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**Physiological and Molecular Characterization of
the Response to Ethylene during Senescence of
Carnation Genotypic Variants**

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Title

Physiological and Molecular Characterization of the Response
to Ethylene during Senescence of Carnation Genotypic Variants

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C. Abstract

The senescence of carnation (*Dianthus caryophyllus* L.) flowers is associated with increased production of the phytohormone ethylene, which in turn serves to initiate and regulate the processes involved in programmed petal death. We investigated the regulation of ethylene production and petal senescence in carnation. Several carnation genotypes were identified that exhibited extended vase-life in comparison to flowers from typical commercial cultivars. The capacity of these genotypes to produce ethylene during postharvest vase-life and to respond to exogenous ethylene was investigated. Several genotypes, represented by 'Sandrosa' and 87-37G produced little ethylene during their postharvest vase-life and as a result failed to exhibit the symptoms (in-rolling and wilting) typical of flowers producing elevated levels of ethylene. These genotypes were further separated by their capacity to respond to exogenous ethylene by both increased ethylene synthesis and premature petal senescence. In one case a genotype (799) was identified that was not capable of responding to exogenous ethylene by either increased ethylene production or premature petal senescence. The regulation of ethylene production during petal senescence was investigated both at the enzyme and gene levels. A full length cDNA was identified for the petal senescence-related ACC synthase gene. Utilizing this, and other ethylene biosynthetic pathway cDNA probes, an increase in both ACC synthase and ACC oxidase mRNAs were detected following ethylene treatment. An increase in ACC oxidase mRNA and enzyme activity was detected within 2-3 h following ethylene treatment, indicating the expression of this gene is an early response to ethylene. An investigation into the expression of novel proteins during petal senescence revealed a number of polypeptides increased in abundance and possibly play a role in the regulation or biochemical processes of senescence. One polypeptide of 70 kDa was identified as being encoded by the previously characterized gene SR12 and possibly represents a β -galactosidase involved in the remobilization of carbohydrates during senescence.

D. Project Objectives

The long range goals of our research as set out in the original proposal are to elucidate the molecular mechanisms involved in the initiation and regulation of petal senescence, and to determine how the phytohormone ethylene is involved in this developmental program. Our research focuses on the senescence of carnation flower petals as this represents a well developed model species for the investigation of ethylene-regulated senescence and is of significant commercial value. The specific objectives of this BARD project were as follows:

- To characterize the patterns of ethylene biosynthesis in cultivars and genotypes of carnation that exhibit increased petal longevity in comparison to 'White Sim' flowers.
- To determine the responsiveness of various carnation cultivars and genotypes to exogenous ethylene.
- To characterize the temporal and ethylene regulation of senescence-related genes in carnation flowers.

E. Body of the Report

Identification and Characterization of Senescence Variant Genotypes

Initially our research focused on the identification and characterization of carnation genotypes that exhibit increased petal longevity in comparison to "typical" carnation flowers. In the US lab, several genotypes were obtained from Dr. Roger Ulinger of the University of Nebraska. These represented selections that Dr. Ulinger had identified as having improved vase life and limited petal in-rolling symptoms. In our lab, the flowers of these plants were evaluated under controlled conditions for symptoms of senescence, including in-rolling of petals, irreversible wilting and necrosis. This analysis identified 3 genotypes that exhibited significant extension of flower vase life in comparison to 'White Sim' flowers (Brandt and Woodson, 1992; Appendix I). The vase life on one genotype (799) was more than twice that of 'White Sim'. In contrast to the senescence of 'White Sim' flowers, the extended vase life genotypes did not exhibit symptoms of petal in-rolling during senescence. Instead, the first symptoms of senescence in these flowers were

typically wilting and necrosis. In contrast to 'White Sim' flowers, the three extended vase life genotypes did not exhibit a dramatic increase in ethylene production during senescence. Furthermore, their senescence was not associated with increased ACC content or ACC oxidase activity as with 'White Sim' flowers. Taken together these results suggest that these genotypes are altered in their capacity to produce ethylene and this in turn results in a delay in senescence. When the flowers of these extended vase life genotypes were exposed to exogenous ethylene (1 μ L/L), 87-37G and 81-2 responded by producing increased ethylene and exhibiting premature symptoms of petal senescence, including in-rolling. In contrast, one genotype, 799, showed no obvious effects of the ethylene treatment. These ethylene treated flowers did not produce increased ethylene and remained attractive 8 days after exposure. These results indicate that the three extended-life genotypes examined likely represent different genetic mutations that affect the postharvest longevity of their flowers. Genotype 799 is impaired in its ability to respond to ethylene by increased ethylene production and premature petal senescence suggesting it might represent an ethylene-response mutant.

The Israeli laboratory examined the senescence behavior of flowers from the carnation cultivar 'Sandrosa' (Mayak and Tirosh, 1993; Appendix II). These flowers were found to be unusual in that they did not exhibit an autocatalytic increase in ethylene production nor did they develop symptoms typical of senescence such as petal in-rolling. However, unlike 799 these flowers were found to be responsive to exogenous ethylene, producing increased ethylene and exhibiting symptoms of premature senescence following exposure. Together with the genotypes identified in the US laboratory, these plants should provide excellent models for investigating the underlying processes of senescence in the presence and absence of ethylene.

Expression of Ethylene Biosynthetic Pathway Transcripts in Senescing Carnation Flowers

The senescence of carnation flower petals is associated with a substantial increase in the biosynthesis of ethylene. In order to begin to elucidate the molecular mechanisms underlying the developmental expression of this increased production of ethylene we set out to clone the genes encoding the enzymes in this biosynthetic pathway. Using a partial ACC synthase cDNA clone amplified from ripe tomato fruit RNA, we were able to identify a full length ACC synthase cDNA clone from a library prepared from senescing carnation petal mRNA (Park, Drory and Woodson, 1992; Appendix III). This cDNA, pCARACC3, encoded an ACC synthase based on amino acid homology with ACC synthases from other

plant species. This ACC synthase cDNA, along with cDNA clones for ACC oxidase and SAM synthetase, was used to investigate the expression of ethylene biosynthetic pathway transcripts in senescence carnation flowers. Both ACC synthase and ACC oxidase mRNAs increased in abundance concomitant with the increase in ethylene production associated with petal senescence (Woodson et al., 1992; Appendix IV). This increase could be prevented by treatment with the ethylene action inhibitor 2,5-norbornadiene. Further, treatment of presenescent flowers with ethylene resulted in increased expression of both ACC synthase and ACC oxidase mRNAs. These results indicate that the autocatalytic regulation of ethylene during petal senescence is at least partly due to the expression of genes encoding both ACC synthase and ACC oxidase. An examination of the spatial regulation of these genes within carnation petals revealed that the expression of ACC synthase was confined to the basal portion of the petal, while ACC oxidase mRNAs could be detected in both the basal and upper portions of petal tissue (Drory, Mayak and Woodson, 1993; Appendix V). This result was consistent with previous research that indicated the basal portion of petals was responsible for the synthesis of ACC and that transport of ACC to the upper petal portion led to the increase in ethylene from this part of the petal.

Regulation of Autocatalytic Ethylene Biosynthesis in Carnation Flower Petals

The increase in ethylene biosynthesis associated with the senescence of carnation petals is thought to be regulated autocatalytically, that is ethylene feeds back to promote its own synthesis. Such an auto-enhancement of the ethylene biosynthetic pathway is common among ripening fruits and senescing flowers; however, the mechanism by which ethylene stimulates its own synthesis is not clear. Studies addressing the regulation of ethylene synthesis by ethylene treatment were undertaken in both the US and Israeli laboratories. In Israel, Dr. Mayak and his colleagues used the petals from cv. Sandrosa to address this question. As described above, these flowers do not normally exhibit increased ethylene during the normal course of their vase-life when held in air, but are capable of responding to exogenous ethylene by increasing the synthesis of ethylene. Their studies (Jiang et al. 1994; Appendix VI) revealed that only a short exposure (1-2 h) to ethylene was required to increase the activity of ACC oxidase. In contrast, 5 h of ethylene treatment was required to induce increased ACC synthase activity. Both activities were partially blocked by inhibitors of transcription and translation, indicating the need for gene expression and *de novo* protein synthesis. Similar studies were conducted in the US laboratory utilizing cDNA probes for both ACC synthase and ACC oxidase. Consistent with the findings of the Israeli group, these results clearly showed a more rapid induction of ACC oxidase

mRNA as compared with ACC synthase (Woodson et al. 1992; Appendix IV). Both the increase in ACC oxidase and ACC synthase mRNA levels in response to ethylene was inhibited by treatment with 2,5-norbornadiene, indicating a requirement for ethylene action. In summary, these results clearly show that ethylene induces ethylene biosynthesis through the transcriptional regulation of both ACC synthase and ACC oxidase genes.

Identification and Characterization of Senescence-Related Proteins from Carnation Petals

While the US laboratory has made significant progress in the identification and characterization of senescence-related genes from carnation, little effort had been directed towards characterization of the function of the proteins they encode. To this end an effort by the Israeli group to identify proteins associated with the onset of petal senescence was initiated. The pattern of proteins isolated from petals treated with air or ethylene and resolved by SDS-PAGE revealed considerable changes (Jiang et al., 1994). Proteins with molecular masses of 70, 60, 35, and 33 kDa increased in response to ethylene, while proteins with molecular masses of 62, 45, 34, 30 and 26 kDa decreased. The 70 kDa polypeptide was purified and polyclonal antibodies prepared against it. The antibodies were used to detect the protein by Western Blotting and this analysis confirmed the ethylene-responsive accumulation of the 70 kDa protein. This protein was subjected to N-terminal sequence analysis and found to be identical to a portion of the predicted protein of the pSR12 cDNA clone previously isolated in Dr. Woodson's laboratory. The purified protein was missing a portion of the predicted n-terminus of the SR12 protein and based on the hydrophobic nature of these sequences it was suggested that this represents a signal peptide removed during processing of the protein *in vivo*. Comparison of the SR12 protein sequences with other proteins in the database revealed homology with β -galactosidases from human and fungi. In order to determine if SR12 encoded a β -galactosidase, the pSR12 cDNA was cloned into an *E. coli* and yeast expression vectors. Expression of the protein was confirmed by SDS-PAGE analysis, but no β -galactosidase was detected. This might be due to a failure of these organisms to properly process the protein. The US laboratory has recently purified β -galactosidase from carnation petals and subjected the purified protein to N-terminal sequence analysis. This revealed the purified β -galactosidase shared significant (80%) homology to the predicted SR12 protein but was not identical. Therefore, the identity of the SR12 gene remains unclear. In addition to the 70 kDa protein, the Israeli group has also tentatively identified the 60 kDa proteins as an isoperoxidase based on cross-reactivity with antibodies raised against a known

isoperoxidase protein. The N-terminal sequence of this protein could not be obtained to confirm this due to its being blocked.

F. Description of Cooperation

The Israeli and US laboratories cooperated extensively during the funding period of this research. The scientists in both laboratories remained in contact through e-mail and fax. The US laboratory employed Dr. Amir Drory as a postdoctoral research associate during this project. Dr. Drory received his Ph.D. from the Hebrew University of Jerusalem Faculty of Agriculture working with Drs. Shimon Mayak and Amihud Borochoy. Dr. Mayak spent a period in the laboratory of Dr. Woodson conducting research with Dr. Drory on the spatial regulation of ethylene biosynthetic pathway genes in carnation petals. The Israeli laboratory shared antibodies with the US lab in an effort to identify the SR12 gene product expressed in *E. coli* and yeast. In turn the US laboratory shared cDNA clones with the Israeli lab to characterize the expression of these genes in the genotypes they were studying.

G. Evaluation of the Research Achievements

Our initial objective was to identify and characterize carnation genotypes exhibiting extended vase life in comparison to 'typical' cultivars. We successfully identified several genotypes meeting these criteria and these were used to describe their patterns of senescence and ethylene production. These genotypes will continue to provide useful genetic material for elucidating the physiological, biochemical and molecular basis for ethylene-responsive senescence in carnation. Indeed, recently the US laboratory has provided these genotypes to several laboratories around the world to encourage others to investigate the basis for this variant senescence behavior. The research project has led to the cloning of an ethylene-responsive ACC synthase from carnation, the first isolated from a senescing tissue undergoing autocatalytic ethylene production. This information has led to the development of strategies to improve the longevity of carnation using antisense technology and genetic engineering. Finally, the purification of proteins from ethylene-treated carnation flowers has, for the first time, given us information of the biochemical function of senescence-related genes and opened up a new area of investigation centered around the remobilization of carbohydrate during petal senescence.

H. List of Publications

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