

For the adaxial surface, within the 14 days of observation, no plastid transition from chromoplast to chloroplast was observed in discs from the control treatment under light. The plastids remained as chromoplasts throughout this period, though at day 10 and day 14, the chromoplasts contained less dense stroma than those evident on day 0 (Figure 6.8 A, B & C). While being hexagonal-shaped on day 7, the plastoglobuli were more spherical in shape on day 10 and day 14. Some primary thylakoids, e.g. type-1 (solid arrow; Figure 6.8 B) and type-2 (dash arrow; Figure 6.8 A & C) were also observed in these chromoplasts on days 7, 10, and 14.

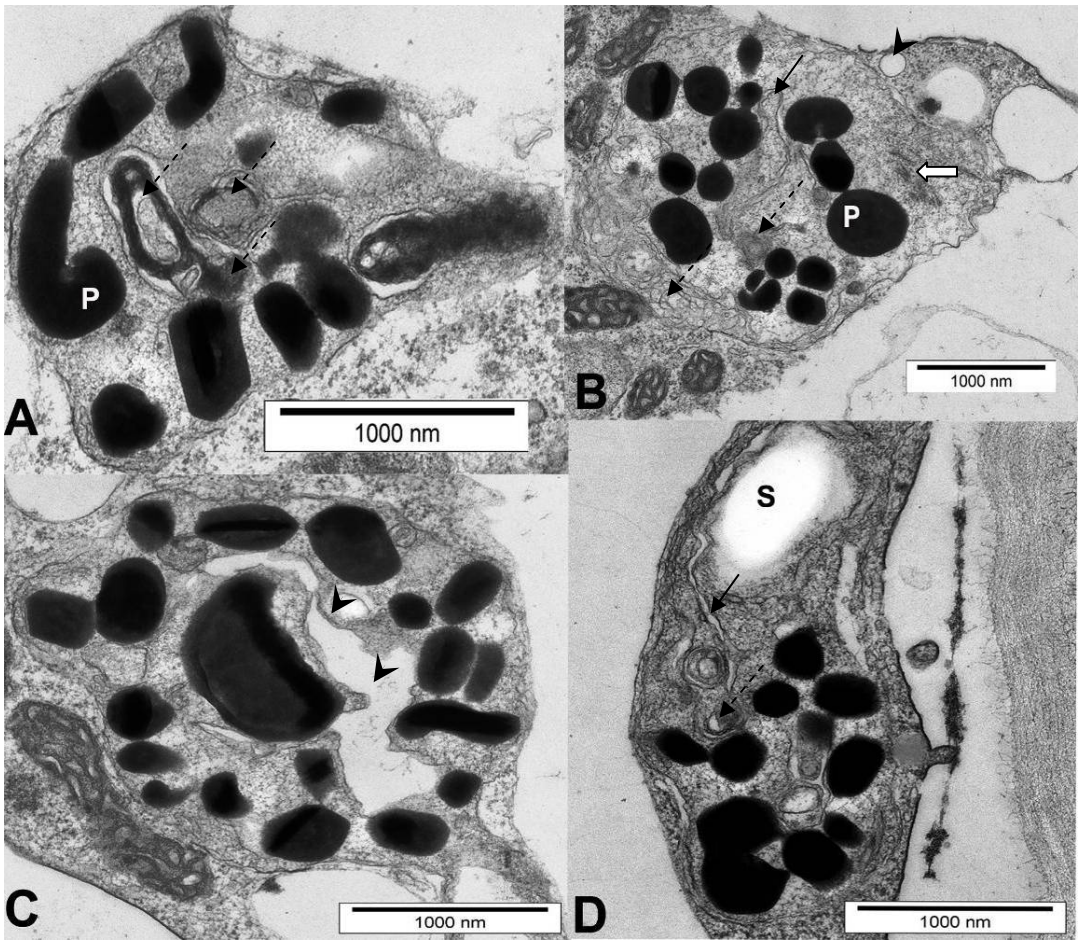

Figure 6.6 Transmission electron micrographs of plastids in subepidermal cell layers of abaxial surface of *Zantedeschia* 'Best Gold' at horticultural harvest-maturity (day 0). A) An oval-shaped chromoplast showing large, irregular size of plastoglobuli and swollen (type-2) thylakoids (dashed arrow); B) round-shaped chromoplast showing both flattened-double-membrane (type-1) thylakoids (solid arrow) and type-4 thylakoid remnants (block arrow); C) round-shaped chromoplast showing a single-membrane-bound body (type-3 thylakoid; arrow head); D) shoe-shaped chromoplast showing the development of both type-1 and type-2 thylakoid membrane. P, plastoglobuli; S, starch grain.

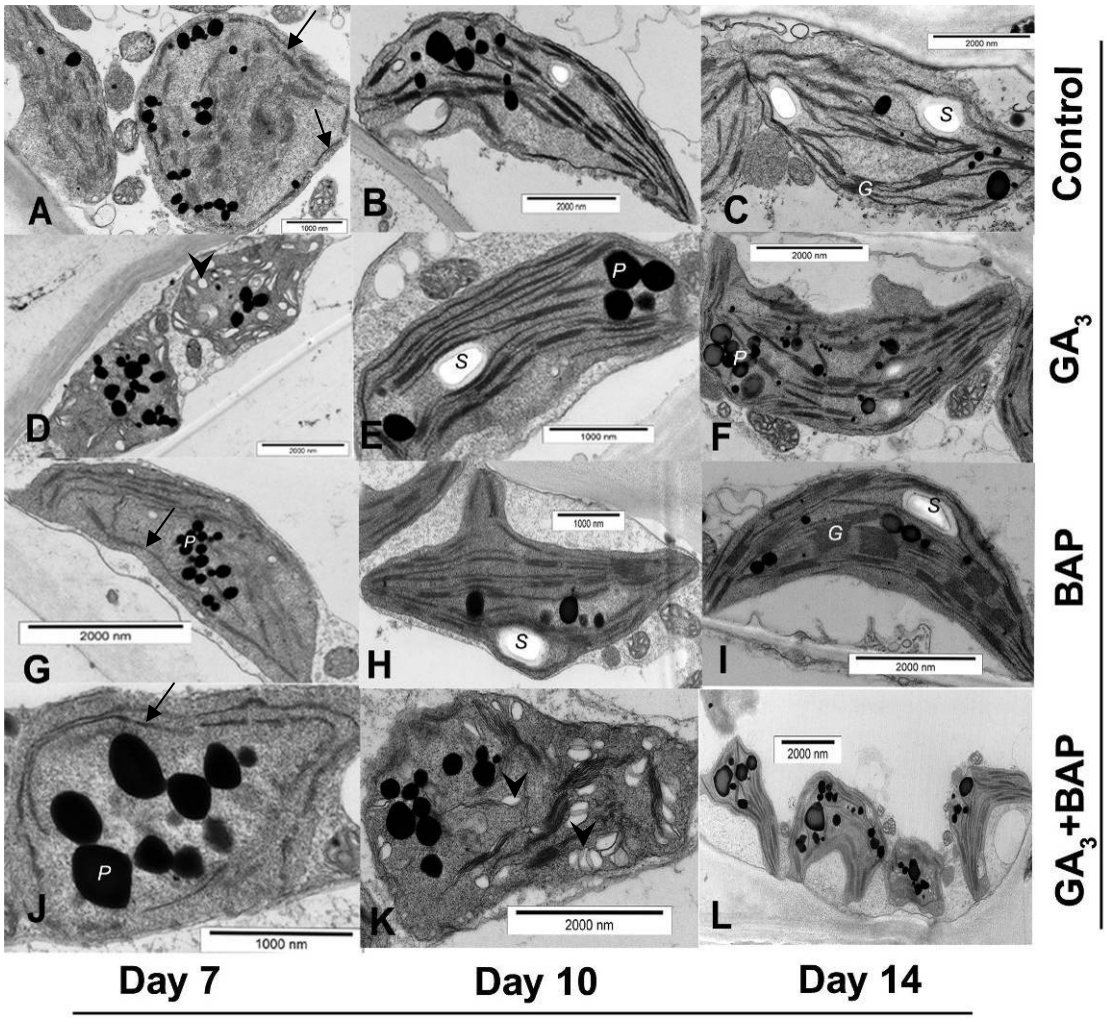

Figure 6.7 Influence of GA_3 and/or BAP in light on the differentiation of plastids within the abaxial surface of spathes in *Zantedeschia* 'Best Gold'. A) day 7, control: chromoplast to chloroplast transition indicated by the various intermediate stages of plastids between chromoplasts and chloroplasts; B) day 10, control: a more developed chloroplast containing some grana stacking; C) day 14, control: a fully developed chloroplast with well-organized thylakoid system; D) day 7, GA_3: plastids mainly occupied by type-3 thylakoids (arrow head) and plastoglobuli; E) day 10, GA_3: similar to B; F) day 14, GA_3: a fully developed chloroplast; G) day 7, BAP: a developing chloroplast characterized by the formation of type-1 thylakoids (solid arrow), type-3 thylakoids and clustered plastoglobuli; H) day 10, BAP: similar to B and E; I) day 14, BAP: a fully developed chloroplast with an intensive thylakoid membrane system; J) day 7, GA_3+BAP: chromoplasts showing an initial stage of chloroplast differentiation with formation of type-1 thylakoids (solid arrow) and retention of plastoglobuli; K) day 10, GA_3+BAP: chromoplast with a decline in plastoglobuli and increase in type-1 and type-2 thylakoids; L) day 14, GA_3+BAP: various stages of plastids between chromoplasts and chloroplasts. *P*, plastoglobuli; *G*, grana; *S*, starch.

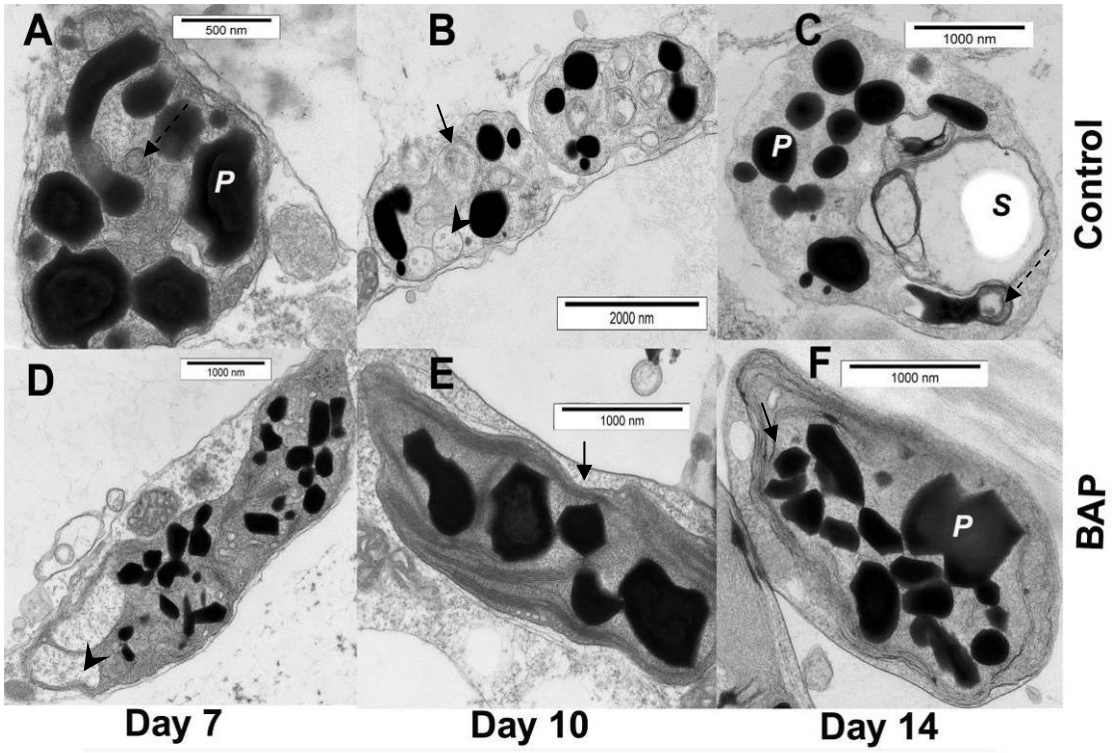

Figure 6.8 Influence of BAP in light on the differentiation of plastids within the adaxial surface of spathes in *Zantedeschia* 'Best Gold'. A) day 7, control: a typical chromoplast containing large and irregular-shaped plastoglobuli; B) day 10, control: chromoplast; C) day 14, control: chromoplast; D) day 7, BAP: elongated chromoplast; E) day 10, BAP: initial conversion of chromoplast to chloroplast showing the formation of thylakoids (type-1) and grana stacking; F) day 14, BAP: plastids showing less thylakoids than those at day 10, BAP. *P*, plastoglobuli; *G*, grana.

6.3.4.2 Hormone treatment effects

In the subepidermal cells of the abaxial surface, by the last sampling time the application of BAP in the presence of light resulted in more developed chloroplasts, compared to those evident in the control treatment. On both day 7 and 10, there was no conspicuous difference in the developmental stages of the plastids between the BAP-treated discs and the control (Figure 6.7 G & H). In contrast, by day 14, the BAP-treated discs had more organized and complete chloroplasts than those evident within the control (Figure 6.7 I). These chloroplasts were crescent-shaped. They contained greater regions of grana, and more condensed stroma, than those of the control. For the adaxial surface of the discs, when treated with BAP in light, the formation of type-1 thylakoids and, in some places, grana stacking were observed (Figure 6.8 E & F). These thylakoids were arranged along the membrane of the inner-envelope and, around the plastoglobuli. The plastoglobuli were large and hexagonal-shaped.

When treated with GA_3 in light, at day 7 the chloroplasts in the abaxial surface were less developed than those in both the control and BAP treatments. Indicative of the initial stage of redifferentiation to chloroplasts, these GA_3-treated plastids were oval-shaped and were occupied by a large number of type-3 thylakoids and plastoglobuli (Figure 6.7 D). The type-3 thylakoids were commonly found along the inner-envelope membrane, close to the plastoglobuli, or among the developing type-1 thylakoids. In some regions of the plastid contents, the type-3 thylakoids elongated to a tubular shape, and appeared to be fused with the type-1 thylakoids, forming grana (refer arrow within enlarged image in Figure 6.10 F). By day 10, the developmental

stage of the chloroplasts in the GA_3-treated discs was similar to that found in the control treatment (Figure 6.7 E). On day 14, the GA_3-treated discs contained a greater number of thylakoids and a denser stroma than that evident in the control treatment (Figure 6.7 F). Plastoglobuli were still present in the plastids, but were less electron-dense, as compared with those evident at earlier sampling times, e.g. at day 7 and day 10. For the adaxial surface, the application of GA_3 in light did not influence plastid differentiation, and the plastids remained as chromoplasts throughout the 14-day period of observation (data not shown).

When treated with GA_3 and BAP simultaneously in light, chloroplast redifferentiation in the abaxial surface of the discs was further delayed, as compared with those treated with GA_3 alone. At day 7, the plastids in the abaxial surface of the BAP+GA_3 treated discs were oval–shaped, and were mainly occupied by the large and electron-dense plastoglobuli (Figure 6.7 J). A few type-1 thylakoids were observed along the inner-envelope membrane, but were less evident than those in the control, BAP- or GA_3-treated discs. Type-3 thylakoids were not observed in GA_3+BAP treated discs until day 10 (Figure 6.7 K). Similar to those observed in GA_3-treated discs, these type-3 thylakoids were grouped, and in close contact with the partially stacked thylakoids. By day 14, various shaped plastids with different levels of thylakoid stacking appeared in the abaxial surface (Figure 6.7 L). During the plastid transition from chromoplast to chloroplast the progressive decrease in the regions of the plastid occupied by the plastoglobuli was coincident with the increase in the number and thickness of thylakoids. Similar to those evident when discs were treated with BAP or GA_3 alone, some of these plastoglobuli were less electron-dense

than those at day 7 and 10. For the adaxial surface, like the control or treatment with GA_3 alone, GA_3+BAP did not alter plastid differentiation, and the plastids remained as chromoplasts throughout the 14-day period of observation (data not shown).

6.3.5 Plastid differentiation in darkness

6.3.5.1 Control treatment

In the absence of light, chromoplasts in the subepidermal cell layers of the abaxial surface of discs in the control treatment showed only the initial signs of differentiation into chloroplasts, i.e. the appearance of primary thylakoids. They were mainly type-1 thylakoids with long double-membrane lamella, and were either positioned along the inner-envelope membrane or near plastoglobuli (Figure 6.9 A & B, solid arrow). Occasionally, a continuum was observed between these developing thylakoids with the inner-envelope membrane (Figure 6.10 B, dashed arrow), and also the plastoglobuli (Figure 6.10 A, solid arrow). The plastids were rounded or oval-shaped, and mainly occupied by the plastoglobuli. These plastoglobuli appeared hexagonal or spherical in shape and varied in size. They were evident throughout the plastids, and were inter-connected to each other through direct adhesion or some tubular extension (Figure 6.10 A & B, arrow head). At this stage of development the characteristics of the various organelles within the plastids were similar to those found in the discs treated with GA_3+BAP for seven days in light (Figure 6.7 J)

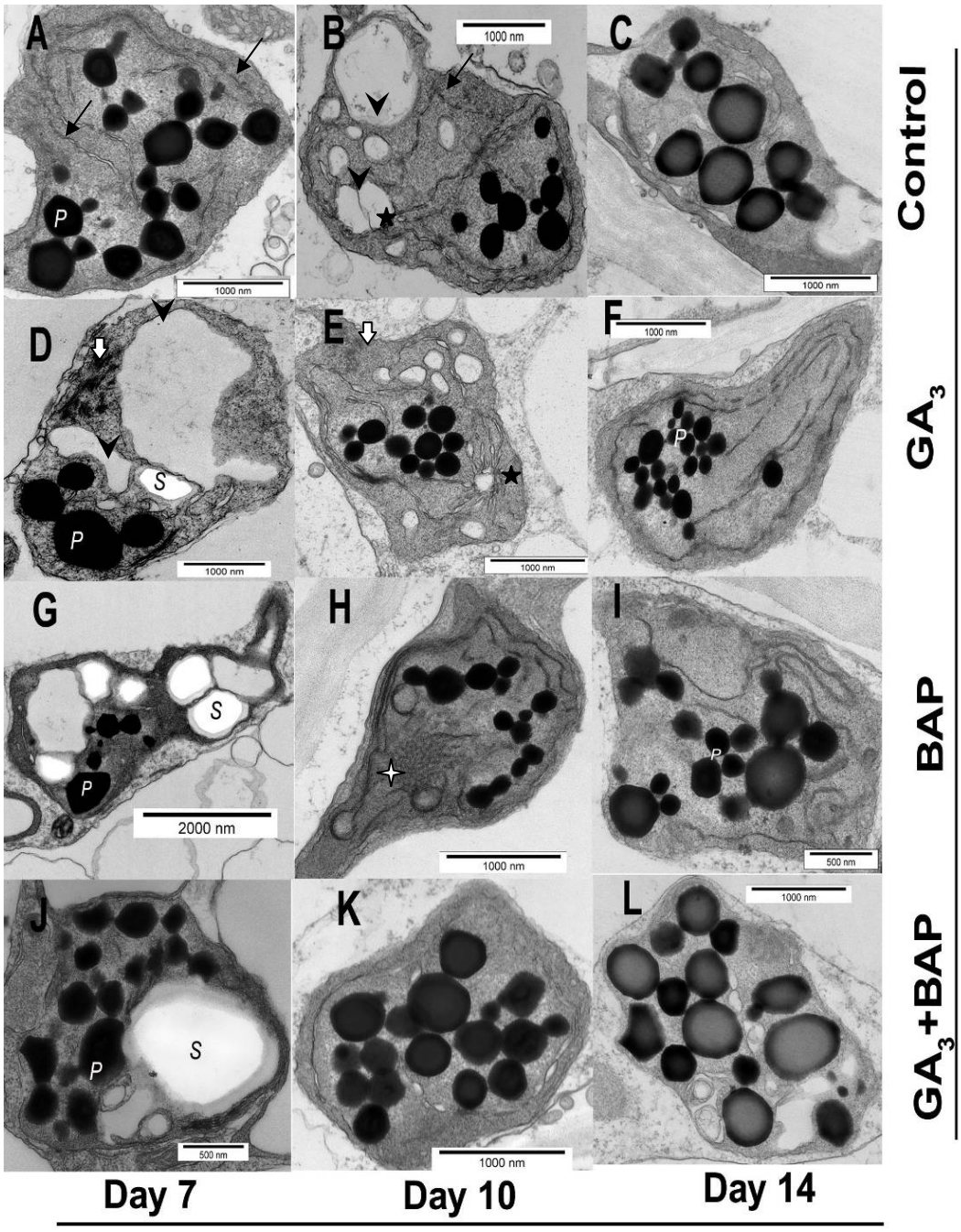

Figure 6.9 Influence of GA_3 and/or BAP in darkness on the differentiation of plastids within the ABAXIAL surface of the spathe in *Zantedeschia* 'Best Gold'. A) day 7, control: initial differentiation of chromoplasts into chloroplasts with the appearance of type-1 thylakoids (solid arrow); B) day 10, control: a further differentiated plastid from chromoplast to chloroplast containing both type-1 and type-3 thylakoids; C) day 14, control: a plastid showing signs of degradation; D) day 7, GA_3: plastids mainly occupied by type-3 thylakoids (arrow head) and plastoglobuli (*P*); E) day 10, GA_3: appearance of small type-3 and a tubular complex of thylakoids (asterisk); F) day 14, GA_3: a partially differentiated chloroplast, but with no grana stacking; G) day 7, BAP: accumulation of large

starch granules and type-3 thylakoids; H) day 10, BAP: initial differentiation of chloroplasts coexisting with a prolamellar-body-like structure (4-point star); I) day 14, BAP: similar to H; J) day 7, GA_3+BAP: plastid remains as chromoplast occupied by large starch granules; K) day 10, GA_3+BAP: chromoplast with disappearance of starch; L) day 14, GA_3+BAP: chromoplast showing less electron-dense plastoglobuli and stroma. *P*, plastoglobuli; *G*, grana; *S*, starch.

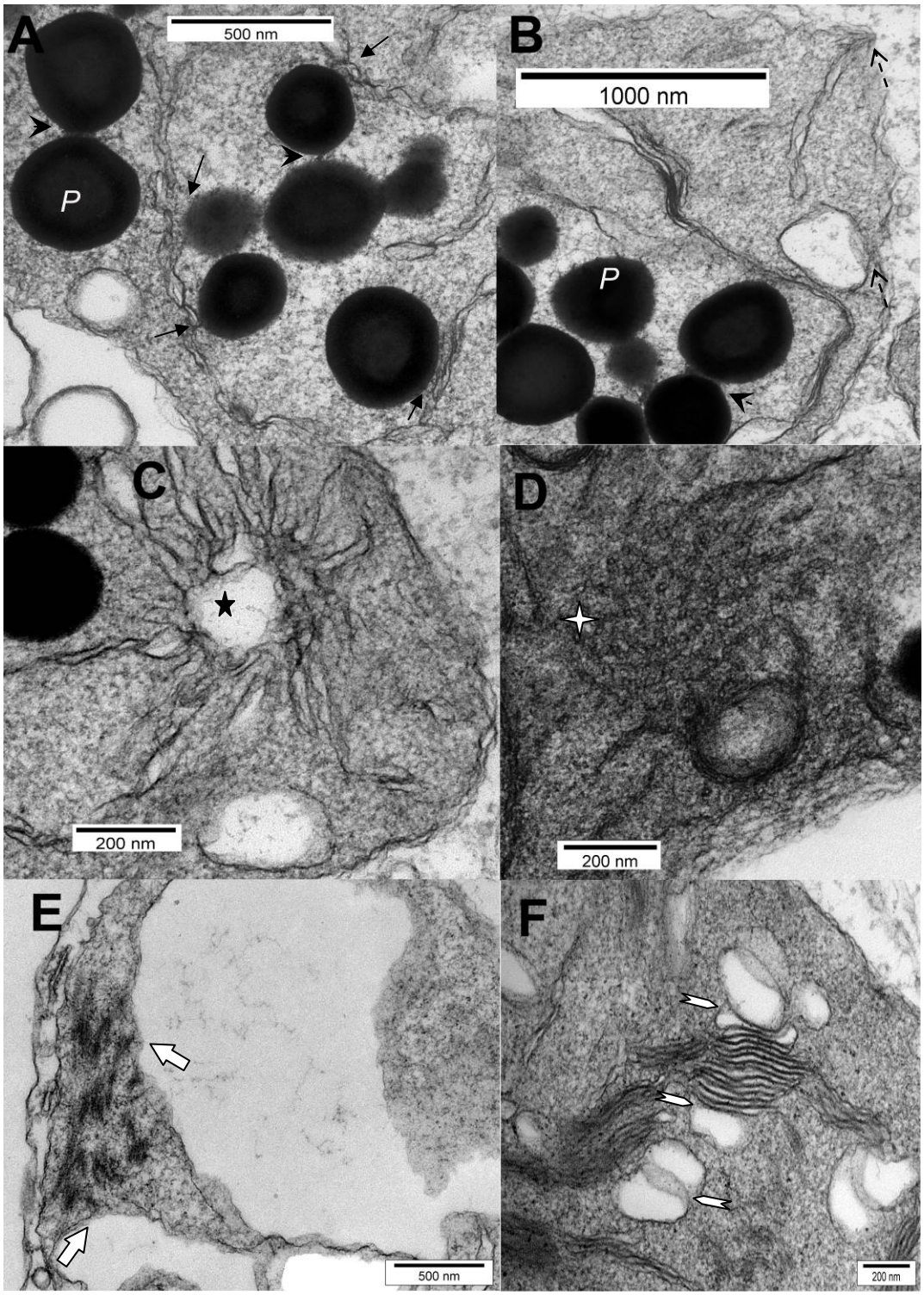

Figure 6.10 Ultrastructural characteristics of plastids in the abaxial surface of *Zantedeschia* 'Best Gold' spathe tissue during re-greening. A) double-membrane primary thylakoids (type-1) showing a close connection with plastoglobuli (solid arrows). Plastoglobuli were inter-connected with each other via direct adhesion or some tubular extension (arrow heads); B) primary thylakoids (type-1 and type-2) showing continuity with inner-envelope membrane (dashed arrows); C)

an enlarged image of a tubular complex, demonstrating the possible origin of type-1 thylakoids from the extension of type-3 thylakoids (asterisk); D) an enlarged image of a prolamellar-body-like structure observed only in the BAP-treated tissue in darkness; E) thylakoid remnants (type-4 thylakoids) with close contact with type-3 thylakoids and the inner-envelope membrane (block arrows); F) type-3 thylakoids were divided and fused with type-1 to form grana (arrows). *P*, plastoglobuli.

After 10 days in darkness, the plastids in the abaxial surface accumulated more primary thylakoids, including type-1 and type-3 (Figure 6.9 B, arrow head). These type-3 thylakoids appeared in different sizes: some were small (about 0.2 μm in diameter), while others were more than 1 μm in diameter, hence occupying a large portion of the plastid volume. These thylakoids were found in close vicinity to the inner-envelope membrane or the type-1 thylakoids. A tubular complex was often observed, constituting one of the likely sites for the formation of type-1 thylakoids (Figure 6.9 B, asterisk). In several cases, it was possible to demonstrate in this tubular complex that the origin of the type-1 form was from the extension of the type-3 thylakoids (asterisk within enlarged image in Figure 6.10 C). At this stage of development the plastoglobuli were still evident and spherical in shape. They clustered together at one side of the plastid, and were surrounded by the type-1 thylakoids. Although the plastids at this stage housed more primary thylakoids, and a less number of plastoglobuli, than those of the plastids at day 7, no grana stacking was observed.

After 14 days in darkness, compared to those found in earlier sampling times, most of plastids in the abaxial surface showed signs of degradation with less electron-dense stroma (Figure 6.9 C). By day 14, the number of the primary thylakoids was reduced; no type-3 thylakoids were observed and only a few type-1 thylakoids remained in the plastids. Along with the disappearance of the thylakoids, the plastoglobuli increased in size, but were less electron-dense than at earlier sampling times within this treatment. The shape of the plastoglobuli at this time resumed to be more hexagonal, rather than spherical.

In contrast to what was evident with the abaxial surface, for the adaxial surface the chromoplasts in subepidermal cells did not show any signs of initial differentiation to chloroplasts over the 14-days of darkness. Chromoplasts in the adaxial surface remained intact for 10 days, before showing signs of degradation, i.e. less electron-dense stroma and plastoglobuli (data not shown).

6.3.5.2 Hormone treatment effects

When treated with BAP in darkness, the chromoplasts in the abaxial surface of discs did not present any evidence of the initial differentiation to chloroplasts until day 10 (Figure 6.9 G). At day 7, the plastids treated with BAP contained more starch granules than the plastids in other treatments. Together with the irregular shaped type-3 thylakoids, these starch granules occupied a large part of the plastid volume. Some hexagonal-shaped and electron-dense plastoglobuli also appeared in the plastids. Compared with the plastids at day 7, at day 10 a number of the type-1 thylakoids had accumulated. They were found along the inner-envelope membrane (Figure 6.9 G). At this stage, a prolamellar-body-like structure was often observed in these BAP-treated plastids in darkness (Figure 6.9 H, 4-pointed star; enlarged image in Figure 6.10 D). At day 14, this structure was not evident, though some type-1 thylakoids were still present. The plastoglobuli increased in size, but reduced in electron density (Figure 6.9 I).

After 7-days of darkness within the GA_3 treatment, no initial differentiation from chromoplasts to chloroplasts was observed in the subepidermal cells of the abaxial surface (Figure 6.9 D). The plastids were distinguished by the typical features of the chromoplasts, i.e. large and highly electron-dense plastoglobuli, and some large

and irregular-shaped type-3 thylakoids (Figure 6.9 D, arrow head). A few type-4 thylakoids were also often embedded in the stroma, and remained in close vicinity of the type-3 thylakoids and/or the inner-envelope membrane (Figure 6.9 D, block arrow). Occasionally, starch granules were present within plastids in the GA_3 treatment.

As the darkness continued for 10 days, the chromoplasts in the abaxial surface of the GA_3-treated discs exhibited evidence of the initial differentiation into chloroplasts. Like those in the control treatment at day 10, these plastids also consisted of both type-1 and type-3 thylakoids (Figure 6.9 E). A tubular complex that showed the origin of the type-1 was from the extension of the type-3 thylakoids, was often observed (Figure 6.9 E, asterisk). After 14 days in darkness, the chromoplasts in the abaxial surface of the GA_3-treated discs had further differentiated into chloroplast-like plastids (Figure 6.9 F). These plastids were characterized by the accumulation of a large number of type-1 thylakoids, which were arranged in parallel, but were not stacked to form grana. The type-3 thylakoids were not evident at this stage, and the plastids were lengthened. Unlike those from the control treatment in darkness, no sign of degradation was observed in these plastids from the GA_3-treatment by day 14.

When treated with GA_3 and BAP simultaneously in darkness, the initial differentiation from chromoplasts to chloroplasts was not observed in the plastids on the abaxial surface of the discs, throughout the 14-day period of observation. At day 7, the plastids still remained in the form of typical chromoplasts, but accumulated large starch granules (Figure 6.9 J). By day 10, the plastids were mainly occupied by large and hexagonal-shaped plastoglobuli (Figure 6.9 K). Occasionally, some type-1

thylakoids were observed along the inner-envelope membrane, but their number was much less than that found in plastids in the GA_3- or BAP-treated, or the control treatments. By day 14, these thylakoids were only present infrequently (Figure 6.9 L), with the plastids being full of large plastoglobuli, and low electron-density.

For the adaxial surface, the application of GA_3 and/or BAP in darkness did not show any notable differences in plastid redifferentiation compared to that evident in the control treatment (data not shown).

6.4 Discussion

6.4.1 Initial differentiation of chloroplasts from chromoplasts is light-independent.

Spathe re-greening in 'Best Gold' required light for the process to complete, although the onset of re-greening, as characterized by plastid transition from chromoplasts to chloroplasts, was initiated in darkness (Figure 6.9). In the absence of light, both abaxial and adaxial surfaces of the discs remained yellow throughout the 14-day period of observation, but the saturation of this yellow colour (i.e. Chroma) progressively reduced after day 7 (Figure 6.2 C & D). On both surfaces, this colour change was associated with the simultaneous decrease in the content of total carotenoid and lack of chlorophyll accumulation (Figure 6.5). In the current experiment the lack of chlorophyll accumulation in darkness was not unexpected. Without light, the enzyme protochlorophyllide reductase (POR) cannot convert protochlorophyllide to chlorophyllide, being the sole light-dependent step in chlorophyll biosynthesis in Angiosperms (Armstrong and Apel, 1998; Armstrong et

al., 1995; Vonwettstein et al., 1995). However, the initial differentiation of chloroplasts from chromoplasts in the abaxial surface held in darkness, e.g. the accumulation of primary thylakoids (Figure 6.9 A & B), was unexpected. This initial development of thylakoids in darkness was similar to the differentiation noted in tissue held in light, at the early stage of spathe re-greening (Figure 6.7 J), and also to that reported during the re-greening of petaloid hypsophylls of *Heliconia aurantiaca* Ghiesbr. and *Guzmania* cf. × *magnifica* Richter (Weidner et al., 1985). In the absence of light, in the current experiment these primary thylakoids appeared unable to further develop to form grana, prior to the occurrence of senescence by day 14. This suggests that the onset of the transition from chromoplast to chloroplast during spathe re-greening on the abaxial surface can be induced in darkness, but the maturation of the chloroplast, i.e. formation of photosynthetic apparatus is light-dependent.

Based on the results presented in the current experiment, it is proposed that light might not have direct control over the onset of re-greening, but does ensure the completion of plastid redifferentiation by stimulating chlorophyll synthesis and grana stacking. In contrast, it is plausible that the initiation of spathe re-greening is dependent upon developmental stage. As such, it is hypothesised that as the spathe matures, the nucleus will receive signals from developmental-factors (e.g. ontogeny) to express chloroplast-related genes which, in turn, lead to the onset of chromoplast-chloroplast differentiation. Its further regulation towards the formation of a functional chloroplast, however, is likely to require developmental signals from plastids, i.e. plastid to nucleus signals (Mochizuki et al., 1996; Susek and Chory, 1992; Vothknecht and Westhoff, 2001). These signals might include the accumulation of chlorophyll or carotenoids in the plastid (Cuttriss et al., 2007; Papenbrock and Grimm,

2001; Susek and Chory, 1992). In darkness, on the abaxial surface, the lack of chlorophyll and gradual loss of carotenoids in chromoplasts, might have provided negative feedback to the nucleus, which in turn led to the discontinuation of chloroplast maturation (Mayfield and Taylor, 1984). As a result, the chromoplasts simply proceeded to senescence (Figure 6.9 C). A further note for consideration is that while proteins were not examined in the current research, light also regulates the synthesis of photosynthetic proteins (e.g. chlorophyll binding proteins) that are required for assembly of the photosynthetic apparatus (Kusnetsov et al., 1994; Pyke, 2009). Hence, without light, the construction of grana cannot be completed. This concept of light mediated biosynthesis of proteins involved in the construction of photosynthetic apparatus further supports the hypothesis that light regulates the formation of a functional chloroplast during the re-greening of spathe tissue.

6.4.2 **Stimulation of re-greening by BAP**

The light-mediated formation of chloroplasts and chlorophyll accumulation during greening or re-greening of plant tissue is influenced by cytokinins (Kusnetsov et al., 1998; Kusnetsov et al., 1994; Yaronskaya et al., 2006; Zavaleta-Mancera et al., 1999a; Zubo et al., 2008). The overlapping roles of light and cytokinins on greening and re-greening processes led to a suggestion that light and cytokinins might exert effects via the same biochemical mechanisms (Thomas et al., 1997). In the current study, in the absence of light, and despite treatment with BAP, the abaxial surface was unable to synthesize chlorophyll and complete chloroplast formation (Figure 6.9 G, H & I). This is in contrast with, in the presence of light, the enhancement of re-greening by BAP toward the later stage of re-greening on the abaxial surface (Figure 6.3 A & B; Figure 6.7 I), and BAP-induction of re-greening in the adaxial surface (Figure 6.4 A;

Figure 6.8 E). These results suggest that BAP is unable to substitute for the role of light in regulation of re-greening and, that the stimulating effect of BAP on re-greening, at least in some aspects, is light-dependent. These findings, at both the biochemical and ultrastructural levels, are in agreement with those focussing on chloroplast transcription in detached leaves of *Hordeum vulgare* L. (Zubo et al., 2008), wherein cytokinin-stimulated transcription of some chloroplast genes required the presence of light. Hence, rather than BAP being an actual component of the pathway, it might exert its effect on one or more components of the light signalling pathway that leads to chloroplast formation.

In the presence of light, it was not until the later stage of re-greening, i.e., 14 days after horticultural harvest-maturity, that BAP affected a colour change, chlorophyll accumulation, and plastid differentiation, in the abaxial surface of the spathe tissue (Figure 6.1; Figure 6.3; Figure 6.7). By day 14, compared to the control, the abaxial surface of the BAP-treated discs was greener, contained higher content of chlorophyll, and a higher degree of grana stacking in the chloroplasts. Similar positive effects of BAP on chlorophyll accumulation and structure of chloroplast have previously been shown in the re-greening of sepals of *H. niger* (Salopek-Sondi et al., 2002), and detached leaves of both *Cucumis sativus* L. (Kovacs et al., 2007) and *N. rustica* (Wilhelmova and Kutik, 1995; Zavaleta-Mancera et al., 1999a; Zavaleta-Mancera et al., 1999b). In the current study, it was unexpected that a positive response of chlorophyll accumulation and chloroplast formation was not observed in the initial stage of re-greening in spathe tissue (i.e., the first week of observation). As shown with leaves of *N. rustica*, BAP enhanced the content of chlorophyll within five days of application (Zavaleta-Mancera et al., 1999a). Criado et al. (2009) also found that

BAP increased the chlorophyll content within 48 h of treatment of leaves of *Triticum aestivum* L.. While surprising that no such enhancement of chlorophyll was detected in the first week, it is worth noting that during this initial stage of re-greening, in the presence of light, the application of BAP promoted the accumulation of carotenoid (particularly neoxanthin and lutein) in the abaxial surface of the spathe (Figure 6.3). Given that neoxanthin and lutein are the end products of β- and α-biosynthetic pathway of carotenoid, respectively, it is reasonable to suggest that the treatment of BAP stimulates the flux of both pathways. Furthermore, several lines of evidence reveal that accumulation of carotenoids assists the construction and stabilization of thylakoid systems (Axelsson et al., 1982; Cuttriss et al., 2007; Park et al., 2002). This raises a question of whether perhaps the positive effect of BAP on chlorophyll accumulation and chloroplast formation, toward the later stage of re-greening, is a result of a BAP-stimulated accumulation of carotenoids during the initial stage of re-greening. Irrespective of this, the influence of BAP on re-greening of spathe tissue differs depending upon the stage of development.

The response of the abaxial surface to BAP during the first week after harvest contradicted that for the adaxial surface, wherein chlorophyll accumulation and chloroplast formation was induced by application of BAP (Figure 6.4; Figure 6.8). This suggests that the response of re-greening to BAP is also tissue-dependent. As discussed in Chapter 5, the diverse responses to BAP between these two surfaces might reflect differences in levels of endogenous cytokinins. The initial level of the endogenous cytokinins on the abaxial surface might have already saturated the process associated with chlorophyll accumulation and plastid differentiation. In contrast, for the adaxial surface, the level of these cytokinins may have been low. If so,

therefore, the exogenous BAP was able to stimulate re-greening in this surface, though it re-greened to a much less extent compared with the abaxial surface. Firn (1986) suggested that the magnitude of the response evoked by plant hormones is not only determined by the concentration of the hormones, but also the number of receptors and the affinity of the receptor for the hormones. It is plausible therefore, that for the adaxial surface, the number of the receptors or their affinity to BAP was lower than that for the abaxial surface, which in turn limited the magnitude of the response in the adaxial surface (i.e., chlorophyll accumulation and chloroplast differentiation) induced by the application of BAP. Further research through hormonal analysis however, is required to address the following questions which now arise:

- Is re-greening of spathe tissue associated with a change in the level of endogenous cytokinins and/or sensitivity of tissue to cytokinins prior to, and during, re-greening?
- If re-greening of spathe tissue is related to an increase in endogenous cytokinin, is the application of an inhibitor of cytokinin biosynthesis able to retard the re-greening?
- Does the abaxial and adaxial surfaces differ in the level of endogenous cytokinins, the number of receptors, and/or the affinity of the receptor to cytokinins?

In the absence of light, within the abaxial surface, the application of BAP promoted an initial accumulation of starch granules and subsequent formation of a prolamellar-body-like structure in the plastids (Figure 6.9 G & H). This differentiation of plastids in darkness was, to some extent, similar to the formation of etioplasts from proplastids, which are mainly occupied by large starch granules (Kusnetsov et al.,

1998; Kusnetsov et al., 1994; Pyke, 2009). They both involve the construction of a prolamellar body, which is a complex of a large amount of POR and lipids. Some workers have shown that BAP was able to increase the level of POR, and also its gene expression (Kusnetsov et al., 1998; Zavaleta-Mancera et al., 1999a). This may partially explain why this prolamellar-body-like structure was only observed in the BAP-treated plastids in this study. In etioplasts, the formation of a prolamellar body is an adaptation of the proplastid to temporary darkness, and renders it primed for rapid conversion to a chloroplast on illumination (Biswal et al., 2003; Pyke, 2009). Similarly in the current study, in response to darkness, BAP might have stimulated an adaptive pathway of re-greening in the spathe tissue by initially storing energy in the form of starch, and then forming a prolamellar body. As a consequence, when light appears, the tissue can promptly recover and complete re-greening more efficiently. The different responses of spathe tissue to BAP, in the absence of light, suggest a complexity in regulation of chloroplast development and chlorophyll accumulation during re-greening of spathe tissue, and open the way for further research into this aspect. For example, by transferring the dark-incubated spathe tissue into light, does the BAP-treated spathe tissue re-green more rapidly than those without the treatment of BAP? Moreover, attempts through a molecular approach could help to clarify whether POR is present prior to and/or during re-greening of spathe tissue and, whether BAP stimulates an increase in the expression of genes and/or proteins of POR in light and darkness.

In summary, the temporal and spatial variations in the response of re-greening to BAP complicate the role of cytokinin in influencing this process. Does cytokinin stimulate re-greening in the spathe tissue of *Zantedeschia*? Yes it can, but it is

dependent on certain prevailing conditions, e.g. the presence/absence of light, the stage of re-greening, and which surface, i.e. abaxial or adaxial.

6.4.3 **Gibberellin temporarily delays re-greening**

The influence of gibberellin on chlorophyll accumulation and plastid differentiation from chromoplasts to chloroplasts has not been extensively reported, and similarly its relation to light in regulation of this process. The retarded development of chloroplasts from proplastids has been previously linked to an increased content of bioactive gibberellin in the shoot apex of *Nicotiana tabacum* L. (Nakano et al., 2003). Similarly, in the current research, in both light and dark conditions, exogenous application of GA_3 enabled a temporary retardation of the differentiation of chloroplasts from chromoplasts, e.g. accumulation of type-1 thylakoids in the abaxial surface of spathe tissue (Figure 6.7 D; Figure 6.9 D). Hence it is concluded that the effect of GA_3 on the onset of this plastid differentiation is light-independent. In the presence of light, a delay in chlorophyll accumulation in the abaxial surface might have been a result of retarded formation of thylakoids, where chlorophyll biosynthesis takes place (Dahlin and Franzen, 1997; Lindsten et al., 1993).

In the current study, it is possible that exogenous GA_3 delays the onset of plastid differentiation and maintains the current status of chromoplasts by directing the nucleus to continue the expression of chromoplast-related genes. Numerous lines of evidence have illustrated that the application of GA_3 rapidly up-regulated some chromoplast-specific carotenoid-associated proteins, e.g. CHRC and CHRD (LibalWeksler et al., 1997; Vainstein et al., 1994; Vishnevetsky et al., 1997). These proteins are bound with carotenoids and lipids to form internal structures of

chromoplasts, e.g. plastoglobuli. The accumulation of these proteins is believed to stabilize the structures of chromoplasts and facilitate sequestration of carotenoids in plastoglobuli (Bartley and Scolnik, 1995). Future research of spathe re-greening could benefit from exploring this line of thinking.

6.4.4 **Combination of cytokinin, gibberellin and darkness offers potential in postponing re-greening**

As presented in Chapter 5, the simultaneous application of BAP and GA_3 was more effective than GA_3 or BAP alone in postponing the time when re-greening became visible in spathe tissue (Figure 5.5), indicating a synergistic effect between BAP and GA_3 in delaying re-greening. Similarly, in the current experiments, this synergistic delay in colour change by the combination of the two hormones was also observed, though to a less degree compared with that found in the previous Chapter (about five days difference in the delay; Figure 5.5; Figure 6.1). In addition, wherein Chapter 5 application of BAP resulted in a slight delay in the time when re-greening was visible, in the current study this treatment exerted no effect on this colour change. Due to variation in growing conditions (e.g. light intensity and temperature), flowers, including spathes of *Zantedeschia*, harvested from different seasons or growing conditions are likely to vary in their initial colour (Ben-Tal and King, 1997; MacKay et al., 1987) and pigment content. These environmental influences may also form the basis for the variation in response to the same hormonal treatment in these two experiments.

During the first week of observation, the synergistic effect in delaying the time when re-greening was visible, by the BAP+GA_3 treatment, was associated with a further retardation of chlorophyll accumulation and chloroplast differentiation from chromoplasts, compared to those induced by GA_3 alone (Figure 6.3 A & B; Figure 6.7 J). Meanwhile, the accumulation of carotenoids, in particular β-carotene, was accelerated compared to that induced by BAP alone (Figure 6.3). Chandler (2009) suggested that synergistic effects can arise when components from independent signaling pathways of separate hormones mutually cooperate (i.e. co-regulate), or the combination of two hormone pathways raises a response above a threshold. In the current study, the synergistic effect between BAP and GA_3 in delaying re-greening is more likely to be an output of mutual cooperation between the two independent pathways, i.e., GA_3-stimulated retardation of chlorophyll accumulation and chloroplast formation, and BAP-stimulated accumulation of carotenoids. While the precise molecular mechanism of this mutual cooperation between BAP- and GA_3-stimulated pathways is unknown, it is possible that as a result of a delay in chloroplast differentiation (i.e., thylakoid accumulation), elevated accumulation of carotenoid induced by BAP was continuously sequestered into plastoglobuli, instead of the thylakoid membrane. The increased sequestration of carotenoid in plastoglobuli in turn stabilized the other components within the plastoglobuli, e.g. lipids, which prevented the export of lipid for thylakoid formation (Axelsson et al., 1982; Dahlin and Timko, 1994). A similar mode of co-regulation between BAP and GA_3 has been previously reported in the processes of dormancy and flowering of *Zantedeschia* 'Best Gold' (Subbaraj et al., 2010). This area of synergistic hormonal effects on re-greening warrants further investigation.

It is worth noting that in the presence of light, the combination BAP and GA_3 in suppressing the onset of re-greening, as characterized by chlorophyll accumulation and plastid differentiation, was temporary. In the presence of light, re-greening was observed in the abaxial surface of BAP+GA_3-treated discs during the second week of observation (Figure 6.1; Figure 6.3; Figure 6.7). Conversely, in the absence of light, the suppression of BAP and GA_3 on plastid differentiation from chromoplast to chloroplast persisted until the occurrence of senescence in the abaxial surface of spathe tissue (Figure 6.9). This leads to the hypothesis that light enables the spathe tissue to overwrite suppression of the re-greening evoked by the simultaneous application of BAP and GA_3. Further studies through biochemical, molecular, and ultrastructural analysis are required to unravel the complex cross talk between light, cytokinin and gibberellin, in influencing the process of re-greening in spathe tissue. The current study reveals the possibility of the existence of this cross talk and, in doing so, raises several new questions:

- Does the balance in the content of endogenous cytokinin and gibberellin determine the developmental status of spathe tissue: e.g. the initiation of re-greening in spathe tissue requires high levels of cytokinin, but low levels of gibberellin?
- Does light-induced re-greening of spathe tissue occur via regulation of the balance in content of cytokinin and gibberellin in spathe tissue; or
- Does light alter the sensitivity of tissue, e.g. number of receptors and affinity (Firn, 1986) to cytokinin and gibberellin at various stages of spathe development?

One of the goals of the ongoing research programme on re-greening in spathe tissue of *Zantedeschia*, was to develop a method for postponing re-greening. The data presented in this thesis so far have indicated many potential ways to delay this process, e.g. application of GA_3 alone or BAP+GA_3, or storing spathes in darkness. Among these three methods, keeping the spathes in darkness appears to be the most effective in delaying re-greening, with discs remaining yellow throughout the 14-day period of observation (Figure 6.2). But this method is not practical for either growers or retailers of 'Best Gold' as cut flowers. Compared with that applied separately, by keeping the spathe tissue in GA_3+BAP solution, as well as in darkness, the re-greening was further delayed and the tissue remained fresh without notable colour change for at least one week. Hence, the question now is whether short-term treatment in BAP+GA_3, in the dark, can be utilised in a horticultural setting, to affect re-greening of entire spathes. This idea was evaluated in Chapter 7.

6.4.5 **Re-greening in spathe tissue: some insights into thylakoid reorganization**

At the level of plastid ultrastructure, re-greening in spathe tissue of 'Best Gold' was characterized by redifferentiation of chloroplasts from chromoplasts. This process involved the reconstruction of thylakoid systems. As transmission electron microscopy cannot undertake live-time imaging, the dynamics of thylakoid reconstitution can only be reassembled based on the observation of sequential micrographs. Irrespective of this limitation, the existence of various states of plastid differentiation from chromoplast to chloroplast, resulting from the various combinations of treatments of light, cytokinin and/or gibberellin, has provided some

insights into understanding the way by which the thylakoid system reconstitutes. In the current study, although the majority of thylakoid system had largely degraded when observing a mature chromoplast, some primary thylakoids persisted, which were evident in four different morphological forms (Section 6.3.4). Similar primary thylakoids have been observed in mature chromoplasts, or an initial stage of chloroplast differentiation, in fruits of *C. sativus* (Prebeg et al., 2008), the epicarp of *Citrus sinensis* (L.) Osbeck. (Mayfield and Huff, 1986), and the spathe of *Z. elliottiana* (Gronegress, 1974). While the exact function of these primary thylakoids in mature chromoplasts remains unclear, it is believed that these primary thylakoids can be recycled for reconstruction of thylakoid system during re-greening of plant tissue (Ljubesic et al., 1991; Prebeg et al., 2008).

During differentiation of chloroplasts from proplastids (i.e. the process of greening), *de novo* synthesis of thylakoids is initiated by the accumulation of long double-membrane lamella, i.e., type-1 thylakoid by invaginations of the inner-envelope membrane (Vothknecht and Westhoff, 2001; Waters and Pyke, 2004). Similarly, the current observations revealed that reconstitution of the thylakoid system during the chromoplast-chloroplast transition in re-greening spathe tissue, also started from the formation of type-1 thylakoids. This was particularly evident in chromoplasts present in discs treated with BAP+GA_3 in light (Figure 6.7 J, solid arrow) or darkness (Figure 6.9 A, solid arrow), wherein the chromoplast-chloroplast transition took place at a slower pace or has commenced but did not complete, compared to that occurring in the control treatment. However, the current study indicated that the ways by which the formation of type-1 thylakoids during the chromoplast-chloroplast transition occurs is less uniform than that proceeding in the

proplastid-chloroplast transition during greening. In addition to *de novo* synthesis of thylakoid by invaginations of the inner-envelope membrane, it is highly probable that the type-1 thylakoids were formed through redifferentiation and multiplication of primary thylakoids present in mature chromoplasts or derived from plastoglobuli. Based on observations within the current study, and in line with the model for thylakoid formation proposed by Prebeg et al. (2008), type-1 thylakoids can possibly be derived by at least four different mechanisms during the initial stage of re-greening in spathe tissue (also illustrated in Figure 6.11):

1. *de novo* synthesis, through invaginations of the inner-envelope membrane (Figure 6.11 **1**). This was evident by the frequent observation of type-1 thylakoids along and, in some places, remaining in contact with, the inner-envelope membrane (Figure 6.7 A, G & J, solid arrow; Figure 6.10 B, dashed arrow). This process of thylakoid formation is also clearly supported in the literature, wherein the inner-envelope membrane is commonly believed to be a major source of primary thylakoids, for the formation of a functional chloroplast (Spurr and Harris, 1968; Thomson et al., 1967; Vothknecht and Westhoff, 2001);
2. direct conversion from type-2 thylakoids (Figure 6.11 **2**). These swollen-membrane thylakoids often contained an extension of a double-membrane lamella that appeared to be in a state of unravelling to become the type-1 form (Figure 6.6, dashed arrow). This process of formation was comparatively rare and has not been previously reported. In the current study, it was only observed in mature chromoplasts or during the initial stage of chloroplast differentiation;

3. derived from type-3 thylakoids (Figure 6.11 **3**). The type-3 form was often irregular or round in shape, and occurred in various sizes. These thylakoids were of different origin; in part they might originate from the inner-envelope membrane when pinched-off as a single membrane body (Figure 6.10 B, dashed arrow), and in part they might be derived from the remnants of granal thylakoids (type-4; Figure 6.10, block arrow). It is possible that small-sized type-3 thylakoids can be directly converted into type-1. This is indicated by the observation of an elongated tubular-membrane body, which appeared to be fused with the type-1 thylakoids (Figure 6.10 F, arrow). For larger-sized type-3 thylakoids, there were at least two possible ways to form type-1 thylakoids:
    i. it was divided or detached into smaller type-3 forms before being converted into the type-1 form (Figure 6.9 B, arrow head; Figure 6.10 F, arrow). Similar mechanisms of this process have also been revealed in re-greening of fruits in *C. sativus* (Prebeg et al., 2008) and of hypsophylls in *Spathiphyllum wallisii* Regel. (Weidner et al., 1985);
    ii. large type-3 thylakoids were also initially able to form a tubular complex with many extrusions of double-membrane lamellar, which were detached to form type-1 (Figure 6.9 B & E, asterisk; Figure 6.10 C, asterisk). This tubular complex, namely a thylakoid plexus (Spurr and Harris, 1968), was also observed during the initial stage of re-greening in hypsophylls of *H. aurantiaca* (Weidner et al., 1985);

4. derived from plastoglobuli (Figure 6.11 **4**). This is evident by observation of the close connection between the plastoglobuli and type-1 thylakoid membranes at all sampling times during plastid differentiation (e.g. Figure 6.10 A, solid arrow). This is also supported by the concomitant decrease in the number of the plastoglobuli and the increase in thylakoid membranes during the chromoplast-chloroplast conversion in spathe tissue. Occasionally, plastoglobuli were clustered together, and were not directly connected to the type-1 thylakoids, but linked to other plastoglobuli in a zigzag configuration (Figure 6.10 A, arrow head). In other systems, the presence of these comprehensive networks has led to a hypothesis that via these channels the plastoglobuli might supply to, or exchange with, the thylakoid, some molecules, e.g. lipids, carotenoids or proteins (Brehelin and Kessler, 2008; Steinmuller and Tevini, 1985; Ytterberg et al., 2006). This hypothesis is further supported by a study conducted by Austin et al. (2006), wherein they revealed that plastoglobuli remain in a physical continuum with the thylakoid membrane via half-lipid bilayers. Carotenoids and other lipid molecules stored in the plastoglobuli were reported to be in a dynamic equilibrium with those in the thylakoid membranes. A similar role of plastoglobuli has also been reported in other studies, e.g. re-greening in fruits of *C. sativus* (Prebeg et al., 2008) and of *C. sinensis* (Mayfield and Huff, 1986), and senescent leaves of tobacco (Zavaleta-Mancera et al., 1999b).

The existence of multiple ways for formation of type-1 thylakoids, in part reflects the natural plasticity of thylakoid membranes within plastids. This

characteristic of the thylakoid enables plastids to undergo dynamic differentiation between various forms (e.g. chromoplast-chloroplast) in order to adapt to changing physiological and environmental conditions.

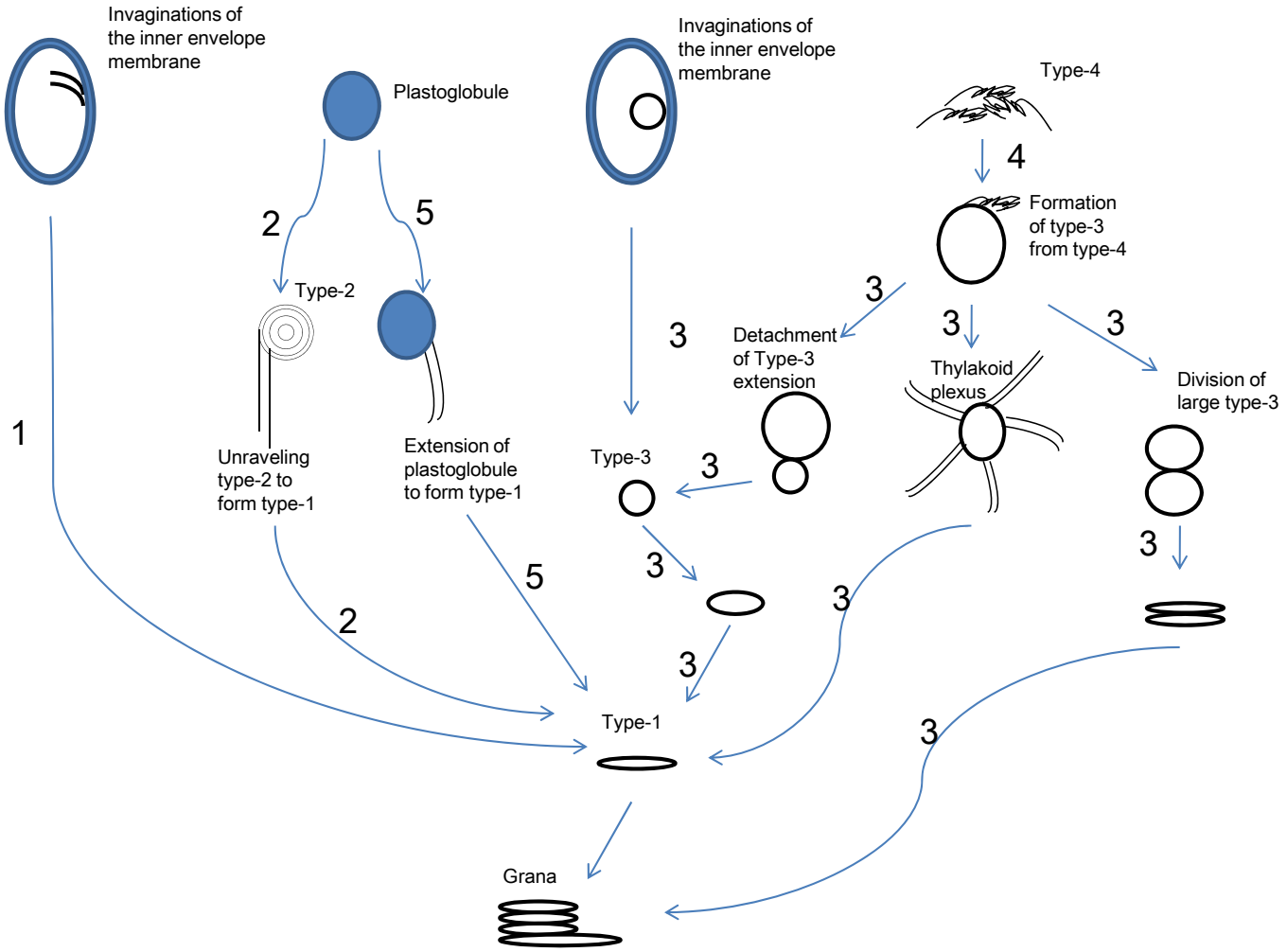

Figure 6.11 Diagrammatic illustration of possible processes by which type-1 thylakoids are formed during the plastid differentiation from chromoplast to chloroplast. The number indicates the process by which type-1 thylakoids are possibly formed (for descriptive details refer Section 6.4.5)

6.5 Conclusion

Re-greening of spathe tissue in 'Best Gold' requires light for the process to complete, but the onset of re-greening, as characterized by formation of type-1 thylakoids, can be induced in darkness. It is therefore proposed, that some other factors (e.g. ontogeny), but not light, have direct control over the onset of re-greening (also illustrated in Figure 6.12). If so, as the spathe develops in maturity, the nucleus will receive signals from the factors of ontogeny to express re-greening-associated genes which, in turn, lead to the onset of chromoplast-chloroplast differentiation. Its further regulation, however, towards the formation of a functional chloroplast, is likely to require the plastid to signal back its developmental state, namely plastid-nucleus signalling. These signals probably include chlorophyll accumulation and/or grana stacking, which are light-dependent (Figure 6.12). Hence, re-greening of spathe tissue is a process that requires the concerted action of external factors (e.g. light), internal factors (e.g. ontogeny), the nucleus and plastids. The role of light in this process is to ensure the completion of re-greening, i.e. formation of functional chloroplasts.

Where do plant hormones (e.g. cytokinin and gibberellin) fit into this proposed mechanism of re-greening (Figure 6.12)? Based on the current studies into the influence of synthetic hormones, i.e. BAP and GA_3, on changes in pigment content and plastid differentiation occurring during the re-greening process, it was suggested that BAP stimulated re-greening by enhancing accumulation of carotenoid and chlorophyll, and also stacking of grana. But the response to BAP was dependent on certain prevailing conditions, e.g. the presence/absence of light, the stage of

development/re-greening, and which surface, i.e. abaxial or adaxial. On the other hand, the application of GA_3 retarded formation of type-1 thylakoids, and thus delayed the onset of re-greening. Hence, a synergistic effect in delaying the onset of re-greening provoked by the combination of BAP and GA_3 was likely to be a result of co-regulation between BAP-stimulated accumulation of carotenoid and GA_3-stimulated retardation of chloroplast differentiation (Figure 6.12). This suppression of re-greening by the combination of BAP and GA_3 however, was soon overcome when light was present. The current results lead to a hypothesis that the induction and progression of re-greening requires a comparatively high level of cytokinin, but low level of gibberellin; light is able to regulate the balance in content of endogenous cytokinin and gibberellin within spathe tissue and/or the sensitivity of tissue to these hormones. Future research through biochemical, molecular and ultrastructural approaches is required to unravel the complex cross talk between light, cytokinin and gibberellin in influencing the process of re-greening.

From a horticultural perspective, the simultaneous application of BAP and GA_3, plus short-term dark incubation, showed potential for delaying spathe re-greening of 'Best Gold'. This horticultural potential with entire flower stems was evaluated in Chapter 7.

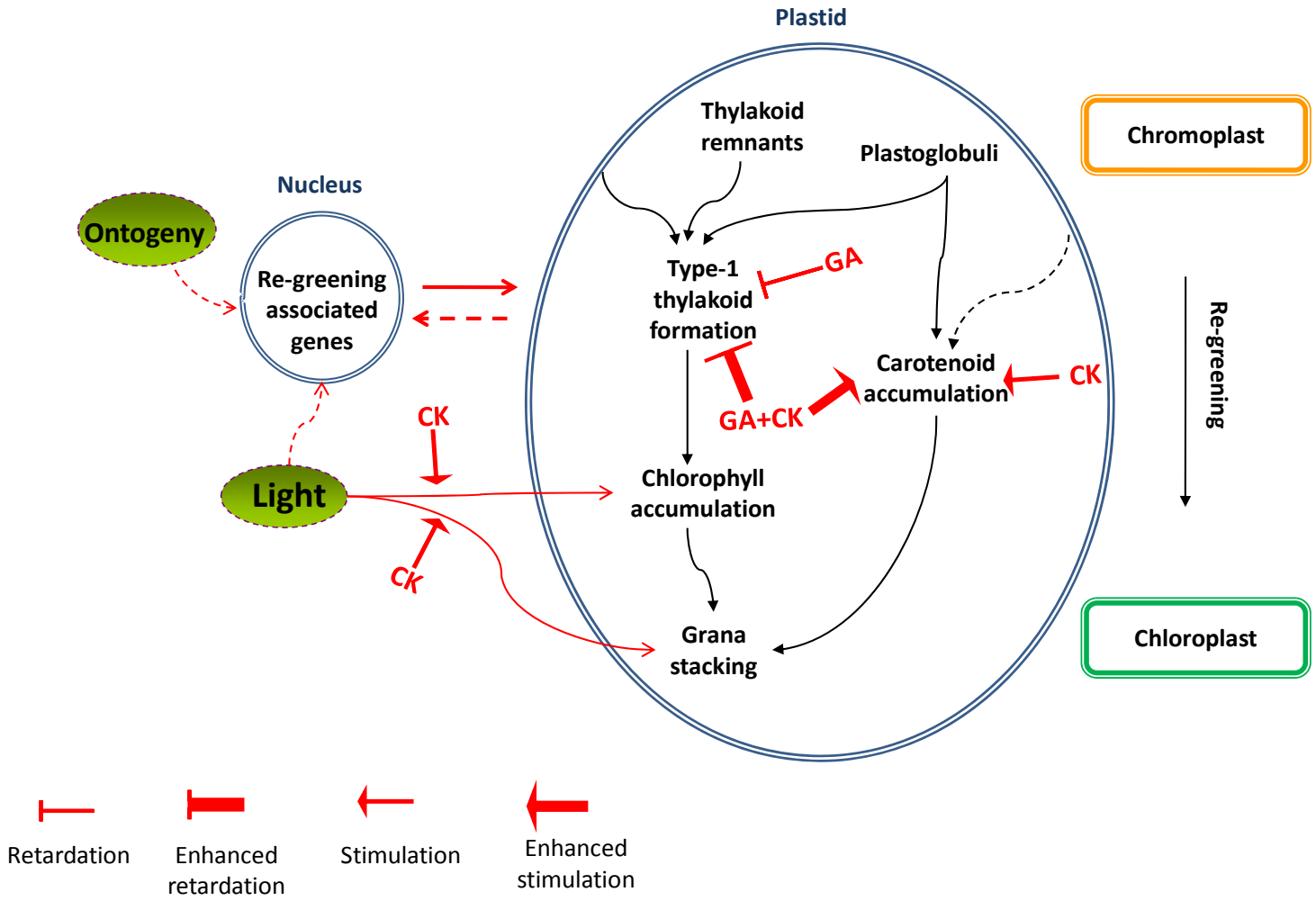

Figure 6.12 Model for the mechanism of light-, cytokinin-, and gibberellin-mediated spathe re-greening of *Zantedeschia* 'Best Gold'. CK: cytokinin; GA: gibberellin.

Chapter 7 Horticultural evaluation of treatments to postpone spathe re-greening in *Zantedeschia* 'Best Gold'

7.1 Introduction

Zantedeschia is a worldwide, commercial, cut flower and pot plant (Funnell, 1993), and is New Zealand's second largest export flower (Statistics New Zealand, 2010). The leaf-like floral structure (spathe) of *Zantedeschia* re-greens after it fully opens and reaches commercial maturity. This re-greening is a primary determinant limiting the postharvest quality for most hybrids of *Zantedeschia*, e.g. 'Best Gold', wherein re-greening begins in two-three days after horticultural harvest-maturity (refer Chapters 2 and 3). Efforts have been undertaken to understand the mechanism of spathe re-greening in *Zantedeschia* and to investigate potential ways of postponing it (Funnell and Downs, 1987; Pais and Neves, 1982-1983; Pais, 1981), but so far no success has been achieved in finding horticulturally relevant treatments.

Synthetic plant hormones have been widely utilized to improve and/or sustain postharvest quality of cut flowers and/or pot plants through application methods of short-term pulsing, continuous vase solution, or spray (Han, 2001; Paull and Chantrachit, 2001; Serek and Reid, 1997; Skutnik et al., 2001; Waterland et al., 2010; Whitman et al., 2001). For example, Paull and Chantrachit (2001) reported that dipping or spraying flowers of *Anthurium andraeanum* Schott with the cytokinin 6-

benzylaminopurine (BAP), prolonged the vase life of flowers by 10 days, and either of these two application methods were equally effective. Furthermore, 10^{-5} M of both BAP and gibberellin (GA_{4+7}) together as a vase solution, or as a pulse treatment for 4 hours at 10^{-4} M of each hormone, effectively prevented leaf yellowing in Oriental lilies (Han, 2001). Similarly, the data presented in Chapters 5 and 6 confirmed with discs of spathe tissue of 'Best Gold' that the process of re-greening can be delayed by application of GA_3 alone or BAP+GA_3, or by storing spathes in darkness.

What, therefore, is now required is to evaluate the performance of the various effective treatments noted in Chapters 5 and 6, under the conditions likely to be encountered within a horticultural context. Among these treatments, keeping spathe tissue in darkness appears to be the most effective in delaying re-greening, with discs of spathe tissue remaining yellow throughout the 14-day period of observation (refer Chapter 6). Even if effective when applied to flowers (spathe plus spadix and peduncle), treatment in darkness is not practical for extended periods for growers, retailers or consumers of 'Best Gold'. However, in a horticultural setting, prior to shipment, short-term storage of flowers in a coolstore, in darkness and low temperature (e.g. 5 ºC), is a common practice for postharvest handling. Given the effectiveness of darkness in postponing re-greening in discs, this raised the question as to whether short-term storage in darkness at 5 ºC is effective in delaying re-greening of flowers of 'Best Gold'. In addition, compared with that applied separately, by keeping the discs in GA_3+BAP solution, as well as in darkness, the re-greening was further delayed and the tissue remained fresh without notable colour change for at least one week (refer Chapter 6). Hence, as a part of an ongoing research programme toward the development of a treatment that can be utilised by growers to delay re-

greening in spathes of *Zantedeschia*, the objectives of the current study were to determine for flowers of 'Best Gold':

1. if BAP+GA_3 as a treatment solution can delay the process of re-greening in the spathe;
2. if the application of BAP+GA_3 as a spray or as a vase solution are equally effective in delaying re-greening of the spathe;
3. if a short-term pulsing in RO water or a solution of BAP+GA_3 in darkness at 5 ºC, delays re-greening of the spathe?

7.2 Materials and Methods

7.2.1 Plant materials and handling

The plant materials used in this study were flowers (spathe plus spadix and peduncle) of 'Best Gold'. They were produced under the same conditions and harvested at the same maturity, as described in Chapter 2 (refer section 2.2.1). Throughout the experiment, the vase-life evaluation room was controlled to the recommended environmental conditions for postharvest evaluation of cut flowers (Reid and Kofranek, 1980), i.e., temperature 20 ± 1 ºC, relative humidity 60 to 70%, and a 12-h (0600 HR – 1800 HR) photoperiod at a light intensity of 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at bench height provided by cool-white fluorescent tubes. Once harvested, and following any pulsing treatment, flowers were held for evaluation in individual vases containing reverse osmosis (RO) water or treatment solutions.

Measurement of the colour of the spathe was conducted as described previously (refer Chapter 3) using a tristimulus colorimeter CM-2600d/2500d (Konica

Minolta, Japan). This comprised recording the colour coordinates of lightness (L*), chroma (C*), and hue angle (H°) at either side of the midrib at the central position of the abaxial surface of each spathe. The colour measurement was repeated every 2 to 4 days for a period of two to three weeks, depending on individual experiments. Based on the results presented in Chapter 2, the change in H° can be used to describe changes in chlorophyll content in the abaxial surface, particularly during the initial stage of spathe re-greening. For the abaxial surface, a value of H° greater than 90º was deemed indicative of a level of accumulation of chlorophyll (i.e. re-greening) visible to the human eye (Iglesias et al., 2001). Hence, for the purposes of evaluating the effectiveness of the treatments in this chapter, changes of H° on the abaxial surface were the primary focus of data interpretation. In some instances, values of L* and C* were also included for further interpretation of treatment effect. The flowers were also visually assessed for any phytotoxic effects (i.e. brown colouration and/or rapid senescence) caused by the treatments.

7.2.2 **Treatments, experiment design and statistical analysis**

Experiment 1 - spray versus vase solution

- Spray: flowers were held in individual vases containing RO water and sprayed daily (≈ 3 ml) with a mixture of BAP+GA_3, both at 10^{-4} M, as a fine mist to cover both the abaxial and adaxial surfaces;
- Vase solution: flowers were held in individual vases containing BAP+GA_3, both at 10^{-4} M, throughout the 17-day period of observation;
- Control: flowers were held in individual vases containing RO water throughout the 17-day period of observation.

Experiment 2 - pulsing versus vase solution

- Pulsing: once harvested, flowers were pulsed with BAP+GA_3, both at 2×10^{-4} M, in darkness at 5 ºC for 24 h. Flowers were then held in individual vases containing RO water for the rest of 15-day period of observation in the vase life evaluation room;
- Dark control: once harvested, flowers were held in RO water in darkness at 5 ºC for 24 h. Flowers were then held in individual vases containing RO water for the rest of 15-day period of observation in the vase life evaluation room;
- Vase solution: once harvested, flowers were held in individual vases containing BAP+GA_3, both at 10^{-4} M, throughout the 15-day period of observation in the vase life evaluation room;
- Control: once harvested, flowers were held in individual vases containing RO water throughout the 15-day period of observation in the vase life evaluation room.

Each individual experiment was conducted as a split-plot design, with the treatment as the main plot, and time (i.e. time from harvest of repeated colour measurement) as the split plot. There were four individual flowers (replicates) for each treatment. Two colour measurements per flower at either side of the midrib were treated as sub-samples. Data were tested initially to ensure they met the requirement for ANOVA using the general linear procedure of SAS (SAS 9.1; SAS Institute, Cary, NC). Where significant ($P < 0.05$) treatment effects were detected, means were separated by using the unrestricted LSD procedure.

7.3 Results

7.3.1 Spray versus vase solution

Within the 17-day period of observation, the colour of the abaxial surface of spathe progressed from golden-yellow, through yellow-green, to green for controls held in RO water. This change in colour was quantitatively described as an increase in H° from 85° to 106°, and a decrease in both L* (80% to 53%) and C* (77% to 47%; $P < 0.05$; Figure 7.1). The application of BAP+GA_3 delayed the time when re-greening became visible (i.e., H° reached a value of 90°), with the magnitude of this delay being greater when applied as a continuous vase solution than as a daily spray to the spathe (Figure 7.1 C). The H° of spathes treated with the BAP+GA_3 vase solution reached a value of 90° on day 7, which was approximately 2 days later than when applied as a spray, and 4 days later than control spathes. Between day 5 and day 12, the H° of spathes treated with BAP+GA_3 either as a vase solution or as a spray was lower than that from the control ($P < 0.05$; Figure 7.1 C). Treatment differences for values of L* and C* were consistent with that for H° (Figure 7.1 A & B). No phytotoxic effects as a result of the treatments were observed on the flowers.

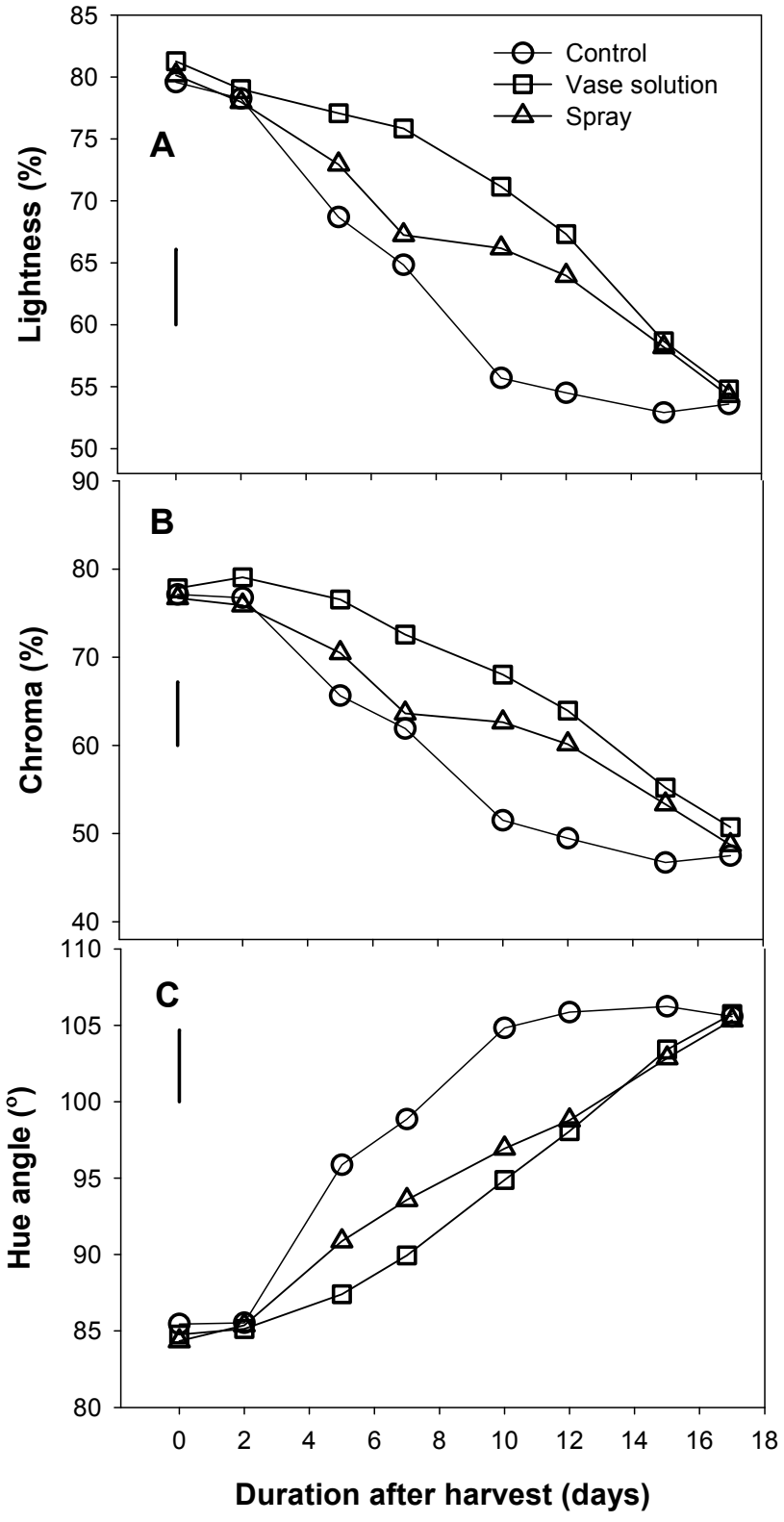

Figure 7.1 Colour coordinates of lightness, chroma and hue angle for the spathe of *Zantedeschia* 'Best Gold', treated with GA_3+BAP either as a spray or vase solution. Controls were held in RO water. Vertical bars represent LSD at $P < 0.05$ (n = 4).

7.3.2 **Pulsing versus vase solution**

The application of BAP+GA_3 either as a continuous vase solution or as a 24-h pulse in darkness at 5 ºC were equally effective in delaying the onset of re-greening for flowers. Compared to the control, both treatment methods of BAP+GA_3 resulted in an approximately 7 to 8 day delay in the time when re-greening was visible, i.e. H° reached a value of 90° (Figure 7.2 C). Within the first 9 days after horticultural harvest-maturity, the spathe of flowers treated with BAP+GA_3 via these two methods remained yellow in colour (Figure 7.3 C & E), corresponding with comparatively stable values of L*, C* and H° during this period ($P > 0.05$; Figure 7.2). By day 15, re-greening was visible to the naked eye in the spathe of BAP+GA_3-treated flowers, but was primarily concentrated in the basal (proximal) area (Figure 7.3 D & F) and, therefore, was not so readily detected by measurements of the tristimulus colorimeter at the central area. This was in contrast with the control flowers held in RO water in light, wherein the entire spathe had become fully re-greened at this time (Figure 7.3 B).

Compared with the control (i.e. held in RO water in the vase-life room throughout the 15-period of observation), a 24-h pulse in darkness in RO water at 5 ºC (i.e. dark control) resulted in a slight delay (approximately 1 day) in the time when re-greening was visible on the spathe (i.e. H° reached the value of 90°; Figure 7.2; Figure 7.3). This delay was enhanced by approximately 7 days when pulsed in a solution of BAP+GA_3 in darkness (Figure 7.2 C).

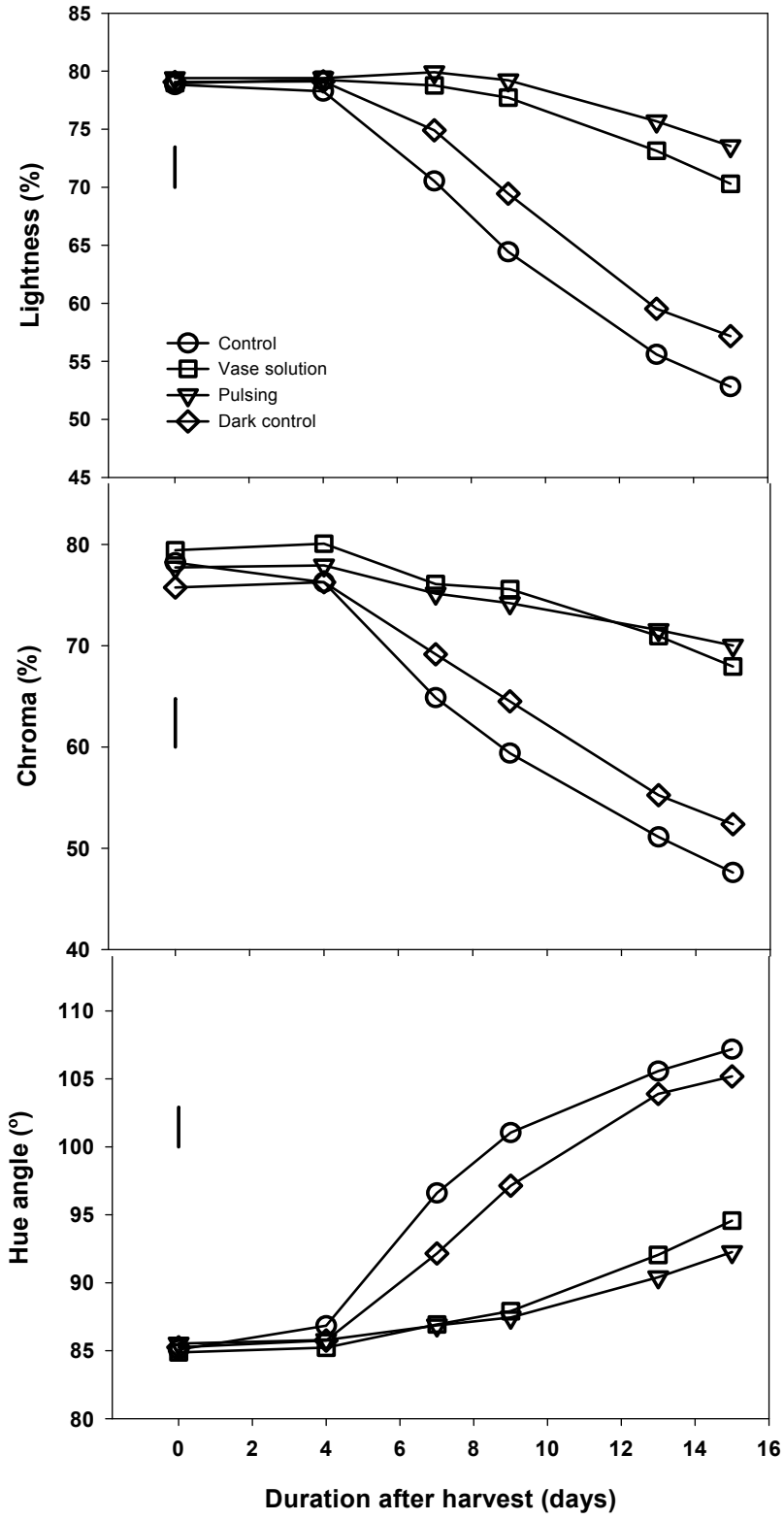

Figure 7.2 Colour coordinates of lightness, chroma and hue angle for the spathe of *Zantedeschia* 'Best Gold', treated with GA_3+BAP either as a 24-h pulse in darkness or a continuous vase solution. The dark-control comprised a 24-h pulse in RO water in darkness. The control was held in RO water in light. Vertical bars represent LSD at $P < 0.05$ (n = 4).

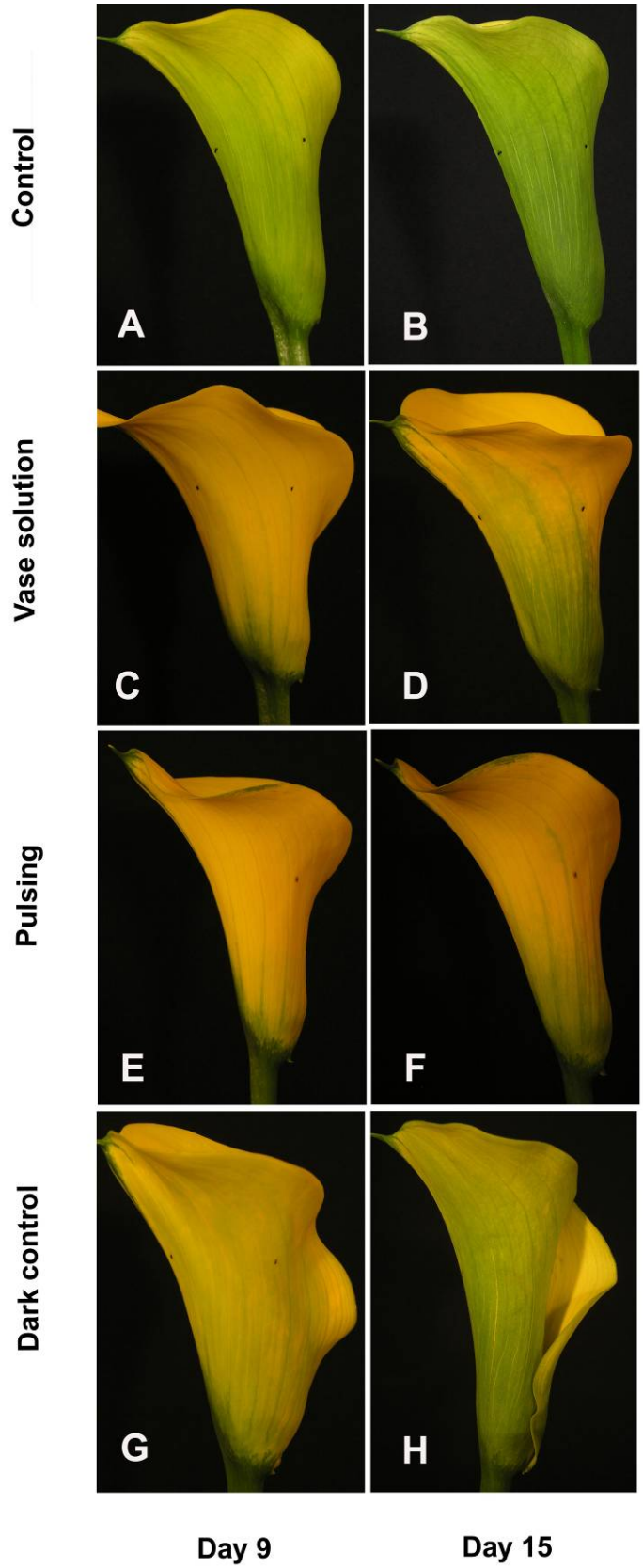

Figure 7.3 Spathes of *Zantedeschia* 'Best Gold' under various treatments (as illustrated in Figure 7.2). Photos taken at day 9 (left) and day 15 (right) after harvest.

7.4 Discussion

One of the goals for the ongoing research programme into re-greening was to develop a method that can be utilized by growers to postpone re-greening. The data presented in Chapter 5 and 6 of this thesis indicated that application of BAP+GA_3 enabled a delay in re-greening for discs of spathe tissue in 'Best Gold'. The current study confirmed that this mixture of BAP and GA_3 was also effective in delaying the re-greening for the entire spathe, without discernible adverse effect on quality. While there have been no previous reports wherein re-greening has been delayed by the joint application of synthetic cytokinin and gibberellins, this combination has been found effective in preventing leaf chlorosis in *Lilium longiflorum* Thunb. (Han, 1997; Whitman et al., 2001), *Viola* × *wittrockiana* Wittrock and *Viola cornuta* L. (Waterland et al., 2010).

In the current study the application of BAP+GA_3, as a daily spray, was less effective in delaying the re-greening than when applied as a continuous vase solution (Figure 7.1). It is possible that without the aid of a surfactant (e.g. Tween 20), which is often added to a spray solution (Ranwala and Miller, 2005; Skutnik et al., 2001), BAP+GA_3 was not efficiently absorbed when sprayed directly onto the surface of spathe. Hence, to potentially improve the efficacy of this means of application further, addition of a surfactant and/or raising the concentration of BAP+GA_3 in the spray solution is worth evaluating in future experiments.

In the current study the response of the entire flower of 'Best Gold' to the application of BAP+GA_3 varied in the two separate experiments. The same combination of BAP and GA_3, each at 10^{-4} M as a continuous vase solution, resulted

in differing degrees of delay in re-greening, i.e. 4 days in Experiment 1 compared with 7 days in Experiment 2 (Figure 7.1 C; Figure 7.2 C). Although the initial colour of the flowers used in these two experiments, reflected by the values of L*, C* and H°, were consistent (Figure 7.1; Figure 7.2), some variation in the stage of development may have existed between the plant material used in these two experiments. The presence of such variation is supported by the fact that re-greening was visible approximately 2 days earlier in the control treatment in Experiment 1 than Experiment 2 (Figure 7.1 C; Figure 7.2 C). Variation in response to hormonal treatments as a result of different developmental stages of plants has been previously reported in *Iris* × *hollandica* L. (Macnish et al.), *Zantedeschia* (Subbaraj et al., 2010) and *V. cornuta* (Waterland et al., 2010). Hence, this inconsistent response of flowers to the mixture of BAP+GA_3 highlights the need to further evaluate the optimal stage of maturity for flowers in *Zantedeschia*, if the treatment effect is to be optimised.

A short-term pulse with hormonal solution has been commonly used to improve postharvest performance for many cut flowers, e.g., *Iris* × *hollandica* (Macnish et al., 2010) and *A. andraeanum* (Paull and Chantrachit, 2001). Similarly, in the current study, although the concentrations were different, application of BAP+GA_3 as a pulsing solution for 24-h was equally effective, compared to that as a continuous vase solution, in delaying the re-greening (by approximately 7 days; Figure 7.2; Figure 7.3). Since re-greening of the spathe is a primary determinant limiting the vase life of *Zantedeschia*, this delay readily suggests a seven-day extension in vase life for 'Best Gold' is achievable. The similar efficacy of these two application methods, in part indicates that via pulsing shortly after harvest, there is an efficient uptake of hormonal compounds through the transpirational stream within the

spathe and peduncle. This uptake is sufficient for a comparatively long-term effect of BAP and GA_3 in delaying re-greening. Despite the results presented here, use of a continuous vase solution might not be the optimal way to treat the flowers, as the capability of the flower to take up any treatment solution gradually declines during the postharvest period. This is supported by fact that within five days after placement in water, some blockage in the water conducting tissue was detected in peduncle tissue of both *Z. aethiopica* and *Z. elliotiana* (Tjia and Funnell, 1986). However, given the limited number of treatment combinations and range of concentrations of BAP+GA_3 evaluated in the current study, a conclusion that pulsing is more efficient than a continuous vase solution in uptake and translocation of treatment compounds is not yet possible. Future research should, therefore, examine the comparative effectiveness of treatment method, duration and concentration.

In a horticultural setting, the means of a short-term pulse with BAP+GA_3 in darkness at 5 ºC offers more potential than that of a continuous vase solution for postponing re-greening of *Zantedeschia*. Firstly, the effectiveness of a short-term pulse in postponing re-greening, affords growers greater control over the quality of flowers that consumers will receive. Secondly, a pulse with BAP+GA_3 in darkness at 5 ºC can easily be included as a part of the postharvest handling for *Zantedeschia* prior to packing and shipment, as current standard practice used in *Zantedeschia* industry in New Zealand is:

- to keep the flowers in a coolstore following harvest;
- to pulse flowers with a solution containing Chrysal L.V.B. (Pokon /chrysal, Naarden, Holland), Florasan (Taranaki Nuchem, New Plymouth)

and dextrose for prevention of " slimy stem" caused by a bacterial soft rot (Funnell, 1994).

Given these two potential advantages, the mixture of BAP and GA_3 as a pulsing solution prior to packing and shipping can potentially be utilized by growers to postpone re-greening in *Zantedeschia* 'Best Gold'. However, before it can be commercialized for growers of *Zantedeschia*, further development of this treatment methodology is required in terms of:

- optimal concentration of the pulsing solution, temperature for pulsing, duration of pulsing, and maturity stage of flowers to be treated;
- effectiveness of pulsing with the mixture followed by a certain period of dry storage, which is usually necessary for shipment;
- effectiveness of the pulse treatment with the addition of other compounds, e.g., sucrose and fungicide, which were recommended to prevent other postharvest problems of *Zantedeschia*, e.g. stem split and *Alternaria* flower spot (Tjia and Funnell, 1985), as well as sucrose by itself delayed re-greening (refer Chapter 5);
- effectiveness of the pulse treatment in delaying re-greening in other hybrids or selections of *Zantedeschia*.

Applying a pulse treatment of water in darkness at 5°C for 24–h, slightly delayed the time when re-greening was visible, but did not alter the pattern of re-greening upon illumination (Figure 7.2). This indicates that the spathe tissue still retains its capability to re-green after a certain period (at least 24-h) of darkness and cool storage. This finding is in line with that previously reported in cotyledons of *Cucurbita pepo* L., wherein the cotyledons re-greened after a 2- or 5-day dark

treatment (Ananieva et al., 2004). As detailed in Chapter 6 of this thesis, during a 7-day dark incubation, the spathe tissue underwent a decline in the content of total carotenoid and an initial differentiation of chloroplast from chromoplast, but no chlorophyll accumulation (Figure 6.5; Figure 6.9). It is therefore highly likely that within the 24-h dark and cool treatment of the experiment presented here, no chlorophyll accumulation occurred in the spathe tissue, as chlorophyll biosynthesis is light dependent (Armstrong and Apel, 1998; Armstrong et al., 1995). It is, however, uncertain whether during the 24-h period the content of carotenoid decreased and chromoplasts initiated their differentiation to chloroplasts. These results from the current experiment, in conjunction with those presented in Chapter 6, have raised some questions for investigation in the future, namely:

1. whether an increased duration of pulsing in BAP+GA_3, while in darkness and low temperature, can, to a certain extent, slow the rate of re-greening or prevent the re-greening upon illumination, without advancing the senescence of the spathe tissue;
2. whether pulsing in darkness and low temperature contribute equally to delaying re-greening?

7.5 Conclusion

The current study confirmed the efficacy of the combination of BAP and GA_3 in delaying re-greening for entire spathes. Among the three means of application of BAP+GA_3, i.e. as a pulse, spray or continuous vase solution, pulsing with a solution of BAP+GA_3 in darkness at 5 ºC appears to offer an effective method to delay re-greening for *Zantedeschia*. However, before it can be commercialized for growers of

Zantedeschia, further development of this treatment methodology is required, e.g. in terms of optimal concentration of the pulsing solution, temperature and duration of pulsing, maturity of flowers to be treated, as well as effectiveness of pulsing with the mixture followed by a certain period of dry storage.

Chapter 8 **General discussion**

The research presented in this thesis was part of an ongoing programme with the goal of providing *Zantedeschia* growers with methods to postpone spathe re-greening. In line with this goal, within this thesis, attempts were made to understand what physiological mechanisms are involved with re-greening, as described by changes in colour, levels and types of pigment, and differentiation of plastids; and how this process is controlled by various factors including fructification, light, cytokinin and gibberellin.

8.1 **What is re-greening about?**

Re-greening of spathe tissue in 'Best Gold' is characterised by regaining of green colouration, firstly seen at the proximal (basal) area on the abaxial surface, and subsequently as it progressed upward as well as outward from the midrib (Chapter 3). Although within the 14 to 21-day period of observation after horticultural harvest-maturity, re-greening was not observed on the adaxial surface, the adaxial surface of the spathe can re-green under normal conditions at a much slower rate compared to the abaxial surface.

The regaining of green colouration in spathe tissue primarily resulted from the accumulation of chlorophyll within the subepidermal layers of the abaxial surface, as reflected by a strong correlation between H° and total chlorophyll content in the surface (Chapters 2 and 3). During this period of increasing chlorophyll content, the

content of total carotenoid was largely retained. At horticultural harvest-maturity, the individual carotenoids present in spathe tissue (mainly lutein, with smaller amounts of other xanthophylls and β-carotene), were typical of those found in mature leaf tissue (Chapter 6). This is in agreement with that reported previously by Lewis et al. (2003). As re-greening progressed, these carotenoids persisted and, in particular, the predominant lutein, remained at a comparatively steady level. It is not surprising that these carotenoids were retained, as they are essential components for formation of chloroplasts (Sundqvist and Dahlin, 1997; Young, 1993a). During re-greening, the change in the content of carotenoid appears to contribute less to the colour of the spathe than the change in chlorophyll content. This is partially due to the fact that within the layers of the subepidermis where chlorophyll and carotenoids coexist, the newly-synthesized chlorophyll appears to mask the visibility of the carotenoids.

From an ultrastructural perspective, the physiological mechanism of re-greening for spathe tissue in 'Best Gold' was characterized by redifferentiation of chloroplasts from chromoplasts, as compared with *de novo* synthesis of chloroplasts from proplastids. This is supported by the observations presented in Chapters 3 and 6, i.e:

1. the presence of the intermediate stage of plastids containing the characteristics of both chloroplast and chromoplast, i.e. a new developing thylakoid system and a large number of plastoglobuli remained from the chromoplast;
2. absence of proplastids in the re-greening tissue;
3. absence of any rupture in the double-membrane envelope of the plastid during the transition from chromoplast to chloroplast.

These findings are in agreement with the previous observations of chloroplast redifferentiation during the re-greening in the spathe of *Zantedeschia elliotiana* (Watson) Engl. (Gronegress, 1974) and the peel of fruits from *Citrus sinensis* (L.) Osbeck. (Thomson et al., 1967). As further, albeit indirect, support of the hypothesis that proplastids are not involved in re-greening of spathe tissue, it is noted that proplastids, as the precursors of all plastid types, are usually present in zygotes and in the meristem of root, stem, leaf and flower tissue (Hudak et al., 2005). The fully mature spathe of 'Best Gold' at horticultural harvest-maturity is therefore, likely to be devoid of meristematic tissue and, therefore, proplastids.

The absence of proplastid to chloroplast differentiation during re-greening of spathe tissue distinguishes this process from greening. As noted in Chapter 1 (Figure 1.3), greening is associated with the formation of chloroplasts from a simple form of plastid, i.e. proplastid, whereas re-greening is associated with the reformation of chloroplasts from a specialized form of plastid (e.g. chromoplasts or amyloplasts).

Despite the comparative uniformity in size and ultrastructure for the chromoplasts present in the subepidermis of both abaxial and adaxial surfaces, and in various areas of the spathe in 'Best Gold' at horticultural harvest-maturity, their fate for subsequent development is tissue-dependent. Unique to this thesis, it was found that as re-greening proceeded, the chromoplasts in the abaxial surface developed into chloroplasts, whereas in the adaxial surface (separated by approximately 10 layers of mesophyll cells), no discernible change in the structural components of chromoplasts was observed (Chapter 3). Additionally, as noted above, re-greening started at the proximal area of the spathe and progressed toward the distal and outer areas away

from the midrib. While not specifically examined in this thesis, it seems plausible that this progressive onset of change in colour is associated with a sequential initiation of plastid differentiation from proximal toward distal areas of the spathe. It is, however, unclear why chromoplasts located in the proximal area (or abaxial surface) are more prompt to be redifferentiated into chloroplast than those located at the central and distal areas (or adaxial surface). One hypothesis is that the signals (e.g. cytokinins) required for induction of re-greening originate from the abaxial surface at the proximal area of the spathe, where some green tissue is retained. Another component of the hypothesis is that despite the comparative uniformity in ultrastructure of chromoplasts, some differences at the molecular level, e.g. amount of chloroplast DNA, RNA or ribosomes remaining in chromoplasts (Herrmann, 1972; Hunt et al., 1986), might exist between the abaxial and adaxial surfaces or between various areas of the spathe. So as to test this hypothesis, through hormonal analysis, ultrastructural observation and molecular approaches, some future research questions considered worth asking are:

1. Do differing areas of spathe tissue encounter spatial and/or temporal variations in the level of endogenous hormones (e.g. cytokinins)?
2. Is there a gradient of chloroplast redifferentiation from chromoplasts as re-greening progresses from proximal to distal areas of the spathe?
3. Do chromoplasts from the differing areas of spathe tissue exhibit spatial and/or temporal variations in the expression of chloroplast-related genes and proteins?

The redifferentiation of chloroplasts during re-greening of spathe tissue, mainly involved the reconstruction of the thylakoid system. As an extension to the

current understanding about how the thylakoid reconstructs, the current study revealed there are at least four possible mechanisms of thylakoid reformation (Figure 6.11). The presence of these multiple mechanisms for thylakoid reformation has not previously been reported in *Zantedeschia*. In addition to *de novo* synthesis of thylakoid by invaginations of the inner-envelope membrane, it is highly probable that the thylakoids were either, derived from redifferentiation and multiplication of primary thylakoids present in mature chromoplasts or, derived from plastoglobuli. The existence of multiple mechanisms for formation of type-1 thylakoids, in part reflects the natural plasticity of thylakoid membranes within plastids. This characteristic of thylakoids enables plastids to undergo dynamic differentiation between various forms (e.g. chromoplast to chloroplast, and *vice versa*) in order to adapt to changing physiological and environmental conditions.

The hypothesis of the existence of multiple mechanisms for thylakoid reorganization during re-greening was primarily based on the observation of physical attachment between the type-1 thylakoid and other types of primary thylakoids, inner-envelope membrane, and plastoglobuli, as well as a concomitant increase in the number of type-1 and decrease in other types of thylakoids and plastoglobuli (Chapters 3 and 6). Further quantitative evidence on dynamic lipid metabolism during thylakoid reorganization is needed to support this hypothesis, e.g. evidence of translocation of lipid metabolites from plastoglobuli to type-1 thylakoids. This may be potentially achieved by using some molecular approaches, e.g. immunolabelling and isotope-labelling combined with techniques of electron and confocal microscopy (Austin et al., 2006; Brehelin and Kessler, 2008; Padham et al., 2007; Ytterberg et al., 2006).

8.2 Why does the spathe re-green?

The data presented in the thesis are unable to provide a precise answer to this question, but some hypotheses are proposed. From morphological point of view, being a sole floral accessory embracing female and male flowers, the spathe may provide some physical protection for the development of fruits (if that occurs). The gradually enrolling of spathe along with the occurrence of re-greening partly substantiates this theory. Re-greening of the spathe prolongs the longevity of spathe tissue and, therefore, ensures the survival of the spathe until the maturation of fruits. On the other hand, by regaining an independent energy source, i.e. functional chloroplasts, re-greening of the spathe tissue potentially provides additional photoassimilates for the development and maturation of fruits on the nearby spadix. Similar to that occurs in *Zantedeschia rehmannii* and *Zantedeschia albomaculata* (Letty, 1973), the maturing fruits of 'Best Gold' embraced by the re-greened spathe enlarge and their weights bends the withering peduncle until the spathe tip touches the ground (pers. comm. Dr. Keith Funnell). The bent and withering peduncle may limit the transportation of photoassimilates from the leaves to the fruits. Hence, additional carbon assimilate supplied by the nearby re-greened spathe can potentially resolve this limitation. Photosynthetic activity has been determined in the re-greened spathe of *Z. aethiopica* (Tavares et al., 1998) and *Spathiphyllum wallisii* (Weidner et al., 1985). Further investigation on the functionality of the redifferentiated chloroplasts within re-greened tissue of 'Best Gold' is required. Also if these chloroplasts are functional, the significance in the flow of carbon assimilates from the re-greened spathe to the developing fruits compared with that from the leaves to the fruits is yet to be clarified.

From plant-insect coevolution point of view, reappearance of green colouration in spathe tissue of 'Best Gold' following the yellow-colour phase might potentially provide a cue to the pollinator that the flowers are no longer reproductive and/or pollination has already occurred, thus allowing the pollinators to forage more efficiently. This explanation for the purpose of re-greening has not been tested in *Zantedeschia*, but has been shown to be plausible in other species that change in floral colour is associated with the fertility of flowers, e.g. *Desmodium setigerum* Harv. (Willmer et al., 2009), *Pedicularis monbeigiana* Bonati (Sun et al., 2005), and *Lantana camara* L. (Weiss, 1991).

In summary, the potential reasons for spathes of *Zantedeschia* to re-green may include prolonging the longevity of the spathe tissue, providing additional photoassimilates to development and maturation of the fruits, and signalling the loss of fertility to the pollinators.

8.3 How re-greening is induced and controlled?

In the current study, investigations into how re-greening is induced and controlled started by testing the hypothesis proposed by Pais and Neves (1982-1983). They postulated that re-greening of spathe tissue in *Z. aethiopica* (L.) Spreng. was induced by cytokinin-like compounds that are produced during fructification, and transferred from the fruits to the spathe. In the research presented in this thesis, the occurrence of re-greening in the spathe of both *Z. aethiopica* and 'Best Gold', following the removal of the spadix prior to pollination, contradicted their hypothesis (Chapter 4). Further to this, the occurrence of re-greening in the spathe of 'Best Gold'

with a spadix naturally absent of female flowers, and also re-greening of pigmented leaves devoid of any true floral tissue, also contradicts their hypothesis. Thus, the current findings indicate that fructification is not necessarily a prerequisite for induction of re-greening, but this does not completely exclude the potential connection between fructification and spathe re-greening. Fructification might indirectly promote chlorophyll accumulation through an unknown mechanism and, therefore, ensure the completion of re-greening in spathe tissue. If re-greening in spathe tissue of *Zantedeschia* is not triggered by fructification, following the testing of Pais and Neves' (1982-1983) hypothesis, the question as to how re-greening is induced, remains.

The physiological mechanism of re-greening of spathe tissue in 'Best Gold' requires light for the process to complete, but the onset of re-greening, as characterized by formation of type-1 thylakoids, can be induced in darkness (Chapter 6). It is therefore proposed that some other factor(s) (e.g. ontogeny), but not light, has(ve) direct control over the induction of re-greening (Figure 8.1). For example, the timing of re-greening may be a genetically pre-programmed event regardless of environmental factors (e.g. light) or internal factors (e.g. fructification). If so, as the spathe matures to a certain stage (e.g. once female or male flowers are no longer viable), the nucleus will receive signals from the ontogenetic factor, to express re-greening-associated genes which, in turn, will lead to the onset of chromoplast to chloroplast redifferentiation. Further development towards the formation of a functional chloroplast, however, is likely to require the plastid to signal back its developmental state, namely plastid-nucleus signalling (Mochizuki et al., 1996; Susek and Chory, 1992). These signals may include chlorophyll accumulation and/or grana

stacking, which are light-dependent and also enhanced by plant hormones (Figure 8.1). Hence, the hypothesis presented in this thesis is that re-greening of spathe tissue is a process that requires the concerted action of environmental factors (e.g. light) and internal factors (e.g. ontogeny, plant hormones, the nucleus and plastids). The role of light in this process is to ensure the completion of re-greening, i.e. formation of functional chloroplasts (Chapter 6).

Where do plant hormones (e.g. cytokinin and gibberellin) fit into this proposed physiological mechanism of re-greening (Figure 8.1)? Based on the current studies into the influence of synthetic hormones, it was found that BAP stimulated re-greening by enhancing the accumulation of carotenoid and chlorophyll, and also stacking of grana (Chapter 6). But the response to BAP was dependent on certain prevailing conditions, e.g. the presence/absence of light, the stage of development/re-greening, and which surface, i.e. abaxial or adaxial. In contrast, the application of GA_3 retarded formation of type-1 thylakoids, and thus delayed the onset of re-greening (Chapter 6). Hence, a synergistic effect in delaying the onset of re-greening provoked by the combination of BAP and GA_3 was likely to be a result of co-regulation between BAP-stimulated accumulation of carotenoid (Figure 6.3) and GA_3-stimulated retardation of chloroplast differentiation (Figure 6.9 and Figure 8.1). This suppression of re-greening by the combination of BAP and GA_3, however, was soon overcome when light was present. The current results lead to a hypothesis that the progression and completion of re-greening requires a comparatively high level of cytokinin, but low level of gibberellin; light may be one factor that is able to regulate the balance in content of endogenous cytokinin and gibberellin within spathe tissue and/or the sensitivity of tissue to these hormones. The presence of these endogenous hormones

subsequently assists the formation of functional chloroplasts. Future research through biochemical, molecular and ultrastructural approaches is required to unravel the complex cross talk between light, cytokinin and gibberellin in influencing the process of re-greening. Some future research questions considered worth asking are:

1. Do levels of endogenous cytokinins and gibberellins in spathe tissue and/or sensitivity of tissue to these hormones vary prior to, and during, re-greening?
2. If yes, when kept in darkness, does the balance of these hormones and/or sensitivity to these hormones change?
3. If re-greening of spathe tissue requires comparatively higher level of cytokinins but lower level of gibberellins, do cytokinin inhibitors or increased endogenous level of gibberellins retard re-greening?

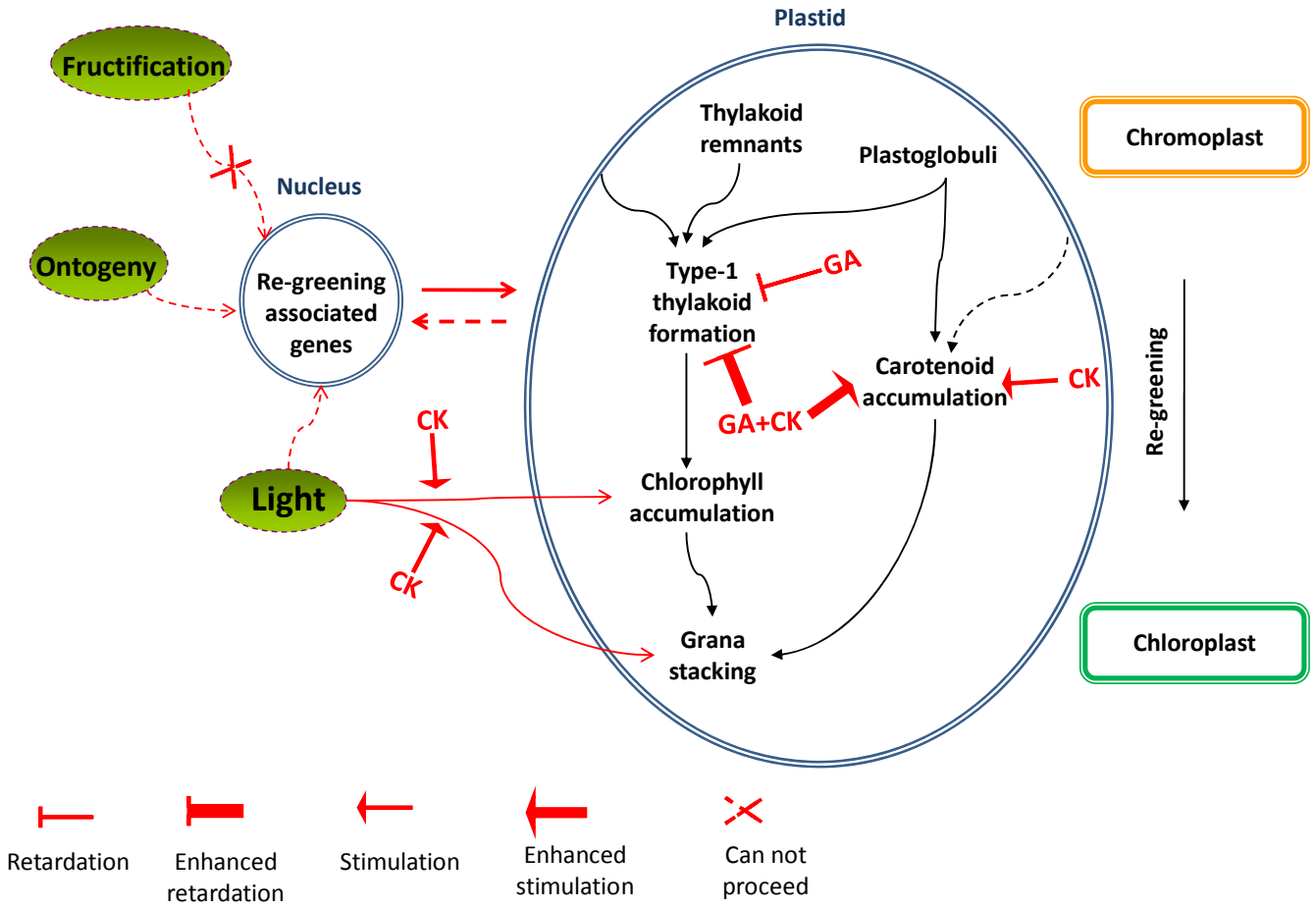

Figure 8.1 Conceptual model for the physiological mechanism of light-, cytokinin-, and gibberellin-mediated re-greening of mature spathes of *Zantedeschia* 'Best Gold'. CK: cytokinin; GA: gibberellin.

8.4 Horticultural application

As a part of an ongoing research programme toward the development of a treatment that can be utilised by growers to delay re-greening in spathes of *Zantedeschia*, the data presented in Chapter 5 and 6 of this thesis indicated that application of BAP+GA_3 enabled a delay in re-greening for discs of spathe tissue in 'Best Gold'. Further studies shown in Chapter 7 confirmed that this mixture of BAP and GA_3 was also effective in delaying the re-greening for the entire spathe by approximately seven days, without discernible adverse effect on flower quality. Among the three means of application of BAP+GA_3, i.e. as a pulse, spray or continuous vase solution, a pulse with BAP+GA_3 in darkness at 5 ºC for 24 hours was the most efficient way to delay re-greening of the entire spathe. This protocol can easily be included as a part of the current standard postharvest handling for *Zantedeschia*. However, before being used commercially by growers or retailers to postpone re-greening of *Zantedeschia*, further development of this treatment methodology is required in terms of:

1. optimal concentration of the pulsing solution, temperature for pulsing, duration of pulsing, and maturity stage of flowers to be treated;
2. effectiveness of pulsing with the mixture when combined with a period of dry storage, which is usually necessary during shipment;
3. effectiveness of the pulse treatment with the addition of other compounds, e.g., sucrose and fungicide, which have been recommended to prevent other postharvest problems of *Zantedeschia*, e.g. stem split and *Alternaria* flower spot (Tjia and Funnell, 1985), as well as sucrose by itself delayed re-greening (refer Chapter 5);

4. effectiveness of the pulse treatment in delaying re-greening in other hybrids or selections of *Zantedeschia*.

Appendix II

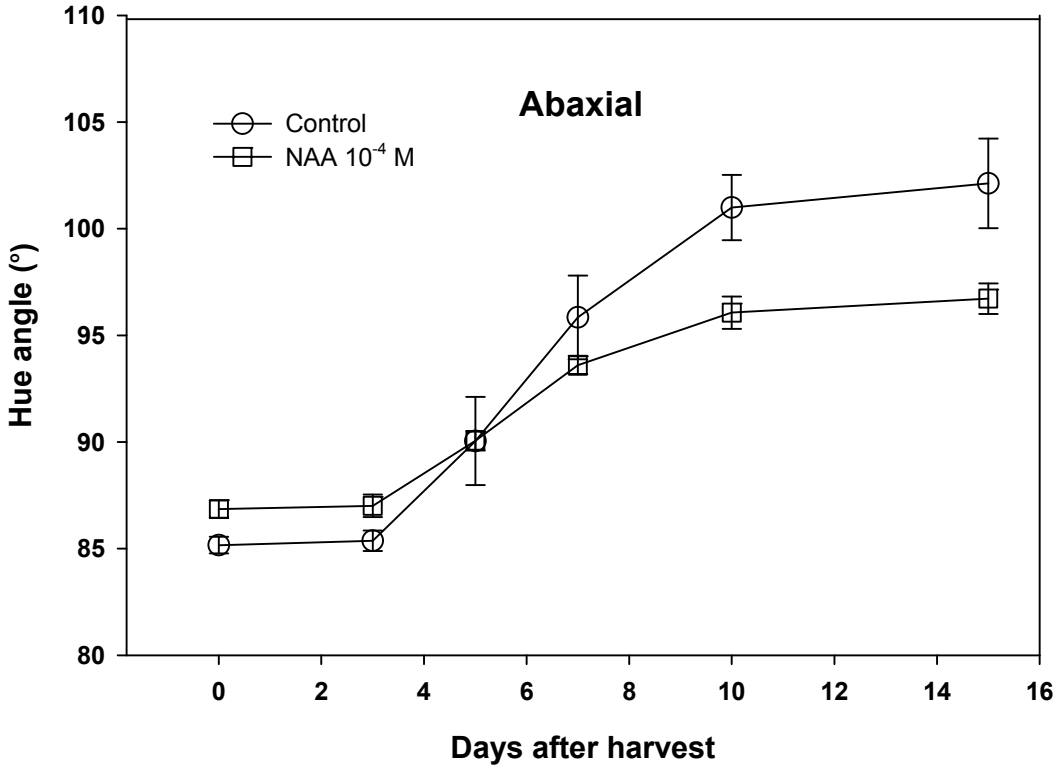

Figure I Change over time in hue angle of the abaxial surface of spathe tissue of *Zantedeschia* 'Best Gold', treated with NAA at the concentration of 10^{-4} M. Each point was the average of four replicates. Vertical bars represent standard errors. Controls were kept in RO water.

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