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**Analysis of Senescence-Inducible Ribonuclease
in Tomato: Gene Regulation and Function**

A. Lers, E. Lomaniec, S. Burd, A. Khalchitski, L. Canetti, P.J. Green

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**Analysis of Senescence-Inducible Ribonuclease in
Tomato: Gene Regulation and Function**

Submitted by

**Amnon Lers¹, Ella Lomaniec¹, Shaul Burd¹, Andrei Khalchitski¹, Lea
Canetti¹ and Pamela J. Green²**

¹Department of Postharvest Science of Fresh Produce, The Volcani Center,
P.O.Box 6, Bet Dagan 50250, Israel; ²MSU-DOE Plant Research Laboratory,
Michigan State University, East Lansing MI 48824-1312 USA

ARO, The Volcani Center

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Address & e-mail of Investigators and Institutions:

Investigators

Principal Investigator:

Amnon Lers

Cooperating Investigators:

Pamela J. Green

Institutions

The Volcani Center,

Dept. of Postharvest

Science of Fresh

Produce. POB 6, Bet

Dagan 50250

Alers@agri.gov.il

Plant Research

Laboratory

Michigan State Univ.

East Lansing MI 48824

Green@pilot.msu.edu

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- Cooperation:** This collaboration was both synergistic and supportive. The synergism stems from the fact that the interests and experience of the two laboratories are not overlapping but complementary. The subject of RNases is of great interest for both laboratories. While the US laboratory has been focusing on the role of RNases in RNA stability the Israeli laboratory is interested in the role and regulation of these enzymes in senescence. The supportive aspect of the collaboration originated from the division of the work. The research tasks were divided between the two laboratories taking advantage the expertise, experience and facilities of each one.

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2. Lers, A., Khalchitski, A., Lomaniec, E., Burd, S. and Green, P. J. (1998)
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3. Pérez-Amador, M. A., Abler, M. L., DeRocher, J, Thompson, D. M., van Hoof, A.,
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nuclease induced during leaf and stem senescence in *Arabidopsis thaliana*. *Plant
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4. Lers A., Lomaniec, A., Burd, S. and Khalchitski, A., (2000) LeNUC1 nuclease is
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leaves. Submitted to *Plant Physiol.*

Abstract

Natural leaf senescence 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. Senescence is regulated by differential gene expression yet, functional characterization of the genes specifically induced and study of their expression control, is still in its infancy. Study of senescence-specific genes is required to allow identification of regulatory elements participating in senescence-induced expression and thus provide insights into the genetic regulation of senescence. A main feature of senescence is the hydrolysis of macromolecules by hydrolases of various types such as RNases and proteases. This study was aimed at analysis of senescence-inducible RNases in tomato with the following objectives: Isolation of senescence-inducible RNase cDNA clones; Expression analyses of RNase genes during senescence; Identification of sequences required for senescence-induced gene expression; Functional analyses of senescence-inducible RNases. We narrowed our aims somewhat to focus on the first three objectives because the budget we were awarded was reduced from that requested. We have expanded our research for identification and characterization of senescence-related RNase/nuclease activities as we thought it will direct us to new RNase/nuclease genes. We have also carried out research in *Arabidopsis* and parsley, which enabled us to draw more general conclusions. We completed the first and second objectives, and have made considerable progress on the remaining two. We have defined growth conditions suitable for this research and defined the physiological and biochemical parameters characteristic to the advance of leaf senescence. In tomato and *Arabidopsis* we have focused on natural leaf senescence. Parsley was used mainly for study of postharvest senescence in detached leaves. We have identified a 41-kD tomato nuclease, LeNUC1, specifically induced during senescence which can degrade both RNA and DNA. This activity could be induced by ethylene in young leaves and was subjected to detailed analysis, which enabled its classification as Nuclease I enzyme. LeNUC1 may be involved in nucleic acid metabolism during tomato leaf senescence. In parsley senescing leaves we identified 2 main senescence-related nuclease activities of 41 and 39-kDa. These activities were induced in both naturally or artificially senescing leaves, could degrade both DNA and RNA and were very similar in their characteristics to the LeNUC1. Two senescence-induced RNase cDNAs were cloned from tomato. One RNase cDNA was identical to the tomato LX RNase while the second corresponded to the LE RNase. Both were demonstrated before to be induced following phosphate starvation of tomato cell culture but nothing was known about their expression or function in plants. LX gene expression was much more senescence specific and ethylene could activate it in detached young leaves. LE gene expression, which could be transiently induced by wounding, appeared to be activated by abscisic acid. We suggest that the LX RNase has a role in RNA catabolism in the final stage of senescence, and LE may be a defense-related protein. Transgenic plants were generated for altering LX gene expression. No major visible alterations in the phenotype were observed so far. Detailed analysis of senescence in these plants is performed currently. The LX promoter was cloned and its analysis is performed currently for identification of senescence-specific regulatory elements. In *Arabidopsis* we have identified and characterized a senescence-associated nuclease I gene, *BFN1*, which is highly expressed during leaf and stem senescence. *BFN1*, is the first example of a senescence-associated gene encoding a nuclease I enzyme as well as the first nuclease I cloned and characterized from *Arabidopsis*. Our progress should provide excellent tools for the continued analysis of regulation and function of senescence-inducible ribonucleases and nucleases in plants. The cloned genes can be used in reverse genetic approaches, already initiated, which can yield a more direct evidence for the function of these enzymes. Another contribution of this research will be in respect to the molecular mechanism, which controls senescence. We had already initiated in this project and will continue to identify and characterize regulatory elements involved in senescence-specific expression of the genes isolated in this work.

Achievements

As detailed in the following report we succeeded in this research project to identify and characterize ribonuclease and nuclease activities which are related to leaf senescence. We have also cloned and characterized cDNAs which represent genes encoding for such enzymes. When we initiated our collaborative research not much information existed about these enzyme/genes. Beside the observation that total RNase and nuclease activities are increased in parallel to progress of senescence there were only few reports on specific senescence-related RNase/nuclease activities and a single report on cloning of a senescence-related cDNA reported by Prof. Green, the US investigator of this project. We believe that the results of this research add new and important information. We have identified *LX*, previously known as phosphate starvation induced RNase gene, as a senescence-induced gene. We have also cloned *BFNI*, which is the first example of a senescence-associated gene encoding a nuclease I enzyme. *BFNI* is also the first nuclease I cloned and characterized from *Arabidopsis*. The strong and specific response of *BFNI* and *LX* indicates that they should be excellent tools in the study of senescence regulation on the molecular level and the degradation of nucleic acid that occurs during this process. Our study on these senescence-related nuclease and RNase activities will be very useful and supply a good basis for understanding the role of these enzymes in degradation of nucleic acid that occurs during this senescence. The cloned genes can be used in reverse genetic approaches, which were already initiated. Such approaches can yield a more direct evidence for the function of these enzymes in senescence on which very limited information is available today. Another contribution of this research will be in respect to the molecular regulatory mechanism, which controls senescence. A study, which had initiated already, is aimed for identification and characterization of the regulatory elements responsible for the senescence specific expression of the genes isolated in this project. Using the information accumulated as a result of this research we will continue to analyze the molecular mechanism/s, which governs senescence-specific gene expression. In the last few years several senescence-related genes were cloned, however, information regarding their control is limited and was obtained only for the *Arabidopsis SAG12* gene.

Strategies designed to control the senescence process in crop plants could have great applied significance. Leaf senescence, which occurs even when growth conditions are near optimal, has a negative influence on yield due to loss of the leaf photosynthetic assimilatory capacity. Postharvest senescence contributes to the losses of quality in flowers, foliage, and vegetables. Key genes, which have the potential to affect the progress of senescence, had already been reported of. Successful application of strategies aimed for manipulation of senescence using these key genes will depend on usage of appropriate promoters. These will allow expression of the chosen genes in the desired time and location. Non-specific expression of those genes may result in deleterious effect on plant development. The molecular studies conducted in our research supply the basis for isolation of these senescence-specific promoters, which could be used for application in transgenic plants of agricultural importance. The characterization of the sequence elements sufficient for direction of senescence-specific

expression could be completed within the 3-year continuation of this program and that could initiate time experiments aimed for application in crop plants.

In this project we have established a true and excellent collaboration which turned to be very fruitful. This collaboration was new to us and BARD funding had a very important role in catalyzing its formation. This collaboration was both synergistic and supportive. The synergism stems from the fact that the interests and experience of the two laboratories are not overlapping but complementary. The subject of RNases is of great interest for both laboratories and each side brought into the project its own experience and knowledge. The US laboratory has been focusing on the role of RNases in RNA stability and brought its experience and knowledge regarding molecular biology of RNases. The Israeli laboratory is interested in the role and regulation of these enzymes in senescence and had gained experience in physiological, biochemical and molecular aspects of leaf senescence. The supportive aspect of the collaboration originated from the division of the work. The research tasks were divided between the two laboratories taking advantage the expertise, experience and facilities of each one in order to obtain the objectives which were common for the two laboratories. The actual experiments themselves were done also in close collaboration. Following are few examples: The initial PCR cloning of the RNases cDNAs were carried-out in the US laboratory by the Israeli principal investigator. The Vectors for generation of the *LX* antisense/overexpression tomato plants were constructed by the US laboratory and used in Israel for transformation of tomato plants, which are also characterized in Israel. The *BFN1* clone isolated by the US laboratory was used in Israel for experiments with tomato. The *Arabidopsis* transgenic plants with altered nuclease/RNase activity will be further characterized currently in Israel. Beside a continuous contact via e-mail for consultant and information exchange mutual visits of the two principal investigators took place. Dr. Lers had spent two periods of about two weeks each in the laboratory of Dr. Green during the first and second years of the project. Dr. Green visited Israel during the 3rd year for discussions about the experimental results and planning of the continuation project. This visit also included meetings with other Israeli investigators. These visits were very helpful and enabled efficient advancement of the research especially in the initial stages. Our collaborative research has advanced to a stage in which a more detailed questions can be answered regarding both the function of RNases and nucleases in senescence and the molecular mechanism which is responsible for senescence specific gene expression.

Background

Agricultural relevance for the study of senescence

Leaf senescence, which occurs even when growth conditions are near optimal, has a negative influence on yield due to loss of the leaf photosynthetic assimilatory capacity. The importance of delayed senescence in increasing the yield of agricultural crops has been confirmed before (Thomas and Smart, 1993). Postharvest senescence contributes to the losses of quality in flowers, foliage, and vegetables. Besides the visible, undesired, yellowing of the tissue, macromolecules degradation may reduce the quality of the stored produce. Postharvest senescence enhances sensitivity to pathogens leading to high losses. Strategies designed to control the senescence process in crop plants could therefore have great applied significance. However, successful design of such strategies will require a more complete understanding of the senescence machinery in higher plants.

Plant senescence is considered to be an active, genetically controlled process in which orderly disassembly of the cell occurs together with the redistribution of released materials. A main feature of the process is the hydrolysis of macromolecules by hydrolases of various types such as RNases and proteases. Our present knowledge is limited with respect to the specific identity and function of enzymes involved in catabolism during senescence. Isolation and characterization of genes encoding for such enzymes will help to understand the degradative and recycling functions associated with the process. Isolation of such genes and characterization of their mode of activation will allow identification of regulatory elements participating in senescence-specific gene expression and thus provide insights into its genetic regulation. This information has a large economical importance in agriculture. Such DNA regulatory elements can be utilized for the regulated expression of desired genes with a senescence retarding activity. Accordingly, this study was designed to elucidate the functional significance of the ribonucleases and nucleases induced during senescence and the regulatory elements that mediate their expression.

Leaf senescence - Leaf senescence is an endogenously controlled degenerative process leading to cell death which involves the synthesis of new RNAs and enzymes (for reviews see Nooden et al., 1997). The senescence process in plants is believed to be a form of programmed cell death (PCD) process (Gan and Amasino, 1997). Senescence occurs at a given time in the life of the leaf even when growth conditions are near optimal, but can also be environmentally induced. During senescence the cellular structure, metabolic activities, and physiological role of the leaf are greatly altered. Prior to senescence the leaf serves as a source of photosynthetic products. As senescence progresses, there is a degeneration of the chloroplasts and the photosynthetic apparatus (Gepstein, 1988), which reduces photosynthetic assimilatory capacity. Nucleic acid, particularly RNA, and protein molecules are catabolyzed and the leaf becomes a source of mobilized carbon, nitrogen, and minerals that are transported out. Differential expression of nuclear-encoded genes is associated with leaf senescence (Gan and Amasino, 1997). Among these genes some were identified as coding for hydrolytic enzymes which probably are

involved in the catabolic processes. Both senescence-promoting and -retarding hormones exist: ethylene and cytokinin may be the most important representatives of these opposing classes.

RNases in plants - RNases of higher plants are grouped into four main enzyme families based on their biochemical properties (for review see Green, 1994). These four types of enzymes designated RNase I, RNase II, Nuclease I, and Exonuclease I. Alternatively they can be classified based on amino acid sequence comparison. Several "S-like" RNases from self-compatible plants have been identified that are related to, but distinguishable from, the S-RNases. Three RNase cDNA clones from *Arabidopsis*, designated *RNS1*, *RNS2* and *RNS3* were isolated in Dr. Green's lab (Taylor and Green, 1991). The identification of S-RNase homologs in this self-compatible species indicated that they were not restricted to self-incompatible species and therefore must play a more fundamental role in plant RNA decay. Other related enzymes include RNases LE and LX from the self-compatible tomato species *Lycopersicon esculentum* (Köck et al., 1994; Loeffler et al., 1993). Amino acid sequence comparison indicates that the S- and S-like RNases comprise separate subclasses within the same RNase superfamily (Green, 1994).

Plants exhibit changes in RNase levels in response to a variety of different endogenous and exogenous stimuli (Green, 1994). Phosphate starvation can induce a number of RNases and RNase genes. These include the tomato LE, and LX (Loeffler et al., 1992), and the *Arabidopsis* *RNS1*, and *RNS2* RNases (Taylor et al., 1993; Bariola et al., 1994). Xylogenesis-associated RNase activity and cDNAs were described in *Zinnia elegans*, which is not detected in senescence (Ye and Dorste, 1996). These RNases are likely to be involved in RNA degradation during this cell death process.

Leaf senescence is associated with an increase in RNase activity. An increase of RNase activity was also reported in detached leaves, a common model system for senescence (Farkas, 1982). Most data suggest that de novo synthesis of proteins is required for the induction of RNase activity. In many of the earlier studies, only changes in total RNase content were measured (Farkas, 1982). The use of RNase activity gels is now the preferred method for investigating the effects of regulatory stimuli on RNases that can be separated electrophoretically (Blank et al., 1982). In tomato McHael and Dove (1968) reported of an RNase activity which is increased almost linearly in old leaves. In addition to tomato, RNases induced during leaf senescence have been studied in other plants. Activity of three RNases during natural and dark-induced senescence of wheat leaves was monitored by Blank and McKeon, (1991). The first RNase gene shown to be induced during senescence was the *RNS2* gene of *Arabidopsis* (Taylor et al., 1993). The *RNS3* and to a lesser extent the *RNS1* transcripts are also induced during senescence (Bariola et al., 1994). The expression of *RNS2* is increased in both leaves and petals of *Arabidopsis*, during senescence, presumably as part of the nutrient remobilization machinery (Taylor et al., 1994).

Methodologies and materials

In this research project we have used mainly three plant systems for our research. Tomato (*Lycopersicon esculentum*) was used mainly for identification and

characterization of RNases/nucleases related to natural leaf senescence. Parsley (*Petroselinum crispum* Mill.) detached leaves were used in studies aimed to detect activities related to postharvest artificial leaf senescence. We have also used, mainly by the US group the model plant *Arabidopsis thaliana*, Columbia ecotype. Growth conditions, and physiological treatments used are described in the papers/manuscript attached. Throughout this project we have undertaken two different supplementing approaches to identify and characterize RNases/nucleases. Activity gel assay was used in order to identify activity of the enzymes and characterize them biochemically. In a molecular approach we have utilized sequence information for cloning of RNase/nuclease genes. To clone the RNases we employed the PCR strategy developed by Taylor and Green, (1991) and used degenerated primers designed based on the highly conserved amino acids regions. We have identified *Arabidopsis* nuclease cDNA by searching the *Arabidopsis* database with N-terminus conserved sequence of plant nuclease. Molecular biology methods used through-out this research including nucleic acid purification, plasmid construction, gel electrophoresis, Southern and Northern blot analysis and PCR based methods were carried-out as described in the appendix attached papers/manuscript. Transformation of *Arabidopsis* was carried-out using the vacuum-infiltration method of *A. tumefaciens* containing the plasmid of interest. Dr. Ron Vunsh of the Plant Genome Center at the Weizmann Institute did tomato plant transformation using *A. tumefaciens* as a service.

Results

1. Nuclease/RNase activities in senescing leaves

1.1. Activities of specific nucleases induced during tomato leaf senescence (*Lers et al., submitted to plant Physiol., appended*).

In order to identify senescence-related nucleases in tomato leaves, an activity gel assay in SDS-PAGE was carried out. Of the three observed RNA-degrading activities only one, of about 41 kDa, was senescence-specific. It was present in leaves at an advanced senescing but not in younger leaves and was termed LeNUC1 (*Lycopersicon esculentum* **nuclease**). The level of a second, 39-kDa activity, termed LeNUC2, was constitutive. A 20-kDa activity seemed to be induced to some extent in the senescing leaves, but other analysis has shown that this area in the gel includes few activities. The activities observed in the tomato leaves differed from those detected in tomato fruits at various ripening stages. We examined the effects of different growth regulators on their activities in detached, mature, green leaves. Ethylene was the only growth regulator, of those tested, which caused an activation of LeNUC1 in the young green leaves. The LeNUC1 activity was not affected by any of the treatments. In the 20-kDa activities area some effect of ethylene was visible which could have been due to induction of a new activity superimposed on the existing 20-kDa activities. In other experiments we identified LX RNase activity in that location. To differentiate between RNase and DNase activities we carried out the gel activity assay using either RNA or DNA as the

substrate. LeNUC1 and LeNUC2 were found to be nucleases, which can degrade both DNA and RNA. The 20-kDa activity could degrade only RNA. Nucleases LeNUC1 and 2 exhibited highest activity at pH 7.5-8.0, whereas the RNase around the 20-kDa RNase position exhibited their highest activity around pH 6.0. Both LeNUC1 and LeNUC2 demonstrated extremely high sensitivity to EDTA, while the RNase activity was more tolerance. Ca^{2+} exhibited a stimulatory effect on the LeNUC1 and LeNUC2 nucleases while only Co^{2+} and Mn^{2+} could restore the activity of LeNUC1 and LeNUC2 following EDTA treatment. Phosphate had an inhibitory effect already at concentration of 10 μM , however, even at the highest phosphate concentration tested LeNUC1 and 2 activities were still observed. No effect was detected on the 20-kDa activity. Both LeNUC1 and 2 were inhibited by PPi even more than by phosphate.

1.2. senescence-related nuclease activity in detached parsley leaves (*Lers et al., in preparation for publication*).

For our study on postharvest senescence, induced during storage of leafy vegetables, we have used parsley as a model system. Immediately upon harvest detached green leaves are incubated in the dark at 25°. Under these conditions the leaves senesce fully within 8-10 days. Progress of senescence in parsley leaves can be accelerated by ethylene application or retarded by treatment with gibberellin (GA_3), or CO_2 as shown before (Lers et al., 1998a). Activity gel assay analysis revealed 3 main senescence-related nuclease activities. These included a 41, 39, and ~20 kDa activities, which increased as senescence progressed (Fig. 1). Comparison of naturally and artificially senescing revealed that the 39 and 41 kDa activities are induced in both while the ~20 kDa activity is less induced in natural senescence (Fig. 2). The 39 and 41 kDa activities were able to degrade both DNA and RNA while the lower MW activity possessed only RNase activity (Fig. 3). Ethylene treatment resulted in enhancement of the nuclease activities compared to the control while both kinetin and GA_3 treatment retarded the rise in their level (Fig. 4). Characterization of the senescence-related nuclease activities in parsley revealed high sensitivity of the 39 and 41-kDa activities toward EDTA, zinc and phosphate and PPi (data not shown) similarly to what was observed for the tomato nucleases. Another similarity to the tomato nucleases was the ions required for activity. In the presence of EDTA Ca^{2+} could not restore the nucleases activities while only Co^{2+} and Mn^{2+} could.

2. Cloning of RNase/nuclease cDNAs and characterization of the expression of the encoding genes.

2.1. Isolation of senescence-inducible RNase cDNA clones and analysis of their expression in tomato (*Lers et al., Senescence-induced RNases in Tomato, Plant Mol. Biol.* 36:438-499, 1998; appended).

Using a PCR based cloning approach we have isolated from tomato two senescence-induced RNase cDNA clones. Each of these cDNAs hybridized to a senescence-induced transcript in Northern analysis. One RNase cDNA was identical to the tomato LX

RNase while the second corresponded to the LE RNase. Both *LX* and *LE* RNase genes were originally demonstrated to be induced following phosphate starvation of tomato cell culture but nothing was known about their expression or function in plants. We observed that the expression of the *LX* and *LE* genes is induced in leaves during an advanced stage of senescence with the *LX* transcript level being much more induced than that of *LE*. Low level expression of the RNase genes was observed in flowers and artificially senescing detached leaves while no expression could be detected in stems, roots, or fruits at different ripening stages. Ethylene activated the *LX* gene expression in detached young leaves while *LE* gene expression, which could be transiently induced by wounding, appeared to be activated by abscisic acid. We suggest that the *LX* RNase has a role in RNA catabolism in the final stage of senescence, and *LE* may function during wounding as a plant defense protein.

2.2. Cloning of cDNAs for senescence-related RNase from parsley (*in progress*).

The PCR cloning approach described above was employed also for cloning senescence-related RNase cDNAs in parsley. We have cloned two different cDNAs of about 200 bps encoding for proteins with high homology to RNases and which hybridized to senescence-induced transcripts (**Fig. 5**). Work is in progress for cloning of the full-length cDNAs and characterization of the expression of the encoding genes.

2.3. Identification of *BFN1*, a bifunctional nuclease induced during leaf and stem senescence in *Arabidopsis thaliana*. (Pérez-Amador *et al.*, *Plant Physiol.*, 122: 169-180, 2000, appended).

Nuclease I enzymes are responsible for the degradation of RNA and single-stranded DNA during several plant growth and developmental processes, including senescence. However, the corresponding genes have not been reported. To identify cDNAs from *Arabidopsis* that encoded nucleases we searched the *Arabidopsis* database using the N-terminus amino acid consensus sequence of nuclease I enzymes from barley (Brown and Ho, 1986; Brown and Ho, 1987) and *Zinnia* (Thelen and Northcote, 1989). We identified an *Arabidopsis* clone from the EST sequence project at Michigan State University (Newman *et al.*, 1994), that contained the sequence -Lys-Glu-Gly-His- in the 5' region of the ORF. The gene corresponding to this clone was designated *BFN1*. We characterized *BFN1* and demonstrated that it is a senescence-associated nuclease I gene. *BFN1* protein shows high similarity to the sequence of a barley nuclease induced during germination and a *Zinnia elegans* nuclease induced during xylogenesis. In transgenic plants overexpressing the *BFN1* cDNA, a new nuclease activity of about 38 kD was detected. Levels of *BFN1* mRNA are extremely low or undetectable in roots, leaves, and stems. In contrast, relatively high *BFN1* mRNA levels are detected in flowers as well as during leaf and stem senescence. *BFN1* nuclease activity is also induced during leaf and stem senescence. In addition, we have isolated two cDNAs from *Zinnia* corresponding to new nuclease I enzymes with senescence-inducible transcripts.

3. Toward analysis of LX function using transgenic plants in which the LX RNase level is altered (*in progress*).

Function of the LX RNase is investigated by following the consequences of changing its level in transgenic plants in which the *LX* gene is overexpressed or inhibited. Appropriate constructs for transformation were prepared and transgenic tomato plants were generated. We used the constitutive 35S promoter for direction of the expression and the E9 terminator was inserted in the 3' end of the cDNA all in the pBI121 transformation vector. In this same vector a *GUS* reporter gene is present as well. More than 20 transgenic plants were already isolated which are currently analyzed for phenotypic consequences. Of these, 5 plants include the *LX* sense over-expression construct and the rest the antisense construct. While expression analysis is being carried out on the T1 plants their seeds were used to examine the segregation of the transgene in the T2 generation using PCR analysis for determining if the T1 plants are homozygous or heterozygous for the transformed construct. We currently have for most of the transformants homozygous plants. We have not used the *GUS* reporter gene for screening since in only few transformed plants *GUS* activity could be detected although the *LX* chimeric gene was present. The reason for this is not clear yet. The steady state level of the *LX* transcript mRNA in the green leaves of different transgenic plants was measured in northern blot analysis to verify that the gene is expressed. As shown in **Figure 6**, for some representative transformants, mRNA is transcribed in some of the plants, while in others it is very low or non-detected. Plants with low level expression were not used for further analysis. In order to examine the consequences to *LX* activity in the overexpressing transformed plants we have carried out activity gel assay. As shown for two transformant, for example in **Figure 7**, we observed higher RNase activity at a position, which agrees with the *LX* protein MW. The effect of *LX* inhibition or overexpression during senescence is currently under investigation and will include evaluation of the consequences to RNA metabolism. In general terms of growth and appearance no significant differences were observed between transgenic and wild type plants.

4. Cloning of the LX gene promoter toward identification of sequences required for senescence-induced gene expression (*in progress*).

We have utilized the *LX* RNase gene for identification of 5' flanking sequences required for senescence-specific gene expression. The preliminary nuclear run-on transcription experiments done indicate that *LX* is transcriptionally induced during senescence. Using PCR we have cloned a DNA fragment containing 0.7 kb of the *LX* gene 5' upstream genomic sequences. This DNA fragment was inserted upstream of the reporter *GUS* gene coding sequence in the pCAMBIA2381Z T-DNA transformation vector which was used for transformation of tomato plants. This vector is a derivative of the pCAMBIA1381Z plasmid (Roberts et al., 1997) in which we exchanged the hygromycin gene coding sequence with that of the kanamycin gene. Transgenic tomato plants harboring this construct were generated. Six plants were already characterized and additional 8 plants, which contain the transgene are currently analyzed. Out of 6

characterized plants two were found to express the *GUS* gene specifically in senescing leaves (Fig. 8), two plants expressed it constitutively at a very high level and in two *GUS* activity was not detected. This inconsistency could be due to lack of all the required regulatory elements in the 0.7 kb DNA fragment which led to large sensitivity to position effect. In a recent report characterizing the senescence-specific *SAG12* promoter it was concluded that the region between -747 to -570 was important for expression during senescence while an enhancer element which markedly enhance the expression is localized in the -1345 to -1181 region (Noh and Amasino, 1999). Thus additional 5' *LX* gene flanking sequences might be needed. We have recently cloned an additional 1 kb DNA fragment and its sequence information is currently used to construct an *LX* promoter-*GUS* gene which will consist of 1.7 kb of the promoter.

Discussion

Little is known about the specific identities, functions or regulation of nucleases involved in senescence. In this research we have initiated a study aimed for identification and characterization of senescence-related RNase and nuclease activities for understanding their function and regulation in this process.

Two nuclease activities were detected in tomato leaves. LeNUC1, was found to be senescence-specific while LeNUC2 was constitutive. The biochemical characteristics of LeNUC1 and LeNUC2 showed high similarity between the two and suggested they belong to the plant nuclease I type enzymes (Bariola and Green, 1997). The only biochemical difference found between them was the fact that LeNUC1 is a glycoprotein. It is possible that LeNUC2 is a glycosylated form of LeNUC1 and that this glycosylation event takes place during senescence. The changes in LeNUC1 nuclease activity resemble the expression pattern observed for the RNase *LX* gene in senescence suggesting that they both respond to the same senescence regulatory mechanism. Thus comparison of their promoters may identify sequence elements required for this control. Previously leaf senescence-associated nuclease was identified in naturally senescing wheat leaves (Blank and McKeon, 1989). However, LeNUC1 does not seem to be related to the wheat nuclease based on its characteristics. LeNUC1 seem not to be related also to the BFN1 nuclease, described in this work. BFN1 shows close similarity in its protein sequence with the nuclease ZEN1 (Aoyagi et al., 1998) which, unlike LeNUC1, is insensitive to reducing agents and is enhanced by Zn^{2+} (Thelen and Northcote, 1989). Southern blot analysis did not detect *BFN1* homologous sequences in tomato, nor could we using PCR and degenerate primers designed according to BFN1/BEN1/ZEN1 conserved sequences, amplify any sequences using tomato DNA.

Nuclease activity in plants has been reported to rise prior to or concomitantly with several programmed cell death (PCD) processes in which DNA degradation or fragmentation is observed (Young and Gallie, 1999; Mittler and Lam, 1995). However, there is no direct evidence yet for the function of any of these nucleases in PCD or to allow relationship between these nucleases and LeNUC1. Recently a caspase-activated DNase was shown to function in degradation of DNA during apoptotic murine cell death (Enari

et al., 1998), however, the function of most nucleases is not clear in either plants or other investigated biological systems.

We have demonstrated that the *LX* gene, previously shown to be induced by Pi-starvation in cultured tomato cells, is induced in senescing tomato leaves. Previous studies were restricted to analysis of *LX* expression under conditions of Pi-starvation in suspension culture cells (Koak et al., 1995; Löffler et al., 1992) and nothing was known concerning expression or function of the *LX* in planta. The tomato *LX*, and the *Arabidopsis* RNS2 and RNS1 RNases share both structural and apparent functional similarities (Taylor et al., 1993). These RNases belong to the S-like RNase group based on their amino acid sequence, and are induced by Pi-starvation and during senescence. The apparent similarity observed between tomato and *Arabidopsis* imply that utilization of the same RNase enzyme system during either Pi-starvation or senescence for Pi-recycling is common to different plants. The observation that *LX* is induced during the late stage of senescence suggests that it has a role in final stage degradation of bulk RNA molecules for Pi recycling. Similarly, the senescence-related nucleases might participate in Pi recycling by employing either their RNase activity for RNA degradation or their DNase activity for degradation of non-functional DNA molecules. A source of such DNA could be the genome of the degenerating chloroplast, of which the level seems to decrease in senescence. Unlike the senescence-associated RNases examined, such as RNS2 (Taylor et al., 1993; Bariola et al., 1994) and *LX*, which are induced also during phosphate starvation, the *BFN1* nuclease is not. This observation suggests that the senescence-associated nucleases are highly specific to this process.

Scientific implications

Knowledge about function of the enzymes that mediate hydrolysis of macromolecules during leaf senescence and the control of senescence-specific gene expression, is very limited. Our research provides new information regarding these issues and forms a solid basis for continuation. The identification of *LX* as a senescence-related RNase provides more evidence for the link between Pi-starvation and phosphate recycling in senescence. *BFN1* is the first example of a senescence-associated gene encoding a nuclease I enzyme. It is also the first nuclease I cloned and characterized from *Arabidopsis*. The properties of *BFN1* and *LX* genes indicate that they will be useful tools in the study of senescence and the degradation of nucleic acid. Characterization of the regulatory elements, which play a role in senescence, initiated in this project, together with recently published information, will enable to understand the basis for senescence-specific gene expression.

Potential impact on agriculture, industry, environment, policy legislation.

The regulatory pathways that govern the expression of senescence related genes, including RNase/nuclease encoding genes, may actually control the progress of senescence and the longevity of the plant tissue. Knowledge about the genetic control of senescence may enable us, in the future, to develop new strategies for the delay of leaf senescence in transgenic plants in which leaf senescence or some of its

characteristics (e.g. macromolecule degradation) will be delayed. A delay in leaf senescence has a large agricultural and commercial value. The biomass of harvested agricultural products could be increased and the postharvest life of the fresh produce could be extended. Successful application of strategies aimed for manipulation of senescence using key genes will depend on usage of appropriate promoters such as that of the *LX* gene. These will allow expression of the chosen genes in the desired time and location. Non-specific expression of those genes may result in deleterious effect on plant development.

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All Publications/patents resulting from the project

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Appendix 1 – Figures

Figure 1. Activity gel analysis of changes in nuclease activities during postharvest senescence of detached parsley leaves. Proteins extracted at different times following harvest were loaded on basis of either equal protein (upper panel) or equal FW equivalent (lower panel).

Figure 2. Nuclease activities in naturally or artificially senescing parsley leaves. Protein samples (15 µg) extracted from artificially senescing leaves following 8 d of dark incubation or from naturally senescing fully yellow leaves were subjected to gel activity assay using RNA as a substrate.

Figure 3. Substrate specificity of parsley leaf nucleases. Protein samples extracted from young or artificially senescing leaves were subjected to gel activity assay using either RNA or DNA as a substrate.

Figure 4. Effect of different growth regulators on the senescence-related nuclease activities in detached young parsley leaves. Leaves were continuously treated with ethylene or treated initially with the other growth regulators as described. Proteins were extracted following 48 h of dark incubation and analyzed using the gel activity assay using RNA as a substrate. Harvest, young leaves frozen immediately upon detachment; control, 48 h dark incubated leaves with no growth regulator application.

Figure 5. Levels of putative RNases mRNA in young (Y) and naturally senescing (S) parsley leaves. Probes used were SP1 and 2 cDNA clones cloned from parsley and which show very high identity in their deduced protein sequence to RNases.

Figure 6. Transcript level of the *LX* antisense gene in young leaves of different transgenic tomato plants. RNA was extracted from green young leaves and following Northern blot analysis and the blot was hybridized with *LX* cDNA probe. Different independent transgenic plants are labeled with letters. The lanes of RNA isolated from control plants senescing or young leaves is indicated.

Figure 7. RNase activities in *LX* gene over-expressing transgenic plants. Proteins were extracted from young green leaves of I and M transgenic plants and subjected to activity gel assay using RNA as a substrate. For each plant we have examined activity in several progenies of the T0 plant, which were screened for the presence of the transgene. Signs of + or - indicate if the plants were found positive or negative for the transgene.

Figure 8. Gus activity in leaf disks of two transgenic plants which contain the *LX* 0.7 kb promoter fused to the *GUS* gene. Leaf disks from young or senescing leaves were subjected to the GUS activity assay using bromo-4-chloro-3-indolyl- β -D-glucoronid acid as a substrate.