**BARD Final Scientific Report
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BARD Project Number: IS-2982-98C

Project Title: The LX Senescence-Induced Ribonuclease in Tomato: Function and Regulation

Investigators

Principal Investigator (PI):

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Institutions

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Nuclease, leaf, promoter, gene expression, tomato, Arabidopsis, parsley.

Abbreviations commonly: PCD, Programmed cell death; PCR, Polymerase chain reaction; 1-MCP, 1-methylcyclopropene

Budget: IS: 165,000\$

US: 85,000\$

Total: 250,000\$

Amnon Lers

Signature
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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)			2	2
Submitted, in review, in preparation	2		1	3
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts	3		4	7
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 of Cooperation:

Cooperation was maintained by exchange of both information and materials between the two laboratories. Transgenic Arabidopsis plants generated in the US were sent to Israel for further physiological and molecular characterization including *RNS2* anti-sensed plants, the knock out mutants in *BFN1* and the *BFN1* over expressing line. Constructs for antisense inhibition or over-expression of *LX* were generated in the US and used in Israel for transformation. These plants are analyzed for the consequences of altering *LX* gene expression. Recently the US laboratory had identified and cloned different ribonucleases possibly related to RNA metabolism in Arabidopsis which were sent to the Israeli for study of their involvement in senescence-related RNA catabolism. This type of cooperation contributed much to the research and will be reported in joint publications.

Patent Summary (numbers)

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

Abstract

Natural leaf senescence, which occurs even when growth conditions are near optimal, has a negative influence on yield. Postharvest induced senescence contributes to the losses of quality in flowers, foliage, and vegetables. Strategies designed to control the senescence process in crop plants could therefore have great applied significance. However, the successful design of such strategies requires a better insight into the senescence machinery and control in higher plants. A main feature of senescence is the hydrolysis of macromolecules by hydrolases of various types such as ribonucleases (RNases) and proteases. Previously we had identified and characterized the tomato *LX* RNase gene demonstrating its transcript to be highly and specifically induced during senescence. This reported study was focused on *LX* but also had broadened our research to other senescence-associated nucleic acids degrading enzymes to learn about their function and the regulation of their encoding genes. Beside tomato we used parsley and Arabidopsis for the study of the bi-functional nuclease which has a role in senescence. The study of different senescence-associated nucleases in few plant systems will allow a more general view on function and regulation of these enzymes in senescence. The specific original proposed objectives included: 1. Study the consequences of alterations in *LX* RNase level on tomato leaf senescence and general development; 2. Analyze stimuli which may participate in senescence-specific activation of the *LX* gene; 3. Clone the senescence-associated BFN1 nuclease gene homologue from tomato. 4. Further characterize the sequences required for senescence-specific gene expression. Homozygous transgenic plants in which *LX* gene was either inhibited or over-expressed were generated. In both of these *LX* mutated plants no major phenotypic consequences were observed, which may suggest that *LX* is not essential for plant growth under optimal growth conditions. Lack of any abnormalities in the *LX* over-expressing lines suggests that special system exist to allow function of the RNase only when needed. Detailed analyses of growth under stress and consequences to RNA metabolism are underway. We have analyzed *LX* expression on the protein level demonstrating that it is involved also in petal senescing. Our results suggest that *LX* is responding to complex regulation involving developmental, organ dependent factors and responds differently to hormonal or environmental stimuli in the different plant organs. The cloned 1.4 kb promoter was cloned and its analysis revealed that probably not all required elements for senescence induction are included. Biochemical analysis of senescence-associated be-functional nucleases in the different plants, tomato, parsley and Arabidopsis, suggests they belong to a sub-class within the type I plant nucleases. The parsley PcNUC1/2 nuclease protein was purified from senescing leaves its activity was studied in vitro revealing endo-, double strand, nucleolytic activity and exo-nucleolytic activity. Its encoding gene was cloned and found to be induced on the mRNA level. The promoter of the related Arabidopsis BFN1 nuclease was shown in both tomato and Arabidopsis to be able to direct senescence-specific expression suggesting that, at least part, the gene is regulated on the transcriptional level and that the mechanism for this senescence-specific regulation is conserved between different plants. Few plants in which the BFN1 gene is mutated were identified which are subjected now to detailed analysis. Our results suggest that the senescence-related nucleic acid degrading enzymes share similarities in both function and regulation between different plants and possibly have important functions in processes un-related to senescence. Still, the function of these enzymes, at least in some cases is not essential to plant development under optimal growth conditions. We are now at the stage which permits in depth investigation of the specific functions and mode of molecular regulation of senescence-associated nucleases with the aid of the research tools developed. The isolated senescence-specific promoter, shown to be active in heterologous plant system, could be utilized in agricultural-related biotechnological applications for retardation of senescence.

Achievements

A main feature of leaf senescence is hydrolysis of macromolecules by hydrolases including ribonucleases (RNases) and nucleases. Our study was focused on the senescence-inducible nucleic acids degrading enzymes to learn about their function and the regulation of their encoding genes. A major focus was given into the study of the previously identified *LX* ribonuclease which is induced during tomato leaf senescence. In this reported research we have broaden our study into other plant systems e.g. parsley and Arabidopsis and a different type nucleic acid degrading enzyme: the bi-functional nuclease. The study of different senescence-associated nucleases in few plant systems will allow a more general view on function and regulation of these enzymes in leaf senescence. The specific objectives as indicated in the original proposals included: 1. Study the consequences of alterations in *LX* RNase level on tomato leaf senescence and general development; 2. Analyze stimuli which may participate in senescence-specific activation of the *LX* gene; 3. Clone the senescence-associated BFN1 nuclease gene homologue from tomato. 4. Further characterize the sequences required for senescence-specific gene expression.

Consequences of alteration in *LX* gene expression - Using the antisense approach we were able to generate transgenic tomato plants in which expression of *LX* ribonuclease was inhibited or constitutively over expressed in non-senescing leaves. Antibodies for the *LX* protein were prepared and used in western blot analyses which in combination with activity gel essays confirmed the changes in *LX* gene expression. In both *LX* inhibited and over-expressed plants no major phenotypic differences were observed which could be attributed to altered *LX* gene expression. Experiments to analyze in more detail possible phenotypic consequences are still underway as *LX* is known to be involved in other programmed cell death processes and response to phosphate (Pi) starvation. We carry out expression on these processes. Experiment designed to test the consequences of alterations to *LX* gene expression included analysis of RNA metabolism, especially in the senescing leaves and effect on growth under transition to Pi limiting conditions. The preliminary results of these experiments can not be reported yet. The presumably normal phenotype may suggest that *LX* ribonuclease is not essential for plant growth under optimal growth conditions. This is not surprising giving that *LX* expression is not constitutive and is induced only under defined developmental stages or in response to external factors. On the other hand the lack of any abnormalities in the *LX* over-expressing lines suggests that plant cells are able to mask the very high amounts of toxic enzyme which can lead to massive cellular RNA degradation. Possibly the enzymes are localized in compartmented sub-cellularly which allow controlled RNA degradation. We have initiated experiments using the *LX* specific antibodies to immuno-localized the *LX* in the cells which may shed some light on their function and substrates.

Control of *LX* gene expression- Using *LX* antibodies we extended our previous finding on senescence induction of the *LX* gene from the RNA to the protein level. Western blot analysis

demonstrated that *LX* mRNA induction was followed with elevation in protein level. This observation support the possibility that most of *LX* gene regulation is carried out on the mRNA level. Following starvation of tomato plants induction of *LX* protein was observed in all organs examined including roots and leaves. We were able also to demonstrate that *LX* gene is senescence-induced also in flowers. Interestingly, in anthers and pistils constitutive high level *LX* expression was observed while senescence induction was restricted to the petals demonstrating that senescence regulation of *LX* is operating in flower petals, as well as, in leaves. The constitutive expression in the anthers and pistils suggests a different, yet undefined, function of *LX* in these organs. We had demonstrated before ethylene induction of the *LX* gene in young leaves on the mRNA level, even before any senescence signs are visible. We were able to demonstrate ethylene induction of *LX* also on the protein level. Still variability in the responsiveness of leaves at different developmental stages was observed with older leaves responding better compared to younger leaves. *LX* expression in roots was found to be constitutive at a low basal level with no apparent effect of ethylene. These results demonstrate that control of *LX* gene expression is highly complex being affected by plant development, as well as, organ dependent factors and responds differently to hormonal or environmental stimuli in the different plant organs. Such complex type of regulation requires appropriate regulatory sequences in the promoter of the gene if such regulation is performed on the transcriptional level. The *LX* gene 1.4 kb 5'-upstream genomic sequences corresponding to the promoter were cloned in front of the *GUS* reporter gene in order to examine its ability to direct senescence-specific gene expression in both tomato and Arabidopsis. In most examined plants senescence-specific *GUS* induction was not detected. It is possible that the regulatory elements required for senescence inducibility are present further up stream in the promoter and are not included in the cloned *LX* promoter used. Since all attempts to clone further upstream sequences were not successful we can not yet test this possibility.

Identification and characterization of senescence-associated nucleases - In our preliminary work with tomato a senescence-associated bi-functional nuclease, LeNUC1, was identified and characterized. We have further identified and characterized a similar nuclease, PcNUC1/2, induced in parsley senescing leaves and also characterized the biochemical characteristics of the Arabidopsis senescence-associated nuclease BFN1. These experiments revealed unique features for these senescence-associated nucleases which define them as a specific group different from other members of the type I nucleases in plants. These nucleases were found to be glycosylated and could degrade both RNA and DNA with optimal activity under basic pH (8.5-9.0). EDTA inhibited their activity which could be restored by addition of Co^{2+} or Mn^{2+} . To get better insight into the function and regulation of this type of nucleases we have purified to homogeneity the PcNUC1/2 nucleases from senescing parsley leaves. The purified nuclease was in vitro to have an endo-nucleolytic activity resulting with double strand breakage of its DNA substrate and is likely to

have also an efficient exo-nucleolytic activity. The sugar moiety of the protein was found not to be essential for its activity and perhaps has a role in enzyme cellular localization. Based on limited amino acid sequencing of the purified protein and using PCR cloning approach the encoding gene from parsley was cloned. The deduced amino acid sequence, as well as the limited determined protein sequence showed high similarity to other senescence-related nucleases including the Arabidopsis BFN1 nuclease. These homologies further support the existence of a unique type of nuclease with a functional role in senescence.

Towards understanding function and regulation of the nucleases in Arabidopsis leaf senescence –

Arabidopsis knockout mutants in the nuclease gene BFN1 were screened for using PCR, Southern and sequence analyses. Individual plants carrying the T-DNA insertion 180 bps downstream of the *BFN1* gene ATG were identified. At the same transposon tagged lines mutated in *BFN1* were identified resulting with a line with the Ds element inserted at 3rd intron of the *BFN1* gene. Homozygous mutants are currently analyzed for phenotypic consequences of the *BFN1* mutations. To learn about the molecular regulation in senescence BFN1 promoter (1.5 kb) was cloned in front of the *GUS* reporter gene and used for transforming both Arabidopsis and tomato. In both plants clear senescence-specific induction of the *GUS* gene was visualized demonstrating that the promoter includes all required regulatory and that, at least part, of senescence-specific regulation is exerted on the transcriptional level. These results suggest that the mechanism governing senescence specific gene expression is conserved between different plants. Experiments are underway for identification of molecular components required for this senescence regulation.

Cooperation - We have continued with our close cooperation by exchange of both information and materials between the two laboratories. The US laboratory had sent to Israel different transgenic Arabidopsis plants which were generated in the US for further physiological and molecular characterization including RNS2 anti-sensed plants, the knock out mutants in BFN1 and the BFN1 over expressing line. Different constructs for antisense inhibition or over-expression of the gene were generated in the US and used in Israel for transformation of tomato. As described in this report these plants are analyzed for the consequences of altering *LX* gene expression. Recently the US laboratory had identified and cloned different ribonucleases possibly related to RNA metabolism in Arabidopsis which were sent to the Israeli laboratory in which they are being examined for their involvement in senescence-related RNA catabolism. This type of cooperation contributed much to the research and will be reported in joint publications.

List of publications

1. Lers, A., Lomaniec, E., Burd, S. and Khalchitski, A. (2001) The characterization of LeNUC1, a nuclease associated with leaf senescence of tomato. *Physiol. Plant.* 112: 176-182.
2. Canetti, L., Lomaniec, E., Elkind, Y. and Lers, A. (2002) Nuclease activities associated with dark-induced and natural leaf senescence in parsley. *Plant Science* 163: 873-880.