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Molecular Approaches to Control Ethylene Response for Improving Shelf Life in Flowers : A Review

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ABSTRACT

"One of the most important characteristics of ornamental plants is flower shelf life. In some plant species, ethylene plays a critical role in flower senescence. Genetic modification targeting genes for ethylene biosynthesis or signalling has improved flower shelf life in several species that exhibit ethylene-dependent flower senescence. Although little is known about the regulatory mechanisms of ethylene-independent petal senescence in flowers, a recent study of Japanese morning glory revealed that EPHEMERAL1 (EPH1), a NAC transcription factor, is a key regulator in ethylene-independent petal senescence. Regardless of the ethylene signal, EPH1 is induced in an age-dependent manner, and suppressing EPH1 expression significantly delays petal senescence. Comprehensive transcriptome analyses of ethylene-dependent petal senescence revealed the involvement of transcription factors, a basic helix-loop-helix protein and a homeodomain-leucine zipper protein, in the transcriptional regulation of the ethylene biosynthesis enzymes. This review summarises molecular aspects of flower senescence and discusses molecular breeding strategies to improve flower shelf life."

Keywords Ethylene, Flower, Programmed cell death, Senescence, Transcription factor.

INTRODUCTION

The shelf life of flowers seems to be an important determinant of the quality of commercial flowers. Consumer value long-lasting flowers, and the distribution industry wishes to reduce deterioration in flower quality throughout the distribution chain. Several techniques have been developed to extend the life of some cut flowers but not many others. To improve inflorescence shelf life, it is necessary to understand the physiology and molecular biology of flower senescence.

Flower shelf life differs between plant species.

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Morning glory flowers, for example, wilt in one day, whereas Phalaenopsis flowers remain open for months. Flower shelf life is thought to be closely related to flowering plant reproductive strategy, as it is an important factor in attracting pollinators (Primack 1985). Flower life in some plant species is terminated by the abscission of flower parts in the presence or absence of petal senescence (Van Doorn 2001). I use the term 'flower senescence' to refer to both petal senescence and the abscission of flower parts in this context. This review focuses primarily on petal senescence. Petal senescence is a type of programmed cell death (PCD), a tightly controlled developmental process (Pennell and Lamb 1997, Rogers 2006, Shibuya and Ichimura 2016, van Doorn and Woltering 2008). In several plants, treatment with cycloheximide, which inhibits protein synthesis, delays petal senescence, indicating that petal senescence is an active process (Shibuya and Ichimura 2016). Because petal senescence is a genetically programmed developmental process, both molecular and traditional breeding could improve flower shelf life. Several reviews on flower senescence have been published (Rogers 2013, Scariot *et al*. 2014, Shahri and Tahir 2014, Shibuya and Ichimura 2016, Shibuya *et al*. 2016, van Doorn and Woltering 2008).

Ethylene response of cut flower

Flower senescence patterns can be classified based on how ethylene is involved: Ethylene dependent and ethylene independent. An autocatalytic increase in endogenous ethylene production causes petal senescence in flowers of plant species with ethylene-dependent senescence (Shibuya 2012, Woltering and van Doorn 1988). In general, inhibiting ethylene biosynthesis or perception delays flower senescence, whereas exogenous ethylene treatment accelerates flower senescence. Other plant species, on the other hand, appear to have little effect on flower senescence when exposed to ethylene (Shibuya 2012, Woltering and van Doorn 1988). Flowers with ethylene-independent senescence typically produce very little ethylene during flower senescence. Treatment with ethylene inhibitors has no effect on flower shelf life and exogenous ethylene has no effect on flower senescence. There are intermediate or mixed patterns of senescence in addition to ethylene-dependent and ethylene-independent senescence (Shibuya 2012). Flowers of Campanula, for example, exhibit ethylenein-dependent senescence in the absence of pollination; however, once pollinated, these flowers begin producing ethylene, resulting in accelerated petal senescence (Kato *et al.* 2002). In flowers of *Mirabilis jalapa* (four-o'clock), endogenous ethylene has little effect on petal senescence but application of exogenous ethylene accelerates it (Xu *et al.* 2008).

Genes involved in ethylene biosynthesis

The ethylene biosynthetic pathway in plants has been characterized, and genes encoding key enzymes have been isolated (Kende 1993, Lin et al. 2009, Yang and Hoffman 1984). Ethylene is synthesized through the following pathway: L-methionine → S-adenosyl-L-methionine → 1-amino-cyclopropane-1-carboxylic acid (ACC) → ethylene. The last two reactions are catalyzed by ACC synthase and ACC oxidase.

ACC synthase (ACS) and ACC oxidase (ACO) are encoded by multigene families and genes encoding these enzymes have been isolated from many ornamental plant species (Shibuya and Ichimura 2016). ACS genes have been isolated, for example, from carnation (Henskens *et al.* 1994, Jones and Woodson 1999, Park *et al*. 1992), geranium (Wang and Arteca 1995), *Phalaenopsis* (Bui and O'Neill 1998), petunia (Lindstrom *et al.* 1999), rose (Wang et al. 2004), snapdragon (Woltering *et al*. 2005), morning glory (Frankowski *et al*. 2009), tree peony (Zhou *et al*. 2013), and *Oncidium* (Shi and Liu 2016). ACO genes have been isolated, for example, from carnation (Tanase *et al.* 2012, Wang and Woodson 1991), *Phalaenopsis* (Nadeau *et al.* 1993), petunia (Tang *et al.* 1993), geranium (Clark *et al.* 1997), snapdragon (Woltering *et al.* 2005), tulip (Momonoi *et al.* 2007), rose (Xue *et al*. 2008), tree peony (Zhou *et al.* 2013), and morning glory (Wilmowicz *et al*. 2014).

ACS and *ACO* genes are differentially regulated in a spatial and temporal-specific manner. In carnation, for example, of the three ACS genes, *DcACS1* is most abundant in petals while *DcACS2* and *DcACS3* are preferentially expressed in styles (Jones and Woodson 1999). Differential expression

of *ACO* genes has also been reported in petunia (Tang *et al.* 1994).

The regulatory mechanisms of the *ACS and ACO* genes during flower senescence are still poorly understood. These genes are regulated by two transcription factors (TFs), homeodomain–leucine zipper (HD-Zip) and basic helix–loop–helix (bHLH). PhHD-Zip, an HD-Zip TF gene, was found to be up-regulated during petal senescence, and PhHD-Zip suppression via virus-induced gene silencing significantly increased flower shelf life in petunia (Chang *et al.* 2014). PhHD-Zip silencing reduced ethylene production as well as the abundance of ACO1, ACO4, and ACS transcripts.

Transgenic approaches to improve flower shelf life by manipulating ethylene biosynthesis and responses

Transgenic techniques targeting genes involved in ethylene biosynthesis and signal transduction have been isolated from several ornamental plants, and flower shelf life can be improved by targeting those genes. Savin *et al.* (1995) used an anti-sense method to create transgenic carnation that suppressed ACO expression in the 1990s. Ethylene production was reduced in the transgenic carnation, and petal senescence was clearly delayed. Untransformed carnation flowers had a vase life of about 5 days from harvest to petal wilting, whereas flowers from transgenic plants had a vase life of 8 to 9 days at 21°C. Transgenic plants with reduced ACS or ACO expression were found to have longer flower shelf life in petunia (Huang et al. 2007a) and torenia (Huang *et al.* 2007b) (Aida *et al*. 1998). Inhibiting ethylene perception, like chemical approaches, is a more efficient way to extend flower life. The introduction of a mutated ethylene receptor gene, such as Arabidopsis etr1-1, is an especially appealing strategy because even a single genetic manipulation can confer ethylene insensitivity in a wide range of heterologous plant species (Wilkinson *et al.* 1997).

This strategy has been used to extend flower shelf life in several ornamental crops, including Campanula (Sriskandarajah *et al.* 2007), carnation (Bovy *et al.* 1999), Kalanchoe (Sanikhani *et al.* 2008), Nemesia (Cui *et al.* 2004), petunia (Wilkinson *et al.* 1997), and torenia (Tanase *et al*. 2012). Nonpollinated flowers in wild-type petunia, for example, last 6.7 days on average, whereas flowers in transgenic plants harbouring etr1-1 last 16.6 days on plants grown at day/night temperatures of 26/21°C (Gubrium *et al.* 2000). In addition to ethylene receptors, suppression of ethylene signalling components such as EIN2 and EIN3 has increased the life of petunia flowers (Shibuya *et al.* 2004, Shibuya and Clark 2006). Aside from ethylene biosynthetic enzymes and signalling components, ectopic expression of Arabidopsis FYF has been shown to delay petal senescence in Eustoma grandiflorum by suppressing ethylene responses (Chen *et al.* 2011). Petunia petal senescence is also delayed when PhHD-Zip and PhFBH4 are suppressed (Chang *et al.* 2014, Yin et al. 2015).

Non pollinated wild-type flowers have a lifespan of about 7 days, whereas suppressing PhFBH4 by expressing the antisense PhFBH4 fragment increases flower shelf life to about 9 days at day/night temperatures of 25/20°C (Yin *et al.* 2015).

It should be noted that physiological side effects in transgenic plants with reduced ethylene sensitivity may limit their commercial use. Transgenic petunia, for example, exhibits inhibited adventitious root formation and a high percentage of premature death (Clark *et al.* 1999, Shibuya *et al.* 2004). These negative side effects are most likely caused by constitutive ethylene insensitivity in transgenic plants, and the key to avoiding them is to use a tissue-specific promoter. Floral Binding Proteini (FBP1) is a petunia floral organ identity gene that is only expressed in petals and stamens (Angenent et al. 1992). With minimal side effects, the FBP1 promoter has been used to control the expression of the etr1-1 gene in transgenic carnation (Bovy *et al.* 1999), Campanula (Sriskandarajah *et al*. 2007), and Kalanchoe (Sanikhani *et al.* 2008). Recently, the InMYB1 promoter from Japanese morning glory was found to function as a petal-specific promoter in a variety of dicots, including Eustoma, chrysanthemum, carnation, Japanese gentian, and stock (Azuma *et al*. 2016). Similarly to the FBP1 promoter, this promoter could be used to improve flower shelf life.

Roles of NAC TFs in the regulation of ethylene-independent petal senescence

Ethylene has little effect on the regulation of petal senescence in some plant species, including lily,

Table 1. Examples of transgenic ornamental plants with prolonged flower longevity.

Plant species	Gene construct	Expression	Reference
Suppression of ethylene biosynthesis			
Carnation (*Dianthus caryophyllus*) "Scania and white sim"	ACO (*D. caryophyllus*)	Silencing (Antisense)	Savin *et al*. (1995)
Carnation (*Dianthus caryophyllus*) "Nora"	*ACO (D. caryophyllus)* CaMV 35S promoter	*Silencing (Sense)*	*Kosugi et al. (2000)*
Petunia (*Petunia hybrida* Hort. Vilm.-Andr.)	ACS/ACO (*Brassica oleracea*) CaMV 35S promoter	Silencing (Antisense)	Huang *et al.* (2007a)
Torenia (Torenia fournieri) 'Crown Mix', 'Crown Blue', and 'White'	ACO (*T. fournieri*) CaMV 35S promote	Silencing (Sense, Antisense)	Aida *et al.* (1998)
Suppression of ethylene signalling			
Campanula (*Campanula carpatica*) 'Blue Uniform'	etr1-1 (*A. thaliana*) Petunia FBP1 promoter	Ectopic Ectopic	Sriskandarajah *et al*. (2007) Bovy *et al.* (1999)
Carnation (*Dianthus caryophyllus*) 'Lena'	etr1-1 (*A. thaliana*) CaMV		
Kalanchoe (*Kalanchoe blossfeldiana*) Debbie'	etr1-1 (*A. thaliana*) Petunia FBP1 promoter	Ectopic	Sanikhani *et al.* (2008)
Nemesia (*Nemesia strumosa*) "genotype White	Cm-ETR1/H69A (*Cucumis melo*) CaMV 35S promoter	Ectopic	Cui *et al.* (2004)
Petunia (*Petunia hybrida* "'Mitchell Diploid"	etr1-1 (*A. thaliana*) CaMV 35S promoter	Ectopic	Wilkinson *et al.* (1997)
Altered expression of transcription factors			
Eustoma (*Eustoma grandiflorum*)	FYF (*A. thaliana*)	Ectopic	Chen *et al*. (2011)
Japanese morning glory (*Ipomoea nil*) 'Violet'	EPH1 (*I. nil*) CaMV 35S promoter	Silencing (RNAi)	Shibuya *et al.* (2014)
Petunia (*Petunia hybrida*) 'Primetime Blue'	PhHD-Zi (*P. hybrida*)	Silencing (VIGS)	Chang *et al.* (2014)

tulip, chrysanthemum, iris, and gladiolus (Woltering and van Doorn 1988, Table 1). Exogenous ethylene treatment does not accelerate petal senescence in these flowers, and chemical inhibition of ethylene biosynthesis or perception does not delay senescence. As a result, petal senescence in these flowers is thought to be regulated by an ethylene-independent pathway.

Using differential screening and microarray analysis to identify genes that regulate PCD during petal senescence, researchers discovered upregulation or downregulation of numerous genes in several plant species, including Hemerocallis (Panavas *et al.* 1999) and Iris (Panavas *et al.* 1999). (van Doorn 2003). However, no cell death-specific genes have yet been identified (van Doorn and Woltering 2008). The lack of effective transformation methods makes determining the function of isolated genes in these plant species difficult.

Because chemical inhibition of ethylene biosynthesis or perception does not delay petal senescence in Japanese morning glory 'Violet,' it is thought to be regulated independently of endogenous ethylene (Shibuya 2012, Shinozaki *et al.* 2011, Yamada *et al.* 2006).

NAC TFs may also play a role in ethylene-dependent petal senescence regulation. Up regulation of NAC TFs in senescing petals has been observed in plants with ethylene-dependent senescence, such as Arabidopsis (Wagstaff *et al*. 2009), wallflower (Price *et al.* 2008), and petunia (Price *et al.* 2008), (Broderick *et al*. 2014). Multiple NAC TF genes were

found to be down regulated in ethylene-insensitive petals where etr1^{-1} expression was induced (Wang *et al.* 2013), implying that these NAC TF genes are regulated by an ethylene signal. Endogenous ethylene induced by pollination or stress may hasten the timing of up regulation of a NAC TF gene, resulting in accelerated petal senescence in ethylene-dependent petal senescence.

CONCLUSION

The role of ethylene in petal senescence has been thoroughly studied. Technically, long-lasting flowers can be produced in plant species that exhibit ethylene-dependent senescence by manipulating genes involved in ethylene biosynthesis or signalling. The most effective way to improve flower shelf life in these plants would be to introduce a mutated ethylene receptor gene, such as Arabidopsis etr1-1, under the control of a petal-specific promoter. The regulatory mechanisms of ethylene-independent petal senescence, on the other hand, have remained unknown. Through studies of Japanese morning glory, a NAC TF, EPH1, was recently identified as a key regulator of ethylene-independent petal senescence. NAC TFs may be a master regulator of PCD that integrates age-dependent (ethylene-independent) and ethylene-dependent signals because they are up regulated in senescing petals of ethylene-dependent species. More research is needed to determine whether NAC TFs are commonly involved in the regulation of petal senescence in both ethylene-independent and ethylene-dependent species.

The emergence of efficient genome editing systems such as CRISPR/Cas 9 has ushered in a new era in ornamental plant molecular breeding. Despite the fact that transgenic ornamental plants with extended flower shelf life have been produced since the 1990s, commercialization has been a challenge. The cost and public acceptance of genetically modified plants could be a barrier. Because it is possible to create knockout mutants for targeted genes that do not harbor transgenes, genome-editing techniques have the potential to change this situation. This technique would be especially effective for a gene that only plays a role in a single phenomenon, because knocking out the gene would not result in undesirable side effects. Information on genome sequences is accumulating in ornamental plant species including carnation (Yagi *et al.* 2014), orchid (Cai *et al.* 2015), petunia (Bombarely *et al.* 2016), Japanese morning glory (Hoshino *et al.* 2016), and sunflower (Badouin *et al.* 2017). Future work will reveal new gene targets of molecular breeding for improving flower shelf life.

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