**BARD Research Grant
Final Scientific Report**

Cover Page

BARD Project Number: IS-3645-04

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Project Title: Study of the regulatory mechanism involved in dark-induced postharvest leaf senescence

Investigators

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Co-Principal Investigator (Co-PI): Susheng Gan

Institutions

Volcani Center

Cornell University

Collaborating Investigators:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Arabidopsis, Transcription factor, promoter

Abbreviations commonly used in the report, in alphabetical order:

ACC, 1-aminocyclopropane-1-carboxylic-acid; KO, knockout; MAPK, Mitogen-Activated Protein Kinase; PCD, Programmed cell death; SAG, Senescence associated gene

Budget: IS: \$ 162,000

US: \$ 133,000

Total: \$ 195,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		1	1	2
Submitted, in review, in preparation		1	1	2
Invited review papers			1	
Book chapters			1	
Books				
Master theses				
Ph.D. theses			1	
Abstracts			6	
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings			2	2
Longer Visits (Sabbaticals)				

Description Cooperation:

The research in the two laboratories was focused in the study of molecular regulation of Arabidopsis dark-induced senescence. We have maintained exchange of information between the two laboratories related to this research. The two laboratories had focused on different candidate genes, thus, a parallel research was performed with the aim to try and see if links between the identified regulatory genes or components could be identified. While the work of the two laboratories cannot be defined as synergistic or supportive we believe they were complementary in that both labs investigated molecular components of senescence regulation with emphasis on dark-induced senescence and resulted with new and interesting knowledge.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract

Postharvest leaf senescence contributes to quality losses in flowers and leafy vegetables. The general goal of this research project was to investigate the regulatory mechanisms involved in dark-induced leaf senescence. The regulatory system involved in senescence induction and control is highly complex and possibly involves a network of senescence promoting pathways responsible for activation of the senescence-associated genes. Pathways involving different internal signals and environmental factors may have distinctive importance in different leaf senescence systems. Darkness is known to have a role in enhancement of postharvest leaf senescence and for getting an insight into its regulatory mechanism/s we have applied molecular genetics and functional genomics approaches. The original objectives were: 1. Identification of dark-induced SAGs in *Arabidopsis* using enhancer/promoter trap lines and microarray approaches; 2. Molecular and functional characterization of the identified genes by analyzing their expression and examining the phenotypes in related knockout mutant plants; 3. Initial studies of promoter sequences for selected early dark-induced SAGs.

Since genomic studies of senescence, with emphasis on dark-induced senescence, were early-on published which included information on potential regulatory genes we decided to use this new information. This is instead of using the uncharacterized enhancer/promoter trap lines as originally planned. We have also focused on specific relevant genes identified in the two laboratories. Based on the available genomic analyses of leaf senescence 10 candidate genes hypothesized to have a regulatory role in dark-induced senescence were subjected to both expression as well as functional analyses. For most of these genes senescence-specific regulation was confirmed, however, functional analyses using knock-out mutants indicated no consequence to senescence progression. The transcription factor *WARK75* was found to be specifically expressed during natural and dark-induced leaf senescence. Functional analysis demonstrated that in detached leaves senescence under darkness was significantly delayed while no phenotypic consequences could be observed on growth and development, including no effect on natural leaf senescence,. Thus, *WARKY75* is suggested to have a role in dark-induced senescence, but not in natural senescence. Another regulatory gene identified to have a role in senescence is *MKK9* encoding for a Mitogen-Activated Protein Kinase Kinase 9 which is upregulated during senescence in harvested leaves as well as in naturally senescing leaves. *MKK9* can specifically phosphorylate another kinase, *MPK6*. Both knockouts of *MKK9* and *MPK6* displayed a significantly senescence delay in harvested leaves and possibly function as a phosphorelay that regulates senescence. To our knowledge, this is the first report that clearly demonstrates the involvement of a MAP kinase pathway in senescence. This research not only revealed a new signal transduction pathway, but more important provided significant insights into the regulatory mechanisms underlying senescence in harvested leaves. In an additional line of research we have employed the promoter of the senescence-induced *BFN1* gene as a handle for identifying components of the regulatory mechanism. This gene was shown to be activated during dark-induced senescence of detached leaves, as well as natural senescence. This was shown by following protein accumulation and promoter activity which demonstrated that this promoter is activated during dark-induced senescence. Analysis of the promoter established that, at least some of the regulatory sequences reside in an 80 bps long fragment of the promoter.

Overall, progress was made in identification of components with a role in dark-induced senescence in this project. Further studies should be done in order to better understand the function of these components and develop approaches for modulating the progress of senescence in crop plants for the benefit of agriculture.

ACHIEVEMENTS

Significance of main scientific achievements or innovations

Analysis of senescence-associated regulatory genes (IS laboratory)

Based on published genomic studies of natural and dark-induced senescence (Buchanan-Wollaston et al., 2005; Lin and Wu, 2004) genes which encode for regulatory proteins were selected for detailed expression and functional analyses. Initially 10 different genes were investigated, narrowed down to 6 genes after further study. These were named *DARK* genes and included: *DARK1* (AT1G52890), No apical meristem family protein - putatively a transcription factor; *DARK2* (AT3G19580), Zinc finger (C2H2 type) protein; *DARK3* (AT5G27420), Zinc finger (C3HC4-type RING finger) family protein; *DARK4* (AT2G47190), MYB family transcription factor; *DARK5* (AT2G33080), Leucine-rich repeat family protein; *DARK6* (At3G15500), NAC-type transcription factor. The results indicated that *DARK5* and *DARK6* exhibited a more senescence-specific expression compared to the other genes examined and were subjected to functional analysis. Detailed physiological studies of the respective mutant lines indicated that neither natural nor dark-induced senescence were affected by the mutations. These results suggest that, at least for the *DARK5* and *6* genes the encoded proteins are possibly not essential for progress of senescence. Another possibility is that other genes can fulfill their function during senescence thus eliminating the possibility to observe an effect on senescence progress when these genes are mutated.

Investigating the role of the transcription factor WRKY75 in senescence (US laboratory)

The *WRKY75* transcription factor gene was found to be specifically expressed during natural and dark-induced leaf senescence in Arabidopsis. T-DNA knockout (KO) mutant lines displayed no changes in growth and development, including natural leaf senescence. However, senescence in detached leaves of KO plants under darkness was significantly delayed in KO plants compared that in wild type plants. Senescence induced by plant hormones such as ABA, ethylene, JA or SA was not altered in the KO plants. Complementation test was preformed which indicated that when a copy of the wild-type *WRKY75* gene was introduced into the KO plants, their phenotype was restored to that of the wild-type. These observations strongly suggest that *WRKY75* is a key regulator of dark-induced senescence in detached leaves and possibly play an important role in dark-induced senescence in detached leaves. To further elucidate *WRKY75* function in senescence and identify genes controlled by this transcription factor microarray analyses was initiated to identify its target genes. These experiments revealed about 400 genes which

expression was changed 2 fold or more and are being continued to further elucidate WRKY75-related regulatory circuit involved in senescence regulation.

Study of senescence-related Mitogen-Activated Protein Kinase (MAPK) signal transduction (US laboratory)

The role and mechanisms of the MAPK signal transduction pathway in harvested senescing leaves as well as in intact plants was investigated. Specifically, a gene called Mitogen-Activated Protein Kinase Kinase 9 (*MKK9*) that was identified to be upregulated during senescence in harvested leaves as well as in naturally senescing. *MKK9* possesses kinase activity and includes an ATP-binding site which is essential for this activity. *MKK9* can specifically phosphorylate another kinase, *MPK6*. Furthermore, *MKK9* was able to enhance the *MPK6* activity in *planta*. Functionally, both knockouts of *MKK9* and *MPK6* displayed a significantly delay dark-induced senescence in detached/harvested leaves while constitutive and inducible overexpression of *MKK9* caused precocious leaf senescence which could be suppressed in *mpk6* knockout background. These observations suggest that *MKK9* and *MPK6* function as a phosphorelay that regulates senescence in detached (and attached) leaves. To our knowledge, this is the first report that clearly demonstrates the involvement of a MAP kinase pathway in senescence of detached (and intact) leaves. This research not only revealed a new signal transduction pathway, but more important provided significant insights into the regulatory mechanisms underlying senescence in harvested leaves.

Identification of a NAC transcription factor associated with senescence (US laboratory)

The expression of *AtNAP* gene, encoding for a NAC transcription factor, was found to be closely associated with the senescence process of Arabidopsis rosette leaves. Functional analysis, using two T-DNA insertion KO lines of this gene, revealed that as a result of the mutations leaf senescence was significantly delayed. The T-DNA KO plants are otherwise normal. The mutant phenotype can be restored to wild-type by the intact *AtNAP*, as well as by its homologs in rice and kidney bean plants that are also upregulated during leaf senescence. Furthermore, inducible overexpression of *AtNAP* causes precocious senescence. These data strongly suggest that *AtNAP* and its homologs play an important role in leaf senescence in Arabidopsis and possibly in other plant species.

Analysis of a senescence specific promoter of the *BFNI* nuclease gene (IS laboratory)

Experiments were performed in order to get an insight into the molecular mechanism/s involved with senescence-specific activation of the *BFNI* gene promoter. The objective was to use this promoter as a handle for identifying components of the senescence molecular regulatory

mechanism. The *BFNI* gene of Arabidopsis encodes a nuclease gene induced in senescence which is also activated during dark-induced senescence of detached leaves. The expression pattern of *BFNI* promoter was analyzed by fusing it to a reporter gene and generating transgenic plants harboring this chimeric construct. These transgenics were analyzed for *GUS* expression. This analysis revealed that both in Arabidopsis and tomato the *BFNI* promoter was active in senescing tissue. Furthermore, activation of the promoter was evident also in tissues undergoing programmed cell death (PCD) indicating that the promoter contains regulatory element/s involved in the PCD regulatory system which might have common features with the regulatory system of senescence.

The analysis of the promoter suggests it probably includes sequences recognized by transacting factors which regulate gene expression during dark-induced senescence. Experiments were initiated to identify the relevant sequences involved in activation of gene expression during dark-induced senescence which could lead to the interacting trans-acting factors. As a first step a deletion analysis of the promoter was performed and a sequence of 80 bps was defined which possibly contain sequences sufficient to enable senescence-specific expression. Work is in progress to verify the ability of this sequence element to confer senescence-specific expression to a minimal promoter. Experiments were also initiated in order to try and identify trans-acting protein factors. Proteins with an affinity to this sequence element were enriched and MS-MS analysis is currently being performed for their identification.

Details of cooperation

The research in the two laboratories was focused in the study of molecular regulation of Arabidopsis dark-induced senescence. We have maintained exchange of information between the two laboratories related to this research. In light of the published genomic studies during the initial phase of the project we have coordinated and agreed to utilize the available information regarding dark-induced, senescence-related gene expression instead of collaborating in the analysis of the enhancer lines and performing our own microarray analysis. The two laboratories had focused on different candidate genes, thus, a parallel research was performed with the aim to try and see if links between the identified regulatory genes or components could be identified. While the work of the two laboratories in this project cannot be defined as synergistic or supportive we believe they were complementary in that both labs investigated molecular components of senescence regulation with emphasis on dark-induced senescence and resulted with new and interesting knowledge which is likely to advance our understanding of senescence and possibly be used in the future for application in crop plants for the benefit of agriculture.

List of Publications

Guo, Y. and S. Gan (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. *Plant J.* 46: 601-612.

Farage-Barhom, S., Burd, S., Sonego, L., Perl-Treves, R., Lers, A. (2008) Expression analysis of the *BFN1* nuclease gene promoter during senescence, abscission and programmed cell death-related processes. *J. Exp. Bot.* 59: 3247–3258.

Zhou, C., Cai, Z. Guo, Y. and S. Gan (2008) A mitogen-activated protein kinase cascade, MKK9-MPK6, plays a significant role in leaf senescence in Arabidopsis. *PNAS* (revised and pending).

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