Characterization and Expression of the Polyamine Biosynthetic Genes from Senescing Carnation Flowers

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1. Floral senescence

Petals and other floral organs are derived from leaves and share common biochemical processes during senescence. Both leaves and flowers exhibit a combination of mobilization, wilting, and abscission during senescence. One difference between these organs is the ability of pollination ti trigger floral senescence. In leaves, stress-induced ethylene can induce a similar rapid deterioration and abscission of the leaf.

Not all flowers use an increase in ethylene production as the signal indicating the end of their functional life. In some cases, externally supplied ethylene does not induce floral senescence. Blossom longevity is controlled by at least two mechanisms. The primary one is aging, the time-dependent developmental program controlling longevity. The second is enhanced ethylene production. Floral senescence followed the rise in ethylene production from many species.

One of the first reports of ethylene-enhanced floral senescence was made with carnation. The carnation has remained a useful model for floral senescence studies because of its well-defined stages of development and economical importance. The sensitivity of carnations to ethylene has been shown to depend on their maturity. Immature carnations (closed blossoms with petals showing through the calyx) did not respond to ethylen treatment while mature flowers did. An ethylene and respiratory climacteric has been associated with floral senescence. It is established that the anticipated sequence of ethylene-generating enzymes participate in the autocatalytic rise in ethylene production. An increase in 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, ACC, ACC oxidase, and ethylene production has been shown to precede the onset of senescence of carnation and other flowers.

Floral senescence can be delayed by treating blossoms with inhibitors of ethylene synthesis and ethylene action. The large number of references which exist on this topic reflects the commercial interests in compounds which can be used to extend vase life. Inhibitors of ethylene synthesis have demonstrated antisenescence activity in flowers. Similarly, inhibitors of ethylene action have extended vase life of numerous species. Ethylene action can also be inhibited by treating flowers with hormones which retard aging in plants. This antisenescent action of auxins, gibberellins, and cytokinins has been found to delay floral senescence.

The senescence of flowers has been described by sleepiness, blasting, wilting, and abscission. Sleepiness is a term normally ascribed to carnations, indicating an inhibition of floral development or reclosing of open flowers. Normal senescence of flowers invloves a combination of perianth inrolling, wilting, and abscission. Other organs such as stamens and styles will also senescence. Petal senescence

is associated with a series of physiological and biochemical changes. These include an increase in hydrolytic enzymes, degradation of macromolecules, increased respiratory activity and a loss of cellular compartmentalization.

2. Interrelationship between ethylene and polyamines

Polyamines form a class of aliphatic amines that are ubiquitous in living organi는, and have been implicated in a wide range of biological processes, including plant growth and development. The major forms are putrescine, spermidine and spermine. Putrescine can be produced directly from ornithine by the action of ornithine decarboxylase (ODC), or indirectly from arginine by arginine decarboxylase (ADC). S-adenosylmethionine decarboxylase (SAMDC) probably is the-rate-limiting activity that provides the aminopropyl moiety that is used by spermidine and spermine synthase, respectively, to convert putrescine to spermidine and spermine (**Fig. 1**). S-adenoslymethionine (SAM) is also a precursor in ethylene biosynthesis thus, increases in polyamine biosynthesis, particulary via SAMDC activity, are likely to affect the rates of ethylene synthesis (**Fig. 1**). Because polyamine concentrations are much higher than those of both ACC and ethylene, changes in polyamines are more likely to affect ACC and ethylene biosynthesis than vice versa.

Polyamines delayed the senescence of cut carnation flowers and reduced ethylene production, endogenous ACC content, and the activities and transcript amounts of ACC synthase, especially senescence-related one (*pCARACC3*), and ACC oxidase. However, methylglyoxal bis-(guanylhydrazone) (MGBG), an irreversible inhibitor of SAMDC, elevated ethylene production, increased activities and amounts of transcripts for ACC synthase and ACC oxidase, and shifted the climacteric pattern of ethylene production ahead by 1 day (**Fig. 2**). By comparing ethylene prduction with the changes of endogenous polyamine levels from control and MGBG- or spermine-treated petals during flower senescence, it was suggested that endogenous polyamines possibly suppress ethylene production.

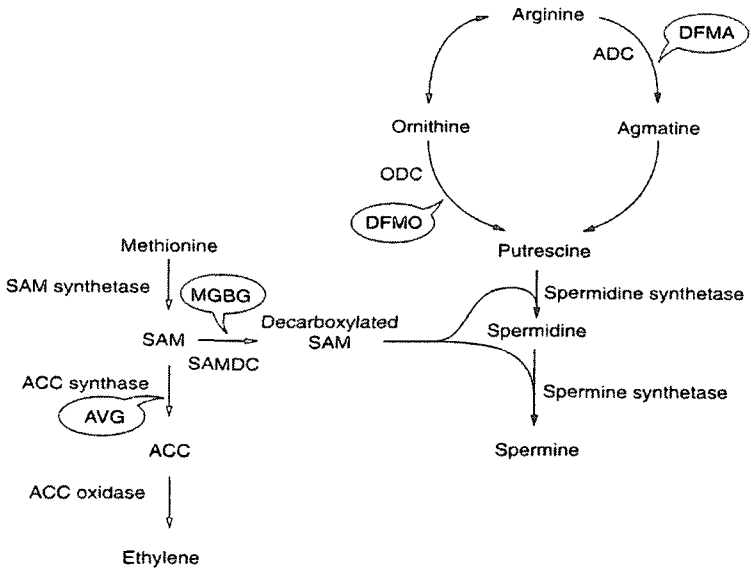

Fig. 1. Polyamine and ethylene biosynthesis pathways and their interrelationships. Inhibitors are indicated in bubbles at their target sites. Abbreviations: SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; MGBG, methylglyoxal-bis-guanyl hydrazone; SAMDC, *S*-adenosylmethionine decarboxylase; ADC, arginine decarboxylase; DFMA, DL-α-difluoromethylarginine; ODC, ornithine decarboxylase; DFMO, DL-α-difluoromethylornithine.

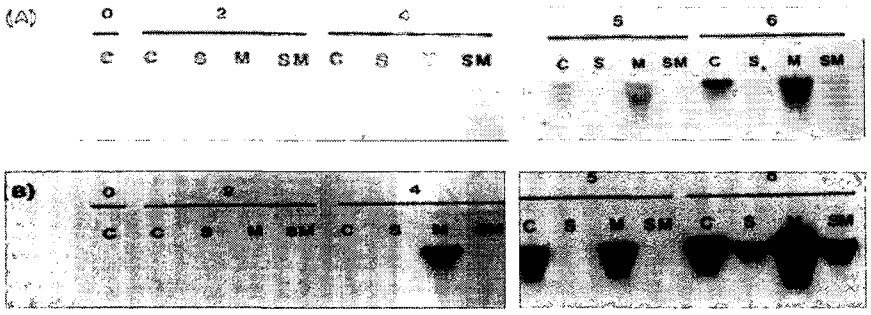

Fig. 2. Accumulation of ACC synthase and ACC oxidase transcripts in the petals of carnation flowers that were held with their stems in deionized water (C), 1 mmol/L spermine (S), 1 mmol/L MGBG (M), or both 1 mmol/L spermine and 1 mmol/L MGBG (SM) through 6 days after anthesis. (A); Senescence-related ACC synthase transcript; (b) ACC oxidase transcripts. Numbers indicate the incubation day after harvest. Twenty micrograms of total RNA were loaded per lane.

3. Polyamines in developmental processes

At cellular pH values, these compounds behave as cations, and can interact with anionic macromolecules such ac DNA, RNA, phospholipids and certain proteins. There is direct evidence that they are essential for growth and development in procaryotes and eucaryotes. Indeed, the use of specific biosynthesis inhibitors has led to claims for a causal relationship between changes in endogenous polyamines levels and growth responses. and their role in the regulation of plant growth and development. A variety of mechanism maintain tight regulation and prevent excessive accumulation of polyamines in many cells and tissues. These include regulation of gene expression, enzymatic activity at several steps in polyamine biosynthesis, enzyme stability, and polyamine degradation and excretion. The relative functions of the two pathways, ODC and ADC, are one of the important questions in polyamine biosynthesis. In general, it has been suggested that changes in ODC activity may regulate cell division in actively growing tissues, whereas ADC may regulate cell extension and secondary metabolic processes. ADC activity levels have been correlated with many abiotic stresses in plants. Putrescine accumulation was stimulated in many stresses such as osmotic, pH, nutrient, UV light, and pollutant.

In some plants, the internal polyamine concentration changes dramatically with other reproductive activity such as normal floral organ morphogenesis, fruit growth, and fruit ripening in particular plant species. In carnations, expression of ADC was also much higher in the reproductive organs of the petal, and was regulated by an organ-specific manner at a transcriptional level. Therefore, polyamines may be essential members of the array of internal components required for floral organ development related to reproductive activity.

Currently, the control of turnover of polyamines by polyamine and diamine oxidase is little understood. One class of plant enzymes that catabolize extracellular di- and polyamines are the copper-containing amine oxidases. Plant copper amine oxidases generally catalyse the oxidation of putrescine at the primary amino group to give 4-aminobutryladehyde, which spontaneously cyclizes to pyrroline, ammonia and hydrogen peroxide (H_2O_2). High activity has been reported in the middle lamella region of pea seedling roots, in the vascular parenchyma cells in the pea epicolty and in lignified tissue. It has been suggested that H_2O_2, generated by oxidation of di- and polyamines, is important in lignification and cross-linking of extensins during normal growth and in response to stress and wounding.

4. Polyamine and ethylene biosynthesis during flower senescence

When cut carnation flowers incubated in water from anthesis to senescence, ethylene production was below detection until the 3rd day, but rapidly increased thereafter to the 6th day and then decreased on 7th day. The levels of spermidine and spermine reached maximum at the 4th day, which increased by about 160% and 140%, respectively, compared with the levels at the starting point of incubation. The levels of spermidine and spermine decreased concurrently with the increase of ethylene production.

To determine the pattern of ADC and SAMDC activity during flower development, we measureed ethylene production and polyamine contents from carnation petals at seven stages (A~ G stage), i. e. bud, opening flower, immature flower, anthesis flower, flower after 3 days of anthesis, flower after 6 days of anthesis, and flower after 9 days of anthesis (**Fig. 3(A)**). Stage A, bud, is the time when the

sepal is not separated along their margin, and stage F is in climacteric flower for ethylene biosynthesis. ADC enzyme activity increased continuously during flower development from a flower bud and reached a peak 6 days after anthesis (Stage F) (**Fig. 3 (B)**). SAMDC activity detected from Stage A, and then gradually decreased to Stage C (**Fig. 3 (C)**). However, after Stage C, SAMDC activity rapidly increased and peaked at Stage E. These increases in polyamine biosynthesis can be responsible for cell expansion in the petals during flower development. An increase in ethylene production was evident from Stage F and peaked at Stage G, when petals showed in-rolling and slight wilting which were due to water stress. An increase in ADC activity at flower senescence induced by water stress may function as the maintenance of cell activity in senescing petals.

Diamine oxidase preferentially oxidaxes putrescine with production of hydrogen peroxide, a signal molecule and subrate for peroxidases. In both vascular tissue and the root cap of Arabidopsis, diamine oxidase gene expression occurs in cells destined to undergo programmed cell death (PCD). During flower senescence, increases in polyamine contents can due to increase of diamine oxidase activity. Reactive oxygen species (ROS) are key triggers of PCD during hypersensitive response and it is possible that ROS represent a general PCD trigger. A tightly regulated mechanism must exist for triggering PCD in cells destined to follow this developmental pathway. It is tempting to speculate that diamine oxidase may play a dual role in PCD by producing H2O2 and by reducing di- and polyamine levels in or around these cells. Recently, it has been shown that amines can induce ROS production by acting as substrates for amine oxidase in tobacco. Local production of H2O2 in a directed manner could intiate PCD and stimulate local protective anti-oxidant genes in surrounding cells. Reducing di- and polyamine levels can also lead to cellular instability in cells destined ti die, for example putrescine strongly counteracts nuclear fragmentation.

(A) (B) (C)

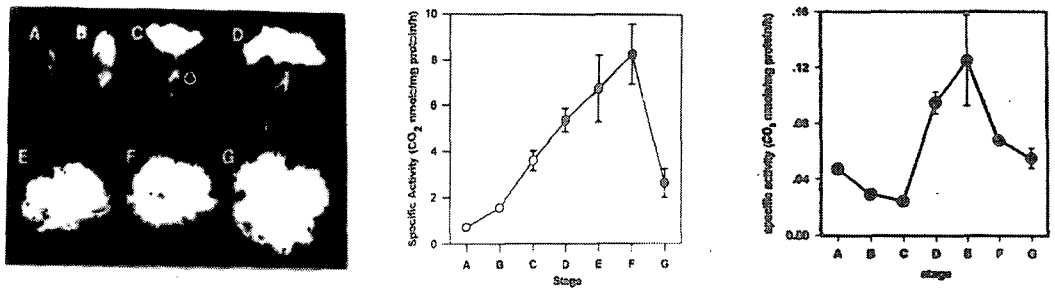

Fig. 3. ADC and SAMDC activity during flower senescence. Flowers were harvested at seven stages (A~G), i.e. bud, opening flower, immature flower, anthesis flower and flower after 3, 6 and 9 days of anthesis. (A) Picture of flowers at seven different stages. (B) The activity of ADC enzyme at various stages. (C) the activity of SAMDC enzyme at varous stages.

5. Cloning and characterization of cDNA and genomic DNA for ADC and SAMDC from senescing carnation flower

From a carnation, we recently isolated ADC genes of two cDNA clones, pCARADC5 and pCARADC8, and one genomic clone, gCARADC8, in which the 5′-UTR was 512 nt long, the 3′-UTR was 268 nt long, and the 5 franking region was 4,207 nt long. The unusually long 5′-UTR of this ADC gene contained a short uORF of 7 amino acids (MQKSLHI) at position 91-114. Only two reported ADC genes from peas and carnations had an uORF in 5′-UTR. Low homology was found in these uORFs of pea and carnation ADC genes. We thought the other ADC genes isolated so far were not enough in length to cover the uORF region.

0Also, we have isolated and sequenced two different SAMDC cDNA clones from carnation petals. The nucleotide sequences of CSDC9 and CSDC16 show 78.3 % identity, and the deduced amino acid sequences of them show 81.7 % identity and 86.5 % similarity. Carnation SAMDC cDNAs have the long transcript leaders of 472 bp and 502 bp for CSDC9 and CSDC16, respectively. CSDC9 has a upstream open reading frame (uORF) of 54-amino acid from residues 152 to 317 and CSDC16 has that of 52-amino acid from residues 156 to 314 in each 5′ -untranslated region. The nucleotide sequences of uORFs in CSDC9 and CSDC16 were identical 89.9 %.

Several ODC genes from human and rat have also uORF, the amino acid sequence of whose was very homologous. However, it is not reported yet that plant ODC gene has uORF, because all plant ODC genes from tobacco and tomato are too short for uORF.

0Taken together, polyamine biosynthetic genes have in generally uORF in their leader sequence. Probably uORF can make an important function.

In an immunoblot experiment, an ADC antibody raised against the purified ADC protein detected two bands of 45- and 33-kDa in a petal extract. This result suggested that a full-length of the 78-kDa polypeptide precursor converted into two polypeptides of molecular weights about 45- and 33-kDa during processing reaction. In vitro transcription/translation experiments showed that the proenzyme of both cDNAs of SAMDC were converted to two polypeptides of the large subunit (calculated 31,544 Da and 32,537 Da, respectively) and the small subunit (calculated 9,704 and 9,041 Da, respectively) after 20 min of translation. We detected a small fragment of 9 kDa in autoradiography. From in vitro transcription/translation, it is suggested that the processing rapidly occurs during translation of protein, and that the processing still occurs after translation but very slowly. The processing of carnation SAMDC enzyme is not stimulated by $Mg2^{+}$ or putrescine.

6. An uORF in its leader sequence may function as a safety machinery in polyamine biosynthesis and give a specificity for different inducer and organ

The scanning model for translational initiation predicts that the translational initiation site is dictated by the location and sequence context of the initiation codon (the scanning model of translation). The 40S preinitiation complex binds to the 5 cap-proximal region of mRNA and migrates downstream until it encounters an AUG in a favorable context. At this point, the 60S ribosomal subunit binds to the

complex, and protein synthesis commences. Most eukaryotic mRNAs have a short 5′-UTR without stable secondary structures and no upstream AUGs. These features enable the preinitiation complex to efficiently scan the 5-region for translation start sites. However, complex 5′-UTR with upstream AUGs and/or the ability to form stable secondary structures are found in about 5% of eukaryotic genes. Strikingly, most of these genes include homeobox genes, proto-oncogenes, transcription factors, and signal transduction components (**Table.1**).

When the second in vitro transcription/translation reactions of *pTnT-Full* were performed with the translated products from the first reaction of *pTnT-Full*, the translation of ADC was almost completely inhibited. It was presumed that the uORF protein was included in the translated products from *pTnT-Full*. This phenomenon showed in the reaction with *SAMDC* gene. The treatment with synthetic heptapeptide MQKSLHI, which was based on the nucleotide sequence of uORF in *gCARADC8*, significantly decreased the amount of translated products from an in vitro transcription/translation of *pTnT-dL*. This heptapeptide also decreased the translation of the luciferase gene in vitro.

We made several constructs of plasmid-acrried fusions of the *gCARADC8* promoter and leader to *gus A*, which contain various leader sequence and promoter, for transforming tobacco plants. In transgenic tobacco, the highest GUS activity get from the construct which did not contain an uORF. However, the leader sequence except for uORF was needed for GUS activity.

These results are suggesting two possibilities. One possibility is that the translated product from a uORF may inhibit the translation of a downstream major ORF of ADC *in trans*. The other possibility is that the translated product of uORF blocked the some step of translational machinery. However, we are doubt about second possibility, because there are not present the sequence homology among the uORFs from the genes for different proteins (**Table 1.**).

The translation of certain uORFs can inhibit translation of the downstream ORF by translation attenuation, and in specific examples, inhibition depends on the amino acid sequence of the uORF encoded peptide. If the translation stopped at stop codon of uORF, the efficiency of reinitiation of translation at downstream start site is usually lowered. Therefore, uORF might function as a translation inhibitor.

It is interesting to note that a small proportion of cellular mRNAs including several proto-oncogenes contain uORFs in their leader regions. The uORF deletions of proto-oncogenes may cause oncogenic conversion. It is quite possible that transcriptional regulation for such genes is not sufficient for a fine control of their expression, and that at the translational level a particular cellular status may be better censored. Therefore, an in vivo modulation of expression in response to some environmental or developmental signals may be involved in the translational regulation of uORF. An activation of the ADC pathway leading to high levels of endogenous putrescine is toxic for the vegetative growth of plants.

The constructs of plasmid-carried fusions of the *gCARADC8* promoter and leader to *gus A* with or without uORF used for transforming tobacco plants. At GUS staining experiments after treatment with several inducers of polyamine biosynthesis such as sucrose and H_2O_2, the transgenic plants produced the highly expression of GUS in the case of the *GUS* construct with uORF, but lowered the GUS activity in that without uORF. Also, the GUS staining appeared at the reproductive organs of anther, stigma, petal and ovary, and the vegetative tissues of hypocotyls, in transgenic plants which contain the construct with uORF. However, if uORF was deleted from those constructs, GUS staining was disappeared from only anther. Therefore, the uORF may also be involved in translational control.

Table 1. Amino acid sequences of uORFs in their leader sequences from different genes

Genes	Species	Number	Sequences
Opaque-2	Maize	1 (3 aa) 2 (21 aa) 3 (20 aa)	MGA MPPTTHHIYGSLPLHCISFNS MGPSPCTASPSIPSVCFSLP
H^+ATPase	Tobacco	1 (5 aa)	MVFLI
Lc	Maize	1 (38 aa)	MEVLALLRCFSSFFLLRLSSIRMPLVRRFTRHRLMI SR
SAMDC	Carnation	1 (54 aa)	MESKGGKKKSSSSSSSSTKSFFAPLGYSIEDLRPK GGIKKFRSAAYSNCARKPS
ADC	Carnation	1 (7 aa)	MQKSLHI
ODC	Human	1 (10 aa)	MGLACGAWAL
Phytochrome	Moss	1 (17 aa) 2 (9 aa)	MKEFSSTSRSLMIVGIY MEEEEDCVP
bZIP	*Antirrinum*	1 (25 aa) 2 (11 aa) 3 (2 aa)	MMLMRRVRVVHSFSVVFLYWFYVFS MFSHELTSIIA MN

7. Toxicity of polyamines at unusually high concentration in trangenic plants

High levels of polyamine biosynthetic genes in transgenic plants might be lethal to plant cells undergoing regeneration as part of the transformation process. For example, potential overexpression of a homologous SAMDC gene in potato has been reported to be lethal They made the sense construct with SAMDC gene without uORF.

The Tet repressor system has been used to regulate expression of the gene in a sense and an antisense orientation. These experiments also displayed increases in levels of SAMDC activity, spermidine, spermine, and intriguing, putrescine.

However, in our system the carnation SAMDC gene with uORF was used for making sense construct for transforming tobacco. The transgenic tobacco was very healthy, and did not show any difference from wild type. The height of oveexpressing transgenic tobacco was a little bit higher than wild type. Therefore, it is suggesting that uORF of SAMDC gene regulated its own capability of spermidine biosynthesis against the high levels of endogenous spermidine.

Some experiments recently evidence to support the concepts that; (1) polyamines or their oxidation products may be initiators of programmed cell death; (2) regulation of polyamine biosynthesis and uptake prevents the accumulation of toxic levels of polyamines; and (3) the antineoplastic effects of polyamine

analogues any be due to the induction of apoptosis in sensitive tumor cells. There has been considerable recent interest in the development of polyamine analogues as therapeutic agents. A series of polyamines with alkyl substituents on the terminal nitrogen atoms have been aynthesized, and many of these have been shown to have potential as anti-tumor agents. These mechanism by which these analogues causes the death of tumor cells is also not fully understood. It is likely that cells are unable to survive with highly elevated levels of polyamine themselves or of the polyamine analogues. This suggestion is consistent with a number of preliminary reports suggesting that accumulation of high levels of the normal polyamines resulting from overexpression of ODC gene led to apotosis with DNA fragmentation. Simarily, accumulation of spermidine with an amplification of ODC gene caused apotosis. When such ODC-overproducing cells were exposed to a hypotonic shock, the transport of exogenous spermidine was greatly increased and, because of the absence of feed-back repression, spermidine accumulated to toxic level. These studies provide support for contention that the powerful homeostasis regulation of the polyamine biosynthetic pathway, which is meadiated by effects on both the synthesis and degradation of key enzymes in polyamine synthesis excretion, may be essential to prevent the accumulation of polyamines to highly toxic levels unless sequestration of polyamines can take place.

It was recently reported in plants that diamine oxidase highly expressed in certain cells that undergo PCD. They showed that DNA fragmentation and cell death occur both in tracheary elements and in lateral root cap cells of Arabidopsis. ROS induced from putrescine by diamine oxidase are key triggers of PCD during senescence.

Therefore, maintenance of the appropriate intracellular concentration of polyamines has been shown to be an essential process in order for cells to proliferate and perform a variety of different functions. Depending upon the needs of the cell and the availability of extracellular polyamines, cells are able to regulate the intracellular concentrations of the polyamines by the enzymatic pathways responsible for their synthesis, interconversion, and degradation or by transport through the membrane in the form of uptake oe export.

Based on the interesting feature that polyamine biosynthetic genes, ADC, ODC, and SAMDC gene, have an uORF, this translational regulator may act as safety machinery for polyamine biosynthesis that will be stringently regulated in an appropriate levels.

8. References

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