The Role of Brassinosteroids in the Regulation of Senescence in *Lilium Orientalis*

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Abstract

Fresh flowers of cut lily plants often have a long postharvest life, but this changes after cold storage, which is often an essential process in the horticultural industry. In many cut lilies, a relatively short period of cold storage of 1 week often leads to early leaf and flower senescence. Early leaf and flower senescence are likely ethylene-dependent, either due to a higher level of ethylene production or a change in ethylene sensitivity. Such changes could be due to alteration in the expression of ethylene biosynthesis, perception, and signalling genes. Brassinosteroids (BRs) can delay the onset of senescence and chilling injury in crops but their effects on chilling tolerance in cut flowers have not been investigated.

In this thesis, I investigated the role of BR and cold storage on cut lilies (*Lilium orientalis*) and the role of ethylene in cold-related injuries. More specifically, I investigated: i) the effects of cold storage and BR on postharvest function and ethylene production in a range of cut lily cultivars (Chapter II); ii) the effects of cold storage and BRs + cold storage on the expression pattern of ethylene related genes and a BRs-related transcription factor gene in cut lilies 'Premium Blonde' and 'Marlon' (Chapter III); and iii) the effects of ethylene on senescence processes and the expression pattern of its biosynthesis, receptor, and signal transduction pathway genes during senescence with and without cold storage in cut lily 'Marlon' (Chapter IV).

The results of chapters 2 and 3 indicated that: 1) the cut lily cultivars had different levels of sensitivity to cold storage; 2) BR decreased ethylene production and the detrimental effects of cold storage; 3) BR treatment resulted in a greater expression *LoBZR1* and a lower expression of most ethylene-related genes compared to cold storage treatment in 'Premium Blonde' and 'Marlon' lilies. Together, these findings suggest that the chilling injuries in cut lilies are ethylene mediated which could be due to either higher ethylene

biosynthesis or higher ethylene sensitivity or a combination of both as a result of alteration in the expression of the related genes. Thus, delayed chilling injuries by application of BR is likely caused by the suppression of the over-expression of ethylene-related genes which results in less ethylene biosynthesis and possibly sensitivity.

To understand whether chilling injuries are ethylene-dependent cut lily 'Marlon' plants in chapter IV were subjected to ethylene and 1-Methylcyclopropene (1-MCP; an ethylene action inhibitor) before and after cold storage. In non-cold-stored plants, ethylene and 1-MCP treatments did not affect postharvest quality, ethylene production, and the expression pattern of the investigated genes. Cold storage led to much earlier leaf yellowing and while bud opening was not affected, flowers senesced and abscised three days earlier. In cold-stored plants, ethylene and 1-MCP had no effects on bud opening and leaf yellowing but flower senescence and abscission were hastened by ethylene and delayed by 1-MCP. Similarly, the expression of all the genes examined and ethylene production were significantly increased by ethylene but significantly decreased by 1-MCP in cold-stored plants, although not at all time points. These results indicate that there is no sensitivity to ethylene in fresh-cut lily 'Marlon'. But the response significantly differs following cold storage, in a tissue-dependant manner as flower senescence and abscission were delayed by 1-MCP but leaf senescence was not. Hence, ethylene does influence flower senescence following cold storage but leaf senescence is under the control of other currently unknown factors.

Together this research indicates that focus on ethylene blockers to prolong postharvest life in lilies will be effective for flowers but not leaves. However, BR application was not seen to be beneficial at a practical level in all cultivars particularly in cultivars with a low level of sensitivity as the delays of senescence were very short.

Declaration

This is to certify that:

1) This thesis comprises only my original work towards the PhD except where indicated in the preface
2) Due acknowledgement has been made in the text to all other material used
3) The thesis is less than 80,000 words in length exclusive of tables, figures, references, and appendices.

Muhammadali Daneshinergi

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Preface

This PhD thesis consists of five chapters, an introduction, three experimental chapters, and a synthesis. The experimental chapters have been prepared in paper format with some overlap in chapter content. Muhammadali Daneshinergi designed the experiments, collected the data in the laboratory, analysed the data and wrote the thesis. The co-authors supervised various stages of the PhD study and contributed in revising the manuscripts.

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Chapter 1

General Introduction

1.1. Senescence

Plants terminate their lives through a self-destructive system known as senescence (Woo et al., 2013). In higher plants, senescence is age-dependent and a type of programmed cell death (PCD) characterised by a series of biochemical and physiological changes such as:

- membrane leakiness,
- a decrease in the level of protective enzymes and an increase in oxidative enzymes;
- activation of nucleases, proteases and wall modifiers;
- degradation of macromolecules (nucleic acids [DNA and RNA], proteins) and organelles (chloroplasts), and
- redistribution of components to other plant parts/organs (Buchanan-Wollaston, 1997; Quirino et al., 2000; Tripathi and Tuteja, 2007).

The degraded and redistributed macromolecules are then used for later development of other organs (Woo et al., 2013). This process is genetically controlled and highly regulated by the expression of many genes known as senescence associated genes (SAGs) which are involved in the biochemical and physiological changes (Biswal and Biswal, 1999; Quirino et al., 2000).

Senescence can be a beneficial process in annual and biennial plants or deciduous trees. In such cases, nutrients are relocated to other parts of the plant, reused in developmental processes and/or stored for later usage, by which plants survive or develop new organs. An example of such a process is the relocation of nutrients to seeds and fruit or storage of nutrients in roots and stems (Woo et al., 2013), which are later used for the growth and development of new plants from seeds such as wheat and barley, or new organs from stems such as flowers and leaves in trees. In the case of many horticultural products however, this process is non-beneficial and undesirable, contributing to a quality loss in organs/plants including fruit, vegetables, cut flowers, and foliage (Reid, 2002b).

1.1.1. Senescence in cut flower

Cut flowers are flowers or flower buds that have been cut from the mother plants (Boodley, 1981). In cut flowers, the quality refers to the appearance of the entire cut stem and quality loss includes leaf and stem yellowing, wilting or abscission of petals or leaves, necrosis, and bending of scapes and stems (geotropic or phototropic) (van Doorn, 2001; Reid, 2002a). The length of the time that a cut flower remains in presentable or fresh in vase form without losing its quality is known as vase life (Anonymous, n.d.), whereas, longevity is defined as to the period of the time during which a flower is open and fresh (Primack, 1985). Shelf life refers the period of the time from cutting a flower until it starts to wilt, loss colour or petals, failure to reach full bloom (Chackal, 1992). Vase life is one of the most important traits in assessing the quality of cut ornamentals which varies from a few hours, a day, several days to several weeks in long-lived flowers (Stead, 1992). After being cut off from their mother plants, cut flowers survive on their own limited resources (da Silva Vieira et al., 2012). But they are subjected to the active process of senescence (Jing and Nam, 2012).

Senescence is known as a common reason of the reduced longevity (Reid, 2002b), which causes significant losses and is a limitation to the marketing of many species of cut flowers during postharvest (Reid, 2002a). In parts of cut flowers, such as leaves and flowers, the senescence process is postmitotic and takes place at the last stage of life cycle (Guo and Gan, 2005; Lim et al., 2007). In these organs, senescence is visible with chlorophyll degradation (degreening), necrosis, wilting and abscission, petal discoloration and fading of blossoms (Reid and Wu, 1992; Lim et al., 2005; Tripathi and Tuteja, 2007). This occurs in response to a series of different factors such as age, reproductive growth, plant hormones/growth regulators, extreme temperature, nutrients, light, and pathogens (Guo and Gan, 2005; Tripathi and Tuteja, 2007; Thomas, 2012; Woo et al., 2013).

1.1.2. Factors affecting senescence

Senescence is related to various internal and external factors. Examples of internal factors are transpiration, respiration, ethylene biosynthesis and sensitivity, biochemical changes, and growth and development. External factors include temperature, moisture, atmosphere, light, gravity, disease, and insects (Reid and Jiang, 2012; El-Ramady et al., 2015). External factors result in biotic or abiotic stresses in plants (Hewett, 2006; Wills et al., 2007; Toivonen and Hodges, 2011; Taylor et al., 2012). These stresses result in early senescence through the activation of cell signalling pathways, accumulation of low molecular weight metabolites, and changes in phytohormone levels such as ethylene (Reid and Kofranek, 1980). However, the level of the changes varies based on species/cultivar, postharvest handling, storage, and distribution conditions (Reid and Kofranek, 1980; Reid and Jiang, 2012). Ultimately, the stresses cause changes in

commodities leading to unmarketable produce and large losses in the horticultural industry (Hewett, 2006; Wills et al., 2007).

1.1.2.1. Dark-induced senescence

Darkness is known as one of the inducers of senescence process in photosynthesis-independent (dark-inducing senescence signalling) and -dependent (sugar starvation) manners (Liebsch and Keech, 2016; Ueda et al., 2020). Chlorophyll loss is the main symptom of dark-induced senescence in many species, (Han, 1997; Weaver and Amasino, 2001; Han and Miller, 2003; Mutui et al., 2005; Hatami and Ghorbanpour, 2013). In photosynthesis-independent manner senescence signalling is mediated by phytochromes (Biswal and Biswal, 1984; Brouwer et al., 2012; Sakuraba et al., 2014). Phytochrome B – red light/far-red light receptor – and PYHTOCHROME INTERACTING FACTORs (PIFs) 4 and 5 as its transcription factors have an important role to play in dark-induced senescence (Ueda et al., 2020). In light, red light converts the phytochromes into their active form (Pfr), but in shading/darkness, far-red light inactivates the phytochromes (Pr) (Franklin, 2008). Activated phytochromes degrade PIFs in a phytochrome B-dependent manner which leads to inactivation of PIF-dependent senescence activation (Liebsch and Keech, 2016). But in dark, PIFs activate ethylene and abscisic acid signalling components such ETHYLENE INSENSITIVE 3 (EIN3), ABSCISIC ACID INSENSITIVE 5 (ABI5), and a transcription factor involved in abscisic acid signal responses ENHANCED EM LEVELS (EEL), which together lead to increase in the expression of a positive senescence-regulating transcription factor ORESARA1 (ORE1) (Khan et al., 2014; Jeong et al., 2016; Liebsch and Keech, 2016; Paik et al., 2017). Then, ORE1 and PIFs decrease the expression of *GOLDEN2-LIKE 1* (*GLK1*) which functions in chloroplast development, and with EIN3,

and ABI5 increase the expression of *STAY-GREEN 1* (*SGR1*) and *NON-YELLOW COLORING 1* (*NYC1*) genes involved in chlorophyll degradation (Qiu et al., 2015; Liebsch and Keech, 2016). Ethylene biosynthesis genes (ACSs) are also activated by ORE1 (Qiu et al., 2015; Liebsch and Keech, 2016; Paik et al., 2017). Other senescence associated genes are also activated by ORE1 (Liebsch and Keech, 2016). PIFs may up-regulate the expression of other transcription factor genes *WRKY22* and *NAP* which are deemed to be involved in dark-induced senescence chlorophyll degradation (Zhou et al., 2011; Fan et al., 2015; Liebsch and Keech, 2016). The expression of *NAP* is also increased in response to abscisic acid (Fan et al., 2015). The complex of PIFs and *ABSCISIC ACID INSENSITIVE 5* (*ABI3*) also increases the expression of *SOMNUS* (*SOM*) gene which is involved in gibberellin biosynthesis inhibition (Paik et al., 2017). SOM (CCCH-type zinc finger protein) decreases the expression of gibberellin biosynthetic genes *GA3ox1* and *GA3ox2* but activates *GA2ox2 a* gibberellin degradation gene which result in low endogenous gibberellin content (Kim et al., 2008; Gabriele et al., 2010). Darkness also leads to senescence in photosynthesis-dependent manner which is due to loss of sugar production (Fujiki et al., 2001; Chrost et al., 2004; Lee et al., 2004; Liebsch and Keech, 2016; Zhaowei et al., 2020). Sugar starvation increases amino acids, fatty acids, and proteins' catabolism, but decreases the activity of enzymes involved in sugar metabolism and respiration, nitrate reduction and assimilation, and protein synthesis (Journet et al., 1986; Brouquisse et al., 1991; Brouquisse et al., 1992; Dieuaide et al., 1992; Tassi et al., 1992; Biswal and Raval, 2013). The loss in sugar production is sensed by SnRK1 - a sucrose nonfermenting-1-related protein kinase - as a sugar sensor (Baena-González et al., 2007) with major regulatory functions in plant metabolism and development, and stress signalling (Li and Sheen, 2016). SnRK1 acts as a heterotrimeric complex which requires KIN10 and KIN11 protein kinases as catalytic α-subunits (Tomé

et al., 2014). KIN10 phosphorylates basic region/leucine zippers (bZIPs), basic-helix-loop-helix (MYC2), and no apical meristem/Arabidopsis transcription activation factor 1 (NAC2/ATAF1) transcription factors which are involved low energy responses (starvation) (Dietrich et al., 2011; Garapati, Feil, et al., 2015; Mair et al., 2015; Qi et al., 2015). bZIP1 and bZIP53 directly bind to the promotors of *PROLINE DEHYDROGENASE* (*ProDH*) and *ASPARAGINE SYNTHETASE1* (*ASN1*) which are involved in amino acid metabolism (Dietrich et al., 2011). MYC2 activate jasmonic acid-induced leaf senescence by binding to the promotor of *SENESCENCE-ASSOCIATED GENE29* (*SAG29*) and activating its expression (Qi et al., 2015). Overexpression of *ATAF1* directly increases the expression of *TREHALASE1* gene (Garapati, Feil, et al., 2015) which encodes a plasma membrane-associated enzyme trehalase (Frison et al., 2007). Trehalase catabolizes trehalose (a nonreducing disaccharide formed of two α-glucose units) into two monomers of glucose (Avonce et al., 2006; Frison et al., 2007). ATAF1 is also a transcriptional regulator of a key enzyme of the biosynthesis of abscisic acid pathway *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*) (Jensen et al., 2013) as increase the expression of ATAF1 leads to increase in the level of abscisic acid by enhancing the expression of *NCED3* (Wu et al., 2009; Jensen et al., 2013). It also directly targets *ORE1* and *GLK1* genes by increasing the expression of *ORE1* and decreasing the expression of *GLK1* (Garapati et al., 2015). ORE1 with ethylene and abscisic acid related elements also regulate the expression of genes (*GLK1*, *SGR1*, and *NYC1*) related to chlorophyll degradation (Qiu et al., 2015; Liebsch and Keech, 2016) and ethylene biosynthesis genes (ACSs) (Qiu et al., 2015; Liebsch and Keech, 2016; Paik et al., 2017) which shows a common pathway of regulating senescence between photosynthesis-independent and -dependent manners, but it is also possible that

they might independently affect the expression of these genes without any integration (Liebsch and Keech, 2016).

1.1.2.2. Role of cytokinins in senescence

Jhj Cytokinins regulate many physiological and development processes in plants from cell division and differentiation to root and shoot growth and senescence (Jameson and Song, 2015; Kieber and Schaller, 2018). Decline in the level of endogenous cytokinins' level either due to age or species difference results in early senescence (Mayak and Halevy, 1970; Lara et al., 2004). While increase in the level of endogenous cytokinins in transgenic plants with a cytokinin biosynthetic gene (*P*(*SAG12*)-*ipt*) delayed senescence (Chang et al., 2003; Zakizadeh et al., 2013). Moreover, exogenous application of cytokinins retarded petal and leaf senescence in cut flowers such as carnation (*Dianthus caryophyllus* L.) (Eisinger, 1977; Bosse and Van Staden, 1989), day lily (*Hemerocallis fulva*) (Gulzar et al., 2003), Dutch iris (*Iris* × *hollandica*) (van Doorn et al., 2013), tulips (*Tulipa* spp.) (van Doorn et al., 2011), rose (*Rosa hybrida* L.) (Lukaszewska et al., 1994), petunia (*Petunia* × *hybrid*) (Trivellini et al., 2015), wintersweet (*Chimonanthus praecox*) (Sui et al., 2015), poppy anemone (*Anemone coronaria*), lisianthus (*Eustoma grandiflorum Eustoma*), and Garden phlox (*Phlox paniculata*) (Chernov et al., 2007). Cytokinins have been reported to delay senescence through ethylene-dependent and independent manners (Eisinger, 1977; Chang et al., 2003; Rogers, 2012). In ethylene-dependant manner, cytokinins delay senescence by reducing ethylene production and/or ethylene sensitivity (Eisinger, 1977; Bosse and Van Staden, 1989; Chang et al., 2003; Zakizadeh et al., 2013). As in carnation flowers (*Dianthus caryophyllus* L.) cytokinin led to less ethylene production and responsiveness and delayed senescence compared to non-

treated flowers (Eisinger, 1977; Mor et al., 1983). In transgenic petunia (*Petunia* x *hybrida* cv V26) (Chang et al., 2003) and miniature potted rose (*Rosa hybrida* cv. Linda) (Zakizadeh et al., 2013), increase in the level of a cytokinin biosynthesis related gene (*P*(*SAG12*)-*ipt*) led to less ethylene production and responsiveness and delayed senescence. The changes in ethylene production and sensitivity could be the result of blocking the conversion of externally applied 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene or suppressing the expression of ethylene-related genes by cytokinins (Mor et al., 1983; Trivellini et al., 2015). However conversely, there are reports showing that cytokinins increase ethylene production and caused early senescence in cut carnations (*Dianthus caryophyllus* L. 'White Sim' and 'Kaly') (Van Staden and Joughin, 1988; Woodson and Brandt, 1991) and rose (*Rosa hybrida* L. 'Sparkle') (Rasouli et al., 2015), carrot (*Daucus carota* L., cell line 'Pisa') (Carimi et al., 2004), and Arabidopsis (*Arabidopsis thaliana* L., cell line 'E157') (Carimi et al., 2004). Such early senescence caused by cytokinin seems to be concentration-dependant. As in cut carnation (*Dianthus caryophyllus* L. 'Kaly') (Van Staden and Joughin, 1988) a low concentration of cytokinins caused early senescence, while in rose (*Rosa hybrida* L. 'Sparkle') (Rasouli et al., 2015), carrot (*Daucus carota* L., cell line 'Pisa') (Carimi et al., 2004), and Arabidopsis (*Arabidopsis thaliana* L., cell line 'E157') (Carimi et al., 2004) high concentrations of cytokinins caused early senescence.

In ethylene-independent manner, cytokinins seems to delay senescence by maintaining membrane integrity (Gulzar et al., 2003), increasing water uptake (Mayak and Halevy, 1974; Gulzar et al., 2003), maintaining the sugar level of tissue (Gulzar et al., 2003), slowing down protein, RNA, and DNA degradation (Osborne, 1962; Mayak and Halevy, 1974; Gulzar et al., 2003), and dry weight reduction (Mayak and Halevy, 1974). In cut rose (*Rosa hybrida* 'Golden Wave'), cytokinins increased water uptake and petal growth

and slowed downed senescence-related processes such an RNA degradation and dry weight reduction (Mayak and Halevy, 1974). In day lily (*Hemerocallis fulva* 'Royal Crown'), cytokinin treatment also delayed senescence by reducing ion leakage, increasing water content, maintaining sugar content of tissue, and slowing down protein degradation (Gulzar et al., 2003).

1.1.2.3. Role of gibberellins in senescence

As another group of plant hormones, gibberellins are involved in senescence process in cut flower. In dandelion leaves (*Taraxacum officinale*), the level of gibberellines was high during leaf growth while it decreased during leaf senescence, suggesting that leaf senescence is related to decrease in level of gibberellines (Fletcher et al., 1969). Similarly, in daisy (*Chrysanthemum morifolium*), the level of gibberellines was high in early stage of the development of the flower but decreased in later stages (Jeffcoat and Cockshull, 1972). Moreover, applied gibberellins delayed senescence process in many cut flower such as carnation flowers (*Dianthus caryophyllus* L.) (Garrod and Harris, 1978; Saks and Van Staden, 1992; Saks and Van Staden, 1993), alstroemeria (*Alstroemeria pelegrina*) (Jordi et al., 1995), Asiatic hybrid lily cultivars ('Vermeer', 'Vivaldi' and 'Marseille') (Ranwala and Miller, 2000), gerbera (*Gerbera jamesonii* 'Ida Red') (Emongor, 2004), satuwa (*Paris polyphylla*) (Yu et al., 2009; Li et al., 2010), cut gladiolus flowers (*Gladiolus grandiflora* Hort.) (Costa et al., 2016), and anthurium (*Anthurium andraeanum* 'Arizona') (do Nascimento Simões et al., 2018). Gibberellins seem to delay senescence directly by inhibiting ethylene (Saks and Van Staden, 1992; Saks and Van Staden, 1993) and abscisic acid production (Yu et al., 2009; Costa et al., 2016) or indirectly by increasing water content (Emongor, 2004), slowing down the decrease of pigments and

total soluble protein, and reducing oxidative stress (Yu et al., 2009; Li et al., 2010; do Nascimento Simões et al., 2018). For instance, in cut carnations (*Dianthus caryophyllus* L.) gibberellins suppressed ethylene production both at early preclimacteric and climacteric stages (Saks and Van Staden, 1992; Saks and Van Staden, 1993) and respiratory climacteric (Saks and Van Staden, 1992) (*Dianthus caryophyllus* L.). Or in satuwa (*Paris polyphylla*) and cut gladiolus flowers (*Gladiolus grandiflora* Hort.) delay in senescence by gibberrellins was reported to be due to decrease in the level and activity of abscisic acid (Yu et al., 2009; Costa et al., 2016). While gibberellins delayed senescence by increasing water content and maintaining flower turgidity, and reducing bent neck gerbera in gerbera (*Gerbera jamesonii* 'Ida Red') (Emongor, 2004). Moreover, gibberelline-treated satuwa (*Paris polyphylla*) had less pigments and total soluble protein degradation, less oxidative stress as a result of reduction in lipid peroxidation and the level of hydrogen peroxide, and changes in the activity of antioxidant and chlorophyllase enzymes (Yu et al., 2009; Li et al., 2010). Similarly, in anthurium (*Anthurium andraeanum* 'Arizona') gibberellins applied in combination with spermine increased phenols level and (do Nascimento Simões et al., 2018).

1.2. Role of ethylene in plant senescence

1.2.1. Ethylene biosynthesis pathway

The ethylene biosynthesis pathway in higher plants has been well studied and documented (Yang and Hoffman, 1984; Kende, 1993; Bleecker and Kende, 2000; Klee, 2004). Ethylene is formed from S-adenosyl methionine (SAM) which is an activated form of the amino acid methionine (Met) (Fig. 1.1.). Methionine is the precursor of many biosynthesis pathways such as ethylene and polyamines (Sauter et al., 2013). In the first

step in the ethylene biosynthesis pathway, methionine is converted to SAM by SAM synthetase enzyme. Then, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). Finally, the ACC is converted to ethylene by ACC oxidase (ACO) (Fig. 1.1.). The conversion of ACC to ethylene is oxygen dependent and under low oxygen conditions the formation of ethylene is inhibited (Yang and Hoffman, 1984; Kende, 1993; Bleecker and Kende, 2000).

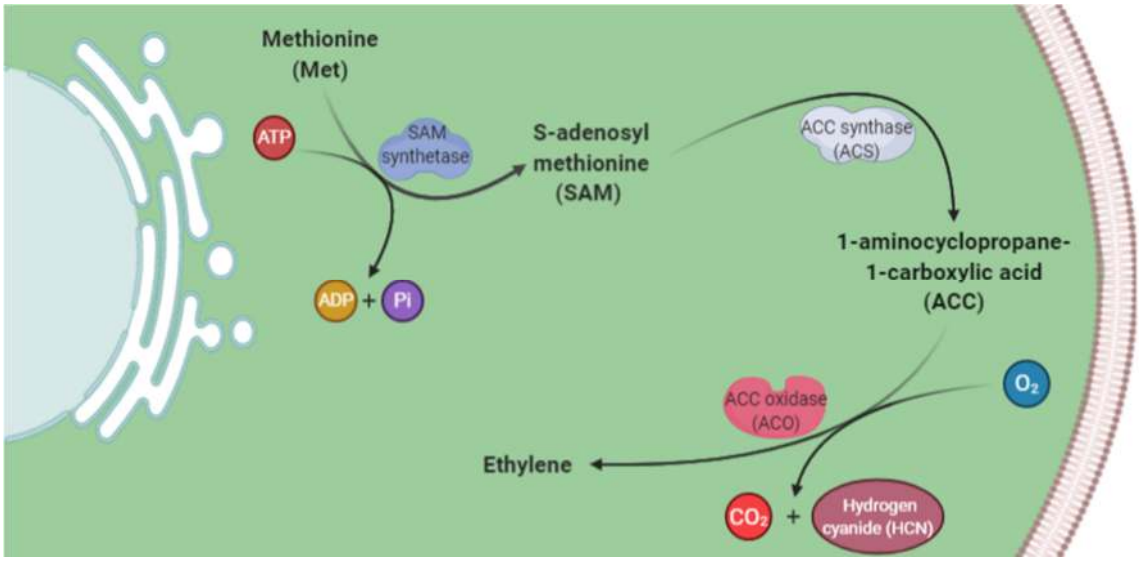

Figure 1.1. Ethylene biosynthesis pathway. Modified from Wang et al. (2002).

Both ACS and ACO are important enzymes in the regulation of ethylene production in plants (Yamagami et al., 2003; Liu et al., 2014). ACC formation from SAM is catalyzed by ACS. The ACC formation is a rate-limiting step in this pathway. ACS is a member of pyridoxal phosphate (PLP) -dependant enzymes that uses vitamin B6 as co-factor to function (De Martinis et al., 2015). In terms of their structural properties, ACS isoforms are classified into three subgroups; these subgroups differ from each other based on the availability of phosphorylation sites in their C-termini (Xu and Zhang, 2014). ACS enzyme is encoded by a medium-sized multi-gene family of several isoforms in different plants (*ACS1-12* in Arabidopsis and less in other plants) (Yamagami et al., 2003; Ma et al., 2005; Liu et al., 2014; De Martinis et al., 2015).

The second important enzyme in this pathway is ACO. ACO regulates the last step of ethylene biosynthesis, which is an oxygen-dependent step. This means a lack of oxygen will result in cessation in ethylene production. ACO is a dioxygenase enzyme that uses iron (Fe^{2+}) and bicarbonate as co-factor and activator, respectively (De Martinis et al., 2015). This enzyme is encoded by a small-sized multi-gene family (*ACO1-5*) (Pech et al., 2010; Van de Poel et al., 2012).

These genes from both families have been isolated from different plants (Oetiker et al., 1997; Ma et al., 2005; Khan, 2006; Van de Poel et al., 2012; Ruduś et al., 2013; Zhou et al., 2013; Liu et al., 2014). The expression pattern of both gene families differ in different tissues and developmental stages in response to environmental factors and hormonal treatments (Liang et al., 1992; Tsuchisaka and Theologis, 2004; Khan, 2006; Arteca and Arteca, 2008; Ruduś et al., 2013; Rodrigues et al., 2014).

1.2.2. Ethylene perception and signal transduction pathway

Ethylene perception and signalling pathway has been well characterized in Arabidopsis (*Arabidopsis thaliana* L.) (Solano and Ecker, 1998; Bleecker, 1999; Stepanova and Alonso, 2005). Different components of this pathway have been identified. Ethylene is sensed by a family of membrane-localized receptor proteins in plants called ethylene receptors, which have similarity to two-component histidine kinase receptors in bacteria. This family comprises of five proteins: ETR1 (ETHYLENE RECEPTOR/RESISTANT/RESPONSE 1), ETR2 (ETHYLENE RECEPTOR/RESPONSE 2), ERS1 (ETHYLENE RESPONSE SENSOR 1), ERS2 (ETHYLENE RESPONSE SENSOR 2) and EIN4 (ETHYLENE INSENSITIVE4) (Solano and Ecker, 1998; Chang and Shockey, 1999; O'Malley et al., 2005; Kim et al., 2011), which have been divided into two subfamilies based on structural properties: I

ETR1 (ETR1 and ERS1) and II ETR2 (ETR2, EIN4, and ERS2) (Fig. 1.2.) (Bleecker, 1999).

These receptors are active in the absence of ethylene, meaning that they negatively affect its associated physiological responses (Fig. 1.2.) (Stepanova and Alonso, 2005). Ethylene binding to these receptors involves copper as co-factor. Upon ethylene binding, these receptors are inactivated, which activates signalling pathway resulting in a chemical signal transduction to the nucleus (Guo and Ecker, 2004).

Signal transduction refers to a cascade of events allowing a signal usually from outside the cell being sensed by the cell (Stewart Jr, 2016). The signal transduction process is carried out by a series of proteins including CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), EIN2 (ETHYLENE INSENSITIVE 2), EIN5 (ETHYLENE INSENSITIVE 5), EIN6 (ETHYLENE INSENSITIVE 6) and EIN7 (ETHYLENE INSENSITIVE 7) (Chang and Shockey, 1999). Like receptors, CTR1 is a negative regulator of ethylene responses and is inactivated in the presence of ethylene. While, EIN2, EIN5, EIN6, and EIN7 positively affect the pathway and responses. CTR1 inactivation leads to the activation of EIN2, which is a transcriptional cascade activator and activates EIN3 and EIN3-like transcription factors, in turn, EIN3 regulates the expression of other transcription factors involved in the pathway such as ERF1 (ETHYLENE RESPONSE FACTOR1) (Fig. 1.2.). Then ERF1 binds to a GCC-box present in the promoters of ethylene and defense-related genes and regulates their expression (Guo and Ecker, 2004), which ultimately, results in ethylene responses (Fig. 1.2.) (Chang and Shockey, 1999; Guo and Ecker, 2004; Stepanova and Alonso, 2005; Frankowski et al., 2007).

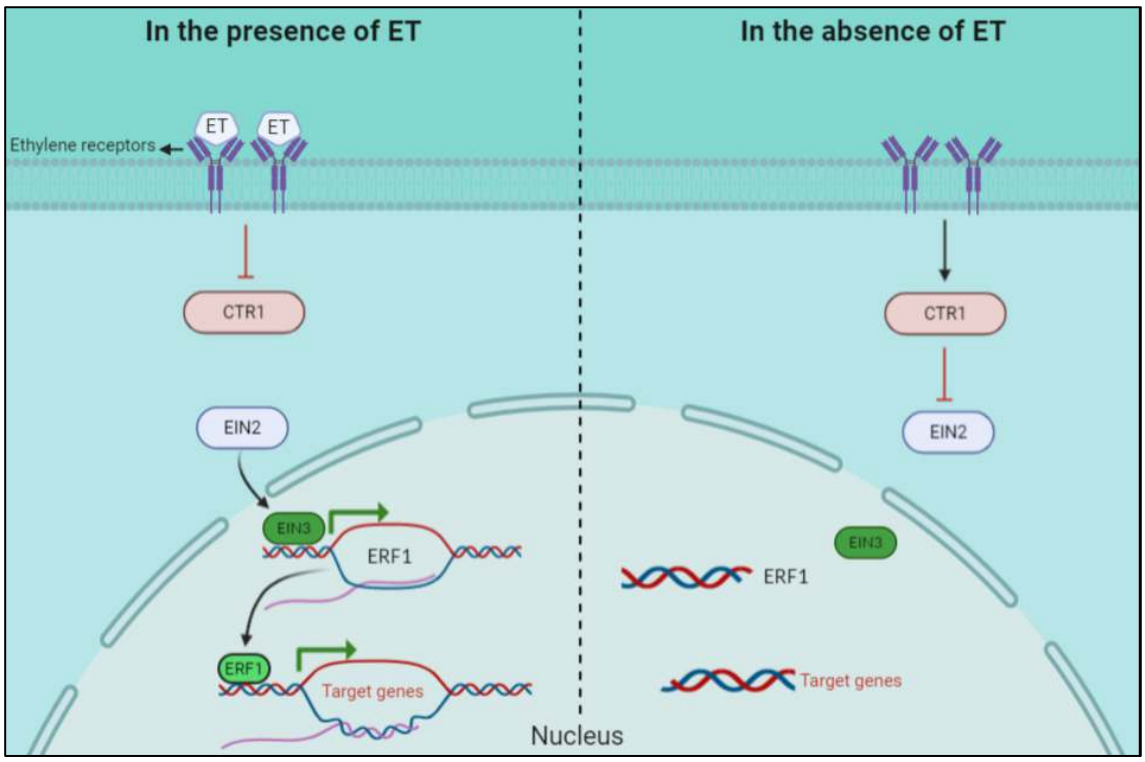

Figure 1.2. Ethylene (ET) signalling pathway. Modified from Wang et al. (2002).

These proteins from receptors to transcription factors are encoded by gene families with a different number of members in different species. Ethylene receptors are encoded by a small family (*ETR1*) of five to six genes (Hua et al., 1998; Klee, 2002). The expression level of receptor genes is differently regulated throughout developmental stages with a negative relationship with genes related to ethylene biosynthesis and responses (Klee, 2002). CTR1 protein is encoded by *CTR1*, a gene acting downstream of the receptors with the same negative impact as receptor genes on the pathway (Kieber et al., 1993).

EIN3 and EIN3-like transcription factors are encoded by *EIN3* and *EIN3-LIKE* (*EIL1*, *EIL2*, and *EIL3*) genes (Chao et al., 1997). ERF1 belongs to a large family of plant-specific transcription factors and is encoded by *ERF1* gene (Fujimoto et al., 2000).

1.2.3. Ethylene regulation of flower senescence

The postharvest life of many horticultural crops is affected by ethylene (Reid, 2002b). Based on the age of plants, species, and sensitivity to ethylene, responses to ethylene are different. These responses range from the ripening of fruit, flowering induction, chlorophyll degradation, abortion, shortening of stems, abscission, dormancy, and epinasty (stems bend) (Blankenship, 2001; Xu and Zhang, 2014).

Ethylene is a natural regulator of flower senescence in many cut flowers (Woltering and Van Doorn, 1988; van Doorn, 2001), and is produced in an autocatalytic manner in all plants/organs with climacteric respiration patterns. After the perception of ethylene by ethylene receptors, activation of its signal transduction cascade affects the expression patterns of ethylene-dependent genes and their mRNA levels in cells (McGrath and Ecker, 1998; Davies, 2004; Serek et al., 2006), which in most cut flowers results in chlorophyll degradation, petal wilting or withering, and abscission (van Doorn, 2001; Ahmadi et al., 2008, 2009). Wilting is caused by the loss of turgor which in turn could be due to water stress in cut flowering stem but withering is caused by a change in colour and also slow dehydration (van Doorn and Woltering, 2008). The underlying mechanism causing both wilting and withering seems to be similar which might be due to xylem inclusion (van Doorn and Woltering, 2008) or ethylene induced activation in the abscission zone at the base of organ and increasing level of enzymes responsible for the hydrolysis of middle lamellae and cell wall in the abscission zone (Rungruchkanont et al., 2007; Estornell et al., 2013; Meir et al., 2015) which block water flow to petals. However, petal wilting/withering might be ethylene-independent (Woltering and Van Doorn 1988; van Doorn, 2001) which could be regulated by other plant growth regulators cytokinins (Zhou *et al.*, 2005; van Doorn and Woltering, 2008), abscisic acid (Panavas *et*

al., 1998), Gibberellins (Costa *et al.*, 2016), auxin (Arrom and Munné-Bosch, 2012) or darkness or sugar starvation (Qi et al., 2015; Liebsch and Keech, 2016). The shedding of plant organs such as flowers and leaves is called abscission or organ separation (Patharkar and Walker, 2018). Abscission could be ethylene-dependent or -independent (Osborne and Sargent, 1976; Woltering and Van Doorn 1988; van Doorn, 2001, 2002). In ethylene-dependent pathway, abscission in regulated by the balance between auxin (abscission blocker) and ethylene (abscission accelerator) but in ethylene-independent pathway other factor regulate the process which is further discussed in section 1.3.

Different types of cut flowers have different capacities in producing ethylene and sensitivity to ethylene. Woltering and Van Doorn (1988) studied the effects of exogenous ethylene in 93 species of cut flowers from 22 families and later van Doorn (2001) further investigated the effects in another 200 species of cut and potted plants. Based on flowers' responses to external ethylene, they classified them as ethylene sensitive and insensitive. Depending on the species, petal senescence was initially shown by petal wilting or abscission. The author observed high sensitivity and very low sensitivity were observed both in monocots and in dicots and a similar response to ethylene within genera and species. In sensitive flowers, ethylene production rises during natural senescence, and exogenous ethylene results in early senescence and reduced longevity. Whereas in insensitive plants there is no significant increase in ethylene production during natural senescence, the occurrence of symptoms is not mediated by ethylene, and even changes caused by exogenous ethylene are not as severe as in sensitive group. In insensitive plants, petal wilting was initial symptom of senescence except for some families (Caryophyllaceae, Campanulaceae, Malvaceae, Orchidaceae, and Dipsacaceae) where petal wilting showed a high sensitivity to ethylene, and wilted petals in sensitive category remained unattached or shattered during the testing period. Plants with low sensitivity

were observed in Compositae, Iridaceae, Amaryllidaceae, and Liliaceae families. In sensitive plants, petal abscission was the initial symptom of senescence without showing any signs of petal wilting. Ethylene sensitivity was mostly found in Caryophyllaceae, Rosaceae, Geraniaceae, Malvaceae, Ranunculaceae, Campanulaceae, Scrophulariaceae, Primulaceae, and Labiatae families. However, in *Cymbidium* (Orchidaceae), coloration of the labellum was used as sensitivity classification but based on wilting, half of the test cultivars had no response to ethylene while the other half showed sensitivity to ethylene.

1.3. Abscission

The shedding of plant organs such as flowers, leaves, fruit that are no longer required is called abscission or organ separation (Patharkar and Walker, 2018). Abscission is a developmentally programmed cellular process which could occur at any given time during plant life (Ascough et al., 2005; Kim, 2014; Olsson and Butenko, 2018). Abscission takes place in a specific region called abscission zone which is comprised of layers of small cells with dense cytoplasm (Sexton and Roberts, 1982; Kim, 2014; Patharkar and Walker, 2018). Abscission process is divided into four phases. The first phase involves the development of abscission zone at the base of an organ, without which the abscission will not happen even in the presence of abscission signals (Osborne and Sargent, 1976; Kim, 2014). The second phase is the activation of abscission signal during which the abscission zone cells acquire the ability to respond to hormonal and developmental abscission signals (Kim, 2014; Kim et al., 2019). The third phase includes the enzymatic hydrolysis of middle lamella by hydrolysis enzymes such as polygalacturonases, beta-galactosidase, and cellulases in the abscission zone which takes place upon receiving abscission signals and results in cell separation (Patharkar and Walker, 2018; Kim et al., 2019). The last phase involves organ abscission and sealing of abscission scar by an epidermal layer in order to protect the exposed cells from pathogen

and water loss (Kim, 2014; Patharkar and Walker, 2018; Kim et al., 2019). Several molecular components involved in these phases have been identified such as BLADE ON PETIOLE 1/2 (*BOP1/2*) genes in Arabidopsis (*Arabidopsis Thaliana* L.) which encode BTP/POZ -ankyrin transcription factors that are involved in many plant development and defense responses (Khan et al., 2014) and necessary for the differentiation of abscission zone cells (McKim et al., 2008) or the IDA-HAE-HSL2 pathway which regulates the abscission activation (Jinn et al., 2000; McKim et al., 2008; Olsson and Butenko, 2018). The analysis of mutants suggests that the peptide ligand INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) binds to a HAE/ HSL2/SOMATIC EMBRYOGENESIS receptor complex and activates it. Upon activation, the receptor complex transmits a signal though MAP-kinase cascade which in turn activates KNOX transcription factors which results in the activation of the expression of genes involved the cell wall degradation enzymes (Olsson and Butenko, 2018; Zhu et al., 2019). Abscission occurs in response to various internal factors such as plant hormones as well as external factors such as temperature, light, relative humidity, water availability, gases, nutrient availability, soil conditions, and pathogen attack relative may affect abscission process (Addicott, 1968; Ascough et al., 2005). External factors also effect various internal physiological conditions and processes such as the level of carbohydrates and plant hormones, respiration, enzymatic reactions, nitrogenous substances availability which in turn may lead to early or delayed abscission (Addicott, 1968).

1.3.1. Role of plant hormones in abscission

Plant hormones auxin, salicylic acid, and brassinosteroids delay abscission (Bornman et al., 1966; Iwahori et al., 1990; Ferrarese et al., 1996; Karimi et al., 2012; Basu et al.,

2013; Meir et al., 2015; de Assis-Gomes et al., 2018). Cytokinins and gibberellins and may delay or accelerate abscission (Osborne and Moss, 1963; Bornman et al., 1966; Sipes and Einset, 1983; Ben-Arie et al., 1985; Domingos et al., 2015; Salleh et al., 2016). Ethylene, abscisic acid, jasmonic acid accelerate organ abscission (Burg, 1968; Cracker and Abeles, 1969; Ueda et al., 1996; Sanikhani et al., 2008; Kim et al., 2013). Plant hormones affect abscission process through regulating enzyme synthesis as abscission retarding hormones increase the production of synthase while abscission accelerating hormones promote the production of hydrolase (Kozlowski and Pallardy, 1997). Auxin and ethylene are primary controlling signals which antagonistically regulate organ abscission (Hong et al., 2000). Auxin acts like a 'brake' in the regulation of abscission as high auxin levels in the abscission zone prevent abscission possibly though decreasing ethylene sensitivity of the abscission zone cells and the level of hydrolytic enzyme (Hong et al., 2000; Dal Degan et al., 2001; Estornell et al., 2013). Reduction in the level of endogenous auxin due to either a normal developmental process or stress response increases the sensitivity of the cells in the abscission zone to ethylene which in turn increases the level of enzymes responsible for the hydrolysing of middle lamellae and finally leads to organ abscission (Rungruchkanont et al., 2007; Estornell et al., 2013; Meir et al., 2015). Decrease in the level of auxin in the abscission zone could also cause premature abscission of organs (Basu et al., 2013). Auxin depletion could be the result of decrease in the biosynthesis and transport of indole-3- acetic acid (IAA), increased autoinhibition of IAA transport, and oxidative IAA catabolism (Meir et al., 2015). The role of auxins has been further confirmed by using auxin antagonists such as 2,3,5-triiodobenzoic acid (TIBA, an inhibitor of auxin transport) or 2-(4-chlorophenoxy)-2-methyl propionic acid (CMPA, an inhibitor of auxin action) as they resulted in organ abscission in *Dendrobium* cv. Miss Teen, the same response that was observed in

ethylene treated flowers (Rungruchkanont et al., 2007). In contrast applying exogenous auxin retarded dark-induced abscission in aspen (*Populus tremula* L. X *P. tremuloides* Michx.; clone T89) (Jin et al., 2015). Ethylene role in abscission contradicts that of auxin and depending on species or cultivar abscission might be ethylene-dependent or -independent (Osborne and Sargent, 1976; van Doorn, 2001, 2002). In ethylene-dependent abscission, ethylene is the primary accelerator of abscission (van Doorn, 2001, 2002). In the ethylene-insensitive mutants of Arabidopsis (*Arabidopsis thaliana* L.) *ein2* and *etr1-1*, ethylene insensitivity leads to retarded abscission (Patterson and Bleecker, 2004). While ethylene exposure leads to early abscission of organs in many species (Woltering and van Doorn, 1988; van Doorn, 2001, 2002). Woltering and van Doorn (1988) studied the effects of exogenous ethylene in 93 species of cut flowers from 22 families and later van Doorn (2001) further investigated the effects in another 200 species of cut and potted plants. Based on flowers' responses to external ethylene, they classified them as species with ethylene-sensitive and -insensitive abscission. Species showing petal abscission without prior wilting were categorised as highly ethylene-sensitive such as species in Caryophyllaceae, Campanulaceae, Orchidaceae, Malvaceae, Rosaceae, Primulaceae, Labiatae, Graniaceae, Ranunculaceae, and Scrophulariaceae families except for *Cymbidium* (Orchidaceae), in which the classification of sensitivity was based on the coloration of labellum and some *Tulipa* cultivars which showed translucency, turgor loss, and desiccation prior to petal fall (Woltering and van Doorn, 1988; van Doorn, 2001, 2002). In ethylene-insensitive abscission, petal abscission occurred prior, concurrently with or after petal wilting and ethylene treatment has no effects on the time to abscission (van Doorn, 2001). This class of abscission was only observed in a few species such as *Saxifraga*, *Tulipa* (three cultivars), and *Lilium* hybrid (van Doorn, 2001). For instance, in *Tulipa* cultivars petal abscission occurred prior to

petal wilting and ethylene did not influence the abscission occurrence even in advanced stages of development. In tulip `Golden Apeldoorn' the petal wilting occurred prior to abscission and abscission was ethylene-insensitive but petal wilting showed a little ethylene sensitivity. In *Saxifraga* species (*S. apiculata*, *S.* x *arendsii* and *S. litacina*), petal abscission happened concurrently with slight wilting. In *Lilium* hybrid `Montenegro' petal abscission took place two days after petal wilting and was ethylene-insensitive (van Doorn, 2001). The role of ethylene in abscission is related to its capacity to affect the degradation and transport of IAA (Kozlowski and Pallardy, 1997). Ethylene changes cell wall metabolism by degradation and new biosynthesis processes (Agustí et al., 2008). Activation of these process are prerequisite for the process of cell elongation during abscission (Addicott, 1982). During abscission the degradation of middle lamella and modification of the primary cell wall take place in order to allow cells in the abscission zone to expand and create the force for organ abscission (Kim et al., 2019). The various cell degrading enzymes such as pecitnases, polygalacturonases and pectate lyases, pectin methylesterases, cellulases, expansins, and xyloglucan endotransglucosylase/hyrolases are expressed to degraded middle lamella and modify cell wall modification process (Meir et al., 2010; Nakano and Ito, 2013; Kim et al., 2019). In ethylene induced senescence, ethylene regulate the transcription level of many genes involved in cell wall hydrolysis and modification, lignin biosynthesis and deposition, lipid transference, protein metabolism, defence, and oxidative stress scavenging (Burns et al., 1998; Joseph Nairn et al., 1998; Agustí et al., 2008; Mishra et al., 2008).

Other plant growth regulators may directly or indirectly affect abscission. For instance, in bean (*Phaseolus vulgaris*) explants, the application of cytokinins to the explants end accelerated abscission but when applied to the abscission zone delayed abscission (Osborne and Moss, 1963). In apple (*Malus domestica* 'Cox's Orange Pippin') cytokinins

accelerated abscission after eight days of inoculation but delayed eighteen days after inoculation in high concentration (Pierik and Abbadi, 1972). Cytokinins seems to affect abscission in an ethylene-independent and -dependent manners as in lemon (*Citrus limon* cv. Lisbon) cytokinins accelerated abscission process without any significant changes in ethylene production (Sipes and Einset, 1983). But in cotton (*Gossypium hirsutum*) abscission process was reported to be regulated through a crosstalk between ethylene and cytokinin as the expression of cytokinin oxidase/dehydrogenase (*CKX*) genes and ethylene-related genes were increased after ethephon exposure with increased level of ethylene but after down regulation of *CKX3* the response to ethylene decreased and abscission delayed (Xu et al., 2019). Gibberellins seem to be ethylene-dependent in affecting abscission process. As in cotton (*Gossypium hirsutum* L.) gibberellins promoted organ abscission in ethylene treated plants (Morgan and Durham, 1975). In Arabidopsis (*Arabidopsis thaliana* L.) mutations in the jasmonic acid receptor, *coronatine insensitive 1* (*coi1*), result in delayed floral organ abscission (Kim et al., 2013). Interestingly, treating these plants with ethylene led to early organ abscission in the mutant plants (Kim et al., 2013). In contrast, treating the *ein2-1* ethylene insensitive mutant with jasmonic acid accelerated organ abscission which indicate that organ abscission is independently regulated by this hormone (Kim et al., 2013). In bean (*Phaseolus vulgaris* L. cv. Masterpiece) jasmonic acid led to early abscission through increasing the activity of cellulase and the degradation of cell wall polysaccharides without increasing ethylene production which indicates ethylene-independent abscission (Ueda et al., 1996). Similarly, in kalanchoe (*Kalanchoe blossfeldiana* Poelln), jasmonic acid led to early organ abscission and increased ethylene production, but treating plants with exogenous ethylene did not induce abscission (Saniewski and Wegrzynowicz-Lesiak, 1994). Abscisic acid has been reported to regulates abscission process through

increasing ethylene production (Cracker and Abeles, 1969; Wilmowicz et al., 2016). Luo et al. (2014) reported that in Arabidopsis (*Arabidopsis thaliana* L.), abscisic acid activated calcium-dependent protein kinases (CDPKs) which in turn phosphorylated the N-termini of 1–aminocyclopropane-1–carboxylate synthases (ACS) which stabilized ACS and increased ethylene prodcution. In lupine (*Lupinus luteus*), abscisic acid also increase the transcriptional level of ethylene biosynthesis genes (*LlACS* and *LlACO*) and the level of 1-aminocyclopropane-1-carboxylic acid (ACC) in the abscission zone cells (Wilmowicz et al., 2016). Salicylic acid may also regulate abscission seemingly in an ethylene dependent matter by inhibiting the conversion of l-aminocyclopropane-l-carboxylic acid to ethylene (Leslie and Romani, 1986). In peach (*Prunus persica* L. Batsch cv. Redhave) and pepper (*Capsicum annuum* var. *longum* (DC.) Sendt) after treating plants with salicylic acid the level of cellulase did not increase following leaf abscission activation while an increase was observed in cellulase expression in ethylene treated plants however this increase was low in the presence of salicylic acid (Ferrarese et al., 1996). Brassinosteroids have also been reported to delay organ abscission (Iwahori et al., 1990; de Assis-Gomes et al., 2018). However brassinteroids effects does not seems to be auxin dependent but ethylene dependent as it was reported that in Arabidopsis (*Arabidopsis Thaliana* L.) exogenous brassinosteroids did not affect auxin which is a necessary signal in abscission retardation but in contrast brassinosteroids decrease ethylene production through suppressing the expression of the genes involved in ethylene production (Lv et al., 2018; Ji et al., 2020). In Arabidopsis (*Arabidopsis thaliana* L.) a direct interaction between BES1 or BZR1 (brassinosteroid-regulated transcription factors) and ACSs (1-aminocyclopropane-1-carboxylic acid synthases enzymes) under low concentrations of BR was observed (Lv et al., 2018). There the activity of *AtACS* promoters was strongly suppressed by the over-expression

of both *BES1* and *BZR1* genes. Similarly, in pear (*P. ussuriensis* cv. Nanguo) and apple (*M. domestica* cv. Golden Delicious), exogenous brassinosteroid suppressed the expression of ethylene production genes (*ACO1* and *ACS1*) and decreased ethylene production (Ji et al., 2020). The author reported that in pear (*P. ussuriensis* cv. Nanguo) BZR1 indirectly suppressed the expression of ethylene production genes (*ACO1* and *ACS1*) through binding to the promotor of transcription factor *PuERF2*. PuERF2 binds to the promotors of ethylene production genes (*ACO1* and *ACS1*) and regulates their expression (Ji et al., 2020).

1.4. Alleviation of ethylene effects

Improving the resistance of flowers to senescence-inducing factors such as ethylene is an ongoing target for postharvest physiologists and flower breeders. Knowledge of the ethylene biosynthesis pathway and mode of action has enabled researchers to apply different methods to alleviate or inhibit the impacts of ethylene on horticultural commodities (Saltveit, 1999). Inhibition of ethylene production and/or ethylene binding and sensitivity results in delayed senescence or longer-lived ethylene-sensitive cut flowers with greater commercial value (Reid and Wu, 1992; Stead, 1992). To date, various approaches, from genetic to environmental and chemical, have been used to alleviate detrimental effects of ethylene on cut flowers and potted plants via inhibiting ethylene biosynthesis and perception, changing tissue responses to ethylene, and avoiding exposure to exogenous ethylene (Serek and Reid, 1997; Saltveit, 1999; Reid and Jiang, 2012; Scariot et al., 2014).

1.4.1. Genetic approaches

Genetic approaches include delaying ethylene-induced senescence directly through regulating ethylene biosynthesis and its signal transduction pathway (Savin et al., 1995; Aida et al., 1998; Bovy et al., 1999; Shaw et al., 2002; Cui et al., 2004; Shibuya et al., 2004; Huang et al., 2007; Sriskandarajah et al., 2007; Sanikhani et al., 2008; Satoh et al., 2008) or indirectly by affecting antagonistic pathways to the ethylene pathway (Chang et al., 2003; Zakizadeh et al., 2013). Although these approaches have been successfully implemented in some ornamentals, there are still limitations and challenges ahead for the use of the genetic approaches. These include difficulties of implementation in some ornamentals, the ineffectiveness of some strategies, the complexity of regulations worldwide, and the time and cost needed for regulatory approval (Chandler and Sanchez, 2012; Scariot et al., 2014; Olsen et al., 2015).

1.4.2. Chemical approaches

Chemical strategies include using ethylene biosynthesis and action inhibitors and other plant growth regulators (Serek and Reid, 1997; Serek et al., 2006; Reid and Jiang, 2012; Scariot et al., 2014). Chemicals have successfully been used to reduce the biosynthesis of ethylene by blocking the components of its biosynthesis pathway (Serek et al., 2006). AVG (1-aminoethoxyvinylglicine) and AOA (aminooxyacetic acid) are the inhibitors of the conversion of SAM to ACC and block the increase in ethylene production (Broun and Mayak, 1981; Serek and Andersen, 1993). Blocking ethylene effects at the receptor level is another way of protecting cut flowers against both endogenous and exogenous ethylene. 2,5-norbornadiene (NBD), silver thiosulphate (STS), diazocyclopentadiene (DACP), and 1-methylcyclopropene (1-MCP) are ethylene action inhibitors (Paliyath et

al., 2009). These compounds prevent the perception of ethylene by its receptors and therefore limit ethylene action (Serek et al., 2006).

Pretreating cut flowers with plant growth regulators is another way of preventing ethylene impacts. Plant growth regulators/hormones interact with one another. This interaction causes alterations in the level and response to a hormone (Santner et al., 2009). Cytokinins and gibberellins have been shown to be very effective in controlling ethylene responses in some cut flowers (Jordi et al., 1995; Danaee et al., 2011; Gholami et al., 2011; van Doorn et al., 2011; van Doorn et al., 2013; Trivellini et al., 2015). These growth regulators modify ethylene synthesis and sensitivity and have been used as commercial vase preservatives for some cut flower species (Serek and Reid, 1997; Rubinstein, 2000). Other growth regulators such as auxin, jasmonic acid, Salicylic acid have had controversial effects on ethylene and have not been proven to be of commercial value in the postharvest industry. Brassinosteroids (BRs) are another class of plant hormone that can have an effect on ethylene production and they will be discussed in section 1.5.2.

1.4.3. Environmental approaches

Environmental approaches include ventilation, low temperature or cold storage (<5 °C), modified and controlled atmosphere, and ethylene scavengers. Cold storage is an effective and frequently used means of maintaining quality, storing additional products, and extending postharvest life (Lyons, 1973; Reid and Jiang, 2012). Cold storage allows easier transportation of crops with less loss from the regions where they are grown to distant markets where they can be consumed (Reid and Jiang, 2012). Cold storage also reduces the growth of pathogens and delays senescence by decreasing plant metabolism (McGlasson et al., 1979; Mitchell, 1992), which provides an opportunity for the

horticultural industry to benefit from the global seasonal variations in food and flower production.

Cold storage has been effectively employed for cooling and storing horticultural commodities such as fruit, vegetables, and ornamentals (flowers) of temperate regions (i.e. crops that are insensitive to cold storage). The cold storage technique regulates ethylene biosynthesis and sensitivity (Martínez-Romero et al., 2007; Reid and Jiang, 2012), but is limited to some species of plants that are tolerant to low temperatures (Reid and Jiang, 2012). Many tropical and subtropical horticultural products, including fruit, vegetables, and ornamentals, are chilling-sensitive and exhibit physiological dysfunctions in response to low and non-freezing temperatures (<15 °C) (Lyons, 1973; Gross et al., 2002; Wang, 2006). In chilling-sensitive cut flowers, exposure to low temperatures causes adverse results such as chilling injuries (CI), high ethylene production and sensitivity, and early senescence (Han and Miller, 2003; Reid and Jiang, 2012).

1.5. Chilling injury

Chilling injury is a term which refers to the physiological disorders, dysfunctions or abnormalities in plants or their products, that occurs within the range of chilling temperatures (<15 °C) (Lyons, 1973; Raison and Lyons, 1986; Wang, 1990; Huang et al., 2006). This term was first suggested by German physiologist, Molisch in 1897, to characterize these damages (Raison and Lyons, 1986). The symptoms of chilling injury are: cellular changes, altered metabolism, reduced plant growth and death, water-soaking of tissue, increased ethylene production and accelerated senescence, increased susceptibility to decay (Saltveit and Morris, 1990; van Doorn and Han, 2011; Lukatkin et al., 2012), failure to open normally and decrease in the length of shelf-life/vase life,

etc. (van Doorn and Han, 2011). These symptoms vary with plants/crops, temperature, duration of exposure, humidity, and postharvest treatments (Saltveit and Morris, 1990; Wang, 2004). Depending on the temperature and duration of exposure, chilling injury might be reversible or irreversible, as, under less exposure to higher temperatures (within the range of chilling temperatures), the injuries may be reversible, while longer exposure to lower temperatures (within the range of chilling temperatures) causes irreversible injuries (Durner, 2013). The development of chilling injury occurs during exposure to chilling temperature, but, the symptoms mostly become evident after transferring crops to warmer temperatures (Saltveit and Morris, 1990; Gross et al., 2002).

Chilling susceptibility leads to a limited growing season, geographic distribution, and storage conditions of plants or their harvested parts (Raison and Lyons, 1986; Wang, 2006). In terms of sensitivity to chilling injury, horticultural commodities are divided into three groups: chilling resistant, chilling sensitive, and slightly chilling sensitive (Wang, 1994a). Many tropical and subtropical horticultural crops including fruits, vegetables, and ornamentals are chilling-sensitive and exhibit physiological dysfunctions in response to low and non-freezing temperatures (<15 °C) (Lyons, 1973; Gross et al., 2002; Wang, 2006).

1.5.1. Chilling injury in cut flowers

Cut flowers and foliage are highly perishable items and should be cooled after cutting and also kept cool throughout the supply chain to minimise deterioration and extend postharvest life. If they are not cooled, they will decay more rapidly, cannot be stored for a longer time, and also cannot be exported to longer distances. The use of refrigeration for storage of cut flowers reduces water loss, respiration rate, ethylene production and sensitivity, infections caused by bacteria and fungi, and delays senescence (da Silva

Vieira et al., 2012). For example, cooling waxflower from 20 °C to 10 °C reduces the respiration rate by about 71%. Further cooling from 10 °C to 0 °C reduces the respiration rate by a further 77%. Most flowers should be held at 0 to 1 °C (Reid, 2002a). But others like tropical or subtropical cut flowers suffer from chilling if grown in temperate climates (Hewett, 2006) or stored and handled under low temperatures (Reid and Jiang, 2012; Prisa et al., 2013). Chilling-sensitive flowers should be held at temperatures above 10 °C. Chilling injuries depend on the plant type, temperature, and storage period (Huang et al., 2006). Wilting, necrosis, browning of petals and bracts, and leaf yellowing are the symptoms of chilling injury in chilling-sensitive cut flowers (Han and Miller, 2003; Reid and Jiang, 2012).

1.5.2. Chilling injury symptoms in cut lilies

Chilling injury is common in many cut lilies and a relatively short period of cold storage (e.g. 1 week) often causes physiological damages such as unopened buds, hastened tepal wilting, induced or increased leaf yellowing, and bud abscission (Han and Miller, 2003; Burchi et al., 2004; van Doorn and Han, 2011). Chilling-induced quality changes vary with the duration of storage (Han, 2001; Han and Miller, 2003; Prisa et al., 2013), temperature (Choi et al., 2014), cultivar (Nell et al., 1998; Han, 2001), maturity level (Han and Miller, 2003; Prisa et al., 2013). Chilling injury symptoms were first observed in cut Asiatic and Oriental lily stems cold-stored for two weeks and then in those cold-stored for one week (Han, 2001). Different cultivars respond to cold temperatures differently. For example, chilling injury of cut oriental lily ‘Acapulco’ was less severe than ‘Stargazer’; the cut Asiatic lily ‘Vivaldi’ was reported to be slightly more susceptible than ‘Geneve’ (Han, 2001). Younger tissues can be more sensitive to chilling

injury, e.g. in cut hybrid lily 'Brindisi' no chilling damage was observed in older floral buds but it was observed in very young floral buds (Prisa et al., 2013).

1.5.3. Chilling injury and role of ethylene

1.5.3.1. Ethylene production and sensitivity in cut lilies

Cut flowers are classified as being climacteric or non-climacteric (Paliyath et al., 2009). In climacteric or ethylene sensitive flowers ethylene production rises during natural senescence and exogenous ethylene results in early senescence and reduced longevity (Woltering and Van Doorn, 1988; van Doorn, 2001). In non-climacteric or insensitive flowers no significant increase in ethylene production is apparent during natural senescence and exogenous ethylene has no or little effects on them (Woltering and Van Doorn, 1988; van Doorn, 2001). For instance, the *Liliaceae* family was reported as ethylene insensitive as most of the tested species in this family showed no or little sensitivity to exogenous ethylene (Woltering and Van Doorn, 1988). Elgar et al. (1999) tested the production of ethylene in several lily cultivars from the three main groups: Asiatic hybrid, Oriental hybrid, and *Lilium longiflorum*. The production of ethylene was close to or below the limits of detection in all tested lily cultivars (Elgar et al., 1999). They did not observe any climacteric increase in ethylene production during seven days and the amount of produced ethylene was undetectable or very low (Elgar et al., 1999). Similarly, Han and Miller (2003) measured the endogenous ethylene production in freshly harvested cut lily 'Stargazer'. They reported that endogenous ethylene was not produced by excised leaves or flowers (Han and Miller, 2003). These findings indicate that cut lilies are non-climacteric or insensitive flowers that do not show any increase in ethylene production during the senescence process or produce a low amount of ethylene which does not affect the senescence process.

1.5.3.2. Effects of ethylene and ethylene inhibitors on cut lilies

Lilies have different responses to exogenous ethylene (van Doorn and Han, 2011). Woltering and van Doorn (1988) reported Asiatic lily 'Brunello' and 'Montenegro' and Oriental lily (*Lilium orientalis*) 'Stargazer' and 'Woodriff's Memory' to have a very low sensitivity to exogenous ethylene (3 μL L^{-1} for 24 h, at 20 °C). Similarly, Han and Miller (2003) investigated the response of Oriental lily 'Stargazer' to exogenous ethylene and found that this cultivar has no sensitivity to exogenous ethylene. They observed that treatment of intact cut stems or freshly harvested excised flowers, buds, leaves with ethylene concentrations as high as 10 μL L^{-1} for 24 h had no effects on flower longevity (Han and Miller, 2003). Elgar et al. (1999) screened three main groups of lilies (Asiatic hybrids, Oriental hybrid, and *L. longiflorum*) for their response to exogenous ethylene. They observed that ethylene had no effects on the vase life of most Oriental hybrids and *L. longiflorum* cultivars but minor effects on some Asiatic hybrids 'Apeldoorn', 'Goldena', 'Elite', and 'Mona' (Elgar et al., 1999). Asiatic hybrids 'Cordelia' and 'Prato' did not show any response to a wide range of ethylene concentrations for varying durations, except at the highest concentration and a longer duration of ethylene exposure (Elgar et al., 1999).

Ethylene inhibitors (STS and 1-MCP) have been tested to understand their effectiveness on preventing effects of ethylene on cut flowers such as cut lilies (Elgar et al., 1999; Han and Miller, 2003; Song and Peng, 2004). But different cultivars responded differently to these inhibitors because their responses to ethylene were different. As these inhibitors will only have effects on quality if there is an ethylene response or effect. For instance, pre-treatment with STS had little effect on the longevity of cut lily flowers exposed to ethylene while pre-treatment with 1-MCP had little or no effects on the vase life of tested

cut lilies, which demonstrated lack of ethylene response in tested lily flowers (Elgar et al., 1999) and in cut lily 'Stargazer' pre-treating with STS and 1-MCP did not improve flower quality as it showed no ethylene response (Han and Miller, 2003). In contrast, in cut lily 'Brussels' pre-treatment with STS before ethylene exposure completely prevented its effects on flower abscission, bud abortion, flowering, floral diameter, vase life, and floral longevity (Song and Peng, 2004) which shows that this cultivar is responsive to ethylene. Thus, these findings indicate that the effectiveness of ethylene inhibitors on cut lilies depends on their response to exogenous ethylene as in ethylene insensitive cultivars have little or no effects on keeping their quality against exogenous ethylene.

1.5.3.3. Effects of low temperature on ethylene production and sensitivity in cut lilies

Low temperature reduces ethylene biosynthesis, but an increase in the production of ethylene has been observed following the removal of low temperature in many chilling sensitive species (Sfakiotakis and Dilley, 1974; Wang and Adams, 1980; Etani and Yoshida, 1987; Larrigaudiere and Vendrell, 1993; Han and Miller, 2003). These changes in the level of ethylene production are caused by the effects of low temperature on ethylene biosynthesis pathway enzymes and their level of mRNA (Wang and Adams, 1980; Etani and Yoshida, 1987; Jackman et al., 1988). Most lilies produce a low or undetectable amount of ethylene during senescence process and show no or little sensitivity to exogenous ethylene (Woltering and Van Doorn, 1988; Elgar et al., 1999; van Doorn, 2001; Han and Miller, 2003), but if cold-stored, they show physiological and biochemical changes (Han and Miller, 2003; Song and Peng, 2004; van Doorn and Han, 2011; Choi et al., 2014).

The effects of low temperature on ethylene production and sensitivity in cut lilies vary within and between species, depending on cultivar/species, they might produce higher or lower amounts of ethylene and even their responses to exogenous ethylene changes as they might show high or low sensitivity to exogenous ethylene (Han and Miller, 2003; Burchi et al., 2004; Song and Peng, 2004). For example, Han and Miller (2003) observed that non-stored cut oriental lily 'Stargazer' produced no/undetectable amount of ethylene during flower senescence and showed no sensitivity to exogenous ethylene but after cold-storage, they produced high amounts of ethylene and became highly sensitive to exogenous ethylene. Similarly, an increase in ethylene production was observed in cut Asiatic lilies 'Elite' after cold storage, whereas no clear increase in ethylene production was observed in cold-stored Asiatic lily 'Prato' (Burchi et al., 2004). Contrarily, the cut Asiatic hybrid lily 'Brussels' has a high sensitivity to exogenous ethylene before cold storage but a low sensitivity after cold storage (Song and Peng 2004).

1.6. Alleviation of chilling injury

Many approaches from genetic and environmental techniques to chemical treatments have been used to prevent chilling injury by either increasing chilling tolerance or reducing the severity of chilling injury symptoms in horticultural crops (Wang, 1994a) (Lukatkin et al., 2012). These include temperature conditioning (Wang, 1994b; Woolf et al., 1995; Woolf et al., 1997; Woolf et al., 2003), intermittent warming (Hruschka, 1970; Kluge et al., 2003; Jing et al., 2009; Biswas et al., 2012), controlled or modified atmosphere storage (Yi Wang and Qi, 1997; Pesis et al., 2000), hypobaric storage (Hu et al., 2012; Song et al., 2016), pre-treatments with plant growth regulators, calcium and other chemical applications (Choehom, 1997; Salvador et al., 2004; Barman et al., 2011; Sayyari et al., 2011; H. Yang et al., 2011; Gao et al., 2015; Gao et al., 2016; Y. Liu et al., 2016), waxing (Choehom, 1997; Mejia Torres et al., 2009; Barman et al., 2011), and

genetic manipulation (Yu et al., 2009; Shu et al., 2011). These techniques alleviate CI by either decreasing the sensitivity of commodities to cold storage or retarding the development of chilling injury symptoms (Wang, 1994a). Chemical treatments alter many biochemical and physiological processes in plant tissues which may in turn change the response of tissues to cold storage (Wang, 1994a). A chemical strategy to reduce the effects of senescence includes the use of ethylene biosynthesis and action inhibitors, and other plant growth regulators (Serek and Reid, 1997; Serek et al., 2006; Reid and Jiang, 2012; Scariot et al., 2014).

1.6.1. Plant growth regulators

Plant hormones regulate plant growth and development through various processes, from cell division to senescence (Ashraf et al., 2010; Reid and Jiang, 2012). They are major developmental and physiological signalling molecules in plants (Stewart Jr, 2016). Signals from outside a cell can be perceived, sometimes by receptors that span the plasma membrane, after stimulation of such receptors, information can be relayed by a series of small molecules or proteins to the cell nucleus, where activation of specific transcription factors can stimulate new gene expression programs which in turn results in the final biological responses to the signal (Stewart Jr, 2016). Plant growth regulators may be natural or artificial and are typically active at very low concentrations. The most important plant growth regulators are auxin, gibberellin, cytokinin, ethylene, abscisic acid, and brassinosteroids (BRs). Other growth regulators often act by modifying the action of natural hormones (Serek and Reid, 1997; Serek et al., 2006).

Plant growth regulators are the most promising group of chemical compounds in increasing the chilling tolerance of chilling sensitive plants (Lukatkin et al., 2012). There

are numerous examples of growth regulators that influence the chilling tolerance of plants (Lipton and Aharoni, 1979; Duncan and Widholm, 1991; Anderson et al., 1994; Lukatkin et al., 2003; Lukatkin and Zauralov, 2009; Ding et al., 2015; Gao et al., 2015; Z. Liu et al., 2016; Mo et al., 2016). Gibberellin treatment alleviated the chilling injury index in cherry tomato (Ding et al., 2015) and peach (Martínez-Romero et al., 2000). Peach fruits treated with gibberellin showed higher firmness and lower ethylene production compared to non-treated ones (Martínez-Romero et al., 2000). Arabidopsis (*Arabidopsis thaliana* L.) treated with exogenous cytokinin grew with normal morphology and displayed relative growth rates greater than control by increasing total cell number under low temperature (Xia et al., 2009). Exogenous cytokinin was also effective in restraining chilling injury in cucumber (*Cucumis sativus* L.) fruit. Treated cucumber fruits had higher levels of chlorophyll, total phenolics, ascorbic acid, and total antioxidant capacity (Chen and Yang, 2013). Controversial results have been reported on the effects of ethylene on chilling injury (Zauberman and Fuchs, 1973; Yuen et al., 1995; Zhou et al., 2001; Pesis et al., 2002). Depending on species the response to exogenous ethylene might be different. But ethylene production is a sign of chilling damage (Zhou et al., 2001). Chilling conditions stimulate ethylene production after transferring from a chilling condition to a warmer temperature (Sfakiotakis and Dilley, 1974; Wang and Adams, 1980; Wang, 1990). Abscisic acid (ABA) was reported to be important in the acquisition of cold tolerance (Carvajal et al., 2017). Treatment of zucchini squash (*Cucurbita pepo* L.) with ABA before storage increased ABA level in its tissues and was also effective in reducing chilling injury (Wang, 1991). ABA was also reported to be effective in preventing chilling injury in cotton (*Gossypium hirsutum*) (Rikin et al., 1979), cucumber (*Cucumis sativus* L.) and wheat (*Triticum aestivum*) seedlings (Flores et al., 1988) subjected to low temperatures. Brassinosteroids have also

been reported to induce tolerance to extreme temperatures and prevent chilling injury in some crops (Bajguz and Hayat, 2009; Wang et al., 2012; Gao et al., 2015; Zhu et al., 2015; Gao et al., 2016). But the effects of BRs on chilling tolerance of cut flowers have not been reported.

1.6.2. Brassinosteroids

Brassinosteroids are involved in developmental and physiological processes, from senescence to stress resistance, through regulating gene expression and the level and sensitivity of other phytohormones (Clouse et al., 1996; Clouse and Sasse, 1998; C. J. Yang et al., 2011). They are found in a variety of organisms, including lower to higher plants (Bajguz and Hayat, 2009) and can be synthesized in vitro (Bartwal et al., 2013). BRs are active at very low concentrations (Clouse and Sasse, 1998; Bajguz and Tretyn, 2003; Bajguz and Hayat, 2009). About 70 BRs have been isolated from plants (Bajguz and Tretyn, 2003). Brassinolide, castasterone, and 24-epibrassinolide are the most important types of BRs (Bartwal et al., 2013).

Plant responses to biotic and abiotic stresses are also regulated by BRs (Bajguz and Hayat, 2009; J Ahammed et al., 2015). BRs have profound effects on reducing the effects of these stresses in plants (Wang et al., 2012; Z. Liu et al., 2016). Studies on the interaction of bacterial, fungal and viral pathogens with BRs showed protection of plants from diseases, such as resistance to rice blast and bacterial blight (Korableva et al., 2002; Nakashita et al., 2003), and viruses and fungi in plants (tomato, cucumber, and tobacco)(Roth et al., 2000). They have also been effective in reducing the effects of salinity and chilling stresses in plants (Liu et al., 2009; Serna et al., 2013; Xi et al., 2013).

1.6.2.1. Chilling injury and role of brassinosteroids

Brassinosteroids have received some attention as a new, non-toxic and ecologically friendly strategy for maintaining postharvest quality and enhancing abiotic stress resistance (Bajguz and Hayat, 2009; Zhu et al., 2013; Zhu et al., 2015). This class of hormone reduces the postharvest loss of horticultural products through affecting ethylene production (in some crops), chlorophyll degradation, chilling tolerance and finally retarding early senescence (Zhu et al., 2010; Wang et al., 2012; Gao et al., 2015; Gao et al., 2016; Z. Liu et al., 2016). Brassinosteroids maintained the postharvest quality of jujube fruits in storage through heightening defense-related enzyme activities (Zhu et al., 2010). Treating bananas with an analogue of BRs decreased necrotic areas (a chilling indication) (Bajguz and Hayat, 2009). Similarly, treating pepper fruit with BRs before storage at low temperature reduced chilling injury of pepper fruit during storage (Wang et al., 2012). Li et al. (2012) observed that mango fruits treated with 10 μM brassinolide (BL) had a higher tolerance to cold temperature of 5 °C. Brassinolide-treated fruits had lower phase transition temperature and higher unsaturation degree of plasma membrane lipids which led to higher fluidity under low temperature (Li et al., 2012). Aghdam et al. (2012) observed less chilling injury under BRs treatment in tomato (*Lycopersicon esculentum* Mill.) fruit, with a reduction in electrolyte leakage and malonyldialdehyde (MDA) content and an increase in proline content. Brassinosteroid-treated tomato (*Lycopersicon esculentum* Mill.) fruit showed lower phospholipase D (PLD) and lipoxygenase (LOX) activities which were reported to be associated with chilling injury in tomato fruit (Aghdam and Mohammadkhani, 2014). Brassinosteroids inhibited PLD and LOX activities, enhanced membrane integrity, and reduced chilling injury in tomato fruits (Aghdam and Mohammadkhani, 2014). Storing eggplants at 1 °C for 15 days resulted in weight loss, electrolyte leakage, MDA content, and higher phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase activities and finally chilling injury

such as pulp browning (Gao et al., 2015). Treatment with BRs alleviated chilling injury in cold stored eggplant which showed less pulp browning (Gao et al., 2015). Taken together, these results indicate that under unfavourable conditions, plants might survive through BR-mediated anti-stress-responses. However, there is not enough information on the effects of BRs on the postharvest quality of cut flowers. If the right concentration and application time are used, not only might natural senescence in cut flowers be delayed, but flowers might also survive extreme temperatures through a BR-mediated anti-stress response.

1.7. Research aims

A relatively short period of cold storage of 1 week often leads to early leaf and then flower senescence in many cut lilies. Changes to postharvest physiology of cut lilies after cold storage are likely ethylene-dependent, either due to a higher level of ethylene production or a change in ethylene sensitivity which is yet unknown. Such changes could be due to alteration in the expression of ethylene biosynthesis, perception, and signalling genes. Given the success of BRs in delaying senescence in postharvest vegetable crops and their effects on ethylene production, in cold-sensitive plants like lilies, BRs might decrease chilling injuries and delay senescence possibly by reducing ethylene production or sensing as a result of alteration in the expression of its biosynthesis or sensitivity related genes. But the effect of BRs' on senescence and ethylene production in cut flowers has not been investigated in detail, particularly at the gene expression level.

Thus, the aims of the current study are:

(i) to determine the effect of cold storage and different concentrations of BR postharvest quality in cut lily cultivars (*Lilium orientalis*) and to determine the optimum concentration of BRs as postharvest treatment;

(ii) to determine the effect of cold storage and optimum concentration of BR on ethylene production cut lilies (*Lilium orientalis*);

(iii) to determine the effects of cold storage and BRs treatment on the expression pattern of ethylene biosynthesis genes and their relationship with ethylene production;

(iv) to determine the effects of cold storage and BRs treatment on the expression pattern of ethylene perception and signalling pathway genes and their role in cold tolerance;

(v) to determine the effects of cold storage and BRs treatment on the expression pattern of the BRs-related transcription factor gene *LoBZR1* and its role in cold tolerance.

(vi) to determine the expression pattern of ethylene biosynthesis, receptor and signal transduction pathways' genes during senescence before and after cold storage in response to exogenous ethylene and 1-MCP in cut lily 'Marlon'.

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Chapter 2

Effect of Brassinosteroids on Chilling Injuries in Cut Lilies

Abstract

Chilling injury is common in many cut flowers and a relatively short period of cold storage (1 week or longer) often leads to accelerated senescence in flowers like lilies. Brassinosteroids (BRs) can delay the onset of senescence and chilling injury in crops but their effects on chilling tolerance in cut flowers have not been investigated. The current study aimed to investigate the effects of Epibrassinolide on chilling tolerance in cut lily cultivars 'Sorbonne', 'Stargazer', 'Premium Blonde', and 'Marlon'. After determining the optimum concentration cut lilies were pre-treated with 8.5 and 10 µM of BR and subsequently cold stored (5 ºC) for seven and ten days, respectively. Plants in the control treatment did not receive BR and were not cold-stored. Cold storage had a negative impact on all measured traits in all cultivars. Plants that were BR treated had less ethylene production in all cultivars. BRs significantly delayed bud opening in 'Stargazer', flower senescence in 'Sorbonne', 'Stargazer' and 'Marlon', flower abscission in all cultivars, and leaf yellowing in 'Sorbonne', 'Premium Blonde', and 'Marlon. BR treatment also significantly increased water uptake and chlorophyll content in 'Sorbonne' and 'Stargazer'. Our data indicate that the cut lily cultivars had different levels of sensitivity to low storage temperatures and that BR decreased the detrimental effects of cold storage possibly in an ethylene dependent manner. However, while the effects of BR were significant, they lead to delays in the onset of senescence by about 1-2 days. Thus, its

application does not seem to be beneficial at a practical level in all cultivars particularly in cultivars with a low level of sensitivity.

2.1. Introduction

Cold storage of plants is often a requirement to ensure that plants or plant parts are preserved when transported long distances to market. However, the cold storage can also lead to chilling injury, which is the physiological damage in plants and plant products as a result of exposure to low but non-freezing temperatures (Jackman *et al*., 1988). A decrease in sensitivity to low temperatures will allow easier transportation of chilling sensitive plants with fewer losses between the regions they are grown and the distant markets where they are consumed (Reid and Jiang, 2012). Furthermore, preventing chilling injury will provide the possibility of longer storage of crops, and the opportunity to supply the products at times of high demand. Garden lilies (*Lilium* spp) are popular and important cut flowers but most cultivars are prone to chilling injury (van Doorn and Han, 2011). Chilling injury symptoms in lilies can include inhibited bud growth, unopen buds, small flowers with twisted tepals, leaf yellowing, shorter time to flower senescence, and abscission (Prisa *et al*., 2013). These symptoms mostly become evident after transferring lilies to warmer temperatures (Han and Miller, 2003; Prisa *et al*., 2013).

The rate of development of chilling injury symptoms varies with cultivar, maturity level, the duration of storage, and temperature (Nell *et al*., 1998; Han, 2001; Han and Miller, 2003; Prisa *et al*., 2013; Choi *et al*., 2014). For instance, cut oriental lily ‘Acapulco’ was reported to be less sensitive to cold stress compared to ‘Stargazer’ (Han, 2001), and cut Asiatic lily ‘Vivaldi’ showed more susceptibility to cold stress than ‘Geneve’ (Han, 2001). In cut hybrid lily ‘Brindisi’ younger tissues were more sensitive to cold stress than older floral buds (Prisa *et al*., 2013). In Oriental lilies ‘Stargazer’ and ‘Acapulco’, two weeks of

cold storage significantly increased the number of blasted flowers, reduced the size of opened flowers and flower longevity, and reduced the time to leaf yellowing (Han, 2001; Han and Miller, 2003).

Low temperature reduces ethylene biosynthesis, but after removal of low temperature, an increase in the production of ethylene has been observed in many chilling sensitive species (Sfakiotakis and Dilley, 1974; Wang and Adams, 1980; Etani and Yoshida, 1987; Larrigaudiere and Vendrell, 1993; Han and Miller, 2003). Most lilies produce a low or undetectable amount of ethylene during the senescence process and show no or little sensitivity to exogenous ethylene (Woltering and Van Doorn, 1988; Elgar *et al*., 1999; van Doorn, 2001; Han and Miller, 2003). The effects of low temperature on ethylene production and sensitivity in cut lilies vary within and between species. Depending on cultivar or species, they might produce a higher or lower amount of ethylene and even their responses to exogenous ethylene changes (Han and Miller, 2003; Burchi *et al*., 2004; Song and Peng, 2004).

Ethylene is a natural regulator of flower senescence in many cut flowers (Woltering and Van Doorn, 1988; van Doorn, 2001). Cut flowers are classified as being climacteric or non-climacteric (Paliyath *et al*., 2009). In climacteric or ethylene sensitive flowers ethylene production rises during natural senescence and exogenous ethylene results in early senescence and reduced longevity (Woltering and Van Doorn, 1988; van Doorn, 2001). While in non-climacteric or insensitive flowers, no significant increase in ethylene production is apparent during natural senescence and exogenous ethylene has no or little effects on them (Woltering and Van Doorn, 1988; van Doorn, 2001). Ethylene is produced in an autocatalytic manner in all plants/organs with a climacteric respiration pattern. After the perception of ethylene by ethylene receptors, activation of its signal transduction cascade affects the expression pattern of ethylene-dependent genes and their mRNA levels

in cells (McGrath and Ecker, 1998; Davies, 2004; Serek *et al*., 2006), which in most cut flowers results in chlorophyll degradation, wilting and abscission (van Doorn, 2001; Ahmadi *et al*., 2008, 2009).

Plant growth regulators can be used to increase the chilling tolerance in chilling sensitive plants (Lukatkin *et al*., 2012). Brassinosteroids (BRs) are a group of plant growth regulators that can decrease the effects of biotic and abiotic stresses in plants (Wang *et al*., 2012; Z. Liu *et al*., 2016). Their production is triggered in response to biotic and abiotic stresses, which can decrease chilling injury and improve growth in plants (Hotta *et al*., 1998; Fujii and Saka, 2001). BRs can delay the occurrence of senescence symptoms and thereby reduce the postharvest loss of horticultural products through affecting ethylene production (in some crops), chlorophyll degradation, chilling tolerance and finally retarding early senescence (Zhu *et al*., 2010; Wang *et al*., 2012; Gao *et al*., 2015; Gao *et al*., 2016; Z. Liu *et al*., 2016). BRs have been reported to be effective in keeping growth under cold stress in crops (He *et al*., 1991; Khripach *et al*., 1998), decreasing chilling injury symptoms in bananas (*Musa* spp.) (Bajguz and Hayat, 2009), and pepper fruit (*C. annuum* L. 'Zhongjiao 7') (Wang *et al*., 2012). However, the effects of BRs on the postharvest quality of cut flowers have not been reported.

The aims of the current study were: (i) to investigate the effects of different concentrations of BR on chilling tolerance and determine the optimum concentration of BR as postharvest treatment; (ii) to determine the effect of cold storage on ethylene production and postharvest quality in cut lily cultivars; and (iii) to determine the effect of optimum concentration of BR on ethylene production and postharvest quality.

2.2. Materials and methods

2.2.1. Plant material and treatments

2.2.1.1. Experiment one: determination of the optimum concentration

Cut lilies (*Lilium orientalis*) 'Sorbonne' and 'Marlon' were selected for this experiment. The flowers were bought from a lily grower in Melbourne to the Burnley campus of the University of Melbourne. The stems were recut under distilled water to the desired height (45 cm). Cut lily 'Sorbonne' stems were treated with 6, 10, and 14 μM of BR for 8 h and then were individually placed in glass vials containing sterilised distilled water and cold-stored in an upright position at 5 °C in dark for ten days (Fig. 6.1S). We then repeated the experiment to obtain a more detailed dose-response curve with the cut lily 'Marlon' that was available at the market at the time. Lily stems were treated with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 μM of BR for 12 h and then individually placed in glass vials containing distilled water and cold-stored in an upright position at 5 °C in 12h Cool White fluorescent light for one week (the light was added to eliminate the effect of darkness as one of the possible reasons of chlorophyll loss). After cold storage, the stems were placed in a ventilated room at 20 ± 2 °C, 58-65% RH, and 12 h Cool White fluorescent light of 20 μmol m^{-2} s^{-1} for postharvest quality assessments. The control treatments received no BR. Postharvest quality characteristics were used to assess the optimum BR concentration, as outlined in section 2.2.2. In non-cold-stored flowers, the day 0 refers to the day that cut stems were placed in individual vials for postharvest quality assessment, in cold-stored flowers the day 0 of the experiment was considered as the day that flowers were moved into the ventilated room after cold storage treatment. Sterilised distilled water was used daily in order to top up the solution in each glass vial.

2.2.1.2. Experiment two: optimum concentration treatment

Cut lilies (*Lilium orientalis*) 'Sorbonne', 'Stargazer', 'Premium Blonde', and 'Marlon' were selected for this experiment. The stems were prepared before treatments as previously described in 2.1.1 above. Based on our previous experiments 8.5 and 10 µM of BR were determined as the optimum concentrations for cut lilies 'Marlon' and 'Sorbonne', respectively. Cut lilies 'Sorbonne' and 'Stargazer' stems were treated with 10 µM of BR for 12 h and then were individually placed in glass vials containing sterilised distilled water and cold-stored in an upright position at 5 °C in dark for 10 days and cut lilies 'Premium Blonde' and 'Marlon' were treated with 8.5 µM BR for 12 h and then were individually placed in glass vials containing sterilised distilled water and cold-stored in an upright position at 5 °C in 12 h Cool White fluorescent light for one week. After cold storage, the stems were placed in a ventilated room at 20 ± 2 °C, 58-65% RH, and 12 h Cool White fluorescent light of 20 µmol m^{-2} s^{-1} for postharvest quality assessments. The control treatments received neither BR nor cold-stored. In non-cold-stored flowers, the day 0 refers to the day that cut stems were placed in individual vials for postharvest quality assessment, in cold-stored flowers the day 0 of the experiment was considered as the day that flowers were moved into the ventilated room after cold storage treatment. Sterilised distilled water was used daily in order to top up the solution in each glass vial.

2.2.2. Postharvest quality characteristics

Data on the time to bud opening, flower senescence, flower abscission, and leaf yellowing were collected daily at the same time. The time to bud opening, flower senescence, and flower abscission were determined in the two lowermost flowers separately from day 0 until each of those processes occurred. The time to leaf yellowing was determined in the two lowermost leaves separately. A bud was considered open when more than one tepal

had moved laterally. Flowers were considered senescent when more than two tepals showed discoloration and desiccation in their tips. Flower abscission was defined by the fall of more than two tepals on a flower. Leaf yellowing was defined as the presence of yellowing in two lowermost leaves on a stem. The day 0 was defined as outlined in section 2.2.1.

2.2.3. Water uptake

Water uptake (WU) was measured daily from day 0 in cut lilies 'Sorbonne' and 'Stargazer' using the following equation:

$$\text{WU (g g}^{-1}\text{ initial fresh weight - FW)} = \frac{B_{n-1} - B_n}{\text{initial FW } (A_0 - B_0)}$$

where B is the weight of vase without cut stem (i.e. vase + solution, g), Bn_{-1} is the weight (g) on the previous day, Bn is the weight (g) at day n, A_0 and B_0 are the weights (g) on day 0 (Çelikel *et al*., 2011).

2.2.4. Leaf chlorophyll content

Leaf chlorophyll content was determined every four days from day 0 using five replicates in cut lilies 'Sorbonne' and 'Stargazer'. Leaf discs from the lowermost leaves were removed using a cork borer and put into a 2 ml microtube, 1 mL 80% ethanol was added to each tube and then the tubes were placed in a water bath at 70 °C for 10 min. Thereafter, the discs were transferred to 50 ml Falcon tubes, topped up to 15 mL with 80% ethanol and kept in a water bath as above until the chlorophyll content was fully extracted (Ahmadi *et al*., 2009). The amount of total chlorophyll was determined using a spectrophotometer at the wavelengths 700, 664, and 647 nm (Lichtenthaler, 1987). Chlorophyll content was

calculated using the following equation: Chlorophyll a+b = 5.24 ($A_{664} - A_{700}$) + 22.24 ($A_{647} - A_{700}$), where A_{700}, A_{664}, and A_{647} are absorbances at the three wavelengths. The day 0 was defined as outlined in section 2.2.1.2.

2.2.5. Ethylene measurement

Ethylene production of flowers was measured every two days from day 0 in all lily cultivars, as individual cut flowers were placed in 2 L air-tight jars for 24 hours at 20 ± 2 °C (Fig. 6.2S). Potassium hydroxide solution (20% w/v) was placed inside the jars to maintain low CO_2 concentrations from respiration during treatments. Gas samples were taken through headspaces using a syringe and were measured using a gas chromatograph (GC) (Shimadzu, Model GC-14B; column Poropak P set at 50°C). The Flame Ionisation Detector was set at 200 °C and the injector temperature was 180°C. The day 0 was defined as outlined in section 2.2.1.2.

2.2.6. Statistical analysis

Experiment 1 had a completely randomised design with four (‘Sorbonne’) and five (‘Marlon’) biological replicates (stems/inflorescences) per treatment and experiment 2 had a completely randomised design with four (‘Sorbonne’ and ‘Stargazer’) and ten (‘Premium Blonde’ and ‘Marlon’) biological replicates (stems/inflorescences). The effects of treatments were analysed using the General Linear Model procedure and the one-way Analysis of Variance (one-way ANOVA) of Genstat software (VSN International, version 18) in experiments one and two, respectively. Means of different treatments were compared using post hoc Tukey test ($P<0.05$).

2.3. Results

2.3.1. Experiment one: determination of the optimum concentration

2.3.1.1. Bud opening

In 'Sorbonne' cold storage bud opening occurred on day 2 which was the shortest time to bud opening among all treatments (data not shown). No significant differences (P>0.05) were observed among treatments (data not shown). In 'Marlon', BR treatments increased the time to bud opening by approximately 1 day (Fig. 2.1A). The longest time to bud opening was observed in 9 µM BR-treated flowers which was significantly higher (P<0.05) than control but had no significant differences (P>0.05) with other BR treatments.

2.3.1.2. Flower senescence

In 'Sorbonne', flower senescence was first observed in control treatment after 6 d, which was significantly lower (P<0.05) than BR treatments (after day 7) (data not shown). The longest time to flower senescence was observed in flowers treated with 10 µM BR after 8 d (data not shown). In 'Marlon' control flowers senesced at 4 days (Fig. 2.1B), BR treatments increased the time to flower senescence (6-7 days) and most of them (3 to - 14 µM BR) had significant differences (P<0.05) with control. Flowers treated with 9 µM BR had the longest time to senescence (7.4 d) followed by 7 µM BR.

2.3.1.3. Flower abscission

In 'Sorbonne', flower abscission was significantly (P<0.05) delayed by BR treatments compared to control (9.2 d) (data not shown). The 10 µM BR treatment had the longest time to flower abscission (11.8 d) (data not shown). In 'Marlon', BR treatments delayed

the time to flower abscission. Flowers of the control showed flower abscission at 8.8 d whereas flowers treated with 9 µM BR had the longest time (11.3 d) to flower abscission. Flower abscission in lilies exposed to BR concentrations from 6 to 12 µM was significantly different (P<0.05) from the control (Fig. 2.1C).

2.3.1.4. Leaf yellowing

In 'Sorbonne', the longest time to leaf yellowing was observed in 10 µM BR (7 d) and showed a significant difference (P<0.05) with other treatments. In control, leaf yellowing appeared after 2 d (data not shown). In 'Marlon', leaf yellowing was delayed in BR-treated flowers. The control showed an early leaf yellowing (6 d) (Fig. 2.1D). The 9 µM BR concentration showed the longest time to leaf yellowing and had a significant difference (P<0.05) compared to control.

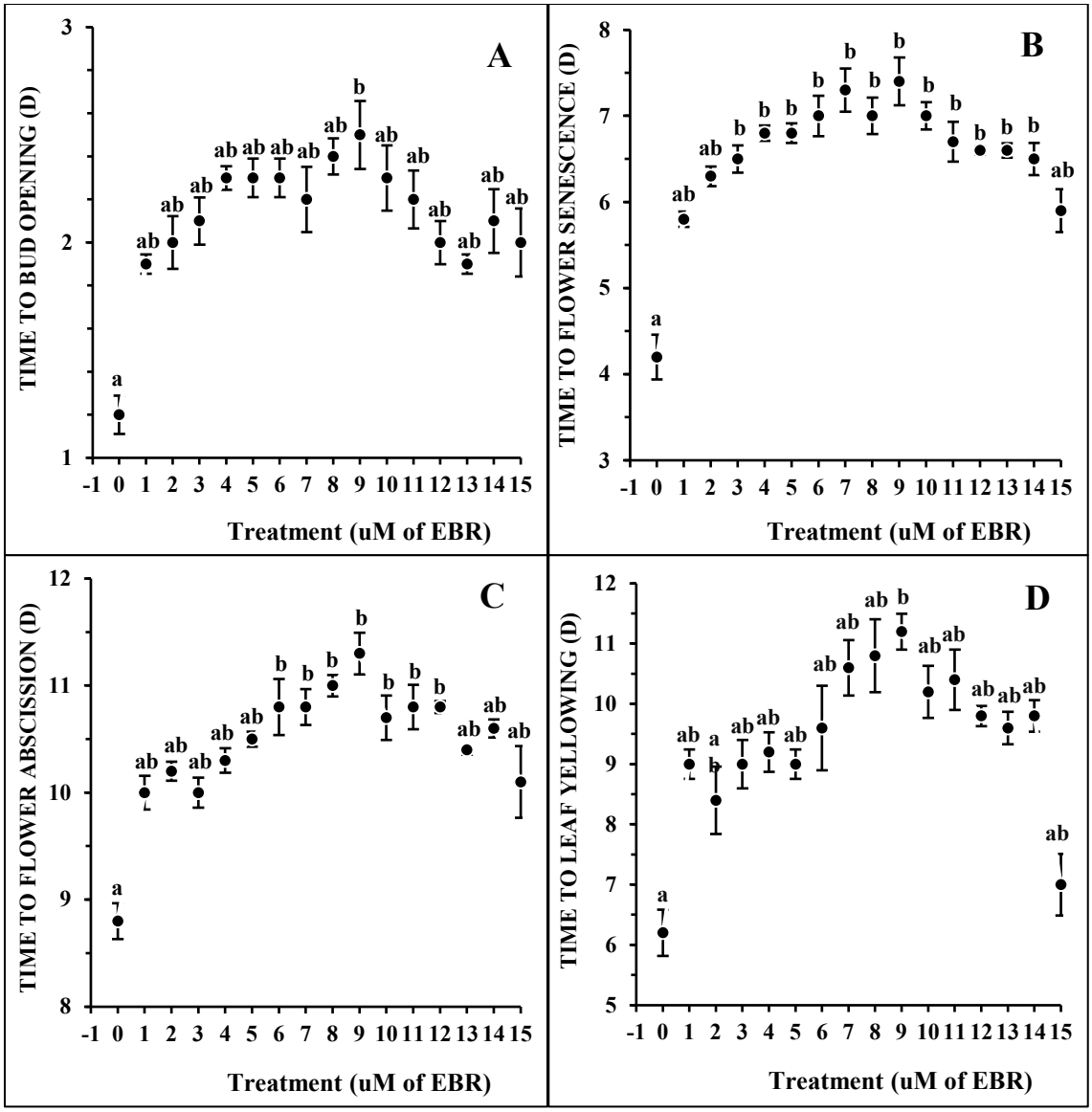


Figure 2.1. Effects of cold storage (5 ºC, one week) and different concentrations (0 to 15 µM) of Brassinosteroid (BR) on postharvest quality indicators in cut lily 'Marlon'. Values are the means (±SE) of five replicates. Data were analysed using one-way analysis of variance (ANOVA), followed by post hoc Tukey test (P<0.05). Data represented by points that do not share the same letter are significantly different (P<0.05).

2.3.2. Experiment 2: Optimum BR concentrations' experiments

2.3.2.1. Bud opening

Cold storage decreased the time to bud opening in all cultivars (Fig. 2.2A). In 'Sorbonne', no significant differences (P>0.05) were observed between cold storage and BR + cold storage treatments. In 'Stargazer', BR + cold storage treatment significantly (P<0.05)

increased the time to bud opening compared to cold storage treatment (Fig. 2.2A). In 'Marlon', cold storage significantly (P<0.05) reduced the time to bud opening (Fig. 2.2A). Control had the longest time to bud opening with significant differences with other treatments (Fig. 2.2A). No significant differences (P>0.05) were observed between cold storage and BR + cold storage treatments (Fig. 2.2A).

2.3.2.2. Flower senescence

Control flowers had a longer time to flower senescence in all cultivars and cold storage decreased the time to flower senescence for all cultivars with the exception of 'Premium Blonde' (Fig. 2.2B). BR + cold storage treatment delayed the time to flower senescence compared to cold storage treatment in all cultivars except in 'Premium Blonde' and the time to flower senescence was similar to the control treatment.

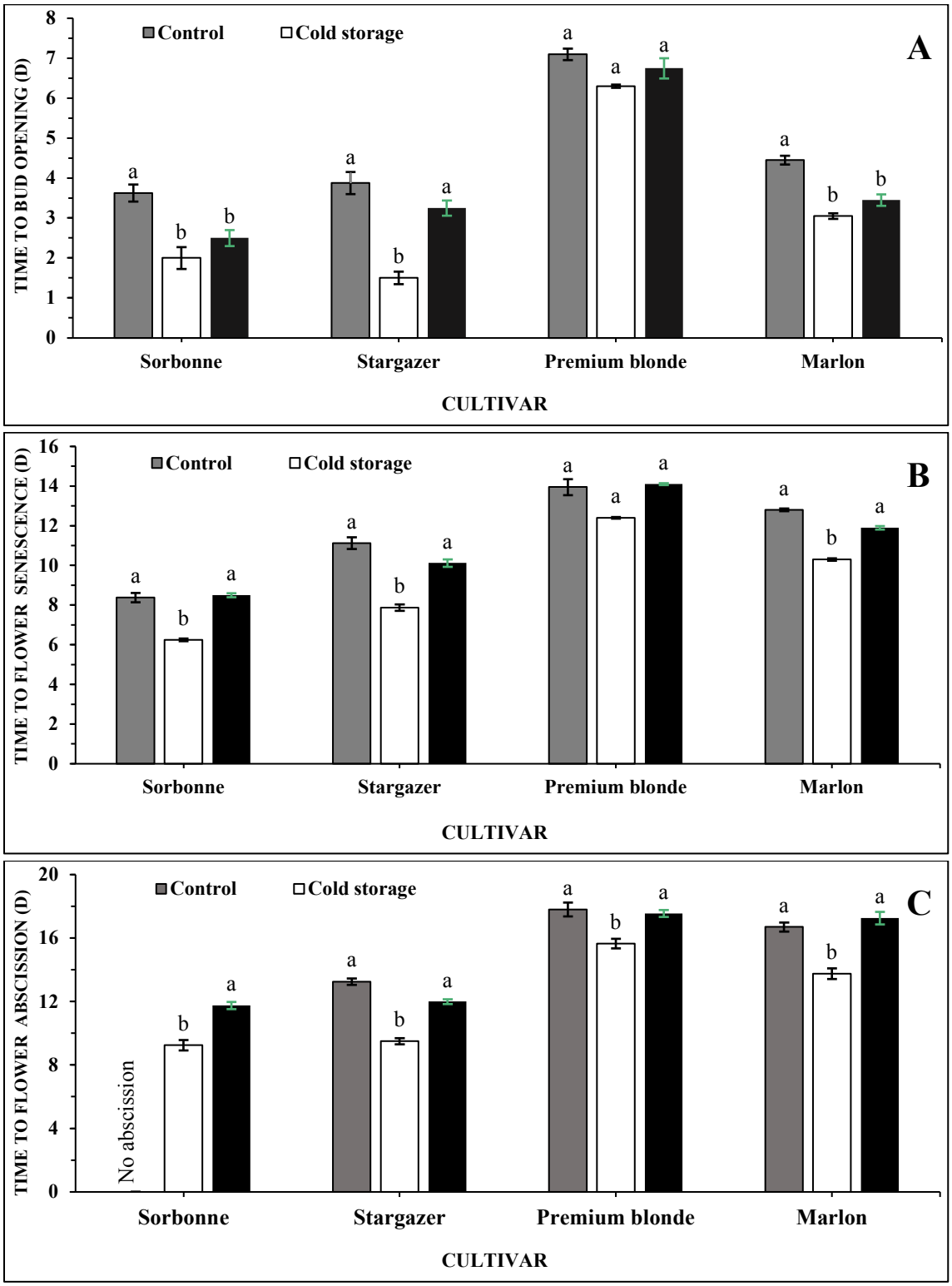
2.3.2.3. Flower abscission

In all cultivars, flower abscission was first observed in cold storage treatment (Fig. 2.2C). BR + cold storage treatment significantly (P<0.05) delayed the detrimental effects of cold storage and resulted in a longer time to flower abscission compared to cold storage treatment in all cultivars (Fig. 2.2C). In 'Sorbonne', control plants showed no abscission of flowers, but it was induced by cold storage treatment (Fig. 2.2C) and in 'Marlon' the BR + cold stored plants showed the longest time to flower abscission of all treatments.

2.3.2.4. Leaf yellowing

Time to leaf yellowing was the shortest in the cold storage treatment and longest in the control (Fig. 2.2D). Leaf yellowing was delayed in BR + cold storage treated flowers, but it was not significantly (P>0.05) different in all cultivars. 'Stargazer' showed no leaf

yellowing during 15 days of postharvest. In 'Stargazer', BR + cold storage treatment did not delay the time to leaf yellowing compared to cold storage treatment (Fig. 2.2D).



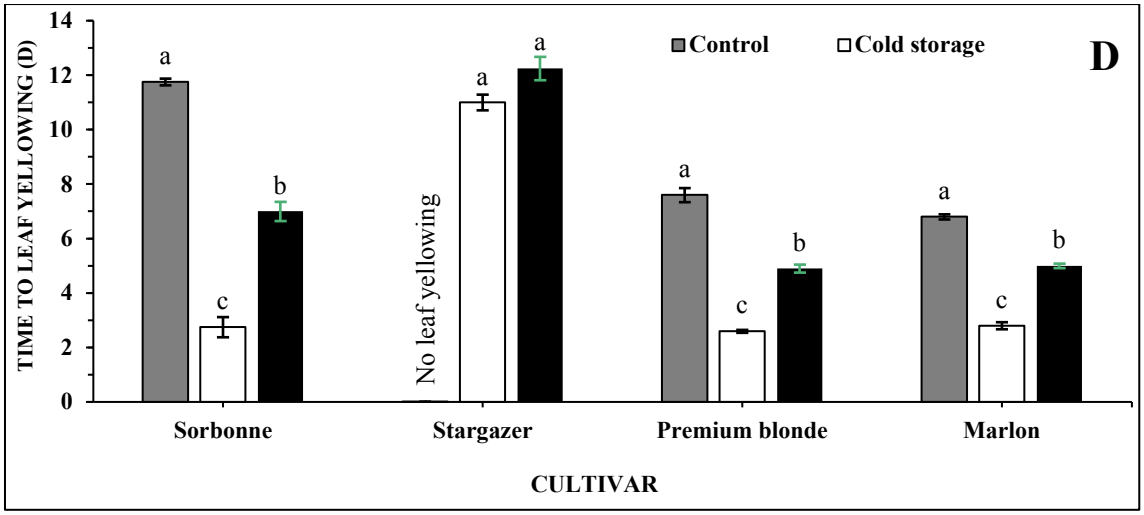


Figure 2.2. Effects of cold storage (5 ºC, seven and ten days) and BR treatments (8.5 and 10 µM) on bud opening (A), flower senescence (B), flower abscission (C), and leaf yellowing (D) in cut lily cultivars. The values of the bars are the means (±SE) of four ('Sorbonne' and 'Stargazer') and ten replicates ('Premium Blonde' and 'Marlon'). Data were analysed using one-way analysis of variance (ANOVA), followed by post hoc Tukey test (P<0.05). Bars marked with different letters are significantly different (P<0.05).

2.3.2.5. Water uptake

In 'Sorbonne', water uptake decreased in all treatments during postharvest life (Fig. 2.3A). BR + cold storage treatment had the highest amount of water uptake amongst all treatments and showed a significant difference (P<0.05) with other treatments. In 'Stargazer', there was no significant difference (P>0.05) between control and BR + cold storage treatments, but a significant difference (P<0.05) was observed between these treatments and cold storage treatment.

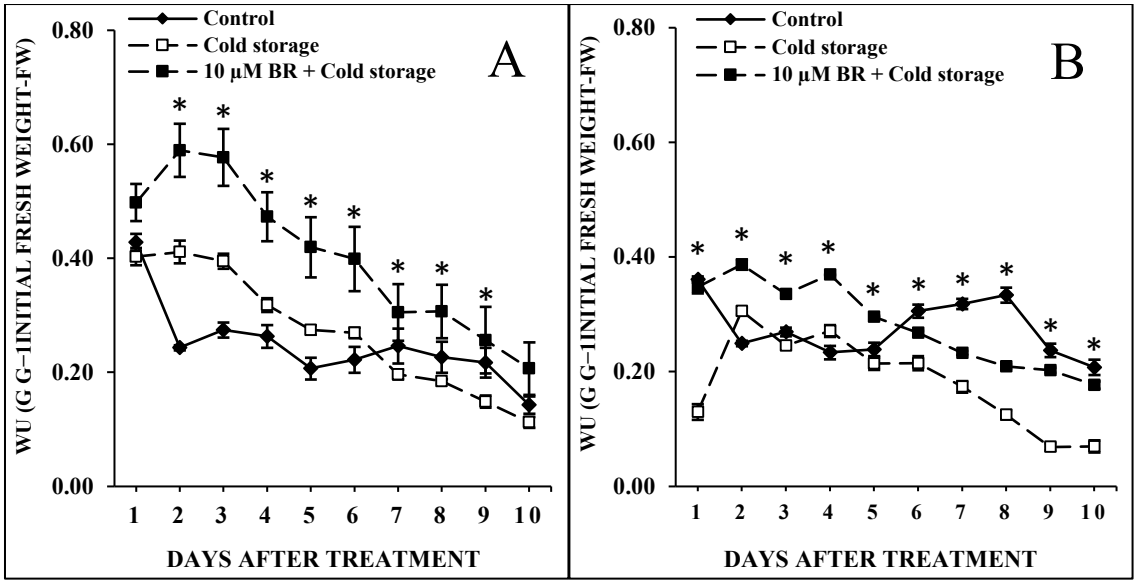


Figure 2.3. Effects of cold storage (5 ºC, ten days) and BR treatments (10 µM) on water uptake in cut lily 'Sorbonne (A) and 'Stargazer' (B) for 10 days after treatment ended. Values are the means (±SE) of four replicates. Data were analysed using one-way analysis of variance (ANOVA), followed by post hoc Tukey test (P<0.05). The asterisks indicate significant differences (P<0.05) between treatments.

2.3.2.6. Chlorophyll content

Chlorophyll content decreased during 8 d of postharvest life in all treatments in both cultivars (Fig. 2.4.). In cold storage treatment chlorophyll content rapidly declined from 100% at the start of the experiment to 8% after 8 days. The decline was less rapid in BR + cold storage treatments and only gradual in the control (70% of initial content after 8 d). In 'Sorbonne', control had the highest chlorophyll content and was significantly different (P<0.05) compared to other treatments. Furthermore, BR + cold storage showed a significantly (P<0.05) higher amount of chlorophyll content compared to cold storage treatment. In 'Stargazer', cold storage showed the lowest amount of chlorophyll content but BR + cold storage treatment significantly (P<0.05) prevented the effects of cold storage on chlorophyll content (Fig. 2.4B).

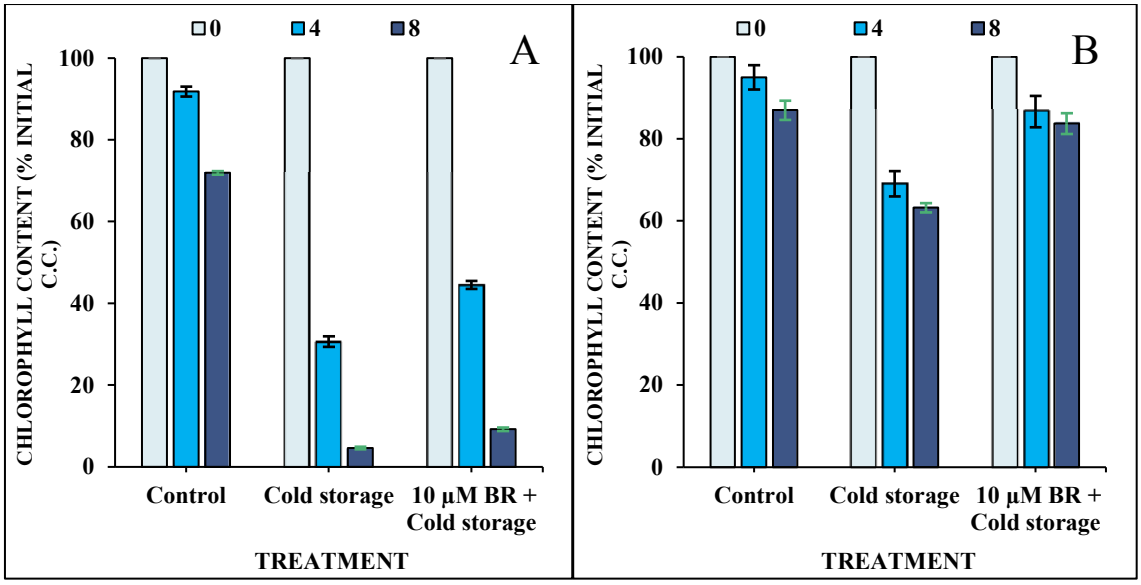


Figure 2.4. Effects of cold storage (5 ºC, ten days) and BR treatments (10 µM) on chlorophyll content in cut lily 'Sorbonne' (A) and 'Stargazer' (B) at 0 (dark grey bars), 4 (open bars) and 8 (closed bars) days after treatment ended. The values of the bars are the means (±SE) of four replicates. Data were analysed using one-way analysis of variance (ANOVA), followed by post hoc Tukey test (P<0.05).

2.3.2.7. Ethylene production

The level of ethylene production differed between all cultivars (Fig. 2.5.). Plants in the control treatment generally produced a lower amount of ethylene compared to the other treatments (Fig. 2.5.). Cold storage induced ethylene production and resulted in higher amounts of ethylene production in all cultivars (Fig. 2.5.). In 'Sorbonne' there were significant differences (P<0.05) between treatments. BR + cold storage significantly (P<0.05) reduced cold-induced ethylene production and had a lower ethylene production than cold storage treatment (Fig. 2.5A). In 'Stargazer', BR + cold storage had a lower amount of ethylene production compared to cold storage treatment (Fig. 2.5B) but the difference in ethylene production was not statistically significant (P>0.05). 'Premium Blonde' (Fig. 2.5C) produced less ethylene amount than 'Marlon' (Fig. 2.5D) across all treatments. Significant differences (P<0.05) were observed between treatments in both cultivars, but not on all days. The BR + cold-stored flowers had less ethylene production than cold-stored ones in both 'Premium Blonde' and 'Marlon' (Fig. 2.5C & D). Cold

storage treatment increased ethylene production and showed the highest amount of ethylene production among all treatments in both cultivars (Fig. 2.5C & D).

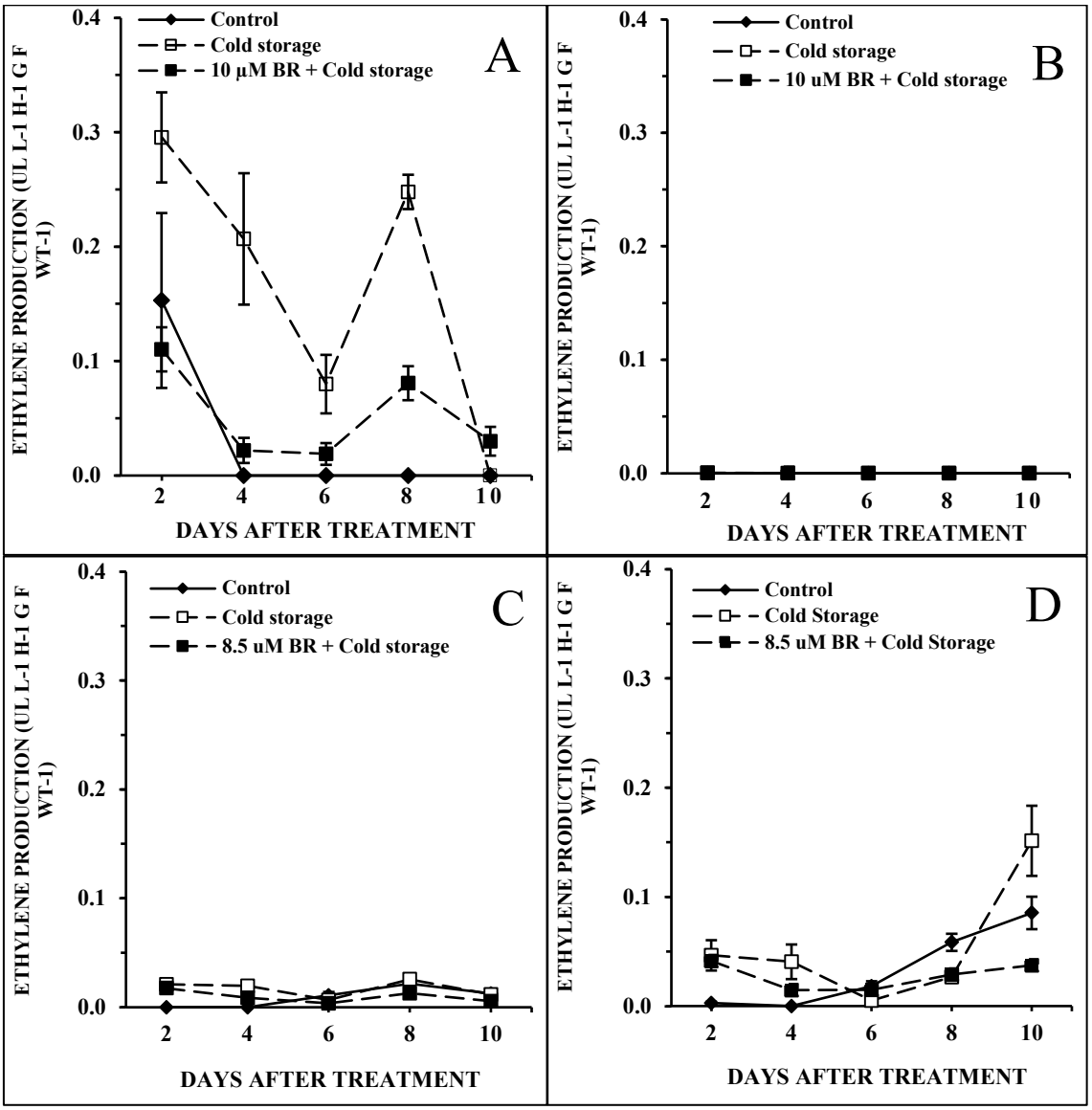


Figure 2.5. Effects of cold storage (5 ºC, seven and ten days) and BR treatments (8.5 and 10 µM) on ethylene production in cut lily cultivars 'Sorbonne (A), 'Stargazer' (B), 'Premium Blonde' (C) and 'Marlon' (D). Values are the means (±SE) of four replicates. Data were analysed using one-way analysis of variance (ANOVA), followed by post hoc Tukey test (*P*<0.05).

2.4. Discussion

In many lilies, a relatively short period of cold storage often causes physiological damages such as unopened buds, hastened tepal wilting, induced or increased leaf yellowing, and promoted bud abscission (Han and Miller, 2003; van Doorn and Han, 2011). Pre-treating

with plant growth regulators is a means of preventing the impacts of low-temperature storage (Choehom, 1997; Salvador *et al*., 2004; Barman *et al*., 2011; Sayyari *et al*., 2011; Gao *et al*., 2015; Gao *et al*., 2016). In this study, we investigated the effects of different concentrations of BR in alleviating chilling injuries in cut lilies cultivars and determined the optimum concentration of BR as postharvest treatment. We observed most of the chilling responses like early chlorophyll loss and flower senescence after cold storage in these cultivars.

All BR concentrations delayed the occurrence of chilling injury and postharvest quality loss (Fig. 2.1.) but we observed the best results at median concentrations of 8.5 and 10 µM BR (Fig. 2.1.). Our data showed that lower concentrations of BR were not sufficient to significantly delay postharvest quality loss and higher concentrations accelerated the quality loss process (Fig. 2.1.). This optimum curve was also observed in other plant species such as red cabbage (*Brassica oleraceae* L.) (Çağ *et al*., 2007), jujube (*Zizyphus jujuba* cv. Huping) (Zhu *et al*., 2010), green bell pepper (*Capsicum annuum* L. cv. 'Zhongjiao 7') (Wang *et al*., 2012), Satsuma mandarin (*Citrus unshiu*) (Zhu *et al*., 2015), and peach fruit (*Prunus persica* Batsch cv 'Qinmi') (Gao *et al*., 2016). For instance, 15 uM of BR was more effective than other BR concentrations (5, 10, and 25 uM) on chilling injury of peach (*Prunus persica* Batsch 'Qinmi') (Gao *et al*. 2016). Wang et al. (2012) reported that BR at the concentrations of 5, 10, and 15 µM effectively reduced chilling injury of pepper fruit during 18-day storage at 3 ˚C, but BR at 15 µM had the best effect. Based on literature and our results, it seems that BR effects on plants are species- and concentration-dependent.

We further investigated the effects of cold storage and optimum concentrations of BR on four lily cultivars: 'Sorbonne', 'Stargazer', 'Premium Blonde', and 'Marlon'. Our data

showed that cold storage caused higher ethylene production and early chilling injuries compared to other treatments (Fig. 2.2 & 5). The response of cut lilies to cold stress seems to be cultivar-dependant as bud opening was not affected in 'Premium Blonde' but in other cultivars it was significantly affected, flowers senescence was significantly affected in 'Sorbonne' and 'Stargazer' but not in other cultivars, and flower abscission and leaf yellowing were significantly affected in all cultivars. This was further confirmed by the reports on cut lily 'Stargazer' (Han and Miller, 2003) and 'Brussels' (Song and Peng, 2004) as two weeks of cold storage changed ethylene production pattern, increased ethylene sensitivity and ethylene exposure advanced flower opening and senescence in 'Stargazer', while, following cold storage, less ethylene sensitivity was observed in cut lily 'Brussels' and ethylene exposure did significantly not affect its longevity. The postharvest quality loss in our study could be ethylene-dependent or -independent. Ethylene-dependent because cultivars with the highest ethylene production had the worst postharvest quality. But it is possible that it could be ethylene independent and ethylene production in these cultivars might be a normal response of lilies to stress condition without any effects on any of the measured characteristics. And the variation in the level of ethylene production among cultivars might be due to different level of their sensitivity to cold stress. Thus, quality loss might have been triggered by other factors such as changes in the concentration of other plant hormones such as cytokinins, and gibberellins or sugar depletion. For instance, decline in the level of endogenous cytokinins and gibberellins which has been reported to led to early senescence (Fletcher et al., 1969; Lara et al., 2004). In transgenic petunia (*Petunia* x *hybrida* cv V26) (Chang et al., 2003) and miniature potted rose (*Rosa hybrida* cv. Linda) (Zakizadeh et al., 2013), increase in the level of endogenous cytokinins delayed senescence. In dandelion leaves (*Taraxacum officinale*), the level of gibberellines was high during leaf growth while it decreased during

leaf senescence, suggesting that leaf senescence is related to decrease in level of gibberellines (Fletcher et al., 1969). Similarly, in daisy (*Chrysanthemum morifolium*), the level of gibberellines was high in early stage of the development of the flower but decreased in later stages (Jeffcoat and Cockshull, 1972). The effects of cytokinins and gibberellins in delaying senescence may be ethylene mediated as there has been reports that these hormone delay senescence process through decreasing ethylene production and sensitivity (Zakizadeh et al., 2013). For instance, Zakizadeh et al. (2013) reported that in miniature potted rose (*Rosa hybrida* cv. Linda) cytokinine led to less ethylene production and responsiveness and delayed senescence. The changes in ethylene production and sensitivity could be the result of blocking the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene or suppressing the expression of ethylene-related genes by cytokinins (Mor et al., 1983; Trivellini et al., 2015). Similar to cytokinins, in cut carnations (*Dianthus caryophyllus* L.) gibberellins suppressed ethylene production both at early preclimacteric and climacteric stages (Saks and Van Staden, 1992; Saks and Van Staden, 1993) and respiratory climacteric (Saks and Van Staden, 1992) which resulted in delayed senescence. But the effects of cytokinins and gibberellins in delaying senescence might be ethylene independent. As there has been reports showing that cytokinins may delay senescence by maintaining membrane integrity (Gulzar et al., 2003), increasing water uptake (Mayak and Halevy, 1974; Gulzar et al., 2003), maintaining the level of sugar in tissue (Gulzar et al., 2003), slowing down protein, RNA, and DNA degradation (Osborne, 1963; Mayak and Halevy, 1974; Gulzar et al., 2003), and dry weight reduction (Mayak and Halevy, 1974). Similarly, gibberellins may delay senescence by increasing water content (Emongor, 2004), slowing down the decrease of pigments and total soluble protein, and reducing oxidative stress (Yu et al., 2009; Li et al., 2010; do Nascimento Simões et al., 2018) which needs to be further investigated. Sugar depletion either due to darkness or lack of sugar supply to the stems

may have also caused early senescence through regulating the activity of region/leucine zippers (bZIPs), basic-helix-loop-helix (MYC2), and no apical meristem/Arabidopsis transcription activation factor 1 (NAC2/ATAF1) transcription factors which are involved low energy responses (starvation) (Dietrich et al., 2011; Garapati, et al., 2015; Mair et al., 2015; Qi et al., 2015). The activation of these transcription factors leads to early senescence as bZIP1 and bZIP53 directly bind to the promotors of *PROLINE DEHYDROGENASE* (*ProDH*) and regulate its expression (Dietrich et al., 2011). ProDH is involved in proline catabolism, higher level of ProDH activity leads to more proline catabolism and early senescence of petal and leaf tissues (Zhang and Becker, 2015). MYC2 activates jasmonic acid-induced leaf senescence by binding to the promotor of *SENESCENCE-ASSOCIATED GENE29* (*SAG29*) and activating its expression (Qi et al., 2015). ATAF1 is a transcriptional regulator of a key enzyme of the biosynthesis of abscisic acid pathway *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*) (Jensen et al., 2013) which leads to increase in the level of abscisic acid by enhancing the expression of *NCED3* (Wu et al., 2009; Jensen et al., 2013). Moreover, it directly targets *ORESARA1* (*ORE1*) and *GOLDEN2-LIKE 1* (*GLK1*) genes by increasing the expression of *ORE1* and decreasing the expression of *GLK1* (Garapati, et al., 2015). ORE1 which is a positive senescence-regulating transcription factor with ethylene and abscisic acid related elements (EIN3 and ABI5) regulate the expression of genes (*GLK1*, *SGR1*, and *NYC1*) related to chlorophyll degradation (Qiu et al., 2015; Liebsch and Keech, 2016) and ethylene biosynthesis genes (ACSs) (Qiu et al., 2015; Liebsch and Keech, 2016; Paik et al., 2017) which lead to early senescence. Flower abscission might have also been ethylene mediated or it might have been caused by other factors. In ethylene dependent manner, it could be due to changes in the level of auxin, as cold storage might have resulted in reduction in the level of auxin which is the primary regulator of organ

abscission (Hong et al., 2000). Reduction in the level of endogenous auxin due to either a normal developmental process or stress response increases the sensitivity of the cells in the abscission zone to ethylene which in turn increase the level of enzymes responsible for the hydrolysing of middle lamellae and finally leads to organ abscission (Rungruchkanont et al., 2007; Estornell et al., 2013; Meir et al., 2015). But it could also be due to changes in the level of other plant growth regualtors such as increase in the level of abscisic acid, and decrease in the levels of Cytokinins and BRs which have been reported to regulate abscission process through an ethylene-dependent manner, as they affect ethylene biosynthesis pathway (Cracker and Abeles, 1969; Leslie and Romani, 1986; Wilmowicz et al., 2016; Lv et al., 2018). For instance, Luo et al. (2014) reported that in Arabidopsis (*Arabidopsis thaliana* L.), abscisic acid activated calcium-dependent protein kinases (CDPKs) which in turn phosphorylated the N-termini of 1–aminocyclopropane-1-carboxylate synthases (ACS) which stabilized ACS and increased ethylene production. Moreover, in Arabidopsis (*Arabidopsis thaliana* L.) a direct interaction between BES1 or BZR1 (brassinosteroid-regulated transcription factors) and ACSs enzymes under low concentration of BRs was observed (Lv et al., 2018) and the activity of *AtACS* promoters was strongly suppressed by the over-expression of both *BES1* and *BZR1* genes. Cytokinins seems to delay abscission via a crosstalk with ethylene as the expression of cytokinin oxidase/dehydrogenase (*CKX*) genes and ethylene-related genes were increased after ethephon exposure with increased level of ethylene but after down regulation of *CKX3* the response to ethylene decreased and abscission delayed (Xu et al., 2019). But the abscission might have been induced by jasmonic acid through an ethylene independent manner as treating the *ein2-1* ethylene insensitive mutant with jasmonic acid accelerated organ abscission which indicate that organ abscission is independently regulated by this hormone (Kim et al., 2013). External factors might have also triggered

abscission particularly darkness in 'Sorbonne' and 'Stargazer' and sugar depletion in all cultivars which in turn is caused by darkness through chlorophyll degradation as source of energy production (Liebsch and Keech, 2016). Darkness and sugar depletion probably cause abscission through both ethylene dependent and independent manners. Darkness and sugar depletion conditions regulate the components of both ethylene and abscisic acid signalling and biosynthesis pathways and increase their production possibly through the activation of an ORE1 mediated pathway which results in the activation of the genes involved in their biosynthesis pathway (Khan et al., 2014; Jeong et al., 2016; Liebsch and Keech, 2016; Paik et al., 2017) which are positive regulators of abscission process. But lack of abscission in 'Sorbonne' before cold storage in our study might be due to lack of an abscission zone at the base of petals which might have been induced by stress condition or an abscission zone might exist and lack of abscission might be due to higher levels of auxin (if abscission is controlled by auxin/ethylene ratio) or low level of jasmonic acid which prevent abscission activation. Overall, it seems that: i) cold stress response in lilies is cultivar-dependant and the response of each cultivar might change at different times and under different conditions; (ii) in each cultivar, the level of response might be ethylene dependent meaning that cultivars with less ethylene production will have better postharvest quality; and/or (iii) the different ethylene production could be just a normal response of cut lilies to cold stress based on their sensitivity level to the stress without any effects on any measured characteristics and it might be under control of other factors such as other plant hormones. But if the changes are triggered by ethylene, this difference in response to cold stress particularly in ethylene production and reaction might be due to the presence/absence of ethylene receptors, the level of presence (Müller *et al*., 2000; Al-Salem and Serek, 2017), active/inactive forms of receptors (Bleecker, 1999), and

developmental stage (Han and Miller, 2003). However, the mechanism behind this is not known and needs further study.

The relationship between the level of chilling injury and the amount of ethylene biosynthesis was further confirmed by BR results. BR decreased ethylene production in all cultivars but its effects were not significant for all cultivars. BR was only effective in cultivars with higher ethylene production such as 'Sorbonne' and resulted in better postharvest quality (Fig. 2.5.). Similarly, Li et al. (2016) observed that BR suppressed the ethylene biosynthesis pathway and significantly increased the net photosynthesis rate and chlorophyll content in pepper. These findings suggest that the effects of BR depend on cultivars' response to cold stress - the effects of BR in stress-sensitive cultivars would be significant. The effectiveness of BRs seems to be related to an alteration in ethylene biosynthesis which might have taken place directly via affecting the expression of some or all genes involved in ethylene biosynthesis pathways (Li *et al*., 2015; Lv *et al*., 2018), or indirectly via affecting the level of ethylene receptors' presence or the expression of many signalling components of other hormonal pathways (Sun *et al*., 2010; Kudryakova *et al*., 2013; Unterholzner *et al*., 2015). For instance, in tomato (*Lycopersicon esculentum* L.), BR changed the level of ethylene production by changing the expression of its biosynthesis pathway genes (e.g. *LeACS2, LeACS4, LeACO1, and LeACO4*) (Zhu *et al*., 2015). Moreover, in Arabidopsis (*Arabidopsis thaliana* L.) a direct interaction between BES1 or BZR1 (brassinosteroid-regulated transcription factors) and ACSs (1-aminocyclopropane-1-carboxylic acid synthases enzymes) was reported under low concentrations of BR (Lv *et al*., 2018), as the activity of *AtACS* promoters was strongly suppressed by the over-expression of both *AtBES1* and *AtBZR1* (Lv *et al*., 2018). Or in another study, it was shown that in Arabidopsis (*Arabidopsis thaliana* L.) BR increases gibberellic acid biosynthesis in a BR-promoted manner as BES1 binded to a regulatory

motif present in the promoters of genes involved in gibberellic acid biosynthesis (*GA20ox1* and *GA3ox1*) and increases their expression (Unterholzner *et al*., 2015). In Arabidopsis (*Arabidopsis thaliana* L.), it was reported that BRs is involved in the regulation of the genes (*AHK2* and *AHK3*) related to cytokinins signalling pathway through an increasing cytokinins level (Kudryakova et al., 2013). Increase in the level of gibberellic acid cytokinins may delay senescence and abscission through an ethylene mediated manner (Saks and Van Staden, 1992) but it could also be ethylene independent by preveting cholorophyll degradation, maintaining membrane integrity, and water uptake of tissues or reducing oxidative stress (Mayak and Halevy, 1974; Gulzar et al., 2003; Yu et al., 2009; Li et al., 2010; do Nascimento Simões et al., 2018). Therefore, prevention of chlorophyll degradation will in turn delay sugar depletion/starvation and sugar deficiency related signalling which will delay both ethylene-dependent or -independent senescence and abscission.

Taken together, these results demonstrate that BR is effective in delaying senescence in most but not all cultivars which could be either ethylene dependent or independent. If ethylene dependent, the difference among cultivars to treatments might be due to different levels of ethylene production or sensitivity but the mechanism behind this is not known. Thus, more work is needed to understand the mechanisms that lead to different cultivar responses. Even in ethylene independent manner, other factors may lead to postharvest quality loss though an ethylene mediated pathway. The results also highlight that BR may not be a ‘game changer’ in enhancing the postharvest quality of cut flowers that are cold stored. The BR treatment delayed the onset of senescence by 1-2 days. And while the results were statistically significant, they probably have less of an impact in the real world.

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Chapter 3

Transcription Analysis of Ethylene- and Brassinosteroid-related Genes in Cold and Brassinosteroid Exposed Lilies

Abstract

Cold storage of cut flowers is often an essential process in the horticultural industry. In many cut lily flowers, cold stress induces ethylene biosynthesis which leads to chilling injuries such as early senescence with unknown molecular effects. Brassinosteroids (BRs) have been reported to induce tolerance to low temperatures in some crops, but their effects on ethylene biosynthesis and the related gene expression are still controversial. This study investigated the effects of cold storage (5°C, ~one week) and BR + cold storage (8.5 μM, 12 h) on cut lilies 'Premium Blonde' and 'Marlon', particularly at molecular and biochemical levels. We monitored display quality characteristics, ethylene production, and the expression pattern of its related genes (*LoACS1*, *LoACO1*, *LoERS1*, *LoETR1*, *LoETR3*, *LoCTR1*, *LoEIN2*, and *LoEIN3*) and *LoBZR1* as BRs-related transcription factor gene. Cold storage caused significant early signs of flower senescence in 'Marlon', and flower abscission and leaf yellowing in both cultivars compared to the BR + cold storage treatment. Ethylene production was significantly higher in cold storage treatment compared to the BR + cold storage one, although not on all observation days. Correspondingly, the expression of most ethylene-related genes was significantly greater in cold stress treatment compared to the BR + cold storage treatment on most observation days in both cultivars. The BRs-related transcription factor gene *LoBZR1* had significantly greater values in BR + cold storage treatment. We conclude that the chilling injuries in cut

lilies are ethylene mediated which could be due to either higher ethylene biosynthesis or sensitivity or a combination of both as a result of alteration in the expression of the related genes. Thus, delayed chilling injuries by application of BRs is likely caused by suppression of the over-expression of ethylene-related genes which results in less ethylene biosynthesis and possibly sensitivity.

3.1. Introduction

The postharvest life of many horticultural crops is affected by ethylene (Reid, 2002b). In most cut flowers ethylene results in chlorophyll degradation, wilting, and abscission (van Doorn, 2001; Ahmadi et al., 2008, 2009). Improving flowers' resistance to senescence-inducing factors such as ethylene is an ongoing target for postharvest physiologists and flower breeders. Knowledge of the ethylene biosynthesis pathway and mode of action has enabled researchers to apply different methods to alleviate or inhibit the impacts of ethylene on horticultural commodities (Saltveit, 1999). To date, various approaches, from genetic to environmental and chemical, have been used to alleviate detrimental effects of ethylene on cut flowers and potted plants via inhibiting ethylene biosynthesis and perception, changing tissue response to ethylene, and avoiding exposure to exogenous ethylene (Serek and Reid, 1997; Saltveit, 1999; Reid and Jiang, 2012; Scariot et al., 2014).

In higher plants, the ethylene biosynthesis pathway has been well-studied and documented (Yang and Hoffman, 1984; Kende, 1993; Bleecker and Kende, 2000; Klee, 2004). Ethylene is formed from S-adenosylmethionine (SAM) by the action of two enzymes, ACC synthase (ACS) and ACC oxidase (ACO). ACS catalyses the conversion of SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase converts the ACC to ethylene (Yang and Hoffman, 1984; Kende, 1989). Ethylene is then sensed by cells

through a family of five membrane-localised receptor proteins in plants called ethylene receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) (Solano and Ecker, 1998; Chang and Shockey, 1999). These receptors are active in the absence of ethylene, upon ethylene binding these receptors are then inactivated, which activates the ethylene signalling pathway (Guo and Ecker, 2004). The signal transduction process is carried out by a series of proteins including CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1), EIN2 (ETHYLENE INSENSITIVE 2), EIN3 (ETHYLENE INSENSITIVE 3) (Chang and Shockey, 1999). Like receptors, CTR1 is inactivated in the presence of ethylene, CTR1 inactivation leads to the activation of EIN2, which is a transcriptional cascade activator and activates EIN3 and EIN3-like transcription factors, in turn, EIN3 regulates the expression of other transcription factors which ultimately, result in cellular, physiological, and metabolic responses (Chang and Shockey, 1999; Guo and Ecker, 2004; Stepanova and Alonso, 2005; Frankowski et al., 2007).

Low-temperature storage (<5 °C) is an effective and highly used environmental approach to maintaining quality and extending postharvest life (Lyons, 1973; Reid and Jiang, 2012). Low temperature reduces ethylene biosynthesis, but after removal of low temperature, an increase in the production of ethylene has often been observed in many chilling sensitive species (Sfakiotakis and Dilley, 1974; Wang and Adams, 1980; Etani and Yoshida, 1987; Larrigaudiere and Vendrell, 1993; Han and Miller, 2003). Many tropical and subtropical plants are chilling-sensitive and exhibit physiological dysfunctions in response to low but non-freezing temperatures (Lyons, 1973; Gross et al., 2002; Wang, 2006). Many cut lilies flowers do not show any increase in ethylene production during the normal senescence process or produce a low amount of ethylene which does not affect the senescence process (Han and Miller, 2003; Burchi et al., 2004). But exposure to low temperatures in lilies can cause adverse effects such as chilling injuries, changes in ethylene production, and

sensitivity which in turn result in early senescence (Han and Miller, 2003; Reid and Jiang, 2012). Hence, low-temperature storage can have detrimental effects on cut lily flowers by decreasing their postharvest life; but the extent of the injuries and biochemical and molecular processes that lead to them are not well understood in lilies.

Brassinosteroids are a group of plant growth regulators that have profound effects on reducing the impact of biotic and abiotic stresses in plants (Wang et al., 2012; Liu et al., 2016). BRs have received much attention as a new strategy for enhancing stress resistance and maintaining postharvest quality in other horticultural produce (Bajguz and Hayat, 2009; Zhu et al., 2013; Zhu et al., 2015). Their production changes in response to biotic and abiotic stresses, which can decrease chilling injuries and improve growth in plants (Hotta et al., 1998; Fujii and Saka, 2001). In maize (*Zea mays* L.) (He et al., 1991) and cucumber (*Cucumis sativus* L.) (Khripach et al., 1998), BR application resulted in more growth under chilling stress, and also decreased chilling injury in bananas (*Musa* spp.) (Bajguz and Hayat, 2009) and pepper fruit (*Capsicum annum* L.) (Wang et al., 2012). BRs maintain quality by affecting ethylene production (in some crops), chlorophyll degradation (Zhu et al., 2010; Wang et al., 2012; Gao et al., 2015; Gao et al., 2016; Liu et al., 2016). In Arabidopsis (*Arabidopsis thaliana* L.) BRs can affect ethylene biosynthesis through an interaction between BES1 or BZR1 (brassinosteroid-regulated transcription factor) and ACSs and the activity of *AtACS* promoters was strongly suppressed by the over-expression of both *BES1* and *BZR1* genes (Lv et al., 2018).

BRs could induce systemic tolerance to biotic and abiotic stresses such as low temperatures, therefore, under unfavourable conditions, plants might survive through BR-mediated anti-stress-responses. In cold-sensitive plants like lilies, BRs might decrease chilling injuries and delay senescence by reducing ethylene production as a result of alteration in its biosynthesis or sensitivity related genes' expression. But the effect of

BRs' on senescence in cut flowers has not been investigated in detail, particularly at the gene expression level. The current study aimed to determine the effects of cold storage and BRs treatment on lily flowers 'Premium Blonde' and 'Marlon' and investigate: i) ethylene production and its role in cold tolerance; ii) the expression pattern of ethylene biosynthesis genes and their relationship with ethylene production; iii) the expression pattern of ethylene perception and signalling pathway genes and their role in cold tolerance; iv) the expression pattern of the BRs-related transcription factor gene *LoBZR1* and its role in cold tolerance.

3.2. Materials and methods

3.2.1. Plant material and treatments

The cut lily (*Lilium orientalis*) cultivars 'Premium Blonde' and 'Marlon' were selected for this study as they were the two cultivars available on the market. We had three different treatments: control, cold storage, and cold storage + BR. The control treatment did not receive any Epibrassinolide (BR) or cold storage treatments and was placed in a ventilated room at 20 ± 2 °C, 58-65% RH, and 12 h Cool White fluorescent light of 20 μmol m^{-2} s^{-1} for postharvest quality assessments. An optimal concentration of 8.5 μM BR was selected based on our preliminary experiment in which various concentrations of BR from 1 to 15 μM were tested. For the cold storage + BR treatment, the stems were recut under distilled water to the desired height (45 cm) and were treated with 8.5 μM of BR for 12 h. Thereafter, they were individually placed in glass vials containing sterilised distilled water and cold-stored in an upright position at 5 °C in 12 h Cool White fluorescent light for one week (Fig. 6.1S). The cold storage treatment did not receive any BR but was also cold stored in an upright position at 5 °C in 12 h Cool White fluorescent light for one week. After cold storage, the stems of both treatments were placed in a ventilated room at 20 ±

2 °C, 58-65% RH, and 12 h Cool White fluorescent light of 20 µmol m^{-2} s^{-1} for postharvest quality assessments. In non-cold-stored flowers the day 0 refers to the day that cut stems were placed in individual vials for postharvest quality assessment, in cold-stored flowers the day 0 of the experiment was considered as the day that flowers were moved into the ventilated room after receiving cold storage treatment. Sterilised distilled water was used daily in order to top up the solution in each glass vial.

3.2.2. Characterization of display quality characteristics

Data on the time to bud opening, flower senescence, flower abscission, and leaf yellowing were collected daily at the same time. The time to bud opening, flower senescence, and flower abscission were determined in the two lowermost flowers separately from day 0 until each of those processes occurred. The time to leaf yellowing was determined in the two lowermost leaves separately. A bud was considered open when more than one tepal had moved laterally. Flowers were considered senescent when more than two tepals showed discoloration and desiccation in their tips. Flower abscission was defined by the fall of more than two tepals on a flower. Leaf yellowing was defined as the presence of yellowing in two lowermost leaves on a stem. The day 0 was defined as outlined in section 3.2.1.

3.2.3. Ethylene measurement

Ethylene production of flowers was measured every two days from day 0, as individual cut flowers were placed in 2 L air-tight jars for 24 hours at 20 ± 2 °C (Fig. 6.2S). Potassium hydroxide solution (20% w/v) was placed inside the jars to maintain low CO_2 concentrations from respiration during treatments. Gas samples were sampled through headspaces using a syringe and were measured using a gas chromatograph (GC)

(Shimadzu, Model GC-14B; column Poropak P set at 50 °C). The Flame Ionisation Detector was set at 200 °C and the injector temperature was 180 °C. The day 0 was defined as outlined in section 3.2.1.

3.2.4. RNA extraction and reverse transcription

Leaf samples of three biological replicates from each treatment were collected every two days from day 0. The samples were stored at −80 °C for future molecular analysis. Total RNA was extracted from leaf samples using the ISOLATE II RNA Plant Kit (Bioline Co.) according to the manufacturer's protocol. The quality and quantity of total RNA were evaluated by fractioning on 1% agarose gel stained with Ethidium Bromide and analysis by a DeNovix DS-11 Spectrophotometer, respectively. Genomic DNA was removed using DNase I (NEB Co.) according to the manufacturer's protocol. To eliminate residual DNase, 0.1 mM of EDTA (NEB Co.) was added to each RNA sample and incubated at 70 ºC for 10 min. RNA samples were stored at −80 °C for long-term storage until processed further. The first strands of cDNA were synthesised using the SensiFAST™ cDNA Synthesis Kit (Bioline Co.) according to the manufacturer's protocol. The day 0 was defined as outlined in section 3.2.1.

3.2.5. Partial isolation of ethylene biosynthesis, perception, and signalling, and brassinosteroid-regulated transcription factor genes and their sequence analysis

To isolate the partial sequences of *LoACO1*, *LoACS1*, *LoETR2*, *LoETR3*, *LoCTR1*, *LoEIN2*, *LoEIN3* and *LoBZR1*, primer pairs were designed for each gene using RNA-seq data in Geneious software (version R11.1) and used in subsequent PCR reactions. An aliquot of each PCR reaction was fractionated on an ethidium bromide-stained 1% (w/v)

agarose gel and the remaining was sequenced by the Australian Genome Research Facility (AGRF, Melbourne, Victoria, Australia). Then the sequences and corresponding amino acids data were compared with database sequences on GenBank using the National Centre for Biotechnology Information (NCBI) BLAST network server (http://www.ncbi.nlm.gov/BLAST). The sequences were aligned using CLUSTALW with default parameters in MEGA software (version 7). The aligned sequence outputs from CLUSTALW were subjected to dendrogram and bootstrap analysis (1000 replicates) using the Maximum likelihood method in MEGA software (version 7). The sequenced nucleotides were submitted to the GenBank under the accession numbers shown in Table 3.1. These sequences were used to design a new set of primers for *qPCR assays* using Geneious software (version R11.1). The new set of primers were tested in separate PCR reactions. An aliquot of each PCR reaction was loaded onto a 1% (w/v) agarose gel to confirm a single product is amplified in the reaction and the remaining was sequenced by AGRF.

Table 3.1. Gene-specific primer pairs used for qPCR.

Gene	Accession number	Primer pair	Sequence (5´-3´)
LoACS1	MN027512	Forward	CCTCCATCGTCTCATCGTCC
		Reverse	CACGGCACCGGAAATTCAAT
LoACO1	MN027510	Forward	GCAATAGAGCGTGTCCCTCC
		Reverse	ATAAGGGCTGCGTGTGAGAC
LoETR2	MN027509	Forward	GAGAGTGAAGGTGAGGGAGAG
		Reverse	AGCAAAGAATCAGGATTCAGCAAC
LoERS1	DQ408428.1	Forward	CTACCGCTGGGTCCTCATCC
		Reverse	TTTAGGAAGAGTTCGCGGGTCTTG
LoETR3	MN027511	Forward	GCTCCAGTTTCGAGTAGTCCA
		Reverse	ACAGAATTGCGTGGTGTGGA
LoCTR1	MN053311	Forward	TACGGATGGCGCTAGATGTG
		Reverse	GGAGCCATCCATTCTGGTGT
LoEIN2	MN053312	Forward	AGCTGATTGACCGAGTTGCT
		Reverse	TCCACAATTCCGCAACGAGA
LoEIN3	MN053313	Forward	CAGCTTAGCAGTGAGAACGGA
		Reverse	CACCAATCTGTGCTTCCACC
LoBZR1	MN654022	Forward	CGCCGACATTCAATCTCATT
		Reverse	CATCAACACCGAAATCATGG
LoGAPDH	MN053314	Forward	CAAGCTCAACGGAATTGCCC
		Reverse	CACCAGTGGCTCATCACACA

3.2.6. qPCR assays

To evaluate mRNA level expression, qPCR assays were performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Co.). The qPCR reaction mixture was made up to a volume of 20 µL containing 20 ng of cDNA template, 10 µl of ITAQ UNIVERSYBR GREEN SUPERMIX (Bio-Rad Co.), 300 nM forward primer, 300 nM reverse primer (Table 3.1.). After 3 min of incubation at 95 °C, the cDNA was amplified by 40 three-step cycles: 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. Specificity of the PCR amplifications was checked with a melting curve analysis (from 65 to 94 °C) following the final cycle. A series of concentrations (5, 10, 20, and 40 ng) of the cDNA template was tested to identify the optimal cDNA concentration that produced cycle threshold values between 18 and 30. Each specific gene expression was quantified by normalization to the housekeeping gene*, LoGAPDH*.

3.2.7. Statistical analysis

The experiment had a completely randomised design with ten biological replicates (stems/inflorescences) per treatment. The effect of treatments was analysed using the one-way Analysis of Variance (one-way ANOVA) using Genstat software (VSN International, version 18). Means of different treatments were compared using post hoc Tukey test ($P<0.05$). In each individual plant sample, the relative quantification of transcript abundance of target genes was determined by the $2^{-\Delta\Delta CT}$ method. Two technical and three biological replicates were used per treatment. Major changes of various genes relative to control were calculated for each replicate of each sample (Ahmadi et al. 2009; Daneshi Nergi and Ahmadi, 2014).

3.3. Results

3.3.1. Display quality characteristics

No significant differences (P>0.05) were observed on bud opening between treatments in both cultivars. In both cultivars, the shortest time to bud opening was observed in the cold storage treatment (Table 3.2.). Flower senescence was first observed in cold storage treatment followed by BR + cold storage treatment in both cultivars (Table 3.2.). The control flowers showed a longer time to flower senescence. The BR + cold storage treatment delayed the time to flower senescence in both cultivars. In 'Premium Blonde' treatments did not have any significant differences (P>0.05). In 'Marlon', cold storage treatment showed the shortest time to flower senescence and had a significant difference (P<0.05) with other treatments (Table 3.2.). Flower abscission was first observed in cold storage treatment in both cultivars. In both cultivars, BR + cold storage treatment delayed the effects of cold storage and resulted in a longer time to flower abscission (Table 3.2.). In 'Premium Blonde', there was not any significant difference (P>0.05) between control and BR + cold storage treatments but they had significant differences (P<0.05) with cold storage treatment (Table 3.2.). In 'Marlon' the BR + cold storage treatment delayed flower abscission and showed a significant difference (P<0.05) with control and cold storage treatments (Table 3.2.). Leaf yellowing was delayed in BR + cold storage treated flowers in both cultivars. The cold storage treatment resulted in a significantly earlier leaf yellowing which appeared after 2 days in both cultivars (Table 3.2.). The control treatment had the longest time to leaf yellowing (~7 days), which was significantly longer than other treatments in both cultivars. In both cultivars, BR + cold storage treatments showed a significantly delayed time to leaf yellowing compared to cold storage (Table 3.2.).

Table 3.2. Effects of cold storage (5ºC, one week) and BR (8.5 µM) on time to bud opening, flower senescence, flower abscission, and leaf yellowing in cut lilies 'Premium Blonde' (PB) and 'Marlon' (M). Values are the means (±SE) of ten replicates. Data were analysed using one-way analysis of variance (ANOVA), followed by post hoc Tukey test (P<0.05). Means in the same column that do not share a letter are significantly different.

Characteristic / *Cultivar* / *Treatment*	*Bud opening*		*Flower senescence*		*Flower abscission*		*Leaf yellowing*	
	PB	*M*	*PB*	*M*	*PB*	*M*	*PB*	*M*
Control	7.1 ± 0.14a	4.45 ± 0.11a	13.95 ± 0.40a	12.8 ± 0.07a	17.8 ± 0.43a	16.7 ± 0.08a	7.6 ± 0.26a	6.8 ± 0.09a
Cold storage	6.3 ± 0.04a	3.05 ± 0.07b	12.4 ± 0.04a	10.3 ± 0.06b	15.65 ± 0.10b	13.75 ± 0.06b	2.6 ± 0.05c	2.8 ± 0.13c
BR + Cold storage	6.75 ± 0.08a	3.45 ± 0.06b	14.1 ± 0.05a	11.6 ± 0.09a	17.55 ± 0.11a	17.25 ± 0.19a	4.9 ± 0.14b	5 ± 0.08b

3.3.2. Ethylene production

The level of ethylene production differed between both cultivars and 'Premium Blonde' produced less ethylene amount than 'Marlon' across all treatments (Fig 3.1.). In both cultivars, the control plants had an undetectable or very low amount of ethylene production for the first four days but then showed a consistent increasing trend until day eight in 'Premium Blonde' and day ten in 'Marlon', whereas all other treatment had higher ethylene production even after two days but did not show a consistent trend over time (Fig. 3.1.). Cold storage had the highest amount of ethylene production among all treatments in both cultivars. BR + cold storage treated flowers had less ethylene production than non-treated ones. Significant differences (P<0.05) were observed between treatments in both cultivars, but not on all days (Fig. 3.1.).

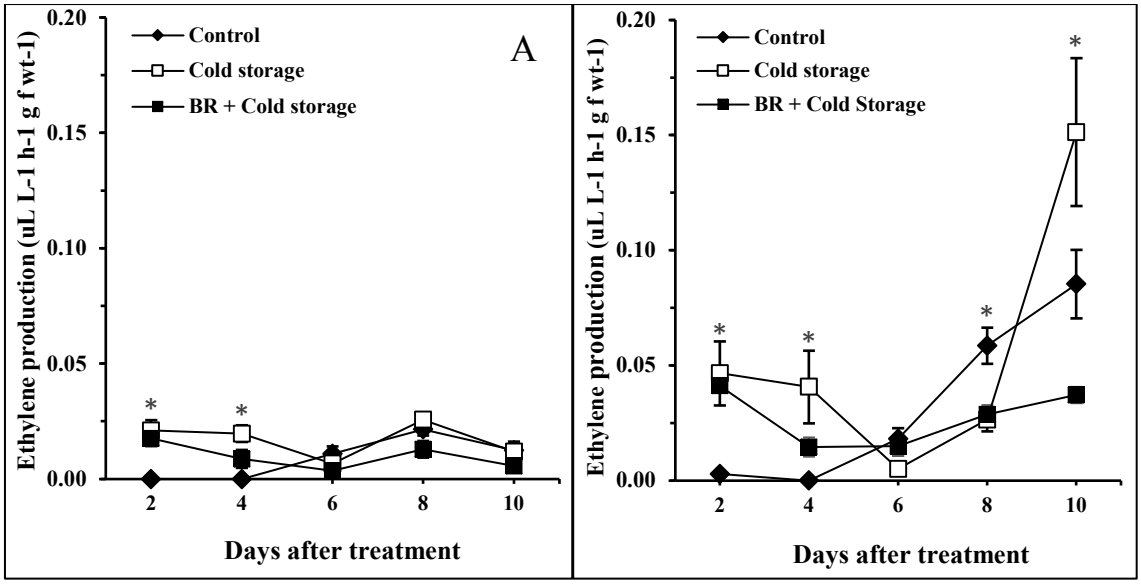


Figure 3.1. Effects of cold storage (5ºC, one week) and BR treatment (8.5 µM) on ethylene production in cut lily cultivars 'Premium Blonde' (A) and 'Marlon' (B). Values are the means (±SE) of five biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (*P*<0.05). The asterisk indicates a significant difference (*P*<0.05) between treatments.

3.3.3. Sequence analysis of the partial-length of the genes

We isolated the partial length cDNAs of *LoBZR1, LoACO1*, *LoACS1*, *LoETR2*, *LoETR3*, *LoCTR1*, *LoEIN2*, and *LoEIN3* genes from lily leaves with the lengths of 631, 606, 495, 626, 782, 716, 911, and 964 bp, respectively. The sequences were submitted to the Genbank under the accession numbers shown in Table 3.1. The blasting of the isolated sequences had similarities with corresponded genes from other species. The alignment results of the deduced amino acid sequences of the genes of interest are as follows: *LoBZR1* showed about 71% similarity to BZR1 from *Elaeis guineensis* (XP_010943157.1), *LoACO1* showed about 54% similarity to ACO1 from *Cinnamomum micranthum f. kanehirae* (accession No. RWR91650.1), *LoACS1* showed about 99% similarity to ACS from *Lilium regale* (accession No. ASV46318.1), *LoETR2* showed 72% similarity to ETR2 from *Elaeis guineensis* (accession No. XP_010918768.1), *LoETR3* showed about 79% similarity to ETR2 from *Phoenix dactylifera* (accession No.

XP_008801310.1) and ETR3 *Elaeis guineensis* (accession No. XP_010935768.1), *LoCTR1* showed about 87% similarity to CTR1 from *Elaeis guineensis* (accession No. XP_010914327.1), *LoEIN2* showed about 99% similarity to EIN2 from *Lilium regale* (accession No. ASV46340.1), and *LoEIN3* showed 99% similarity to EIN3 from *Lilium regale* (accession No. ASV46341.1).

Phylogenetic analyses revealed that *LoBZR1* is closely related to *Arachis ipaensis* (XP_016185891.1) (Fig. 6.3SA), *LoACO1* is closer to *Cinnamomum micranthum* f. kanehirae (accession No. RWR91650.1) and *Ziziphus jujuba* (accession No. XP_015887914.1) (Fig. 6.3SB), *LoACS1* is closer to *Nelumbo nucifera* (accession No. XP_010261352.1), *Narcissus tazetta* subsp. chinensis (accession No. ACZ54911.3), and *Pistacia vera* (accession No. XP_031249662.1) (Fig. 6.3SC), *LoETR2* is closer to *Zea mays* (accession No. AQK42858.1) *Sorghum bicolor* (accession No. XP_002446098.1) (Fig. 6.3SD), *LoETR3* is closer to (accession No. XP_020247532.1) (Fig. 6.3SE), *LoCTR1* is closer to *Solanum pennellii* (accession No. XP_027772422.1) (Fig. 6.3SF), *LoEIN2* is closely related to *Lilium regale* (accession No. ASV46340.1) (Fig. 6.3SG), and *LoEIN3* is closer to *Oryza sativa* Japonica Group (accession XP_015612094.1) (Fig. 6.3SH).

3.3.4. Expression of ethylene biosynthesis pathway genes

The expression of both *LoACO1* was always greater in cold storage plants compared to BR + cold storage treated ones (Fig. 3.2.). Significant differences ($P<0.05$) were observed between treatments in both cultivars particularly in 'Marlon' towards the end of the experiment (Fig. 3.2.). A similar trend was also observed for *LoACS1* with significant differences ($P<0.05$) between treatments in both cultivars, although not on all days particularly in 'Premium Blonde' (Fig. 3.2.).

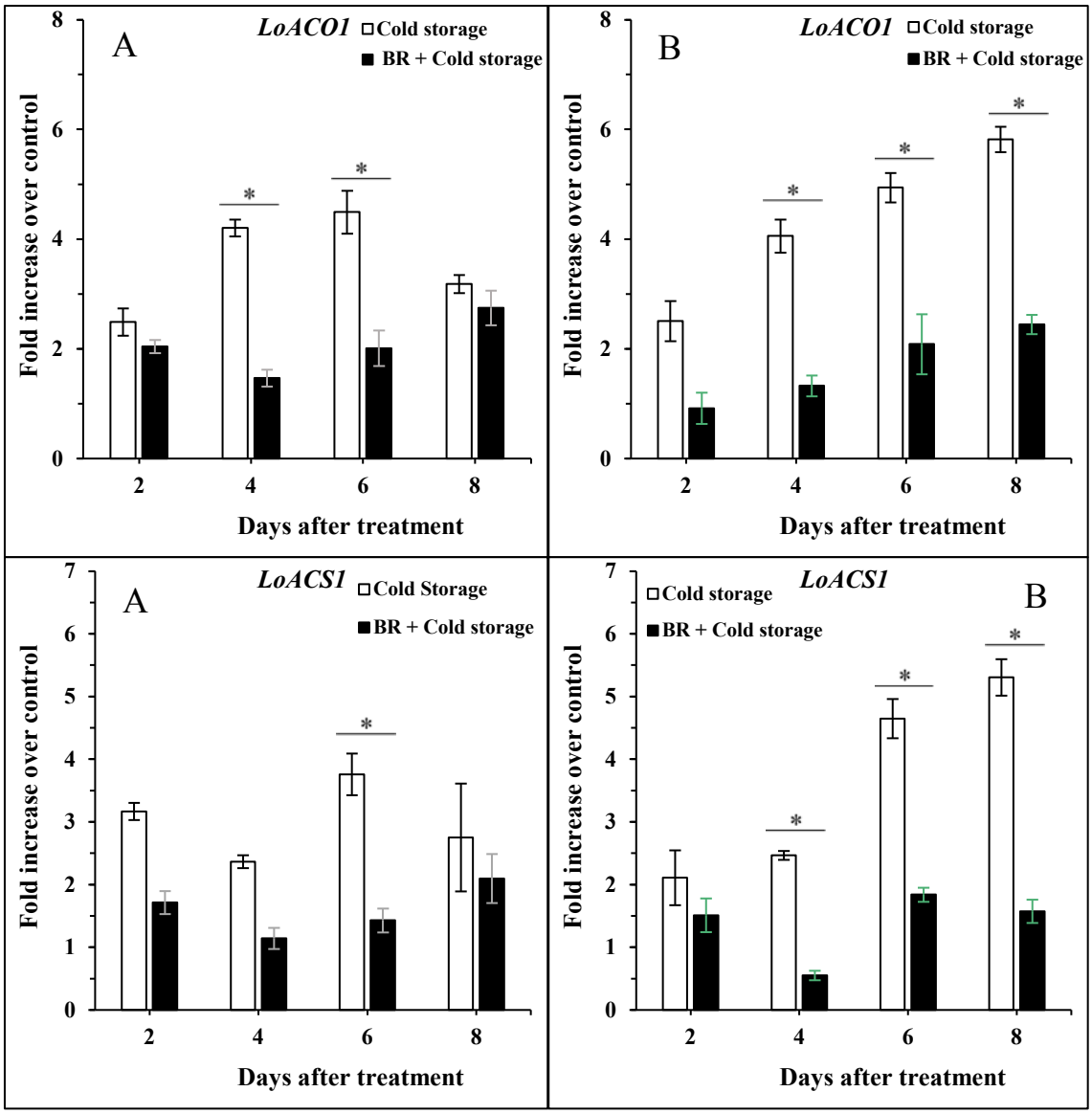
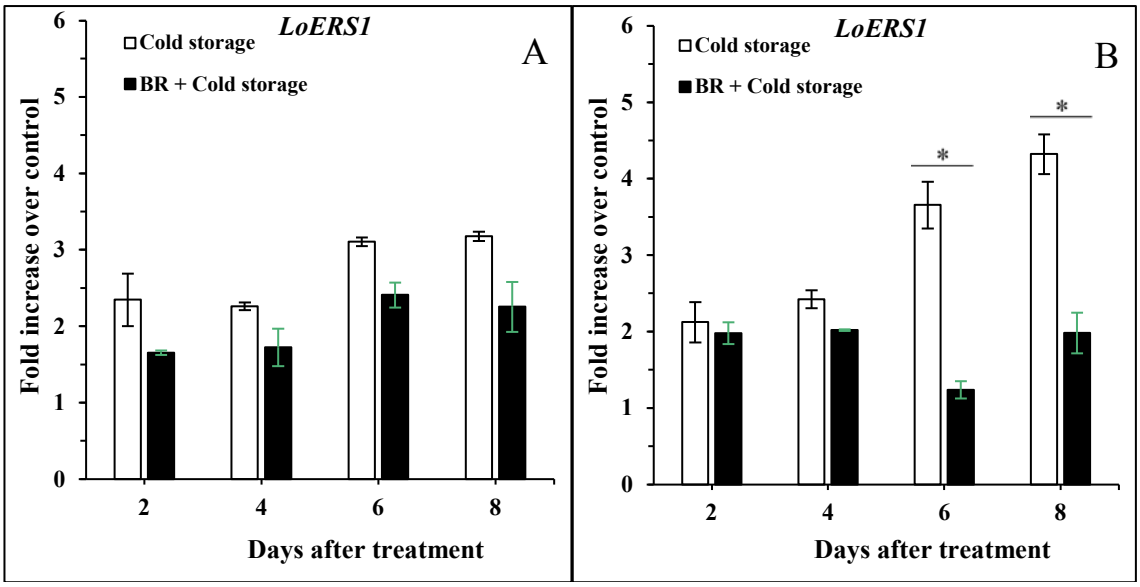


Figure 3.2. Effects of cold storage (5ºC, one week) and BR treatment (8.5 µM) on the expression of ethylene biosynthesis pathway genes (*ACO1* & *ACS1*) in cut lily 'Premium Blonde' (A) and 'Marlon' (B). The fold change expression of the genes was calculated relative to the untreated sample as control at the same time after normalization to the *LoGAPDH*, a housekeeping gene. The expression level in control samples is defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test ($P<0.05$). The asterisk indicates a significant difference ($P<0.05$) between treatments.

3.3.5. Expression of ethylene perception genes

Cold storage induced the expression of ethylene receptor (*LoERS1*, *LoETR2*, and *LoETR3*) genes in both cultivars, but BR decreased the expression of these genes (Fig. 3.3.). The expression of *LoERS1* was greater in cold storage plants compared to BR +

cold storage treated ones in both cultivars across all days, but significant differences (P<0.05) were only observed in 'Marlon' towards the end of the experiment. A similar trend was also observed for *LoETR2*, but here significant differences (P<0.05) were observed in both cultivars, although not on all days. The expression of *LoETR3* was always greater in cold storage treated plants than BR treated ones in 'Premium Blonde' with a consistent increasing trend. But in 'Marlon', it did not show a consistent increasing trend over time and after two days and at the end of the experiment, BR + cold storage treated plants had higher expression than cold storage treated ones (Fig. 3.3.). Significant differences (P<0.05) were observed between treatments on the expression of *LoETR3* in both cultivars but not on all days (Fig. 3.3.).



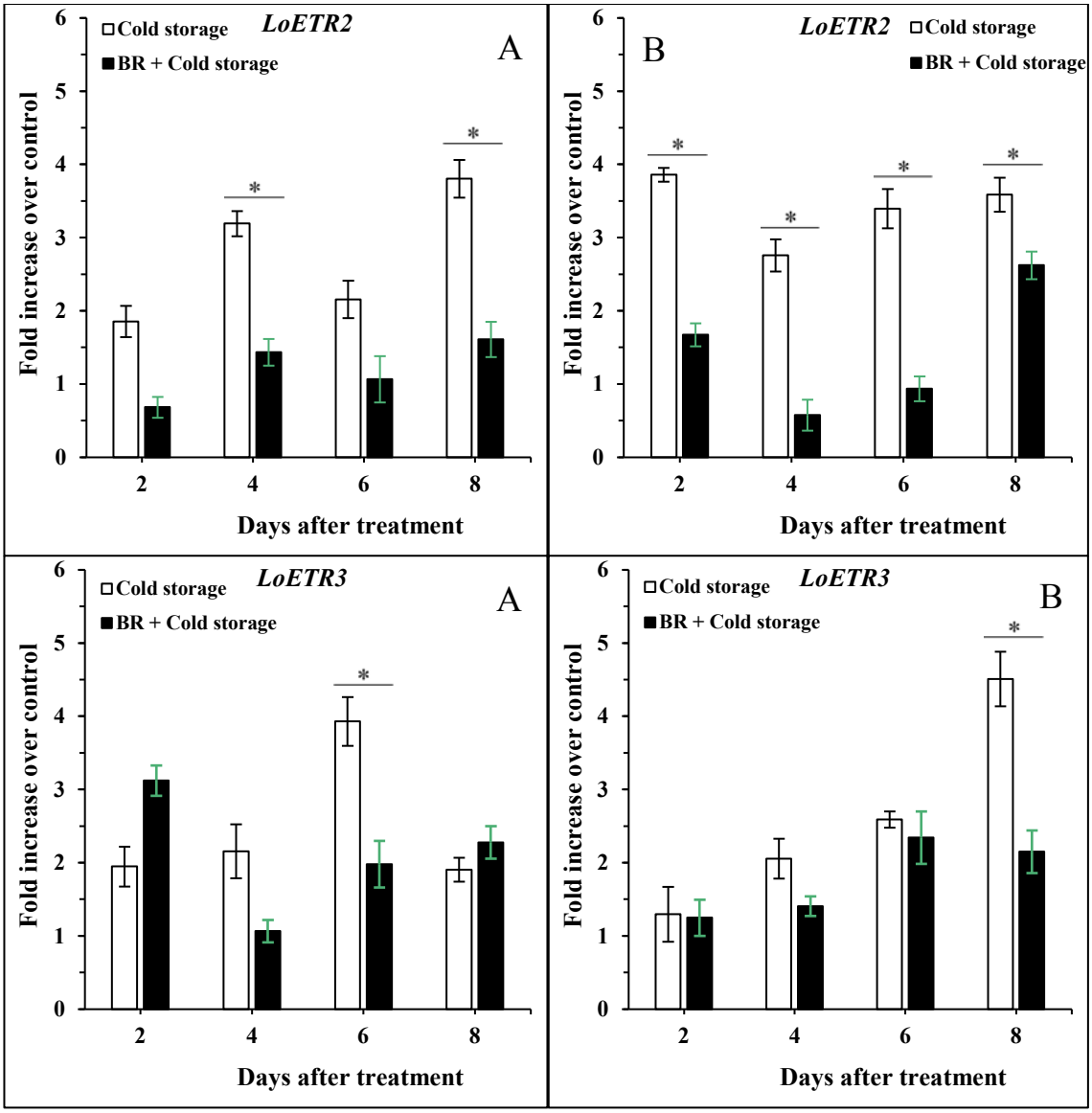
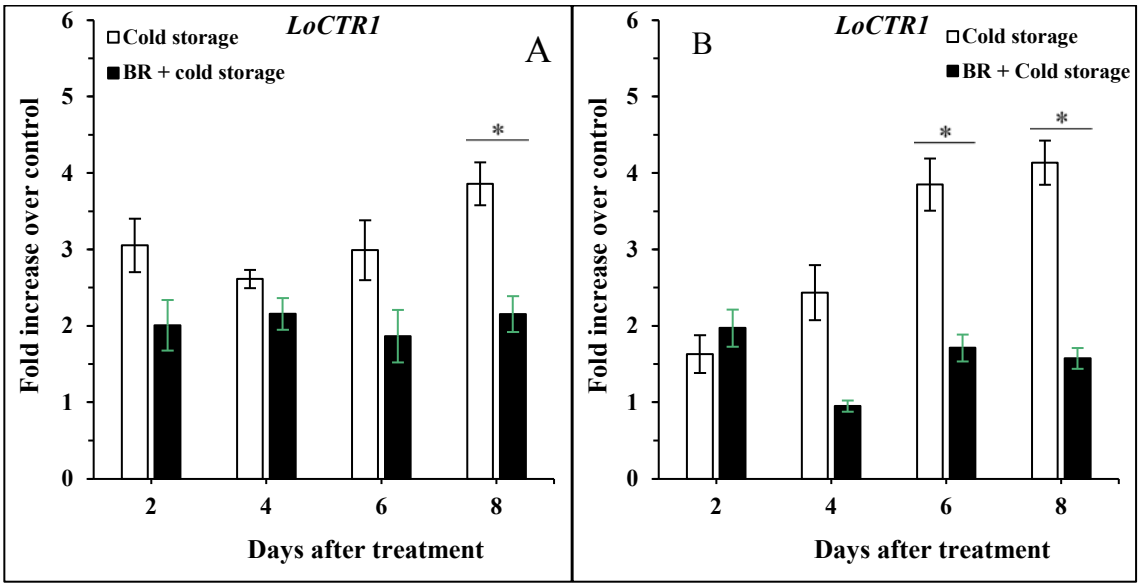


Figure 3.3. Effects cold storage (5ºC, one week) and BR treatment (8.5 µM) on the expression of ethylene perception genes in cut lily 'Premium Blonde' (A) and 'Marlon' (B). The fold change expression of the genes was calculated relative to the untreated sample as control at the same time after normalization to the *LoGAPDH*, a housekeeping gene. The expression level in control samples is defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (*P*<0.05). The asterisk indicates a significant difference (*P*<0.05) between treatments.

3.3.6. Expression of ethylene signal transduction pathway genes

Cold stored plants had a greater expression of signal transduction pathway genes in both cultivars (*LoCTR1*, *LoEIN2*, and *LoEIN3*), and BR + cold storage treated plants had a lower expression of these genes (Fig. 3.4.). The expression level of *LoCTR1* was greater in cold storage treated flowers than BR + cold storage treated ones on all days except for

day two in 'Marlon' (Fig. 3.4.). No consistent trend of expression was observed over time to both treatments in 'Premium Blonde' and BR treatment in 'Marlon' (Fig. 3.4.). In both cultivars, significant differences (P<0.05) were only observed towards the end of the experiment. Similarly, a higher expression of *LoEIN2* was observed in cold storage treated flowers compared to BR + cold storage treated ones particularly in the middle of the experiment with a consistent trend over the period of the experiment in both cultivars (Fig. 3.4.). But the response to BR + cold storage was not consistent over time in both cultivars (Fig. 3.4.). There were significant differences (P<0.05) between treatments, although not on all days in both cultivars (Fig. 3.4.). Cold storage treatment also caused a higher expression of *LoEIN3* compared to BR + cold storage treatment on all days in both cultivars with significant differences (P<0.05) between treatments on some days (Fig. 3.4.).



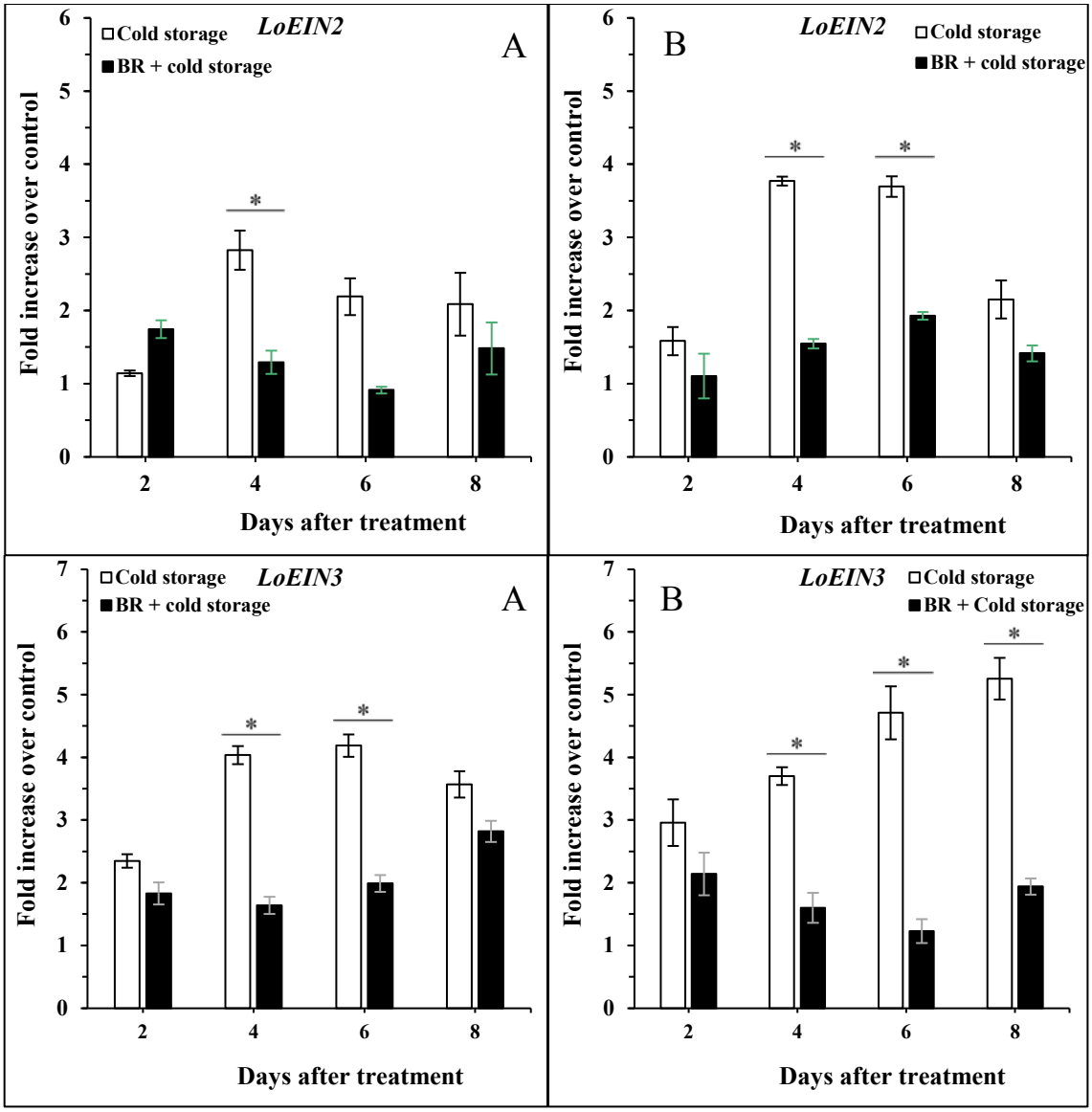


Figure 3.4. Effects cold storage (5ºC, one week) and BR treatment (8.5 µM) on the expression of ethylene signal transduction pathway genes in cut lily 'Premium Blonde' (A) and 'Marlon' (B). The fold change expression of the genes was calculated relative to the untreated sample as control at the same time after normalization to the *LoGAPDH*, a housekeeping gene. The expression level in control samples is defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (*P*<0.05). The asterisk indicates a significant difference (*P*<0.05) between treatments.

3.3.7. Expression of brassinosteroid-regulated transcription factor gene

Cold storage and BR + cold storage induced the expression of *LoBZR1* gene in both cultivars compared to the cold storage treatment (Fig 3.5.). In both cultivars, the expression was greater in BR + cold storage treated plants than cold storage treated ones with significant differences ($P<0.05$) between them, although not on all days.

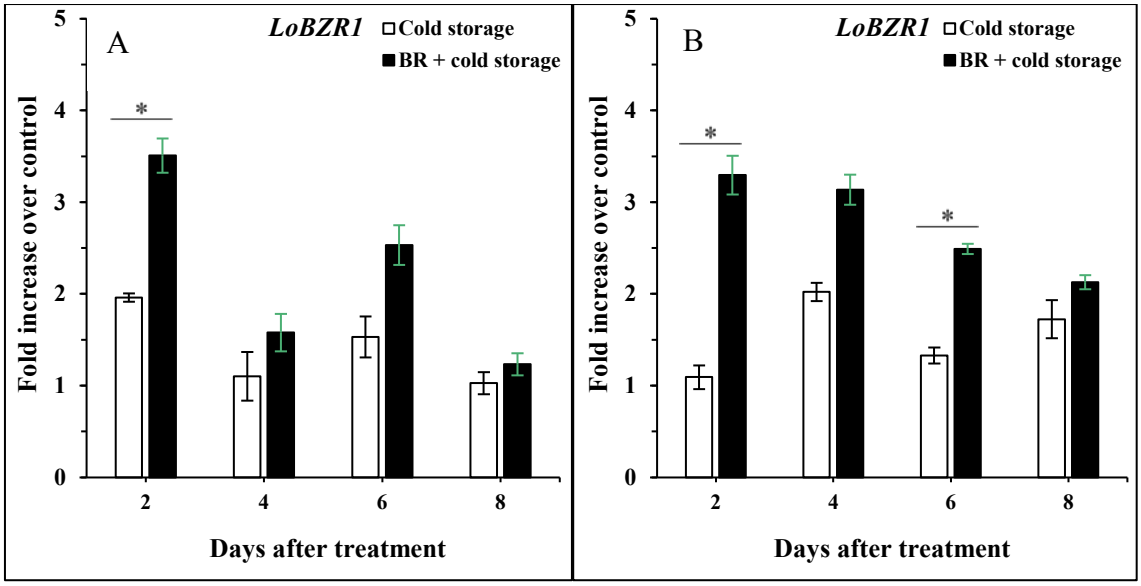


Figure 3.5. Effects cold storage (5ºC, one week) and BR treatment (8.5 µM) on the expression of *LoBZR1* gene in cut lily 'Premium Blonde' (A) and 'Marlon' (B). The fold change expression of the genes was calculated relative to the untreated sample as control at the same time after normalization to the *LoGAPDH*, a housekeeping gene. The expression level in control samples is defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (*P*<0.05). The asterisk indicates a significant difference (*P*<0.05) between treatments.

3.4. Discussion

This study aimed to evaluate the effects of cold storage and BR on postharvest quality, hormonal content and gene expression patterns during postharvest life to understand the role of ethylene, its related genes and *LoBZR1* in cold storage tolerance ('Premium Blonde' and 'Marlon').

To examine the gene expression pattern, we partially isolated two ethylene biosynthesis pathway genes (*LoACO1* and *LoACS1*) from cut lilies. *LoACO1* had a closer protein sequence to the stout camphor tree (*Cinnamomum micranthum f. kanehirae*) ACO1 (Chaw et al., 2019) and *LoACS1* had a closer protein sequence to the regal lily (*Lilium regale*) (https://www.ncbi.nlm.nih.gov/protein/ASV46318.1/) ACS1. Two ethylene receptor genes (*LoETR2* and *LoETR3*) were also partially isolated. *LoETR2* showed the highest protein sequence similarity to the oil palm (*Elaeis guineensis*)

(https://www.ncbi.nlm.nih.gov/protein/743776711) ETR2 and *LoETR3* had a closer protein similarity to the date palm (*Phoenix dactylifera*) (https://www.ncbi.nlm.nih.gov/protein/672162963) ETR2 and the oil palm (*Elaeis guineensis*) (https://www.ncbi.nlm.nih.gov/protein/743835280) ETR3. Moreover, three ethylene signalling genes (*LoCTR1*, *LoEIN2*, and *LoEIN3*) were partially isolated. *LoCTR1* showed a high protein sequence similarity to the oil palm (*Elaeis guineensis*) CTR1 (https://www.ncbi.nlm.nih.gov/protein/743768102) and one BR-related transcription factor gene (*LoBZR1*) were isolated. *LoEIN2* had a closer protein similarity to the regal lily (*Lilium regale*) EIN2 (https://www.ncbi.nlm.nih.gov/protein/1236612668) an *LoEIN3* showed had high protein similarity to the regal lily (*Lilium regale*) EIN3 (https://www.ncbi.nlm.nih.gov/protein/1236612670). We also partially isolated one BR-related transcription factor *LoBZR1* which had a high protein similarity to showed about 71% similarity to from the oil palm (*Elaeis guineensis*) BZR1 (https://www.ncbi.nlm.nih.gov/protein/XP_010943157.1/).

3.4.1. Ethylene biosynthesis and its role in cold tolerance

Our data showed that cold storage treated flowers had an increased ethylene biosynthesis (Fig. 3.1.) and an early deterioration of postharvest quality compared to the BR treated ones in both cultivars (Table 3.1.). These findings indicate that ethylene overproduction is a cold-storage related response and likely a key trigger in inducing early senescence in these lilies. Consistent with our findings, Han and Miller (2003) observed that following cold storage, cut lily 'Stargazer' had advanced flower opening and senescence which was reported to be due to higher ethylene production and sensitivity, but ethylene exposure did not advance leaf yellowing. Ethylene exposure was also reported to advance flower

senescence and abscission, and even leaf yellowing and abscission in other cut lilies (Elgar et al., 1999; Çelikel et al., 2002). Flower senescence could also be triggered by other factors. For instance, decline in the level of endogenous cytokinins and gibberellins (Fletcher et al., 1969; Lara et al., 2004), or sugar level of tissues (Dietrich et al., 2011; Garapati, et al., 2015). These factors may regulate senescence processes both ethylene dependently and independently. In ethylene dependent manner, they regulate the expression of ethylene related genes and therefore affect ethylene-induced senescence and abscission processes (Mor et al., 1983; Trivellini et al., 2015; Xu et al., 2019). For instance, in miniature potted rose (*Rosa hybrida* cv. Linda) cytokinins decreased ethylene production and responsiveness and delayed senescence (Zakizadeh et al., 2013). Gibberellins also suppressed ethylene production both stages in cut carnations (*Dianthus caryophyllus* L.) and delayed senescence (Saks and Van Staden, 1992; Saks and Van Staden, 1993). Or sugar deficiency leads to upregulation of region/leucine zippers (bZIPs) and no apical meristem/Arabidopsis transcription activation factor 1 (NAC2/ATAF1) transcription factors which are involved low energy responses (starvation) (Dietrich et al., 2011; Garapati, et al., 2015; Mair et al., 2015; Qi et al., 2015). The activation of these transcription factors leads to early senescence as bZIP1 and bZIP53 directly bind to the promotors of *PROLINE DEHYDROGENASE* (*ProDH*) and regulate its expression which is involved in proline catabolism (Dietrich et al., 2011). Higher level of ProDH activity leads to more proline catabolism and early senescence of petal and leaf tissues (Zhang and Becker, 2015). ATAF1 is a transcriptional regulator of a key enzyme of the biosynthesis of abscisic acid pathway *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*) (Jensen et al., 2013) which leads to increase in the level of abscisic acid by enhancing the expression of *NCED3* (Wu et al., 2009; Jensen et al., 2013). Moreover, it directly targets *ORESARA1* (*ORE1*) and *GOLDEN2-LIKE 1* (*GLK1*)

genes by increasing the expression of *ORE1* and decreasing the expression of *GLK1* (Garapati, et al., 2015). ORE1 which is a positive senescence-regulating transcription factor with ethylene and abscisic acid related elements (EIN3 and ABI5) regulate the expression of genes (*GLK1*, *SGR1*, and *NYC1*) related to chlorophyll degradation (Qiu et al., 2015; Liebsch and Keech, 2016) and ethylene biosynthesis genes (ACSs) (Qiu et al., 2015; Liebsch and Keech, 2016; Paik et al., 2017) which lead to early senescence. In ethylene independent manner, it could also cytokinins and gibberellins could delay senescence by preventing cholorophyll degradation, maintaining membrane integrity, and water uptake of tissues or reducing oxidative stress (Mayak and Halevy, 1974; Gulzar et al., 2003; Yu et al., 2009; Li et al., 2010; do Nascimento Simões et al., 2018). Sugar depletion also regulates the activity of basic-helix-loop-helix (MYC2) transcription factor which activates jasmonic acid-induced leaf senescence by binding to the promotor of *SENESCENCE-ASSOCIATED GENE29* (*SAG29*) and activating its expression (Qi et al., 2015). Prevention of chlorophyll degradation by cytokinins and gebberellins will in turn delay sugar depletion/starvation and sugar deficiency related signalling which will delay both ethylene-dependent or -independent senescence and abscission. Flower abscission might have also been ethylene mediated or it might have been caused by other factors. Abscission is more regulated by the auxin/ethylene ration however other factors including other plant growth regulators may accelerate or delay this process independently or through affecting the auxin/ethylene ratio (Cracker and Abeles, 1969; Leslie and Romani, 1986; Meir et al., 2015; Wilmowicz et al., 2016; Lv et al., 2018). Any decrease in the level of endogenous auxin due to either a normal developmental process or stress response enhances the ethylene sensitivity of the cells in the abscission zone which leads to abscission (Rungruchkanont et al., 2007; Estornell et al., 2013; Meir et al., 2015). In Arabidopsis (*Arabidopsis thaliana* L.), it was reported that abscisic acid increased

ethylene production and caused early abscission by activating calcium-dependent protein kinases (CDPKs) which in turn phosphorylates the N-termini of 1–aminocyclopropane-1-carboxylate synthases (ACS) which stabilized ACS (Luo et al., 2014). Or the expression of *AtACS* promoters was strongly suppressed by the over-expression of both *BES1* and *BZR1* (brassinosteroid-regulated transcription factors) genes which delays ethylene related effects (Lv et al., 2018). Cytokinins seems to delay abscission through a positive reationship between the expression of cytokinin oxidase/dehydrogenase (*CKX*) genes and ethylene-related genes where down regulation of *CKX3* led to decreased ethylene response and delayed abscission (Xu et al., 2019). But jasmonic acid plays a different role and induces abscission through an ethylene independent manner which was shown by treating the *ein2-1* ethylene insensitive mutant with jasmonic acid where it accelerated organ abscission which indicate (Kim et al., 2013). External factors might have also triggered abscission particularly darkness in 'Sorbonne' and 'Stargazer' and sugar depletion in all cultivars which in turn is caused by darkness through chlorophyll degradation as source of energy production (Liebsch and Keech, 2016). Sugar depletion may also be the reason of abscission through as sugar starvation activates ORE1 through sugar related signalling which in turn regulates the components of both ethylene and abscisic acid signalling and biosynthesis pathways (Khan et al., 2014; Jeong et al., 2016; Liebsch and Keech, 2016; Paik et al., 2017). Taken together, these data indicate that ethylene overproduction in cold-stored flowers not only is a normal response of cut lilies to stress condition but depending on cultivar, early senescence and abscission might be partially or fully ethylene mediated or may not be ethylene mediated but could be triggered by other factors which needs to be further investigated using ethylene and anti-ethylene agents.

3.4.2. Expression pattern of ethylene biosynthesis genes and their relationship with ethylene production

Like the changes in ethylene production, the expression of ethylene biosynthesis genes (*LoACO1* and *LoACS1*) increased by cold storage and was suppressed by BR + cold storage treatments (Fig. 3.2.). These results indicate that ethylene production may directly related to the expression of the biosynthesis genes as we observed a higher expression of these genes and higher ethylene production and vice versa particularly in 'Marlon'. This was also reported for *ACSs* octuple Arabidopsis (*Arabidopsis thaliana* L.) mutant and *ACO1* antisense tomato (*Lecopersicum esculentum* Mill) plants as they produced less ethylene and significantly delayed leaf yellowing (John et al., 1995; Tsuchisaka et al., 2009). These results, combined with our finding concerning the ethylene overproduction, suggest that the expression of these genes increased in response to cold storage in lilies and higher ethylene production due to greater expression of biosynthesis genes which ultimately, due to negative role of ethylene in flower senescence process, leads to early senescence. However, there was an exception in 'Premium Blonde' where the changes at ethylene production level between treatments were greater than the changes in gene expression level particularly for *LoACS1* (Fig. 3.2.).

3.4.3. Expression pattern of ethylene perception and signalling pathway genes and their role in cold tolerance

The expression of ethylene receptors (*LoERS1*, *LoETR2,* and *LoETR3*), and signalling pathway (*LoCTR1*, *LoEIN2,* and *LoEIN3*) increased by cold storage and was suppressed by BR + cold storage treatments (Fig. 3.3 & 4). The changes in the expression of the receptor genes may also account for the changes in the response of lilies to cold storage

and BR + cold storage. The in the expression of the receptor genes increase could also be a response to higher ethylene production after cold storage. A greater ethylene exposure results in the occupation of existing receptors on cell membranes by ethylene, which triggers the expression of the receptors repress ethylene signalling and prevent an excessive response to ethylene (Hua and Meyerowitz, 1998). Therefore, Given the negative role of the receptors in ethylene signalling pathway and by comparing the level of ethylene biosynthesis (Fig. 3.1.), chilling injuries (Table 3.1.) and the level of the expression of the genes (Fig. 3.3.) under the treatments, it is more likely that this increase is a preventive response to higher amounts. This was confirmed in gain-of-function ethylene receptor mutants (*etr1-1* and *ein4-1*) of Arabidopsis (*Arabidopsis thaliana* L.) which exhibited increased freezing tolerance (Shi et al., 2012). Like receptors, the *LoCTR1* overexpressed under cold storage but repressed under BR (Fig. 3.4.). CTR1 is a negative regulator of the ethylene signal transduction pathway and the presence of ethylene inactivates CTR1 and activates response pathways downstream from CTR1 (Kieber et al., 1993; Arora, 2005). Thus, the induction in the expression of *LoCTR1* in cold-stored flowers might be a response to higher ethylene production as a result of chilling injuries and a damping mechanism to temper the increase in ethylene biosynthesis and finally delay senescence in lilies. This was also reported in *ctr1-1* mutant Arabidopsis which exhibited reduced freezing tolerance and CTR1 was considered as a positive regulator in freezing tolerance (Shi et al., 2012). Therefore, these data indicate that *LoCTR1* might increase cold storage tolerance in cut lilies possibly by repressing the ethylene signalling pathway. Adversely, given the positive role of EIN2 and EIN3 in the ethylene signalling pathway, the higher expression of *LoEIN2* and *LoEIN3* genes might have caused more sensitivity to low temperatures and early senescence in cold-stored lilies. As in Arabidopsis, cold stress resulted in the overexpression of *AtEIN3* and

accumulation of EIN3 which caused a reduction in freezing tolerance possibly through binding to specific elements in cold tolerance-related genes' promoters and repressing their expression (Shi et al., 2012). Whereas, the mutants of both *EIN2* and *EIN3* genes had a stay-green phenotype during leaf senescence (Chao et al., 1997; Oh et al., 1997). Moreover, Therefore, it seems that these genes play a negative regulatory role in cold response in cut lilies.

3.4.4. Expression pattern of the BR related transcription factor gene LoBZR1 and its role in cold tolerance

The expression of *LoBZR1* in the cold storage treatment was low compared to the BR + cold storage treatment (Fig. 3.5.). This indicates that BR might have altered ethylene biosynthesis and the related gene expression through increasing the expression of *LoBZR1* in cut lilies. This was reported in Arabidopsis (*Arabidopsis thaliana* L.) where a direct interaction between BES1 or BZR1 and ACSs (1-aminocyclopropane-1-carboxylic acid synthases enzymes) under low concentrations of BRs was observed (Lv et al., 2018). There the activity of *AtACS* promoters was strongly suppressed by the over-expression of both *BES1* and *BZR1* genes. But it is possible that BRs might have indirectly affected the expression of ethylene related genes by regulating the expression of other plant growth regulators which repress the expression of ethylene related genes such cytokinins and gibberellins (Unterholzner et al., 2015; Kudryakova et al., 2013). For instance, in Arabidopsis (*Arabidopsis thaliana* L.) it was shown that by BRs increased gibberellic acid biosynthesis through binding of BES1/BZR1 to a the promoters of *GA20ox1* and *GA3ox1* genes and increases their expression which are gibberellic acid biosynthesis genes (Unterholzner et al., 2015). In Arabidopsis (*Arabidopsis thaliana* L.), it was also reported that BRs also regulated the expression of the genes

(*AHK2* and *AHK3*) related to cytokinins signalling pathway and increased cytokinins level (Kudryakova et al., 2013). It seems that there is a negative relationship between *LoBZR1* and ethylene related genes either directly or indirect through other plant growth regulators but a positive relationship between the expression of *LoBZR1* and lily response to low temperatures. Thus, it is possible that BR might have reduced cold storage effects through affecting the expression of ethylene biosynthesis genes and reducing ethylene production, and as a result of that had less receptor and other gene expressions. But there is another possibility that BR might have first decreased the expression of ethylene receptors and thus resulted in less available ethylene binding sites, and finally less ethylene sensing and related physiological responses which needs to be further investigated.

Taken together, the data presented here show cold storage induced chilling injuries possibly ethylene mediated in cut lilies but it could have been triggered by other factors such as other plant growth regulators or sugar depletion. If ethylene mediated, it could be due to either higher ethylene biosynthesis or higher ethylene sensitivity or a combination of both as a result of alteration in the expression of the related genes. In this case, the effect of BR could be due to suppressing the over-expression of ethylene-related genes and reducing ethylene biosynthesis and possibly ethylene sensitivity both directly or indirectly through other mechanisms. But this needs to be further investigated using ethylene and anti-ethylene agents to clarify the role of ethylene in quality loss following cold storage in lilies at physiological and molecular levels.

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Chapter 4

Ethylene Biosynthesis and Sensitivity Before and After Cold Stress in Cut Lily 'Marlon'

Abstract

Fresh flowers of cut lily plants often have a long postharvest life and produce undetectable levels of ethylene. This changes after cold storage, when lilies produce a higher amount of ethylene with a very early leaf and then flower senescence. It is likely that changes to postharvest physiology are ethylene-dependent, either due to a higher level of ethylene production or a change in ethylene sensitivity. Such changes could be due to alteration in the expression of ethylene biosynthesis, perception, and signalling genes. Therefore, we investigated the transcriptional regulation of ethylene biosynthesis, perception, and signalling pathways in response to cold storage in the cut lily cultivar 'Marlon'. Cut lily 'Marlon' stems were subjected to three treatments: 1) 1-Methylcyclopropene (1-MCP; an ethylene action inhibitor) (10 nL L^{-1}), 2) ethylene (10 µL L^{-1}), and 3) 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}) for 12 h before and after cold storing (5 °C and 12 h Cool White fluorescent light for one week). Control plants were not cold-stored and did not receive 1-MCP or ethylene. We measured the time to bud opening, flower senescence and abscission and leaf senescence, ethylene production, and the expression of its biosynthesis (*LoACO1* and *LoACS1*), perception (*LoERS1, LoETR2,* and *LoETR3*), and signalling (*LoCTR1*, *LoEIN2*, and *LoEIN3*) genes. In non-cold-stored plants, ethylene and 1-MCP treatments had no effects on postharvest quality, ethylene production, and the expression pattern of the investigated genes. Cold storage led to much earlier leaf yellowing (from 8 to 4 days), and while bud opening was not affected, flowers senesced

and abscised 3 days earlier. In cold-stored plants, ethylene and 1-MCP had no effects on bud opening and leaf yellowing but flower senescence and abscission were hastened by ethylene and delayed by 1-MCP. Similarly, the expression of all the genes examined and ethylene production were significantly increased by ethylene but significantly decreased by 1-MCP in cold-stored plants, although not at all time points measured. In cold-stored plants, 1-MCP treatment significantly delayed flower senescence and decreased the expression of some ethylene related genes on some days. These findings indicate that there is no sensitivity to ethylene in fresh-cut lily 'Marlon'. But the response significantly differs following cold storage, in a tissue-dependant manner as flower senescence and abscission were delayed by 1-MCP but leaf senescence was not. Hence, ethylene does influence flower senescence following cold storage but leaf senescence is under the control of other factors. Our results indicate that treatments that focus on ethylene blockers to prolong postharvest life in lilies will be effective for flowers but not leaves.

4.1. Introduction

As a simple gaseous plant hormone, ethylene is endogenously produced by almost all plants, including cut flowers, and is involved in many physiological processes from abscission and ripening to senescence and responses to stresses (Watada, 1986; Abeles et al., 1992; Lacey and Binder, 2014). Most lilies produce a low or undetectable amount of ethylene during the senescence process and show no or little sensitivity to exogenous ethylene (Woltering and Van Doorn, 1988; Elgar et al., 1999; van Doorn, 2001; Han and Miller, 2003). But if cold-stored, like other species sensitive to low temperature, lilies show physiological and biochemical changes (Han and Miller, 2003; Song and Peng, 2004; van Doorn and Han, 2011; Choi et al., 2014).

Low temperature reduces ethylene biosynthesis, but after removal of low temperature, an increase in the production of ethylene has been observed in many chilling sensitive species (Sfakiotakis and Dilley, 1974; Wang and Adams, 1980; Etani and Yoshida, 1987; Larrigaudiere and Vendrell, 1993; Han and Miller, 2003). Exposure to low temperatures causes adverse effects such as shorter postharvest life (early leaf yellowing and flower senescence), changes in ethylene production, and sensitivity in lilies (Han and Miller, 2003; Reid and Jiang, 2012). The effects of low temperature on ethylene production and sensitivity in cut lilies vary within and between species (Han and Miller, 2003; Burchi et al., 2004; Song and Peng, 2004). For example, Han and Miller (2003) observed that non-stored cut oriental lily 'Stargazer' produced no/undetectable amount of ethylene during flower senescence and showed no sensitivity to exogenous ethylene, but after cold-storing, they produced a high amount of ethylene and became highly sensitive to exogenous ethylene. Similarly, an increase in ethylene production was observed in cut Asiatic lilies 'Elite' after storage, whereas no clear increase in ethylene production was observed in cold-stored Asiatic lilies 'Prato' (Burchi et al., 2004). In our previous experiment, we also observed a low amount of ethylene production in non-cold-stored lilies but a higher amount of ethylene in cold-stored cut lilies 'Sorbonne', 'Stargazer', 'Marlon' and 'Premium blonde' (unpublished data). In contrast, Song and Peng (2004) reported that cut Asiatic hybrid lily 'Brussels' has a high sensitivity to exogenous ethylene before cold storage but a low sensitivity after cold storage.

The cold storage-induced changes in ethylene production and sensitivity could be due to alteration in the expression of ethylene related genes (biosynthesis, perception, and signalling). For instance, in tomato (*Solanum lycopersicum* L.), pear (*Pyrus communis* L.), and grapefruit (*Citrus paradisi*), cold storage increased the expression of ethylene biosynthesis genes (*ACSs* and *ACOs*) which led to the accumulation of ethylene

biosynthesis pathway enzymes (ACC synthase and ACC oxidase) and finally higher ethylene production (Lelievre et al., 1997; Zhao et al., 2013; Lado et al., 2014). Changes in the expression of receptors (*ETRs* and *ERSs*) may also account for the changes in ethylene response as their expression levels are differently regulated in response to external stimuli (Klee, 2002), which affects the presence/absence of ethylene receptors and ethylene response. Depending on species, cold storage can up- or down-regulate the expression of ethylene receptor genes (El-Sharkawy et al., 2003) Lado et al., 2014; (Zou et al., 2014). There is an inverse correlation between the level of ethylene receptors and ethylene sensitivity as a higher level of the receptors will result in lower sensitivity to ethylene, and vice versa (Klee, 2002). Ethylene response might also be due to changes in the ethylene signal transduction pathway (Müller et al., 2000), suggesting that the expression of genes (*CTRs* and *EINs*) involved in this pathway is the major target of regulation. Alterations in the expression of these genes affect ethylene response as *ctr1* mutant Arabidopsis plants showed constitutive expression of ethylene-regulated genes (Kieber et al., 1993) and loss-of-function mutations in *EIN2* led to ethylene insensitivity indicating EIN2 as a key regulator in ethylene responses (Guzman and Ecker, 1990). Similarly, *ein3* mutants in Arabidopsis showed a loss of ethylene-mediated effects from gene expression to senescence (Chao et al., 1997). It has been reported that cold storage results in the accumulation of EIN3 protein in an EIN2-dependent manner (Potuschak et al. 2003; Shi et al. 2012). In papaya (*Carica papaya* L.) and peach fruit (*Prunus persica* L. Batsch) the expression of *EIN2* and *EIN3* genes is up-regulated in response to low temperatures (Begheldo et al., 2008; Zou et al., 2014). The overexpression of *EIN2* and *EIN3* leads to constitutive ethylene responses (Klee, 2004). Although much progress has been made in understanding the role of ethylene in

cut lilies, no information on the expression profiles of ethylene biosynthesis, perception and signalling genes in response to low-temperature storage in cut lilies is available.

1-Methylcyclopropene (1-MCP) is a competitive inhibitor for ethylene receptors which has enabled scientists to understand the role of ethylene in plants (Blankenship and Dole, 2003). It has a high affinity to permanently bind to ethylene receptors and prevents the perception of ethylene by its receptors, consequently suppressing ethylene response (Sisler and Serek, 1997; Serek et al., 2006). 1-MCP by itself or in combination with other growth regulators has been effective in reducing ethylene production and prolonging postharvest life in cut lilies and other plants (Çelikel et al., 2002; Han and Miller, 2003; Wei et al., 2018), albeit sometimes in a tissue-specific manner (Han and Miller, 2003).

The current experiment aimed to investigate the expression pattern of ethylene biosynthesis, receptor, and signal transduction pathway genes during senescence with and without cold storage and in response to exogenous ethylene and 1-MCP in cut lily 'Marlon'. More specifically, we aimed to understand: i) the role of ethylene in senescence in cold-stored and non-stored plants; ii) the expression pattern of ethylene perception genes and their role in ethylene response in cold-stored and non-stored plants; iii) the expression pattern of ethylene signalling genes and their role in ethylene response in cold-stored and non-cold-stored plants; and iv) the effect of exogenous ethylene on endogenous ethylene biosynthesis and its biosynthesis-related gene expression in cold-stored and non-cold-stored plants.

4.2. Materials and Methods

4.2.1. Plant material and treatments application

We sourced cut lily flowers (*Lilium orientalis*) 'Marlon' at a commercial lily greenhouse in Melbourne. The stems were recut under distilled water to the desired height of 45 cm.

We had two storage treatments where half of the plants were immediately (without storing) placed in individual vials containing sterilised distilled water into gas-tight Perspex tubes sealed with water and subjected randomly to the following four treatments for 12 h: 1) control (no 1-MCP and ethylene), 2) 1-MCP (10 nL L^{-1}), 3) ethylene (10 µL L^{-1}), 4) 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}). The experiment was conducted at 20 ± 2 °C, 58-65% RH and 20 µmol m^{-2} $s^{-}1$ using Cool White fluorescent light. 1-MCP was prepared according to the manufacturer, AgroFresh. Ethylene was injected into the tubs using a gas tight syringe through the headspace. The tubs were subsequently vented outside the treatment room to avoid any cross-contamination between treatments. Potassium hydroxide solution (20% w/v) was placed inside the tubs to maintain low CO_2 concentrations from respiration during treatments. Next, the treated plants were kept in a ventilated room at 20 ± 2 °C, 58-65% RH and 20 µmol m^{-2} $s^{-}1$ using Cool White fluorescent light for 12 h for about sixteen days for postharvest quality assessment. The day 0 of the experiment was considered as the day that flowers were kept in the ventilated room after receiving ethylene and 1-MCP treatments. Sterilised distilled water was used daily in order to top up the solution in each glass vial.

The other half of the plants were individually placed in glass vials containing sterilised distilled water and cold-stored in an upright position at 5 °C under 12 h light (Cool White fluorescent) and 12 h darkness (LD 12:12) for one week (Fig. 6.1S). Next, the stems were placed into gas-tight Perspex tubes sealed with water and subjected randomly to the same treatments as above following the same procedure. Finally, postharvest quality assessment was carried out in a ventilated room at 20 ± 2 °C, 58-65% RH and 20 µmol m^{-2} $s^{-}1$ using Cool White fluorescent light for 12 h for 10 days. The preparation of 1-MCP was carried out according to the manufacturer's protocol, AgroFresh. A 100

ml potassium hydroxide solution (20% w/v) was placed inside the tubs to maintain low CO_2 concentrations from respiration during treatments. The day 0 of the experiment was considered as the day that flowers were kept the ventilated room after receiving cold storage treatment and then ethylene and 1-MCP treatments. Sterilised distilled water was used daily in order to top up the solution in each glass vial.

4.2.2. Characterization of display quality characteristics

Data on the time to bud opening, flower senescence, flower abscission, and leaf yellowing were collected daily at the same time. The time to bud opening, flower senescence, and flower abscission were determined in the two lowermost flowers separately from day 0 until each of those processes occurred. The time to leaf yellowing was determined in the two lowermost leaves separately. A bud was considered open when more than one tepal had moved laterally. Flowers were considered senescent when more than two tepals showed discoloration and desiccation in their tips. Flower abscission was defined by the fall of more than two tepals on a flower. Leaf yellowing was defined as the presence of yellowing in two lowermost leaves on a stem. The day 0 was defined as outlined in section 4.2.1.

4.2.3. Ethylene measurement

Ethylene production of flowers was measured every two days from day 0 until day ten, as individual cut flowers were placed in 2 L air-tight jars for 24 hours at 20 ± 2 °C (Fig. 6.2S). Potassium hydroxide solution (20% w/v) was placed inside the jars to maintain low CO_2 concentrations from respiration during treatments. Gas samples were taken from the headspace using a syringe and were measured by injecting in a gas chromatograph (GC) (Shimadzu, Model GC-14B; column Poropak P set at 50 °C). The Flame Ionisation

Detector was set at 200 °C and the injector temperature was 180 °C. The day 0 was defined as outlined in section 4.2.1.

4.2.4. RNA extraction and reverse transcription

Leaf samples of three biological replicates from each treatment were collected every two from day 0 days until day eight. The samples were stored at −80 °C for future molecular analysis. Total RNA was extracted from leaf samples using the ISOLATE II RNA Plant Kit (Bioline Co.) according to the manufacturer's protocol. The quality and quantity of total RNA were evaluated by fractioning on 1% agarose gel stained with Ethidium Bromide and analysis by a DeNovix DS-11 Spectrophotometer, respectively. Genomic DNA was removed using DNase I (NEB Co.) according to the manufacturer's protocol. To eliminate residual DNase, 0.1 mM of EDTA (NEB Co.) was added to each RNA sample and incubated at 70 ºC for 10 min. RNA samples were stored at −80 °C for long-term storage until processed further. First-strand cDNA was synthesised using the SensiFAST™ cDNA Synthesis Kit (Bioline Co.) according to the manufacturer's protocol. The day 0 was defined as outlined in section 4.2.1.

4.2.5. qPCR assays

To evaluate mRNA level expression, qPCR assays were performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Co.). The PCR reaction mixture was made up to a volume of 20 µL containing 20 ng of cDNA template, 10 µl of ITAQ UNIVERSYBR GREEN SUPERMIX (Bio-Rad Co.), 20 nM forward primer, 20 nM reverse primer (Table 4.1.). After 3 min of incubation at 95 °C, the cDNA was amplified by 40 three-step cycles: 30 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. Specificity of the PCR amplifications was checked with a melting curve analysis (from 65 to 94 °C)

following completion of the final cycle. Each specific gene expression was quantified by normalization to the housekeeping gene*, LoGAPDH*.

Table 4.1. Gene-specific primer pairs used for qPCR.

Gene	Accession number	Primer pair	Sequence (5´-3´)
LoACS1	MN027512	Forward	CCTCCATCGTCTCATCGTCC
		Reverse	CACGGCACCGGAAATTCAAT
LoACO1	MN027510	Forward	GCAATAGAGCGTGTCCCTCC
		Reverse	ATAAGGGCTGCGTGTGAGAC
LoETR2	MN027509	Forward	GAGAGTGAAGGTGAGGGAGAG
		Reverse	AGCAAAGAATCAGGATTCAGCAAC
LoERS1	DQ408428.1	Forward	CTACCGCTGGGTCCTCATCC
		Reverse	TTTAGGAAGAGTTCGCGGGTCTTG
LoETR3	MN027511	Forward	GCTCCAGTTTCGAGTAGTCCA
		Reverse	ACAGAATTGCGTGGTGTGGA
LoCTR1	MN053311	Forward	TACGGATGGCGCTAGATGTG
		Reverse	GGAGCCATCCATTCTGGTGT
LoEIN2	MN053312	Forward	AGCTGATTGACCGAGTTGCT
		Reverse	TCCACAATTCCGCAACGAGA
LoEIN3	MN053313	Forward	CAGCTTAGCAGTGAGAACGGA
		Reverse	CACCAATCTGTGCTTCCACC
LoGAPDH	MN053314	Forward	CAAGCTCAACGGAATTGCCC
		Reverse	CACCAGTGGCTCATCACACA

4.2.6. Statistical analysis

The experiment had a completely randomized design with ten biological replicates (stems/inflorescences) per treatment. Testing the data for normality and homogeneity of the variances, the effect of treatments was analysed using the one-way Analysis of Variance (one-way ANOVA) of Genstat software (VSN International, version 18). Means of different treatments were compared using Tukey post hoc test at $P<0.05$. In each plant sample, the relative quantification of transcript abundance of target genes was determined by the $2^{-\Delta\Delta CT}$ method (Livak, 1997). Two technical and three biological replicates were used per treatment. Major changes of various genes relative to the control were calculated for each replicate of each sample (Ahmadi et al. 2009; Daneshi Nergi and Ahmadi, 2014).

4.3. Results

4.3.1. Display quality characteristics

The display characteristics of lily flowers were not uniformly influenced by cold storage, 1-MCP, and ethylene treatments. There were no statistically significant differences ($P>0.05$) on bud opening among all the treatments in non-cold-stored and cold-stored plants (Table 4.2.). Plants that were not cold stored had flower senescence after 11-12 days but there were no significant differences ($P>0.05$) between treatments. In the cold-stored plants, flowers started to senesce 2-3 days earlier in most treatments and this difference was statistically significant ($P<0.05$) compared to non-stored plants. The exception were plants in the cold-stored + 1-MCP treatment, where flowers senesced after 11 days, similarly to non-cold-stored plants (Table 4.2.). Cold storage + ethylene treated plants had the first sign of flower senescence (7.7 days) and had significant differences ($P<0.05$) with cold-stored + 1-MCP and cold + 1-MCP + ethylene. Whereas, cold storage + 1-MCP delayed flower senescence and showed a significant difference ($P<0.05$) with cold storage treatment. Cold + 1-MCP + ethylene treatment also delayed flower senescence, but it had no significant difference ($P>0.05$) with cold storage treatment.

In non-cold-stored plants, flowers started to abscise around day 15 of the experiment (Table 4.2.) with no significant differences ($P>0.05$) between treatments. In the cold-stored plants, flowers started to abscise 2-4 days earlier and this difference was statistically significant ($P<0.05$) compared to non-stored plants. The exception was again the cold-stored + 1-MCP treatment, where flowers abscised around day 14. Cold-stored plants that had ethylene treatments, had the shortest time to flower abscission (10.6 days) followed by cold storage and cold storage + 1-MCP + ethylene treatments with no significant differences ($P>0.05$) between them. Cold storage plants that were subjected

to 1-MCP had a significant delay in flower abscission compared to cold storage + ethylene and cold storage treated plants but not cold storage + 1-MCP + ethylene treatment.

In non-cold-stored plants, leaf yellowing was observed after 8 days in all treatments (Table 4.2.) with no significant differences (P>0.05) between treatments. In the cold-stored plants, the time to leaf yellowing was reduced to 3-4 days, showing statistically significant differences (P<0.05) with non-stored plants. No significant differences (P>0.05) were observed between treatments after cold storage.

Table 4.2. Effects of 1-MCP (10 nL L^{-1}), ethylene (E; 10 µL L^{-1}) and 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}) on time to bud opening (BO), flower senescence (FS), flower abscission (FA), and leaf yellowing (LY) before and after cold storage (C; 5 ºC, one week) in cut lily 'Marlon'. Values are the means (±SE) of five biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (P<0.05). Values in the same column that do not share a letter are significantly different.

Characteristic / *Treatment*	*Bud opening*	*Flower senescence*	*Flower abscission*	*Leaf yellowing*
Control	3.9 ± 0.08a	11.7 ± 0.13a	15 ± 0.1a	8.2 ± 0.3a
1-MCP	3.8 ± 0.08a	11.8 ± 0.08a	15.2 ± 0.09a	8.5 ± 0.17a
Ethylene	3.7 ± 0.05a	11.5 ± 0.08a	14.9 ± 0.1a	8 ± 0.14a
1-MCP + ethylene	3.9 ± 0.04a	11.6 ± 0.16a	14.9 ± 0.23a	8.4 ± 0.3a
C	3 ± 0.12a	8.9 ± 0.13cd	11.8 ± 0.13b	3.4 ± 0.08b
C + 1-MCP	3.6 ± 0.04a	10.8 ± 0.18ab	13.8 ± 0.14a	4.1 ± 0.10b
C + Ethylene	2.7 ± 0.15a	7.7 ± 0.19d	10.6 ± 0.16b	3 ± 0.02b
C + 1-MCP + ethylene	3.2 ± 0.13a	9.4 ± 0.18bc	12.3 ± 0.17ab	3.6 ± 0.02b

4.3.2. Ethylene production

In non-cold-stored flowers, the level of ethylene production was low in all treatments (Fig. 4.1.) and there were no significant differences (P>0.05) between treatments. In cold-stored flowers, we observed a climacteric increase in ethylene production with the greatest production observed after 8 days and a sharp decline in production thereafter (Fig. 4.1.). Significant differences (P<0.05) were observed between treatments after cold

storage on days 6 and 8. As cold-stored flowers subject to ethylene pre-treatment had the highest amount of total ethylene production among all treatments and showed significant differences (P<0.05) with cold storage + 1-MCP and cold storage + 1-MCP + ethylene treatments but not with other cold storage treatment on both days. Whereas, cold-stored flowers pre-treated with 1-MCP had the lowest amount of total ethylene production and showed significant differences (P<0.05) with cold storage treatment on both days. A significant difference (P<0.05) was observed between cold storage and cold storage + 1-MCP + ethylene treatments on day 6. Cold storage + 1-MCP and cold storage + 1-MCP + ethylene treatments had no significant differences (P>0.05).

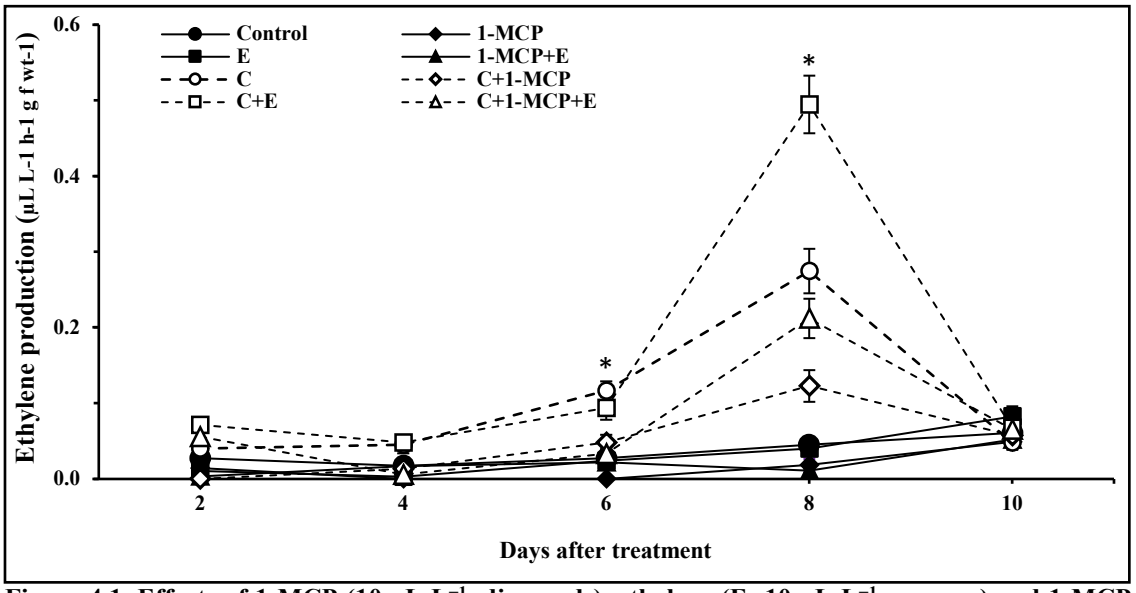
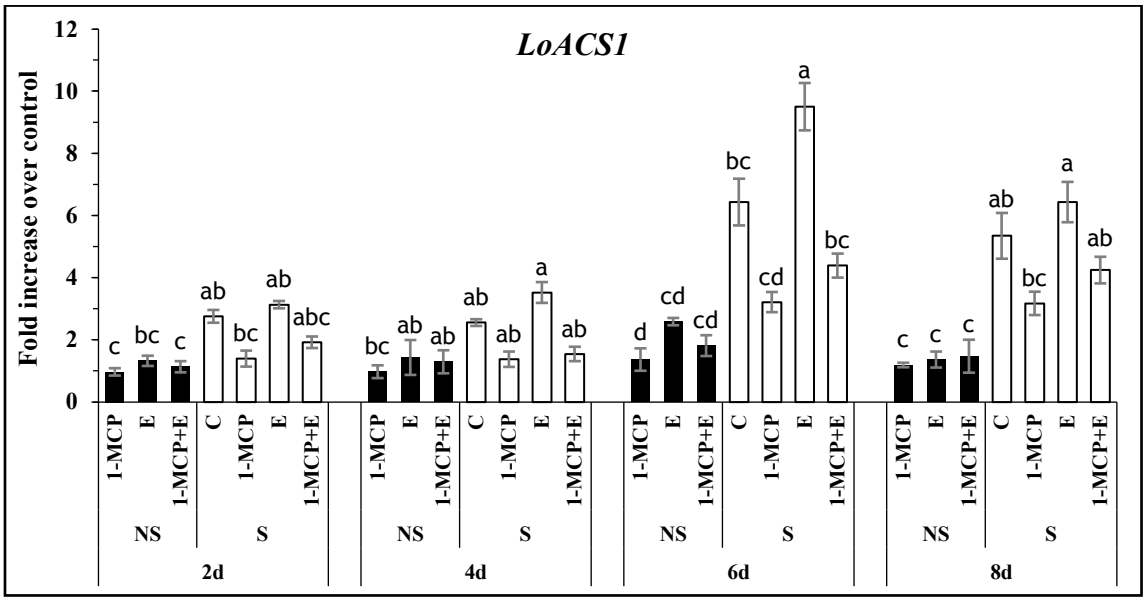


Figure 4.1. Effects of 1-MCP (10 nL L^{-1}, diamonds), ethylene (E; 10 µL L^{-1}, squares) and 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}, triangles) on ethylene production in non-cold-stored (solid lines, closed symbols) and cold-stored (dashed lines, open symbols; C; 5ºC, one week) cut lily 'Marlon'. Values are the means (±SE) of five biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (P<0.05). The asterisk indicates a significant difference (P<0.05) between treatments.

4.3.3. Expression of ethylene biosynthesis pathway genes

Like the changes in the level of ethylene production, the ethylene biosynthesis pathway genes (*LoACO1* and *LoACS1*) had two different patterns of expression and were not

uniformly affected by the same treatments in cold-stored and non-cold-stored flowers (Fig. 4.2.). In non-cold-stored flowers, the expressions of *LoACO1* and *LoACS1* were low in all treatments (Fig. 4.2.) and there were no significant differences ($P>0.05$) between treatments. Cold storage led to an increase in the expression of both *LoACO1* and *LoACS1* compared to non-stored ones with an increasing trend until day six (Fig. 4.2.). This difference was statistically significant ($P<0.05$), although not on all days (Fig. 4.2.). Significant differences ($P<0.05$) were also observed between treatments in stored flowers on some days (Fig. 4.2.) where ethylene treatment induced the expression of *LoACO1* and *LoACS1* and resulted in the highest level of expression among all treatments after cold storage and showed a significant difference ($P<0.05$) with plants treated with 1-MCP. In cold-stored plants, 1-MCP suppressed the expression of *LoACO1* and *LoACS1* even in the presence of external ethylene on some days.



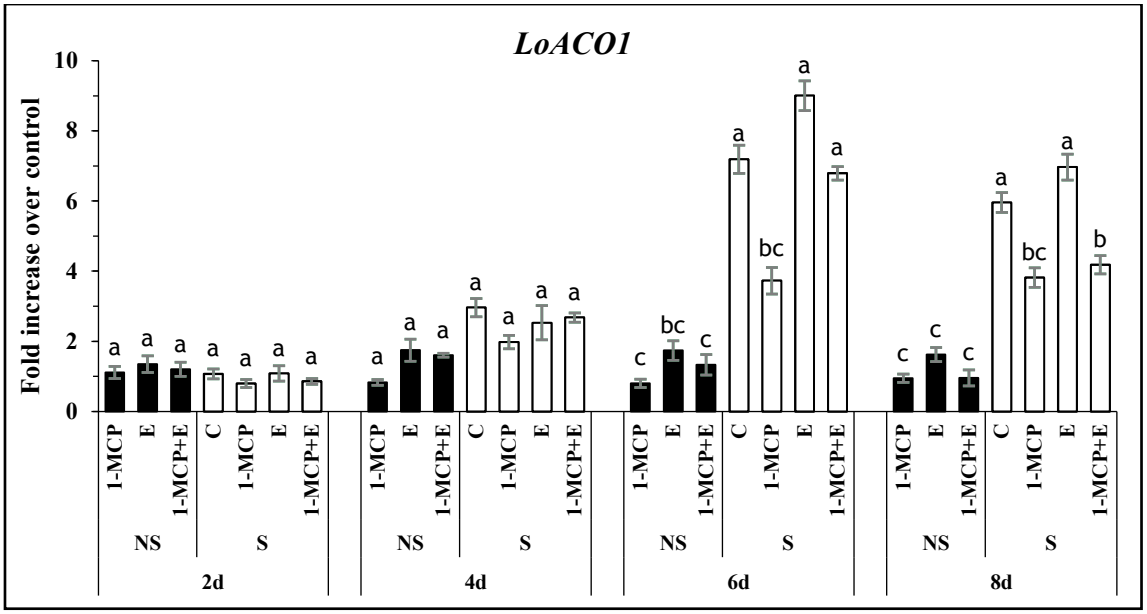
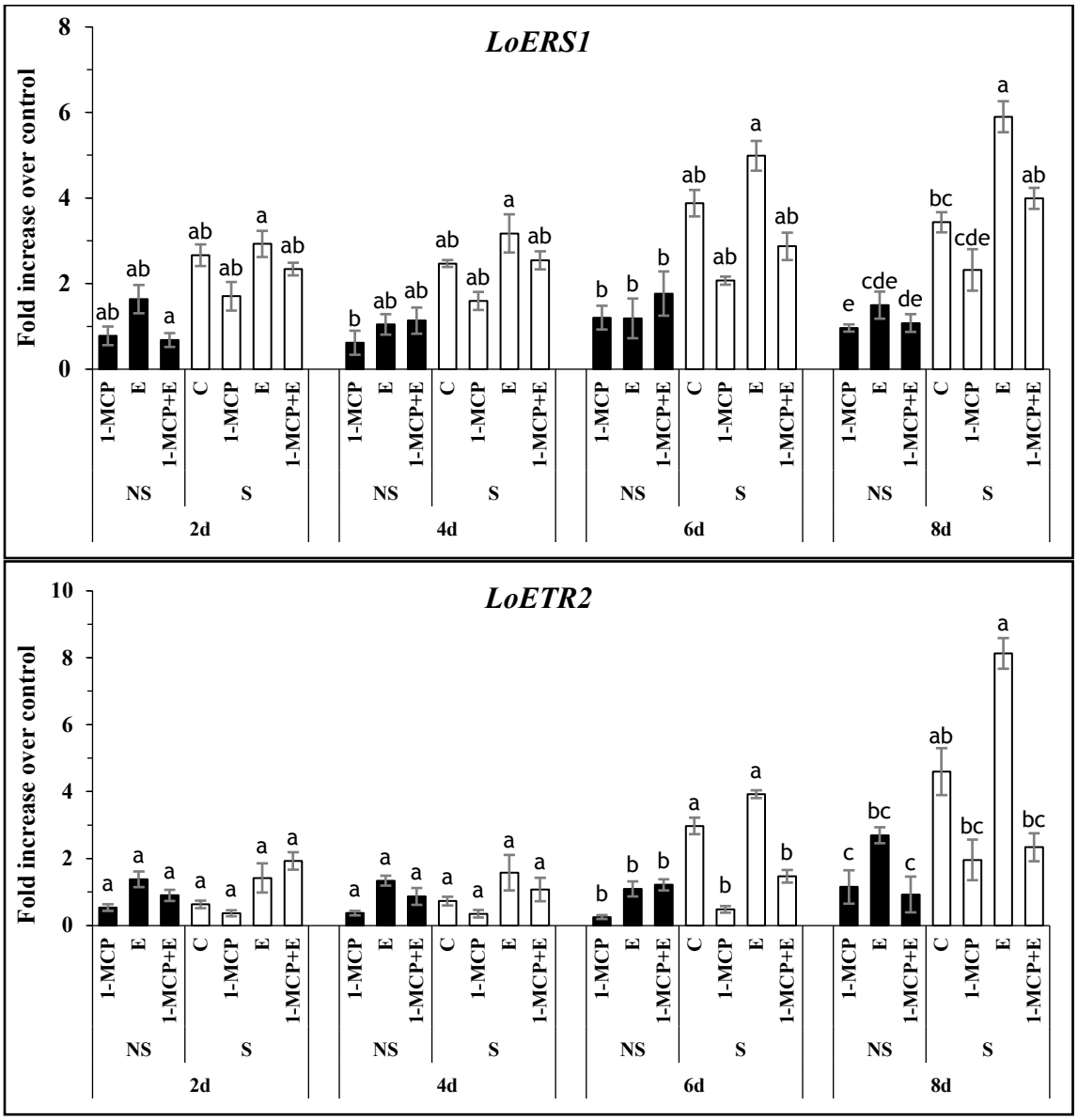


Figure 4.2. Effects of 1-MCP (10 nL L^{-1}), ethylene (E; 10 µL L^{-1}) and 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}) on the expression of ethylene biosynthesis pathway genes in non-cold-stored (NS; ■ closed bars) and cold-stored (S; □ open bars; cold-stored (C) at 5 ºC, one week) cut lily 'Marlon'. The fold change expression of *LoACO1* and *LoACS1* was calculated relative to the control at the same time (untreated sample) after normalization to the *LoGAPDH* gene. The expression level in the control was defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test ($P<0.05$). Bars that do not share a letter are significantly different.

4.3.4. Expression of ethylene receptor genes

Non-cold-stored plants had a constantly low expression of *LoERS1*, *LoETR2*, and *LoETR3* (Fig. 4.3.) and there were no significant differences ($P>0.05$) between treatments. In cold-stored plants the expression of these genes was always greater compared to non-cold-stored ones (Fig. 4.3.). The level of expression of these genes increased in cold-stored plants towards the end of the experiment (Fig. 4.3.) as we observed statistically significant differences ($P<0.05$) between non-cold-stored and cold-stored plants, although not on all days and between all treatments (Fig. 4.3.). In cold-stored plants we observed significant differences ($P<0.05$) in the expression of *LoERS1*, *LoETR2*, and *LoETR3* between treatments at the end of the experiment (Fig. 4.3.). Ethylene exposure increased the expression of *LoERS1*, *LoETR2*, and *LoETR3* in cold-

stored plants, while the induction of gene expression caused by cold storage with or without ethylene treatment was significantly reversed by 1-MCP treatment.



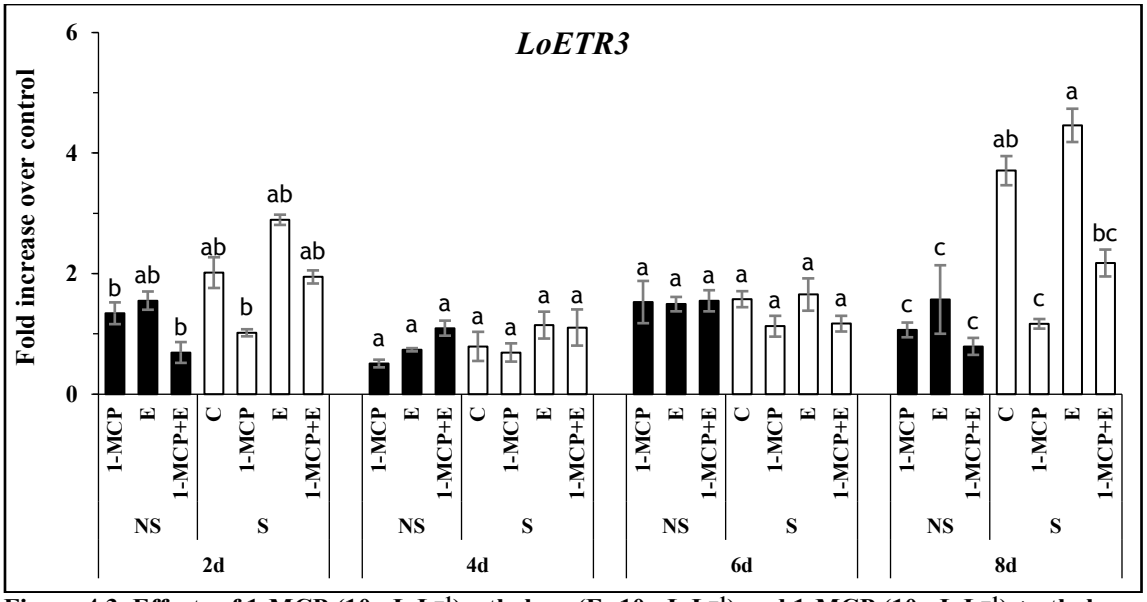


Figure 4.3. Effects of 1-MCP (10 nL L^{-1}), ethylene (E; 10 µL L^{-1}) and 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}) on the expression of ethylene perception genes in non-cold-stored (NS; ■, closed bars) and cold-stored (S; □ open bars; cold-stored (C) at 5 ºC, one week) cut lily 'Marlon'. The fold change expression of *LoERS1, LoETR2,* and *LoETR3* was calculated relative to the control at the same time (untreated sample) after normalization to the *LoGAPDH* gene. The expression level in the control was defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test (P<0.05). Bars that do not share a letter are significantly different.

4.3.5. Expression of signal transduction pathway genes

Non-cold-stored plants had a constant low expression of *LoCTR1*, *LoEIN2*, and *LoEIN3* and there were no statistically significant differences (P>0.05) between treatments (Fig. 4.4.). Cold storage led to an increase in the expression of *LoCTR1*, *LoEIN2*, and *LoEIN3* genes. In cold-stored plants, the non-1-MCP treatments in the cold-stored plants led to greater expression of *LoCTR1*, *LoEIN2*, and *LoEIN3* than the 1-MCP treatments but this was not statistically significant (P<0.05) for all the genes (Fig. 4.4.). There were no significant differences (P>0.05) among treatments for the expression of *LoCTR1* and *LoEIN3* while cold storage + 1-MCP treatment showed significant differences (P<0.05) with cold storage and cold storage + ethylene treatments on the expression of *LoEIN2* on day four.

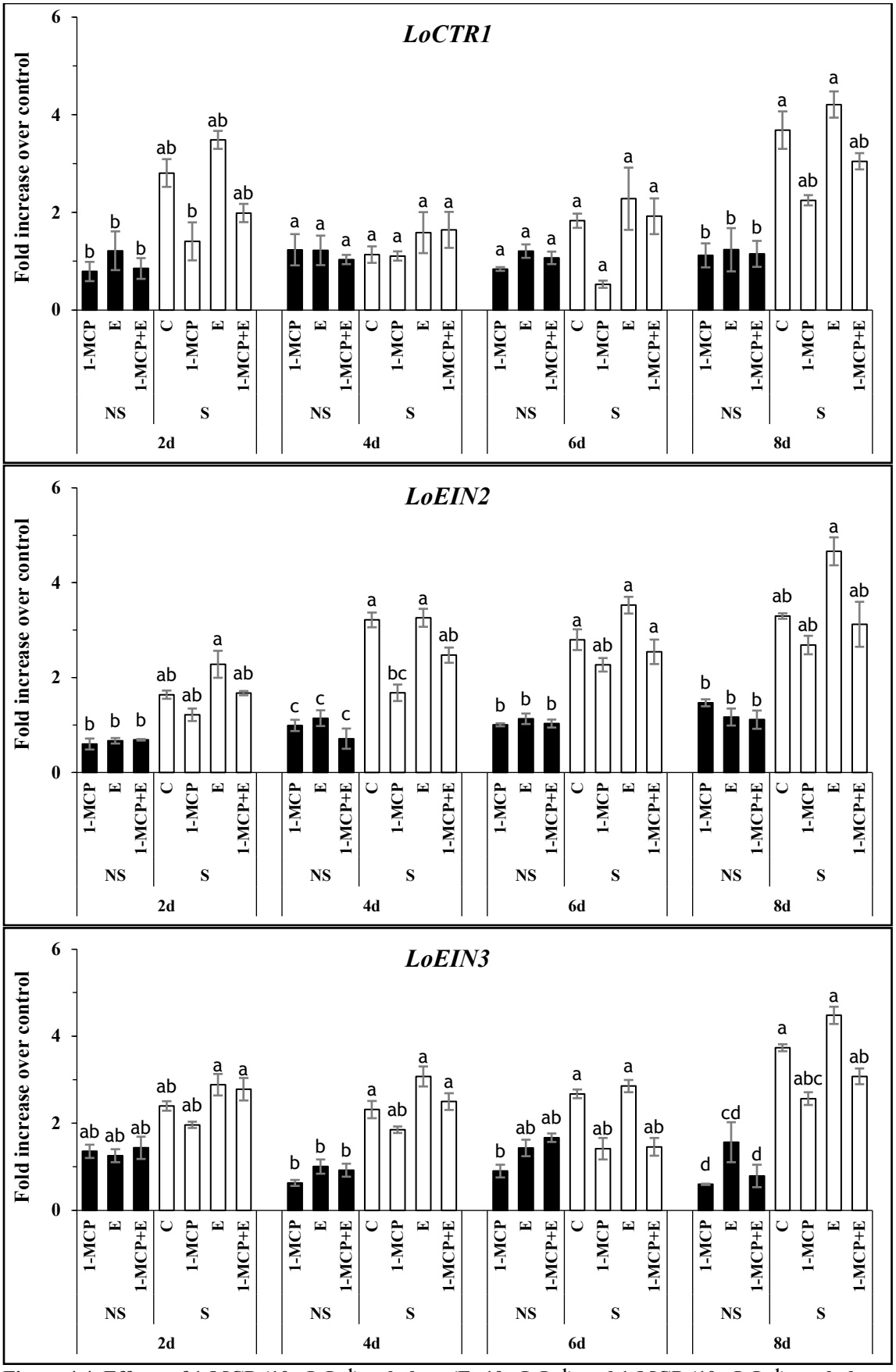


Figure 4.4. Effects of 1-MCP (10 nL L^{-1}), ethylene (E; 10 µL L^{-1}) and 1-MCP (10 nL L^{-1}) + ethylene (10 µL L^{-1}) on the expression of ethylene signal transduction pathway genes in non-cold-stored (NS;

■ closed bars) and cold-stored (S; □ open bars; cold-stored (C) at 5 ºC, one week) cut lily 'Marlon'. The fold change expression of *LoCTR1*, *LoEIN2*, and *LoEIN3* was calculated relative to the control at the same time (untreated sample) after normalization to the *LoGAPDH* gene. The expression level in the control was defined as 1. Values are the means (±SE) of three biological replicates. Data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test ($P<0.05$). Bars that do not share a letter are significantly different.

4.4. Discussion

4.4.1. The role of ethylene in senescence in cold-stored and non-cold-stored plants.

In non-cold-stored plants, ethylene or 1-MCP treatments had no effect on bud opening, flower senescence and abscission, and leaf senescence. Cold storage led to a significant reduction in the time to flower senescence (2.8 days) and abscission (3.2 days), and leaf senescence (4.1 days), but bud opening was not significantly affected by cold storage. The time to flower senescence and abscission was further shortened by exogenous ethylene but prolonged by 1-MCP treatment in cold-stored plants. Both 1-MCP and ethylene treatments had no effects on time to bud opening and leaf senescence in cold-stored plants (Table 4.2.). A similar pattern of response to cold storage, 1-MCP, and ethylene treatments was observed in cut lily 'Stargazer', where flower senescence and abscission were delayed by 1-MCP, but bud opening and leaf yellowing were not (Han and Miller (2003). An increase in ethylene production was also observed in cut Asiatic lilies 'Elite' after storage, whereas no clear increase in ethylene production was observed in cold-stored Asiatic lilies 'Prato' (Burchi et al., 2004) but ethylene did not have a triggering role in senescence in both cultivars after cold-stored (Burchi *et al*., 2004) Similarly, in cold-stored cut Asiatic hybrid lily 'Brussels', ethylene treatment did not lead to chilling induced injuries (Song and Peng, 2004). Collectively, these data indicate that the response to cold storage and ethylene is tissue- and species-dependent and could also change in a tissue at different times during developmental stages. The lack of response

by buds to cold storage in cut lily 'Marlon' could be due to lower sensitivity of petals to low temperatures at bud opening (Payton et al., 1996). However, the lack of response of buds to ethylene and 1-MCP treatments seems to be due to lower sensitivity of petals to ethylene at the pre-senescent stage. Alternatively, bud opening might be under the control of other unknown factors which needs further investigation. The early flower senescence and abscission in cold-stored plants that received ethylene indicates that the response of petals to ethylene changes over time. Changes in ethylene response could be due to cold activated existing ethylene receptors possibly through a conformational change which results in ethylene sensing in cold-stored plants or cold storage might have led to the regeneration of new and active receptors which result in ethylene sensing. Ethylene sensing suppresses the expression of genes responsible for producing aquaporin proteins which act as water channel proteins in vacuolar and plasma membranes (Ma et al., 2008) and therefore limits water uptake by cells which could cause early senescence. Moreover, in terms of petal abscission, it could also be due to cold induced changes in the level of auxin in the abscission zone. Cold storage might have resulted in early reduction in the level of auxin which is the primary regulator of organ abscission (Hong et al., 2000). Reduction in the level of endogenous auxin due to either a normal developmental process or stress response increases the sensitivity of the cells in the abscission zone to ethylene which in turn increase the level of enzymes responsible for the hydrolysing of middle lamellae which limits water flow and finally leads to organ abscission (Rungruchkanont et al., 2007; Estornell et al., 2013; Meir et al., 2015). A different response was observed in leaves, where ethylene and 1-MCP had no effect on time to leaf yellowing (leaf senescence) in cold-stored plants and leaves started to yellow much earlier compared to non-cold-stored plants. Although, in our previous experiments we observed that cultivars with high levels of ethylene production had early leaf yellowing and vice versa, the

findings of this chapter indicate that leaf yellowing is not regulated by ethylene. In fact, it might be triggered by another factor such as ethylene-independent pathway regulated by cytokinins and gibberelins or sugar-induced jasmonic acid related leaf senescence (Qi et al., 2015). For instance, decline in the level of endogenous cytokinins and gibberellins which has been reported to led to early senescence (Fletcher et al., 1969; Lara et al., 2004) probably by affecting membrane integrity (Gulzar et al., 2003), water uptake (Mayak and Halevy, 1974; Gulzar et al., 2003; Emongor, 2004), the level of sugar in tissue (Gulzar et al., 2003), protein, RNA, and DNA degradation (Osborne, 1963; Mayak and Halevy, 1974; Gulzar et al., 2003), dry weight (Mayak and Halevy, 1974), and pigments content and oxidative stress (Yu et al., 2009; Li et al., 2010; do Nascimento Simões et al., 2018). Or sugar depletion either due to darkness or lack of sugar supply to the stems may have also caused ethylene-independent early leaf senescence through regulating the activity of region/leucine zippers basic-helix-loop-helix (MYC2) transcription factors which is in involved low energy responses (starvation) (Qi et al., 2015). MYC2 activates jasmonic acid-induced leaf senescence by binding to the promotor of *SENESCENCE-ASSOCIATED GENE29* (*SAG29*) which leads to its expression and early leaf senescence (Qi et al., 2015) which needs to be further investigated.

4.4.2. The expression pattern of ethylene perception genes and their role in ethylene response in cold-stored and non-cold-stored plants.

To clarify the molecular basis for the difference in non-cold-stored and cold-stored plants, the expression pattern of genes related to ethylene receptors (*LoERS1*, *LoETR2*, and *LoETR3*) was investigated in leaves. There was a low level of expression of the receptor genes in non-cold-stored plants (Fig. 4.3.). Such a low level of expression is expected to make the plants more sensitive to ethylene, as a negative relationship between

the level of receptors and ethylene sensitivity has previously been reported (Klee, 2002). However, non-cold-stored plants showed insensitivity to ethylene. This lack of response in non-cold-stored plants despite low expression of ethylene receptor genes suggests that these receptors exist but they might be in an inactive form (Han and Miller, 2003). Conversely, a significant induction was observed in the expression of ethylene receptor genes in cold-stored lilies (Fig. 4.3.). Moreover, a further increase was caused by ethylene treatment in cold-stored lilies, whereas the expression of these genes was decreased in cold-stored plants that received 1-MCP. A similar pattern of response under cold stress conditions was also reported in soybean (*Glycine max* 'Williams') (Robison et al., 2019). These data indicate that in cut lily 'Marlon', cold storage had activated the existing receptors possibly through a conformational change and therefore resulted in ethylene sensing in cold-stored plants. It is also possible that cold storage had led to the regeneration of new and active receptors, resulting in ethylene sensing. Further changes in the expression of receptor genes in cold-stored cut lilies under ethylene and 1-MCP treatments could possibly be a response to the level of ethylene perception by the receptors. As a greater ethylene exposure results in the occupation of existing receptors on cell membranes by ethylene, which triggers the expression of the receptor genes to prevent an excessive response to ethylene (Hua and Meyerowitz, 1998) and vice versa, if involved in the senescence process. Moreover, having a similar pattern of expression like that of ethylene signalling genes (*LoEIN2* and *LoEIN3*) despite their opposite roles in ethylene response particularly on days with high and significant expression levels seems to be a response to higher expression of ethylene signalling genes particularly when the ethylene response exceeds its threshold level. Higher presence of ethylene and therefore more ethylene perception will result in a signalling pathway that increases the expression of many senescence associated genes including its signalling genes (*LoEIN2* and

LOEIN3). But after occupation of all the available receptors and also after surpassing the ethylene threshold response which may differ from one species to another, in order to avoid excessive response new receptors are produced (Hua and Meyerowitz, 1998) which is through increase in their related genes expression. It is possible that the expression of these genes might coincide with the higher expression of ethylene signalling genes at the beginning of this response but differ in the later stages of response as new receptor will start to supress ethylene signalling pathways and genes expression levels.

4.4.3. The expression pattern of ethylene signalling genes and their role in ethylene response in cold-stored and non-cold-stored plants.

To further understand the molecular causes of the changes in ethylene response, the expression pattern of signal transduction pathway genes (*LoCTR1*, *LoEIN2*, and *LoEIN3*) was monitored in leaves. Like the receptor genes, the expression of ethylene signal transduction pathway genes (*LoCTR1, LoEIN2*, and *LoEIN3*) was also significantly higher in cold-stored plants than non-cold-stored plants (Fig. 4.4.). This difference was greater in cold-stored plants that received ethylene and lower in 1-MCP treated cold-stored plants. The difference in the expression of *LoCTR1* in non-cold-stored and cold-stored plants under different treatments appears to be related to ethylene sensing at the receptor level as it had low expression in non-cold-stored plants (Fig. 4.4.). CTR1 acts as a downstream ethylene receptor and is a negative regulator in the ethylene signal transduction pathway and is inactivated in the presence of ethylene (Kieber et al., 1993; Arora, 2005). Therefore, ethylene perception will induce its expression which is a normal plant response. This was further confirmed by previous reports on cut rose (*Rosa hybrida*) 'Samantha' (Ma et al., 2006) and Arabidopsis (Shakeel et al., 2015). As in cut rose 'Samantha', ethylene induced the expression of *CTR1* (Ma et al., 2006), and in *etr1-*

1 Arabidopsis, *CTR1* transcript was reduced compared with wild type suggesting the role of ethylene in maintaining *CTR1* expression (Shakeel et al., 2015). This means lack of ethylene sensing either as a result of a mutant in the receptors or receptor occupancy by 1-MCP will result in no and/or a low level of expression of *LoCTR1* gene and vice versa which is due to its negative role in the ethylene signalling pathway. *LoEIN2*, and *LoEIN3* had the same response to the treatments in both cold-stored and non-cold-stored plants (Fig. 4.4.). A similar response of *EINs* to cold storage was observed in soybean (*Glycine max* 'Williams') (Robison et al., 2019). EIN2 and EIN3 act downstream of CTR1 and are positive regulators of the ethylene signalling pathway. The difference in response in cold-stored and non-cold-stored lilies also seems to be related to ethylene sensing by the receptors. It has been reported that the absence of ethylene leads to the degradation of EIN2 and EIN3 (Guo and Ecker, 2003), which results in a lack of ethylene signalling, ethylene-responsive gene expression and finally ethylene response. Thus, in cold-stored plants, it seems that cold-induced ethylene sensing has led to EIN2 and EIN3 stabilizing and therefore ethylene signalling and higher expression of downstream genes including *EINs, ASC1 and ACO1* (Fig. 4.4.) and finally more ethylene response including higher ethylene production. This was further confirmed by our 1-MCP results (Fig. 4.4.) where lack of ethylene perception led to lower expression of these genes. Collectively, these data indicate that the changes in the expression pattern of signal transduction pathway genes (*LoCTR1*, *LoEIN2*, and *LoEIN3*) are possibly triggered by the changes in the level of ethylene sensing.

4.4.4. The effect of exogenous ethylene on endogenous ethylene biosynthesis and its biosynthesis-related gene expression in cold-stored and non-cold-stored plants

We also investigated ethylene biosynthesis and its related gene expression. Non-cold-stored plants had no increase in ethylene biosynthesis, while cold-stored plants had higher ethylene production in a climacteric production pattern (Fig. 4.1.). Ethylene exposure of cold-stored plants further increased ethylene production, whereas 1-MCP led to a lower ethylene production in stored plants (Fig. 4.1.). Han and Miller (2003) also reported that non-stored cut oriental lily 'Stargazer' produced an undetectable amount of ethylene during flower senescence and showed no response to exogenous ethylene and 1-MCP, but after cold-storing, they produced a higher amount of ethylene and even become highly sensitive to exogenous ethylene. Such a change in the level and pattern of ethylene production in lilies seems to be due to a higher expression of its biosynthesis pathway genes as we observed in cut lily 'Marlon' (Fig. 4.2.), similar to that of other plant species (Lelievre et al., 1997) which results in the accumulation of ethylene biosynthesis pathway enzymes (ACC synthase and ACC oxidase) and higher ethylene levels. Then the produced ethylene, after being sensed by ethylene receptors, might have led to signal transduction by signalling components (*CTR1*, *EIN2*, and *EIN3*) and expression of downstream genes. This is further confirmed by our 1-MCP results as a lower expression of these genes in 1-MCP treated plants led to less ethylene production.

4.5. Conclusion

Taken together, the response of cut lilies to ethylene differs before and after cold storage in a tissue-specific manner. Ethylene only accelerates flower senescence in cold-stored flowers without having effects on their leaf yellowing process which defines it as a multifactor-controlled process. In non-stored flowers, ethylene has no effects on both flower and leaf senescence processes which shows that this cultivar is not ethylene sensitive which is possibly due to lack or incomplete ethylene perception at the receptor

level. In cold-stored plants, 1-MCP was effective in delaying the senescence of flowers but not leaf senescence which indicates that it cannot be considered as a postharvest treatment alone but should be used in combination with other chemicals to prevent leaf yellowing. We did not measure the expression of ethylene related genes in petals, which is the limitation of the current study. But given the role of ethylene in flower senescence and the receptor genes' expression pattern during senescence in leaves, it is likely that cold storage leads to changes in ethylene perception in petals possibly through either activation of available receptors or formation of new receptors as a result of increased expression of the related genes and therefore caused ethylene and 1-MCP response. Hence, the response of lily plants to cold storage and the sensitivity to ethylene differs between different plant organs as ethylene does influence flower senescence following cold storage but not leaf senescence. This makes the application of ethylene blockers to prolong postharvest life in lilies more difficult as they will be effective for flowers but not leaves.

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Chapter 5

Synthesis of key results and implications for future research

In this thesis, I investigated the effects of different and optimum concentrations of brassinosteroid (BR) on postharvest quality characteristics and ethylene production in cold-stored cut lilies 'Stargazer', 'Sorbonne', 'Premium Blonde' and 'Marlon' (Chapter 2); the effects of optimum concentration of BR on cold-stored cut lilies 'Premium Blonde' and 'Marlon' at physiological, biochemical and molecular levels (Chapter 3); and the effects of ethylene and 1-MCP on cut lily 'Marlon' at physiological, biochemical and molecular levels before and after cold storage (Chapter 4). In this synthesis, I will summarise the key findings of the research chapters and discuss them in relation to other studies and finally make some recommendations for future research.

5.1. Summary of key findings

Chilling injury is common in many cut lilies and a relatively short period of cold storage (~1 week) often leads to physiological damages in these flowers. Brassinosteroids (BRs) have been reported to induce tolerance to extreme temperatures and prevent chilling injury in plants (Wang et al., 2012; Z. Liu et al., 2016), whereas their effectiveness to influence chilling tolerance in cut flowers has not been investigated. The response of plants to BRs often is concentration dependent. I first subjected cut lilies to different concentrations of BR (1-15 μM) and discovered an optimum concentration of BR where the effects were most prominent (8.5 μM). In the first experiment, BR significantly delayed a range of postharvest quality indicators. Leaf yellowing – one of the first signs

of chilling injury - was delayed in 'Sorbonne', 'Premium Blonde', and 'Marlon'. Bud opening was delayed in 'Stargazer', flower senescence in 'Sorbonne', 'Stargazer' and 'Marlon', and flower abscission was delayed in all cultivars. BR also led to a significant increase in water uptake and high chlorophyll content in 'Sorbonne' and 'Stargazer' and led to lower ethylene production in all cultivars. However, while BR treatment delayed the onset of senescence significantly, the 1-2 days delay does not present a major delay in the onset of flower senescence. Thus, its application does not seem to be beneficial at a practical level in all cultivars, particularly in cultivars with low level of sensitivity to cold storage.

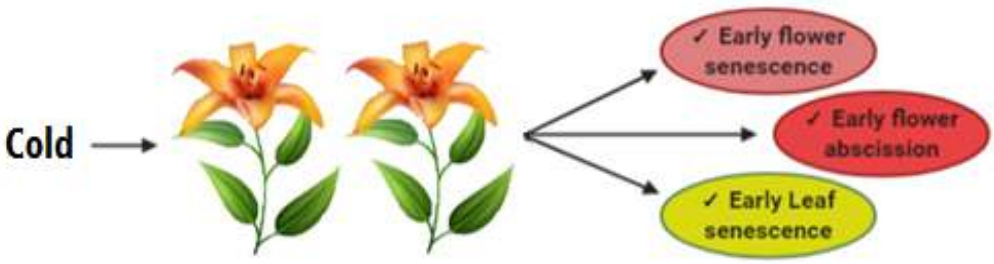

Figure 5.1. Effect of cold storage (5 ºC, one week) on quality characteristics in cut lilies.

In the second experiment I investigated the effects of cold storage and optimum concentration of BR (8.5 µM) at a molecular level. I analysed the expression of ethylene biosynthesis genes (*LoACO1* and *LoACS1*), perception genes (*LoERS1, LoETR2* and *LoETR3*) and signalling genes (*LoCTR1*, *LoEIN2*, *LoEIN3*), and the BR-related transcription factor 1 gene (*LoBZR1*) in 'Premium Blonde' and 'Marlon'. I also monitored time to bud opening, flower senescence and abscission, leaf yellowing, and ethylene production. Cold storage in non-treated flowers led to a significantly higher

ethylene production and significantly earlier flower senescence in 'Marlon', and earlier flower abscission and leaf yellowing in both cultivars compared to BR treated ones. Correspondingly, the expression of most ethylene-related genes was significantly greater in cold storage treatment compared to the BR + cold storage treatment on most observation days in both cultivars. The BR-related transcription factor gene *LoBZR1* had significantly greater expression in BR + cold storage treatment. This experiment suggests that the chilling injuries in cut lilies are ethylene mediated which could be due to either higher ethylene biosynthesis or higher ethylene sensitivity or a combination of both as a result of alteration in the expression of the related genes. Thus, delayed chilling injuries by application of BR is likely caused by the suppression of the over-expression of ethylene-related genes which results in less ethylene biosynthesis and possibly sensitivity.

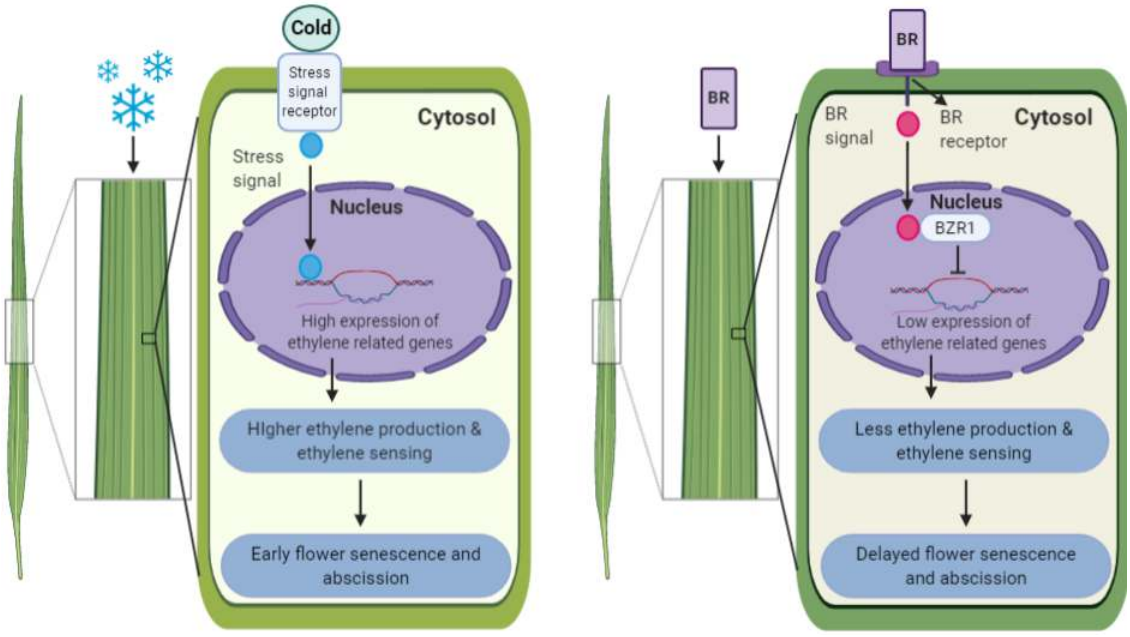

Figure 5.2. Conceptual diagram of the effects of cold storage (5 ºC, one week) and BR treatment (8.5 µM) on the expression of ethylene related genes, ethylene production, and flower and leaf senescence in cut lily 'Premium Blonde' and 'Marlon'.

The third experiment was designed to investigate whether i) chilling related injuries are ethylene-dependent either due to a higher level of ethylene production or a change in ethylene sensitivity and ii) if such changes are due to alteration in the expression of ethylene biosynthesis, perception, and signalling genes. I subjected cut lily 'Marlon' to ethylene and the ethylene action inhibitor 1-Methylcyclopropene (1-MCP) before and after cold storage. I measured the time to bud opening, flower senescence and abscission and leaf senescence, ethylene production, and the expression of its biosynthesis (*LoACO1* and *LoACS1*), perception (*LoERS1, LoETR2,* and *LoETR3*), and signalling (*LoCTR1*, *LoEIN2*, and *LoEIN3*) genes. In non-cold-stored plants ethylene and 1-MCP treatments had no effect on postharvest quality, ethylene production and the expression pattern of the investigated genes. Cold storage led to much earlier leaf yellowing (from 4 to 8 days), and while bud opening was not affected, flowers senesced and abscised three days earlier (Fig. 5.3.). In cold-stored plants, ethylene and 1-MCP did not affect bud opening and leaf yellowing but flower senescence and abscission were hastened by ethylene and delayed by 1-MCP. Similarly, the expression of all genes and ethylene production were significantly increased by ethylene but significantly decreased by 1-MCP in cold-stored plants although not on all days. In cold-stored plants, 1-MCP treatment significantly delayed flower senescence and decreased the expression of some of ethylene related genes on some days. These results indicate that there is no sensitivity to ethylene in fresh cut non-cold-stored lily 'Marlon'. This changes following cold storage, particularly in a tissue-dependant manner, as flower senescence and abscission were delayed by 1-MCP but leaf senescence was not. Hence, ethylene does influence flower senescence following cold storage, but leaf senescence is under the control of other factors. Hence, treatments that focus on ethylene blockers to prolong postharvest life in lilies will be effective for flowers but not leaves.

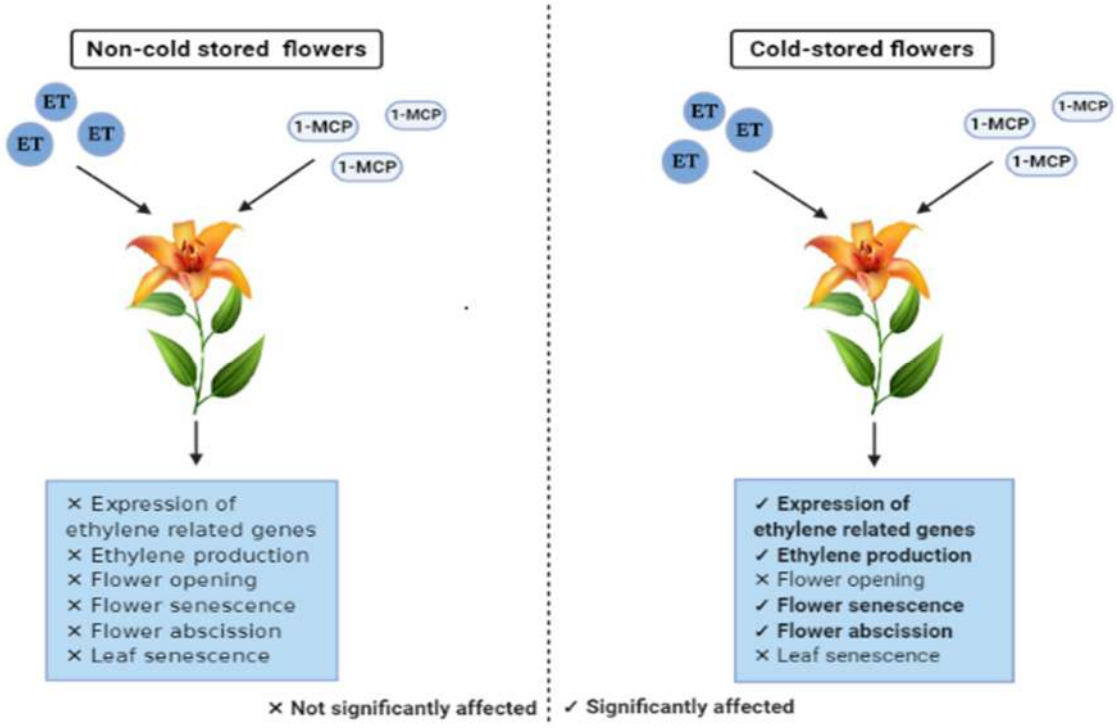

Figure 5.3. Effects of ethylene (ET) and 1-MCP on the expression of ethylene related genes, ethylene production, and quality characteristics in cut lily cultivars 'Marlon' before and after cold storage (5 º C, one week).

5.2. Ethylene production in cold-stored lilies and its role in chilling injuries

Ethylene plays a central role in stress response and senescence in many plants and ethylene biosynthesis and receptors are often stimulated in response to biotic and abiotic stresses such as cold temperatures (Yang and Hoffman, 1994; Dong *et al*., 2001). The results of my second (Table 5.1.) and fourth chapters showed induction in ethylene production in cold-stored cut lilies. The observed increase in ethylene production in response to cold storage is in line with other cold storage studies (Han and Miller, 2003; Burchi *et al*., 2004). The increase in ethylene production in my studies is likely due to a higher expression of its biosynthesis pathway genes as observed in cut lily 'Marlon' (Fig. 5.2.) which is consistent with the results of other studies (Lelievre *et al*., 1997; Tian *et al*., 2002; Zou *et al*., 2014). However, I did not measure the activity of ethylene biosynthesis pathway enzymes – thus the higher ethylene production could also be cold

storage stimulated activity of existing ethylene biosynthesis pathway enzymes which would also lead to a higher level of ethylene biosynthesis (Lelievre *et al*., 1997; Kim *et al*., 2004). Further analysis in my fourth chapter on the role of ethylene in chilling injuries using ethylene and 1-MCP showed that ethylene involvement in chilling injuries is developmental and tissue-dependent: ethylene and 1-MCP had no effects on flowers at bud opening stage and the leaf yellowing process but significantly affected flower senescence and abscission in cut lily 'Marlon'. However, the response and mechanism may vary from one cultivar to another. There have been only a few studies on the role of ethylene in chilling related injuries and different lily cultivars can respond differently to cold storage. For instance, cold storage caused early leaf yellowing in cut lily 'Stargazer' in our study and also in (Han and Miller, 2003). Whereas, in cold-stored cut Asiatic hybrid lily 'Brussels', ethylene treatment did not lead to chilling induced injuries (Song and Peng, 2004). Even in Asiatic lilies 'Elite' and 'Prato' ethylene did not have a triggering role in senescence in cold-stored plants (Burchi *et al*., 2004). These data indicate that the induction in ethylene production might always be observed in cold-stored lilies as a sign of chilling injuries, but it may not always be a key driver of chilling injuries as it is dependent on the developmental stage, tissue, and cultivar.

Table 5.1. Effects of cold storage on quality characteristics and ethylene production in cut lilies. ✓ indicates significant and × non-significant changes.

Cultivar / ***Characteristics***	*'Sorbonne'*	*'Stargazer'*	*'Premium Blonde'*	*'Marlon'*
Bud opening	✓	✓	×	×
Flower senescence	✓	✓	×	✓
Flower abscission	✓	✓	✓	✓
Leaf yellowing	✓	✓	✓	✓
Ethylene production	✓	×	✓	✓

5.3. BR reduces chilling injuries in both ethylene dependent and independent manners

The results of my third and fourth chapters provide a clearer understanding of the role of BR in reducing chilling related injuries in cut lily 'Marlon' (Table 5.2.). Cold storage did not affect bud opening but led to a significant reduction in the time to leaf and flower senescence and abscission, while BR led to a significant delay in leaf and flower senescence and abscission. The time to flower senescence and abscission in cold-stored plants was further shortened by exogenous ethylene but prolonged by 1-MCP treatment. But both 1-MCP and ethylene treatments had no effect on time to bud opening and leaf senescence in cold-stored plants. These results indicate that the delay of chilling injuries by BR has both ethylene dependent and independent pathways. In the ethylene-dependent pathway, BR leads to an overexpression of *LoBZR1* which in turn suppresses the expression of ethylene biosynthesis-related genes (Fig. 5.4.). This results in less ethylene production and related damages in ethylene-responsive tissues (Table 5.3.), which is in line with the results of other studies (Li *et al*., 2016; Zhu *et al*., 2015; Lv *et al*., 2018). Although I did not measure ethylene independent components but based on the observations of other studies from other species, ethylene independent effects may be due to the activation of phenolic and proline biosynthesis and inhibition of oxidative stress (Aghdam *et al*., 2012; Gao *et al*., 2016; Liu *et al*., 2016), elicitation of an antioxidant response (Liu *et al*., 2011; Wang *et al*., 2012) which is partially mediated abscisic acid (Liu *et al*., 2011). Furthermore, ethylene independent effects may be due to enhanced enzyme activities related to energy (H+-ATPase, Ca(2+)-ATPase, succinate dehydrogenase, and cytochrome C oxidase) (Liu *et al*., 2016), or modulating the activity of cell wall-related enzyme pectin methylesterases through regulating its related gene's (*PME41*) expression (Qu *et al*., 2011) which needs to be further investigated in lilies. I

did not investigate ethylene role in the senescence process in other cut lilies and to my knowledge, there are no studies investigating the effects of BR on other lilies. But based on my data, the response to BR might change from one cultivar to another possibly depending on their responses to ethylene.

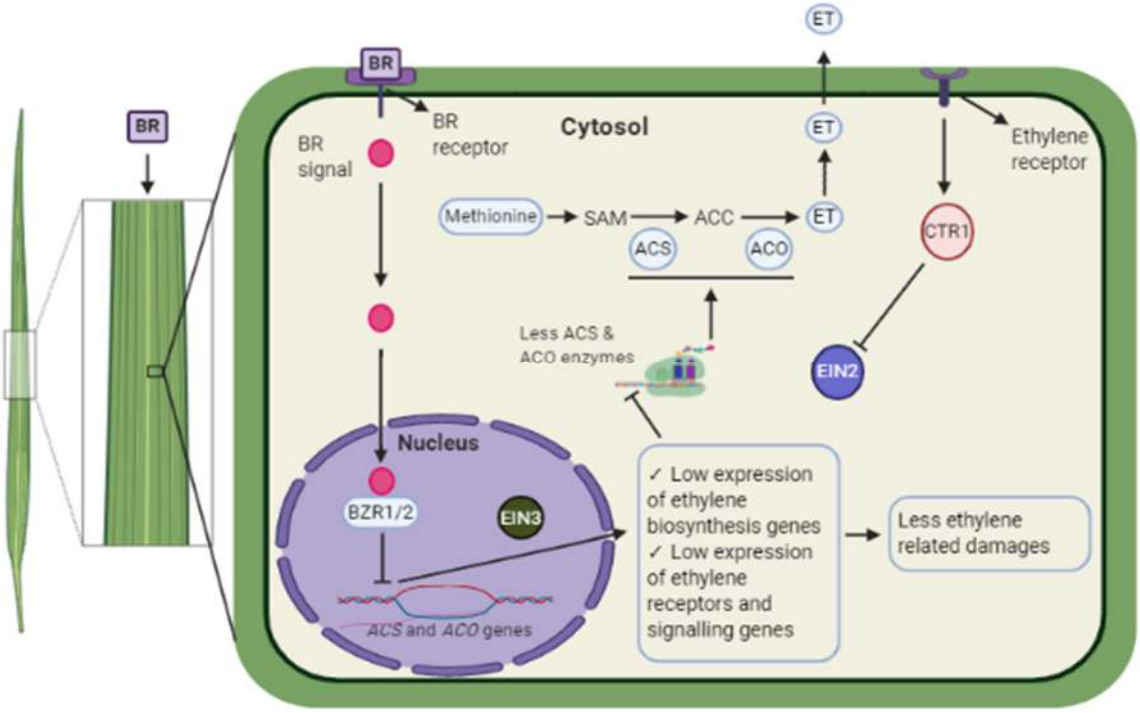

Figure 5.4. Conceptual diagram of the effect of BR treatment (8.5 µM) on the expression of ethylene related genes, ethylene production, and flower in cut lily 'Marlon'. BR perception by cell results in signal transduction to the nucleus which in turn decreases the expression of ethylene related genes and therefore less ethylene biosynthesis enzymes and less ethylene production. Low amount of ethylene results in less occupation of ethylene receptors. Unoccupied receptors repress ethylene signalling to nucleus and therefore prevent ethylene response by cell/tissue.

Table 5.2. Effects of BR, ethylene, and 1-MCP on quality characteristics in cold-stored cut lily 'Marlon'. ✓ and ×indicate significant and non-significant effects.

Treatment / ***Characteristic***	*BR*	*Ethylene*	*1-MCP*
Bud opening	×	×	×
Flower senescence	✓	✓	✓
Flower abscission	✓	✓	✓
Leaf yellowing	✓	×	×
Ethylene production	✓	✓	✓

Table 5.3. Relationship between BR and ethylene biosynthesis genes with the level of chilling injury.

Characteristic / ***Treatment***	*LoBZR1*	*LoACO1 & LoACS1*	*Chilling injuries*
Cold storage	low	high	high
BR	high	low	low

5.4. Are different ethylene responses due to the availability of ethylene receptors?

The results of the fourth chapter demonstrate the possible mechanism behind the difference in ethylene response before and after cold storage in cut lilies. There was a low level of expression of the ethylene receptor genes in non-cold-stored plants, but cold storage led to a significant induction of the expression of ethylene receptor genes (Fig 5.5.). The availability of ethylene receptor sites does not seem to be the underlying reason behind the difference in ethylene response. Ethylene receptors had a lower expression before cold storage. Based on the generally accepted model of the receptor activity in plants, such a low level of expression should have made the plants more responsive, as there is a negative relationship between the level of receptors and ethylene responsiveness of a tissue (Klee, 2002), but non-cold-stored plants did not show responsiveness to ethylene. Such a response demonstrates that these receptors exist but they might be in an inactive form (Han and Miller, 2003). The ethylene responsiveness of the cold-stored flowers indicates that cold temperatures may have led to an activation of existing receptors and therefore ethylene responsiveness by plants at the receptor level. But it is also possible that cold storage has led to the regeneration of new and active receptors in cold-stored plants, which results in ethylene responsiveness at receptor level which is in line with previous findings on soybean (*Glycine max* 'Williams') (Robison *et al*., 2019). However, I did not measure the expression level of the receptor genes in petals. But given the role of ethylene in flower senescence and the receptor gene

expression pattern during senescence in leaves, it is likely that cold storage has led to changes in ethylene perception in petals possibly through either the activation of available receptors or formation of new receptors as a result of increased expression of the related genes and therefore caused ethylene and 1-MCP responses.

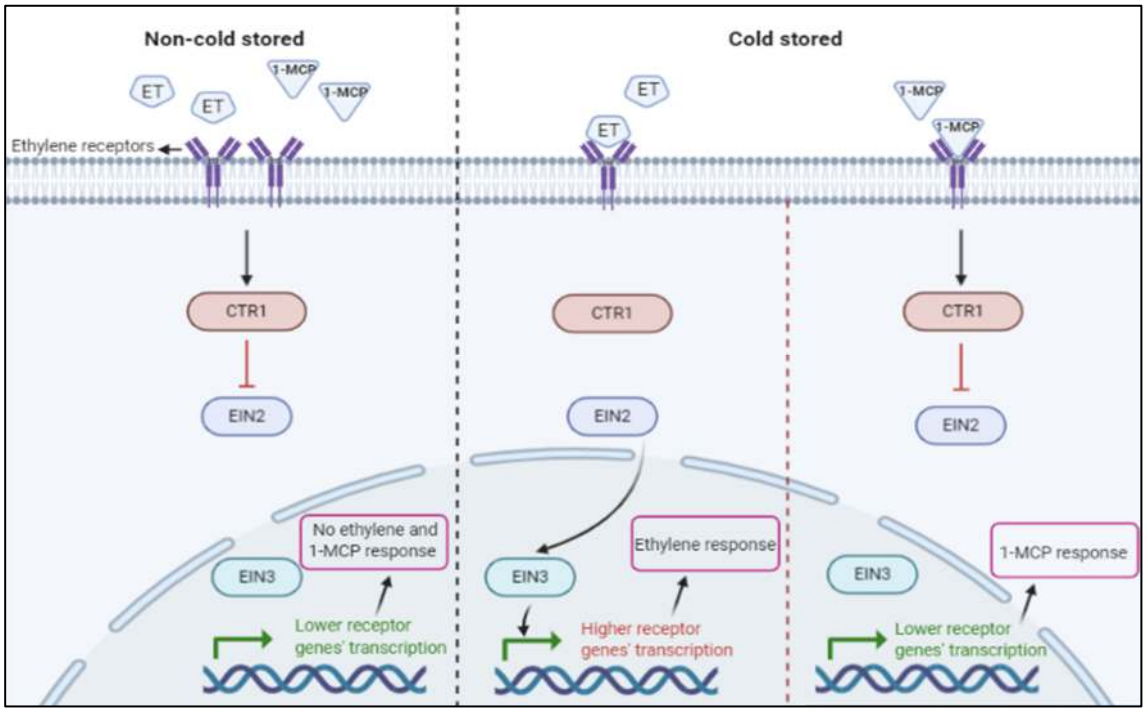

Figure 5.5. Conceptual diagram of the response to ethylene (ET) and 1-MCP before and after cold storage and its relationship with the expression of ethylene receptor genes' expression in cut lily 'Marlon'.

5.5. Advance in knowledge and future research directions

The results of the present study demonstrated that the response of cut lilies to cold storage is cultivar dependant and cold storage causes an induction in ethylene biosynthesis and also changes in the ethylene response. Cultivars with a high level of sensitivity will have a higher ethylene production possibly due to a higher expression of ethylene biosynthesis pathway genes which aligns with the observations of other studies (Lelievre et al., 1997; Tian et al., 2002; Zou et al., 2014). But it could also be cold storage stimulated the activity of existing ethylene biosynthesis pathway enzymes that lead to a higher level of ethylene biosynthesis (Lelievre et al., 1997; Kim et al., 2004), which raises a future research

question. Cold storage also led to changes in ethylene sensitivity but in a tissue-specific manner, which means that the damages could be partially ethylene dependent (Han and Miller, 2003). But this could change between cultivars as in other cultivars the damages could be ethylene independent (Song and Peng, 2004), or ethylene might play an indirect role in such changes (Burchi *et al*., 2004). Such changes raise the question if receptor availability is the underlying factor? My data indicated that the receptor site availability is not the reason for the changes in ethylene sensitivity. Rather changes in receptor form or regeneration of new and active receptors seem to be the key reason for the observed changes in ethylene response in the studied cut lily cultivar. Thus, further research could investigate if the responses are caused by changes in the form of the receptors.

My thesis also confirms that BR was effective in reducing chilling injuries (Wang et al. 2012; Gao et al. 2016), but in a concentration-dependent manner. However, the level of effects varied from one cultivar to another cultivar, indicating that it is also cultivar dependent. The effect of BR is partially caused through hormonal interaction with ethylene, which seems to be in an ethylene dependent manner, possibly through interaction between the components of biosynthesis pathways of the hormones. But this needs to be further investigated in other cultivars as there is a difference among cultivars regarding the role of ethylene in the senescence process. Moreover, BR also had ethylene independent component/components in delaying chilling injuries which are yet unknown in lilies, however, there are many reports in other species (Liu *et al*., 2011; Qu *et al*., 2011; Aghdam *et al*., 2012; Wang *et al*., 2012; Gao *et al*., 2016; Liu *et al*., 2016). Further research is necessary to understand the underlying factors in lilies. Overall, while the effects of BR were significant, it is not recommended to use BR as postharvest treatment because it did not lead to major delays in the onset of senescence in all cultivars. In addition, the effectiveness of BR may change depending on the production conditions

and the level of ethylene response and its role in the senescence process, which needs to be further investigated in different cultivars. Thus, its application does not seem to be beneficial at a practical level in all cultivars particularly in cultivars with a low level of sensitivity.

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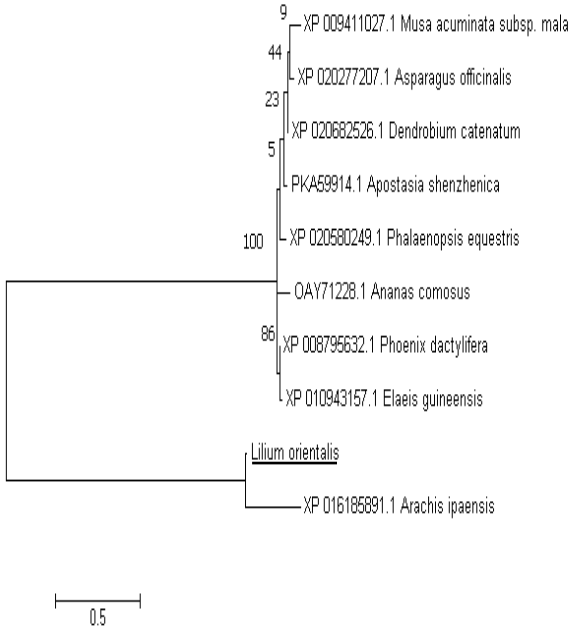
Chapter 6

Supplementary

Figure 6.1. Cold storing (5 ºC, seven and ten days) BR-treated (8.5 and 10 µM for 8 h) cut lily stems.

Figure 6.2. Endogenous ethylene production of flowers in cut lily cultivars in 2 L air-tight jars for 24 hours at 20 ± 2 °C. Potassium hydroxide solution (20% w/v) was placed inside the jars to maintain low CO2 concentrations from respiration during treatments. Gas samples were taken through headspaces using a syringe.

A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XP 020682526.1 Dendrobium catenatum
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OAY71228.1 Ananas comosus
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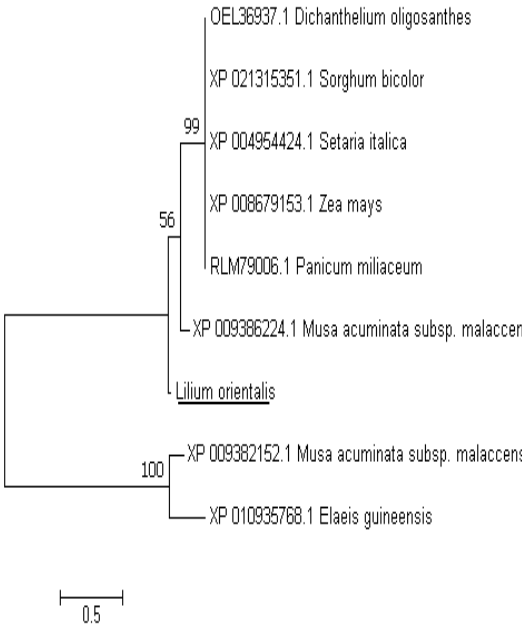
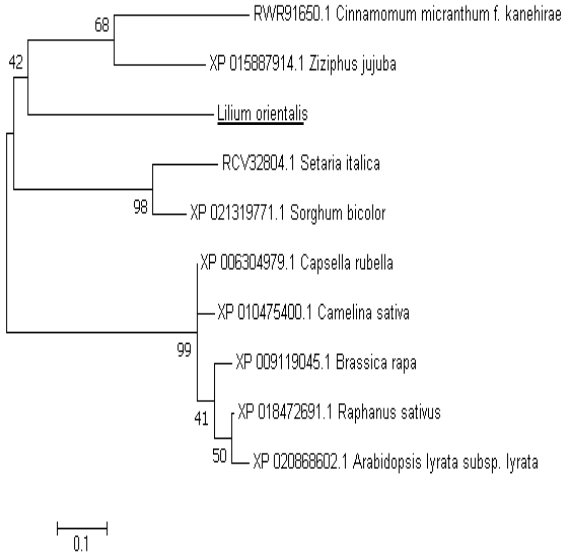
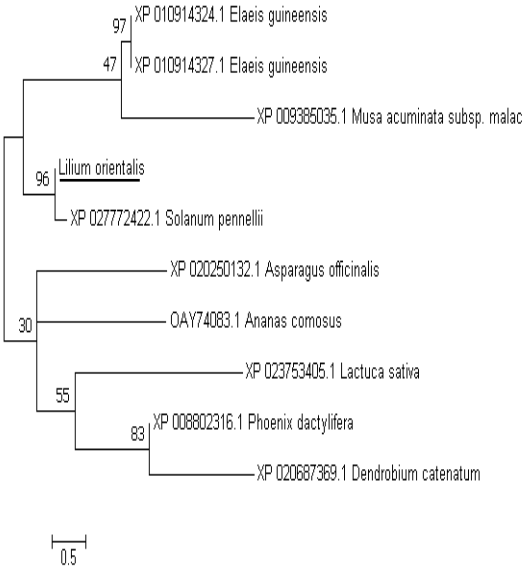

OEL36937.1 Dichanthelium oligosanthes
XP 021315351.1 Sorghum bicolor
99
XP 004954424.1 Setaria italica
XP 008679153.1 Zea mays
56
RLM79006.1 Panicum miliaceum
XP 009386224.1 Musa acuminata subsp. malaccen
Lilium orientalis
XP 009382152.1 Musa acuminata subsp. malaccens
100
XP 010935768.1 Elaeis guineensis
0.5

B


RWR91650.1 Cinnamomum micranthum f. kanehirae
68
42
XP 015887914.1 Ziziphus jujuba
Lilium orientalis
RCV32804.1 Setaria italica
98
XP 021319771.1 Sorghum bicolor
XP 006304979.1 Capsella rubella
XP 010475400.1 Camelina sativa
99
XP 009119045.1 Brassica rapa
41
XP 018472691.1 Raphanus sativus
50
XP 020868602.1 Arabidopsis lyrata subsp. lyrata
0.1

F


97
XP 010914324.1 Elaeis guineensis
47
XP 010914327.1 Elaeis guineensis
XP 009385035.1 Musa acuminata subsp. malac
96
Lilium orientalis
XP 027772422.1 Solanum pennellii
XP 020250132.1 Asparagus officinalis
30
OAY74083.1 Ananas comosus
XP 023753405.1 Lactuca sativa
55
83
XP 008802316.1 Phoenix dactylifera
XP 020687369.1 Dendrobium catenatum
0.5

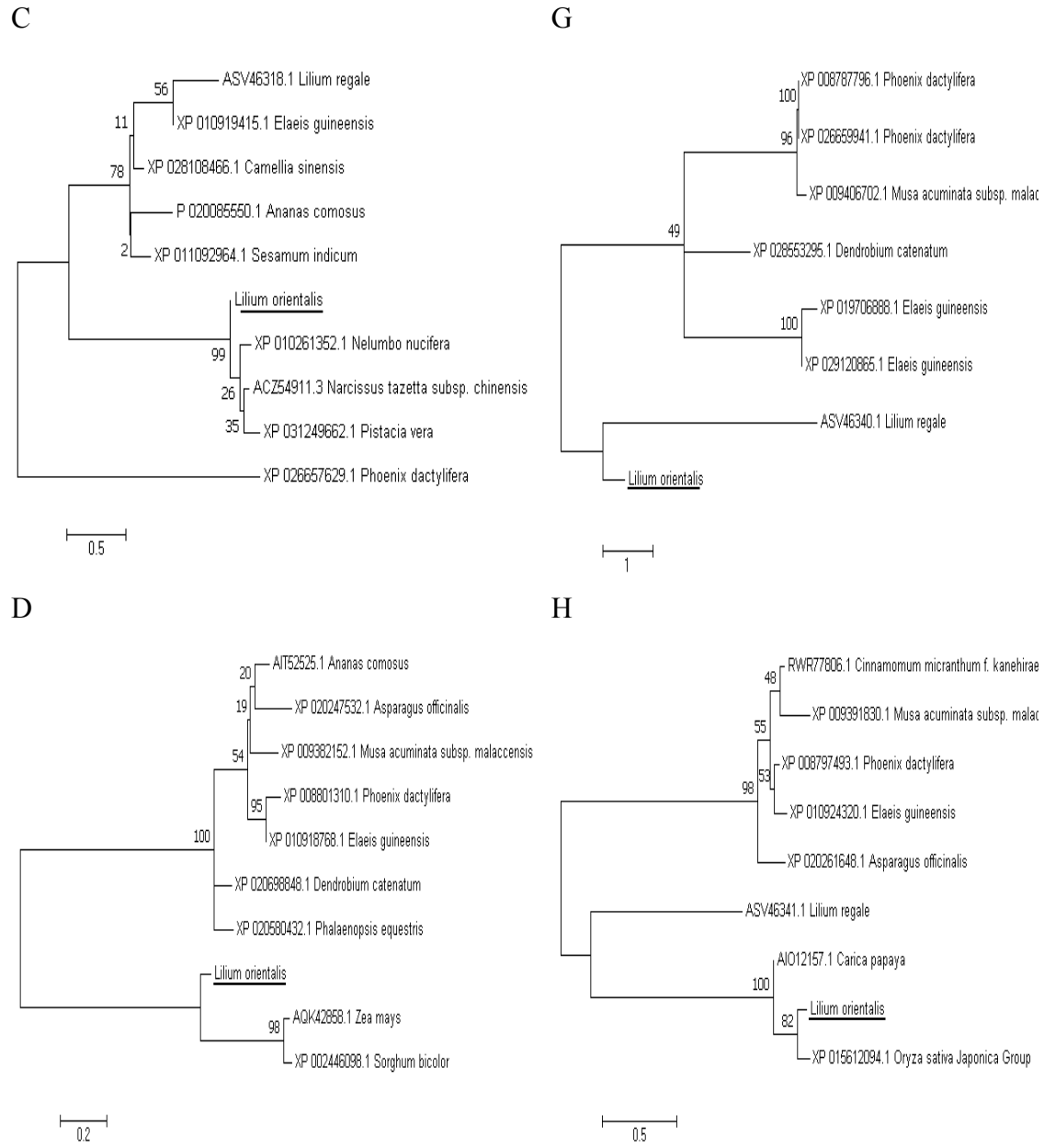


Figure 6.3. Dendrograms of *LoBZR1* (A), *LoACO1* (B), *LoACS1* (C), *LoETR2* (D), *LoETR3* (E), *LoCTR1* (F), *LoEIN2* (G), and *LoEIN3* (H) genes from *Lilium orientalis* with orthologs from other plants species. The evolutionary trees were inferred using the Maximum likelihood method in MEGA software (version 7). The percentage of trees in which the associated taxa clustered together is shown above the branches.