Suppression of LX Ribonuclease in Tomato Results in a Delay of Leaf Senescence and Abscission \[W\]

Amnon Lers*, Lilian Sonego, Pamela J. Green, and Shaul Burd

Department of Postharvest Science of Fresh Produce, Agricultural Research Organization, Volcani Center, Bet Dagan 50250, Israel (A.L., L.S., S.B.); and Delaware Biotechnology Institute, University of Delaware, Newark, Delaware 19711 (P.J.G.)

Although present in different organisms and conserved in their protein sequence, the biological functions of T2 ribonucleases (RNase) are generally unknown. Tomato (*Lycopersicon esculentum*) LX is a T2/S-like RNase and its expression is known to be associated with phosphate starvation, ethylene responses, and senescence and programmed cell death. In this study, LX function was investigated using antisense tomato plants in which the LX protein level was reduced. LX protein levels normally become elevated when leaves senesce and antisense inhibition of LX retarded the progression of senescence. Moreover, we observed a marked delay of leaf abscission in LX-deficient plants. This correlated with specific induction of LX protein in the tomato mature abscission zone tissue. LX RNase gene regulation and the consequences of antisense inhibition indicate that LX has an important functional role in both abscission and senescence.

The T2 RNases (EC 3.1.27.1) are secreted endoribonucleases that are found outside cells or in compartments of the endomembrane system with no absolute substrate base specificity (Irie and Ohgi, 2001; Deshpande and Shankar, 2002). First isolated from fungi, proteins in this family have subsequently been identified in a wide variety of organisms, ranging from viruses and bacteria to mammals; thus, it is the most broadly distributed family of RNA-degrading enzymes known (Irie, 1997, 1999; Deshpande and Shankar, 2002). Although biological functions of various T2 RNases have been postulated on the basis of enzyme location or regulation of gene expression, the physiological roles of these enzymes are generally unknown. RNases have been hypothesized to have roles in RNA turnover and metabolism and also to be involved in nutrition and viral pathogenicity. A novel function in regulation of cell membrane permeability was suggested for the yeast (*Saccharomyces cerevisiae*) Rny1 T2 RNase (MaIntosh et al., 2001).

The only plant enzymes in the T2 family for which the in vivo role is known are the S-RNases, which are involved in gametophytic self incompatibility in plants (Lee et al., 1994; Murfett et al., 1994; Golz et al., 1995) and require RNAse activity for their functioning (Huang et al., 1994). The other major group of T2 plant RNases, the S-like RNases, has been found in all plants that were examined for their presence, which is indicative of an important function; however, the nature of this function is largely unknown in plants, as in other organisms.

S-like RNase genes are often expressed in diverse organs and in response to different environmental conditions (Green, 1994; Bariola and Green, 1997). According to these expression patterns and potential or identified localizations, various possible functions were attributed to these enzymes in plants. Based on the effects of pathogen attack or wounding, roles for RNases in defense were suggested: induction of the tobacco (*Nicotiana tabacum*) NE gene by a pathogen (Galiana et al., 1997), which could inhibit plant pathogen hyphal growth (Hugot et al., 2002); induction of the tobacco NGR1 and NGR3 RNases by wounding and *Tobacco mosaic virus*, respectively (Kurata et al., 2002; Hayashi et al., 2003); local and systemic wound induction of the Arabidopsis (*Arabidopsis thaliana*) RNS1 (LeBrasseur et al., 2002), and wound induction of the tomato (*Lycopersicon esculentum*) LE (Lers et al., 1998; Gross et al., 2004) and tobacco NW (Kariu et al., 1998) RNases. An effect on the activity of RNases has also been observed in plants, following abiotic stresses such as ozone and salinity (Gomes-Filho and Sodek, 1988; Booker, 2004). An observed decrease in RNase activity in wheat (*Triticum aestivum*) following heat shock was hypothesized to have a role in the observed increase in mRNA stability (Chang and Gallie, 1997), whereas reduction in activity of specific RNases in response to low-O2 stress in maize (*Zea mays*) roots was suggested to facilitate conservation of nontranslating ribosomes and poorly translated mRNA (Fennoy et al., 1997).

The presence of vacuole-localized RNases and RNA (Boller and Kende, 1979; Abel and Glund, 1986, 1987;
Role of RNase LX in Abscission and Senescence

Abel et al., 1990) has led to the suggestion that vacuoles may participate in nucleic acid turnover. Secretion of RNases from the embryo and aleurone into the endosperm during germination (Barker et al., 1974; Rogers and Rogers, 1999) is thought to aid mobilization of stored RNA. The induction of S-like RNases by senescence in Arabidopsis (Taylor et al., 1993; Bariola et al., 1994) and tomato (Lers et al., 1998), and by phosphate (Pi) starvation in several plant species (Taylor et al., 1993; Bariola et al., 1994; Köck et al., 1995; Dodds et al., 1996) suggests a role in nutrient recycling that comprises scavenging Pi from RNA. In Arabidopsis, three genes, RNS1, RNS2, and RNS3, each with a unique pattern of expression, were described (Taylor et al., 1993; Bariola et al., 1994). Antisense-mediated decrease in the expression of the Arabidopsis RNases RNS1 or RNS2 genes, associated with senescence and Pi starvation, was sufficient to induce anthocyanin overproduction, indicating dependency on the activity of these enzymes for optimal plant growth (Bariola et al., 1999).

The expression of genes encoding for nucleic acid-degrading enzymes was described for several types of programmed cell death (PCD) processes in plants (Mittler and Lam, 1997; Aoyagi et al., 1998; Sugiyama et al., 2000; Kuriyama and Fukuda, 2002). The ZEN1 nuclease was even demonstrated to be responsible for degradation of nuclear DNA during PCD of tracheary elements (Ito and Fukuda, 2002). RNases have been reported to be induced in PCD processes, such as tracheary element differentiation (Thelen and Northcote, 1989; Ye and Droste, 1996), aleurone cell death (Rogers and Rogers, 1999), and endosperm development (Young and Gallie, 2000). The PCD-associated RNases might be part of the death execution machinery or may be responsible for removal of RNA from the dying cells.

The tomato LX S-like RNase was originally identified as a Pi-induced RNase (Löffler et al., 1992). Expression of the LX gene was highly induced during Pi starvation (Köck et al., 1995; Bosse and Köck, 1998; Abel and Köck, 2001), during advanced stages of leaf senescence, and upon ethylene treatment in young leaves (Lers et al., 1998). The involvement of LX in PCD processes is also suggested by its expression during seed germination and xylem differentiation (Lehmann et al., 2001). To investigate more directly the function of LX, we have generated LX-deficient antisense tomato plants and our results regarding LX gene expression and the consequences of its inhibition support the hypothesis of an important and novel role in the advancement of both abscission and senescence.

RESULTS

LX RNase Protein Is Induced during Senescence and Its Level Is Reduced in the LX Antisense Lines

To detect and measure LX protein levels during development and following modulation of LX gene expression, we generated polyclonal antibodies specific for the LX protein that was overexpressed in bacterial cells. The specificity of the antibodies was demonstrated by lack of cross-reaction with any induced protein following western-blot analysis performed with tomato leaf proteins extracted from wounded leaves (data not shown). The LE RNase, which shares the highest degree of amino acid sequence similarity with LX among known tomato proteins, is induced by wounding (Lers et al., 1998; Gross et al., 2004). Furthermore, as outlined below, the pattern of LX protein regulation matched the pattern of LX expression measured before for the transcript (Köck et al., 1995; Lers et al., 1998; Lehmann et al., 2001). These antibodies were used to follow LX protein levels in western-blot analysis, performed with proteins extracted from tomato leaves at various senescence stages. The results shown in Figure 1A confirm that the antibodies raised detected the senescence-induced LX protein with a kinetic similar to the induction of the transcript demonstrated before (Lers et al., 1998). Induction of LX protein was

![Figure 1](image-url)

Figure 1. Induction of the LX protein level during senescence and in response to ethylene and its inhibition in LX antisense lines. A, Regulation of the LX protein level during leaf senescence. Proteins extracted from leaves at different stages of natural senescence from very young leaf (L5) up to fully yellowed leaf (L1). The arrow indicates the progress of senescence. Proteins extracted from the same fresh weight of leaves with six developed leaves were exposed to ethylene in air at 2 μL L⁻¹ for 24 h, followed by air streaming. B, Changes in LX protein level in young leaves following ethylene treatment. Proteins extracted from leaves at different positions on the stem and at different times were used in western-blot analysis for measuring the LX protein level. L1 to L4, Leaves at position 1 (lowest) to position 4 on the stem, respectively. C, Comparison of LX protein level between wild-type and antisense lines in young leaves following ethylene treatment. Intact 6-week-old plants were subjected to ethylene (10 μL L⁻¹) treatment for 18 h. Protein samples (10 μg) extracted from young leaves at two different positions were subjected to western-blot analysis. SL, Senescing leaf; VF36, wild-type control; A2, H9, and T2, independent LX antisense transgenic lines.
also visualized during senescence of petals (Supplemental Fig. S1).

The LX antibodies were further used to determine whether induction of the LX gene by ethylene in nonsenescing leaves occurred at the protein level, as predicted from our previous analysis showing induction at the mRNA level (Lers et al., 1998). Intact soil-grown young tomato plants, with six developed leaves, were exposed to 10 μL L⁻¹ ethylene on air in a continuous gas flow-through system. Proteins extracted from four leaves at different positions on the stem, from position 1 (the first leaf at the lower position that was fully expanded and green with no signs of yellowing) up to position 4, were used in western-blot analysis. Proteins extracted from stem, cotyledon, and root tissues were also used. The analysis revealed induction of LX protein within 24 h into the ethylene treatment (Fig. 1B). The LX level increased in a leaf age-dependent manner, but, after an additional 24 h under air, high levels of LX protein were observed in leaves in all positions.

Antisense plants were generated to investigate the consequences of inhibiting the expression of the LX gene. Tomato VF36 plants were transformed with an LX antisense construct. The transforming vector, pLXAS9-11, was based on the pBI121 plasmid in which the full cDNA of LX was cloned in inverse orientation under the direction of a doubly enhanced cauliflower mosaic virus (CaMV) 35S promoter and the E9 terminator. Plants homozygous for the antisense transgene were identified by PCR analysis and further screened for the effects on LX gene expression. The LX protein level was measured with western-blot analysis and the LX antibodies we generated. Initially, the level of LX was compared between senescing, same-age leaves of wild-type and antisense plants. In some of the antisense lines, the LX protein level was found to be reduced (data not shown). To more accurately quantify and compare the LX protein level between wild-type and LX antisense plants, the LX level was measured following its induction by ethylene in young green leaves. Young tomato plants, about 6 weeks old and harboring seven or eight leaves, were treated by streaming ethylene in air at 10 μL L⁻¹ for 18 h. The plants were grown on perlite with a complete artificial nutrient supply. Total proteins were extracted from the second and third leaves and used for measurement of LX protein by western-blot analysis. In three independent LX antisense lines—A2, H9, and T2—the LX level was reduced markedly compared with that observed in wild-type plants (Fig. 1C). LX gene expression was also measured by reverse transcription-PCR, which revealed a reduced LX transcript level in the antisense lines compared with wild-type plants (data not shown). These three LX antisense lines, A2, H9, and T2, were used for the remainder of this study.

Inhibition of LX Gene Expression Results with Anthocyanin Accumulation during Growth under Pi-Limiting Conditions

When wild-type tomato and the three antisense lines were grown under optimal growth conditions, either on soil or on perlite fully supplemented with nutrients, no obvious phenotypic differences were detected. Next, we compared the growth of the wild-type and the LX-deficient lines under conditions of Pi deficiency. Tomato seeds were germinated on perlite and irrigated with full nutrient solution. Once cotyledons had emerged, irrigation was continued with Pi-deficient nutrient solution. Leaves and cotyledons of the antisense lines appeared more purple than those of the wild type, especially in the veins, which suggests that anthocyanin was elevated in the antisense plants. To quantify anthocyanin levels, leaf disc samples were harvested from the first true leaf of each plant and anthocyanin and chlorophyll levels were measured. The results shown in Figure 2 demonstrate significantly higher anthocyanin levels in the three LX-deficient lines than in the wild type, whereas the chlorophyll content showed no significant difference. When Pi starvation was imposed at a later stage of plant development, anthocyanin induction was observed mainly in the new leaves, which had developed after the application of Pi starvation (data not shown).

Senescence Is Delayed in LX-Deficient Plants

To determine whether inhibition of LX gene expression affected senescence, the progress of senescence was compared between the wild type and three antisense lines. Tomato plants were grown in the greenhouse in propagation trays with fertilizer included in the soil and irrigated with tap water. Following about 4 weeks of growth under these conditions, the plants developed four true leaves, but cotyledons in some of them had entered senescence, as indicated by the initiation of yellowing. At this stage, the cotyledons...
from all the plants were collected for analysis. Following 3 weeks of further growth, when the first true leaves in some of the plants had begun to senesce and significant yellowing was apparent, the first true leaves of all the plants were collected for analysis. Leaf or cotyledon tissue was homogenized and used for quantifying the levels of chlorophyll and total soluble protein as senescence markers and for measuring the LX protein level by western-blot analysis. Samples for all three measurements were taken from the same tissue homogenate.

These analyses revealed higher chlorophyll and protein content relative to fresh weight in both the cotyledons (Fig. 3A) and first leaves (Fig. 3B) in the three antisense lines than in the wild type, indicating a delay in senescence in the antisense lines. The delay in senescence in the three antisense lines was also manifested in a slower decline, compared to wild type, in the levels of the large and small subunits of the Rubisco enzyme (data not shown). Western-blot analysis of the protein extracts confirmed the higher level of the LX RNase protein in the wild-type plants than in the three LX-deficient antisense lines. To examine the effect of LX inhibition on senescence that was induced under different growth conditions, we have compared the initiation of leaf senescence between wild-type and LX-deficient plants under Pi starvation. Tomato plants were grown in perlite and watered with full nutrient solution for 5 weeks and then with Pi-deficient solution. Following a further 3 weeks, there was a visible difference between wild-type and LX antisense transgenic plants in the progress of senescence. Fixed-area leaf discs were sampled from the first true leaves of the plants and chlorophyll and protein were extracted and quantified. Both chlorophyll and protein content were found to be much lower in the leaves of the wild type than in the three lines of LX-deficient antisense plants (Fig. 3C).

Leaf Abscission Is Delayed in LX-Deficient Plants

We observed that when LX-deficient and wild-type plants were germinated and grown in perlite under Pi-limiting conditions or were irrigated with water

![Figure 3](image-url)
with no nutrient supply, the cotyledons and leaves of the young plants abscised much earlier in the wild-type plants than in the antisense ones (Fig. 4A). Abscission occurred before the appearance of any signs of senescence in the abscised cotyledons or leaves so that early abscission seemed to be unrelated to the effect of LX inhibition on senescence. Furthermore, in many cases, signs of epinasty were observed in the nutrient-limited plants, which suggest that high levels of ethylene were synthesized in response to stress conditions. Ethylene is known to have a key role in the induction of abscission processes in plants and we hypothesized that the observed delay in cotyledon/leaf abscission in the LX-deficient antisense plants may have resulted from lack of a sufficiently high LX level. To establish and quantify the difference between LX-deficient antisense and wild-type plants in the occurrence of abscission, we have measured the difference in the time needed for cotyledon and leaf detachment following induction of the process. The wild-type and three LX-deficient antisense lines were grown on soil under optimal growth conditions for about 6 weeks, until five or six leaves had developed. To induce abscission, we initially removed the leaf blades (deblading) from the three lower leaves in each plant, but not from the cotyledons. Deblading is known to accelerate petiole abscission because it reduces the auxin supply. After 36 h, plants were treated with ethylene at 2 μL L⁻¹ for 36 h and then returned to the greenhouse. Immediately following ethylene treatment (designated as time zero of the experiment) and on each subsequent day, the numbers of abscising cotyledons and petioles were counted and the results were presented as percentages of the initial total numbers in each line (Fig. 4, B and C).

The cotyledons were more sensitive than the leaves to the abscission-inducing ethylene treatment. Differences in the percentage of abscising cotyledons were observed immediately following ethylene treatment: Whereas more than 50% of the cotyledons abscised...
from the wild-type plant, less than 10% of the cotyledons abscised in each of the three antisense LX-deficient lines (Fig. 4B). After 1 d, the differences were less pronounced, but cotyledon abscission was still lower in transgenic than in wild-type plants. After 3 d, cotyledon abscission reached 90% to 100% in all lines (Fig. 4B). Abscission of the petioles occurred at a much reduced rate compared to that observed for the cotyledons: Immediately following ethylene treatment, about 10% petiole abscission was observed in wild-type plants, but none in any of the LX-deficient lines (Fig. 4C) and, after 3 d, petiole abscission was more than 80% in the wild-type plants compared with 10% to 40% in the three antisense lines (Fig. 4C). Even after 10 d, a difference in the progress of abscission was maintained between the wild-type and the antisense lines and only after 19 d following the treatment were there comparable levels of petiole abscission in the various tomato lines (Fig. 4C). The duration of the abscission delay varied among the three LX-deficient lines, with the A2 and T2 lines showing a more inhibited phenotype than the H9 line for both cotyledon and leaf abscission (Fig. 4, B and C).

In additional experiments aimed at comparing abscission between wild-type and LX-deficient plants, more mature tomato plants were used and abscission was induced only by deblading. The plants were grown on soil in the greenhouse for about 3 months, by which time they had initiated flowering and had developed about 10 mature leaves. Deblading was applied to the leaves in positions 5 to 8, counting from the base of the plant upward, leaving 5-cm-long petioles. The total number of petioles that abscised from each position was counted separately for each line and the abscission levels are presented as percentages that abscised out of the total number of debladed petioles (Fig. 5). The three LX-deficient lines exhibited pronounced delay in the abscission of petioles from all positions compared with that of wild-type plants (Fig. 5). The difference in the progress of the petiole abscission process was more evident in the higher positions, where the induction of the process was slower than in the more mature, lower positions. For example, among the petioles in the eighth position, by 20 d after leaf deblading, wild-type abscission had reached 100%, compared with about 10% for the H9 line, and no abscission in the A2 and H2 LX-deficient lines (Fig. 5). Interestingly, in this experiment, as well as in the previous one (Fig. 4, B and C), the H9 line seemed to be less inhibited in abscission progress than the two other lines, A2 and T2 (Fig. 5).

**LX Protein Level Is Specifically Induced in the Abscission Zone**

The observed influence of LX inhibition on the progress of abscission raised the possibility that LX RNase is involved in this process. Experiments were...
performed to examine the possibility that LX is expressed in the abscission zone (AZ) in association with the progress of abscission. We used LX-specific antibodies to measure the level of LX protein in mature and young AZs and in adjacent tissue that served as a control. Tissue slices of 2- to 3-mm thickness that included the AZ cells were sampled from leaf petioles that were approaching abscission and from young leaf petioles. As a control tissue, an adjacent slice of the petiole a few millimeters away from the AZ was sampled as well. Proteins were extracted from pooled tissue slices from different plants and, following electrophoresis, LX levels were measured by western-blot analysis.

The results shown in Figure 6A indicated high LX protein levels in the tissue that included the mature AZ, whereas much less of the protein was observed in the nearby petiole control tissue (Fig. 6A). In the young AZ, as well as in the nearby tissue, LX protein levels were very low (Fig. 6A). Additional experiments in which LX protein level was measured in the AZ at different stages during its development revealed that LX protein accumulation is not a late event that occurs just a few days before actual abscission, but that it is initiated as much as 2 weeks before abscission takes place and is induced to a higher level as abscission approaches (data not shown). We compared the levels of LX protein accumulation in the AZs of wild-type and of LX-deficient plants: Slices of tissue that included the AZs and nearby petiole tissue were sampled from petioles of leaves at the same stage of advanced senescence, and western-blot analysis of the extracted proteins revealed higher levels of LX protein in the AZs of wild-type plants than in those of the antisense plants (Fig. 6B). Similar analysis of LX protein levels in the AZs of ripe tomato fruit demonstrated induction of LX in the fruit pedicle AZ similar to that in the leaf AZ, as well as reduced levels of LX in antisense plants (Fig. 6C). The reduction in the LX protein level was observed mainly in the A2 and T2 lines; it was less in the H9 line in which, as mentioned above, there was less retardation of abscission than in the other two lines.

To examine whether AZ-related expression of an RNase occurs in a different plant, tree tobacco (Nico-

tiana glauca) plants were examined for the presence of a related RNase associated with both leaf senescence and abscission. When tomato LX antibodies were used in western-blot analysis of proteins extracted from senescing or young tobacco leaves, a protein with a similar molecular weight to that of LX was detected specifically in senescing leaves (Fig. 7A). This tobacco LX-related protein was found to be induced in the tobacco leaf AZ compared to its level in the nearby petiole tissue (Fig. 7B).

**DISCUSSION**

Although widespread among a variety of different organisms, the in vivo functions of the T2 RNases are largely unknown. In only one case has a function been attributed to T2 RNases in plants, that of the S-RNases involved in self incompatibility. Our results demonstrate the involvement of a T2/S-like RNase, the LX, in plant abscission, as well as provide evidence for a function in senescence. Previously, a role for RNases in senescence was only implied on the basis of correlations between activation of gene expression or enzyme activity and senescence. Retardation of leaf senescence was observed in LX-deficient antisense plants, suggesting a link between RNA degradation and senescence progress. The involvement of LX RNase in
Role of RNase LX in Abscission and Senescence

abscission is supported by both its specific expression in the AZ and the marked delay of leaf abscission observed in the LX-deficient antisense plants. The finding of LX involvement in abscission suggests the association of a new group of hydrolytic enzymes, namely, nucleic acid-degrading enzymes, in abscission.

Induction of the LX Protein in Petal Senescence and in Response to Ethylene

The tomato LX RNase was identified originally as a Pi starvation-induced RNase (Loffler et al., 1992), which suggests its involvement in Pi metabolism and recycling. This hypothesis is further supported by induction of LX during senescence (Lers et al., 1998). Our present analysis of LX protein levels revealed high induction, especially in the late stages of leaf senescence and in response to ethylene in young leaves (Fig. 1). This is consistent with regulation of LX mRNA levels and suggestive of regulation mainly at the RNA level. The induction of LX protein in tomato flower petal senescence was also demonstrated by Lehmann et al. (2001). We extended this by separating petals from the other floral parts and observed that, besides its senescence-associated expression in petals, LX was highly expressed in a senescence-independent manner in the stamen plus pistil (Supplemental Fig. S1). This suggests a function of the LX RNase in processes unrelated to senescence. Induction of LX protein by external ethylene in green leaves followed a similar pattern to that previously observed for its mRNA (Lers et al., 1998), but clear dependency on the developmental stage of the leaf was observed: The younger the leaf, the more time required for induction by ethylene. This leaf-age dependency might be explained by differences among the leaves of different ages in their ethylene-sensing system or in the machinery responsible for LX activation. Ethylene treatment seemed to induce LX in the roots too, but to a lesser extent than in the leaves, whereas no detectable LX protein was observed in the stem (Supplemental Fig. S1).

Overall, the observed expression pattern of LX suggests a complex regulation of this gene in response to tissue identity, developmental stage, hormone action, and environmental conditions.

LX Protein Level Was Reduced in Antisense Plants and Results in Anthocyanin Accumulation

The expression pattern of LX suggests that it has a function in the response to Pi-limiting conditions, PCD-related processes, and senescence. The antisense tomato lines developed in this study to examine the consequences of inhibiting LX expression had strongly reduced levels of LX protein, but residual LX protein could be detected. Tomato plants with reduced LX protein levels developed without major phenotypic alteration under optimal growth conditions and only when they were exposed to Pi-limiting stress conditions did the limiting level of LX result in elevated anthocyanin levels compared to that measured in the wild type, indicating stress while chlorophyll levels remained similar (Fig. 2). Elevated anthocyanin levels were also reported for RNS1- or RNS2-deficient Arabidopsis plants made using antisense constructs (Bariola et al., 1999). The stress indicated by anthocyanin accumulation in LX-deficient plants grown under Pi-limiting conditions could be indicative of an important role for RNase in scavenging Pi from cellular RNA. The finding of an increase in anthocyanin level, mainly under Pi-limiting and not under optimal growth conditions, supports the hypothesis of a function for LX in Pi reutilization, although we cannot rule out the possibility that the elevated anthocyanin levels resulted from a combined effect of limited Pi and unrelated stress imposed by the reduced level of LX.

Leaf Senescence Is Delayed in LX-Deficient Plants

The negative influence of reduced LX RNase levels on the progression of leaf senescence that we observed seems unlikely to be a consequence of a possible function of LX in Pi reutilization. If, in the antisense plants, the reduced level of LX results in elevated stress due to Pi limitation, we would expect senescence to be accelerated because nutrient limitation is known to induce senescence. However, we observed delayed senescence in LX-deficient plants compared with the wild type whether the process was induced either by general nutrient limitation caused by reduced soil volume or by reduced Pi supply. Retardation of leaf senescence was manifested in higher levels of both chlorophyll and protein content. This senescence delay effect of LX deficiency was fairly unexpected. Because the LX gene was found to be induced at a relatively late stage of senescence, we did not anticipate that reducing its level would result in retardation of leaf senescence at such an early stage. One possible explanation is that there is cross talk (i.e.
Leaf Abscission Is Delayed in LX-Deficient Plants

A clear delay in leaf abscission was observed in the LX-deficient antisense plants relative to wild-type plants. The involvement of LX RNase in the abscission process is an unexpected finding. To our knowledge, no relationship between RNases and abscission was reported before. Abscission is a natural part of plant development in which leaves, flowers, or fruits separate from the plant (Bleecker and Patterson, 1997; Patterson, 2001; Roberts et al., 2002). The AZ has a crucial role in the process; it is composed of several layers of differentiated cells that acquire competence to respond to ethylene and auxin, hormones known to induce or inhibit abscission, respectively. The central role of ethylene is indicated by its ability to induce premature abscission (Taylor and Whitelaw, 2001). Organ separation occurs within the AZ as a result of the hydrolysis of the middle lamella. Few genes have been described that are induced and function in the AZ. Cell wall-degrading hydrolytic enzymes, including polygalacturonases and \( \beta \)-1,4-glucanases (cellulases), have been hypothesized to have a central role in the process (Greenberg et al., 1975; Lashbrook et al., 1998; Brumell et al., 1999; Roberts et al., 2002). Other genes induced in the AZ are expansins (Cho and Cosgrove, 2000; Belfield et al., 2005), pathogenesis-related proteins (Eyal et al., 1993; Coupe et al., 1997), and metallothionines (Coupe et al., 1995); however, their roles are not clear.

In this study, the effects of reduced LX levels on abscission were demonstrated during various stages of plant development and they do not seem to be related to a function of LX in senescence. Retarded abscission of cotyledons was observed in very young antisense plants when abscission was accelerated by nutrient limitation. It is likely that enhanced production of stress ethylene, which led to detectable leaf epinasty, resulted in induction of abscission in the non-senescent cotyledons. Retardation of cotyledon and petiole abscission was observed in 6-week-old antisense plants following debblading and ethylene application, a treatment that did not result in senescence in the abscising cotyledons or petioles.

As the kinetics of abscission became more moderate, the differences in abscission rates between LX-deficient and wild-type plants increased. Comparison between the effects of LX inhibition on abscission of the eighth petiole and on that of the more mature fifth petiole (Fig. 5) revealed a greater difference between antisense and wild-type plants in the younger eighth petiole. These differences might result from elevated expression of the endogenous LX gene in mature AZs possibly due to increased ethylene sensitivity. This elevated expression could then diminish differences in the level of the LX protein between antisense and wild-type plants. The antisense H9 line exhibited less inhibition of abscission in most of the experiments in accordance with less suppression of the LX gene observed in this line.

LX Protein Is Specifically Induced in the AZ

Specific induction of LX in the AZs of both leaves and fruits further supports its role in the abscission process. The tissue print analysis indicated expression in an area that seems to include more than the few cell layers known to be included in the AZ. However, such spreading of the signal could result from the pressure imposed on the tissue during the printing process. More accurate experiments, such as immunocytochemistry, will be carried out to determine the specific cell localization of the LX protein in the AZs. Induction of LX-related, senescence-associated RNase in the tobacco leaf AZ suggests that this enzyme is involved in abscission in tobacco as well.

Could LX Function in Abscission Be Related to a PCD Process?

Both our functional and expression studies indicate that LX RNase has a role in the abscission process; however, the specific function of LX is not clear yet. On the basis of gene expression analysis, LX was hypothesized to function in PCD-related processes (Lehmann et al., 2001). One possible hypothesis for the demonstrated involvement of LX in abscission would be the occurrence of a PCD process in the AZ whose normal progression is a prerequisite for the occurrence of abscission in tomato and in which LX had a role.
Viability staining of the AZ tissue with Evans blue in both tomato and tobacco demonstrated the presence of dead cells around the AZ prior to the occurrence of abscission (A. Lers, S. Burd, and L. Sonogo, unpublished data), but more evidence is required to demonstrate the occurrence of a PCD process during abscission. We have also observed specific induction of protease and nuclease activities in tomato and tobacco AZs and activation of the Arabidopsis senescence-associated nuclease BFN1 promoter in AZs of Arabidopsis flower petals (S. Burd, S. Farage Barhom, and A. Lers, unpublished data). These observations further support the hypothesis of PCD occurrence in abscission. Nucleases and proteases have been found previously to be involved in PCD processes in plants (Beers et al., 2000; Sugiyama et al., 2000; Woltering, 2004; Lam, 2005).

The possible involvement of PCD in the abscission process has been discussed in several reviews (Sexton and Roberts, 1982; Beers, 1997; Gonzalez-Carranza et al., 1998; Roberts, 2000; Morgan and Drew, 2004). Interestingly, the term apoptosis, which has become a generic term for PCD processes, was originally applied to the shedding of leaves or petals mediated by abscission (Kerr and Harmon, 1991). It was suggested that the occurrence of cell death during abscission is more likely to be a result of cell separation rather than its cause (Sexton and Roberts, 1982; Morgan and Drew, 2004). It was argued that cells that separate must remain metabolically functional or wall loosening would not take place (Beers, 1997; Roberts, 2000). Nevertheless, evidence was accumulating to support the hypothesis that more than a single class of AZ cells might exist and that those cells immediately adjacent to the separation site might lose their viability (Evensen et al., 1993; Tirlapur et al., 1995; van Doorn and Stead, 1997; Roberts, 2000; Kitajima et al., 2003; Macnich et al., 2005).

The demonstrated involvement of an RNase in abscission opens up a new avenue in the study of the abscission process, which so far was mostly associated with cell wall-degrading enzymes. We believe this finding may lead to new insight on both the general function of RNases in plants and the mechanism of the abscission process and further studies of LX and other nucleases is required.

MATERIALS AND METHODS
Plant Growth Conditions and Treatments
Tomato (Lycopersicon esculentum) line VF36 was used throughout the study. Tomato seeds were germinated on perlite support at 28°C in the dark and, after 3 d, were transferred to light. About a week later, when cotyledons had fully developed, the seedlings were transferred to 12-cm containers filled with either perlite or HR1 artificial soil (Hagarin Ltd). The plants were grown in a greenhouse under a controlled temperature of 25°C and natural daylight. The plants grown on perlite were watered with nutrient solution, with or without Pi, as described by Bosse and Kock (1998).

Abscission was induced by removal of the leaf blade with a sharp razor, leaving most of the petiole intact. For ethylene application, intact tomato plants in their containers were placed for the required time in sealed transparent Perspex jars fitted with inlet and outlet ports and connected to a flow-through system. Ethylene mixed with air was bubbled through sterile water to maintain humidity in the jars, and the flow rate was maintained at 100 mL min⁻¹.

Chlorophyll, Protein, and Anthocyanin Quantification
Chlorophyll and protein were quantified in the same tissue extract. One to three discs, 7 mm in diameter, were sampled from the leaves, placed in a microtube in 150 μL of extraction buffer (50 mM Tris-HCl, pH 7.5, 0.1% [w/v] SDS, and 10% [w/v] polyvinylpyrrolidone), and ground with a fitting pestle and a motorized drill. Chlorophyll was extracted from a sample of the solution with 80% acetone and its content was measured spectrophotometrically according to Porra et al. (1989). The protein content was determined with the Bradford assay, performed with a protein assay kit (Bio-Rad). Anthocyanin was extracted and measured as described (Bariola et al., 1999).

Generation of Transgenic Antisense Plants
To generate the LX antisense plant transformation vector pB9-11, the entire LX cDNA (accession no. X79338) was first fused in the antisense orientation between the doubly enhanced CaMV 35S promoter and the E9 terminator in plasmid p1185 (Diehn et al., 1998) following removal of the globin coding region. The 35S antisense LX-E9 expression cassette was inserted in the HindIII site of pB121 (Jefferson, 1987) so that the cassette was oriented in the same direction relative to the β-glucuronidase gene of pB121. The chimera construct was transferred via Agrobacterium tumefaciens strain EHA105 to cotyledons of tomato var. VF36 according to McCormick (1991). Kanamycin-resistant T0 plants were analyzed by PCR with specific LX and β-glucuronidase primers to verify the presence of the antisense gene. Homozygous lines were established and T3 or T4 lines were used for the experiments described. Based on Southern-blot analysis in all the antisense lines, a single copy of the transgene was introduced into the genome.

Antibody Preparation
LX sequences corresponding to amino acids 25 to 211 of the protein (accession no. X79338) were fused to a His tag, overexpressed in bacteria, and used for rabbit immunization. The appropriate 562-bp-long DNA was synthesized by PCR and cloned in the EcoRI site of the pET22(+)+ vector (Novagen) by means of specific primers extended by the EcoRI recognition site: LXRI primer (5′-CGAATTCACAAGTTGTCTACTTCTTAGG-TGTTC-3′) and LXRIB primer (5′-GACCTATTTTAAAGAAAACCGGT- TAACC-3′). The LX expression vector was transformed into Escherichia coli ER2566 strain and protein synthesis was induced by 1 mM isopropyl-β-D-galactoside. Induced cells were collected by centrifugation and protein was extracted with BugBuster lysis buffer according to the manufacturer’s instructions (Novagen). Purification of the overexpressed partial LX protein fused to a His tag was performed with the His bind Quick 900 cartridge (Novagen) in conjunction with the His bind buffer kit (Novagen), according to the manufacturer’s manual and in the presence of 8 M urea. The eluted protein sample was concentrated with a Vivavisin 20 concentrator (10,000 molecular weight cutoff; Vivascience). Rabbits were immunized by three injections with the purified partial LX protein: the first injection with 1 mg and the second and third injections with 0.5 mg each at intervals of 4 weeks.

SDS-PAGE and Immunoblot Analyses
Protein for immunoblot analysis was extracted from one to three leaf discs (7-mm diameter) or from stem segments. The tissue was homogenized in the presence of 2-fold (v/w) extraction buffer (50 mM Tris-HCL, pH 7.5, 0.1% [w/v] SDS, 10% [w/v] polyvinylpyrrolidone, and 1 mM phenylmethlysulfonyl fluoride) in a microtube by means of a fitting pestle and a motorized drill. Following 15-min centrifugation in a microfuge at 4°C, the soluble protein extract was assayed for protein content with the Bradford assay (Bio-Rad) and stored at −80°C. For analysis of proteins in experiments that involved senescing tissue, gel loading was based on fixed volumes of samples that contained proteins extracted from equal fresh weights of tissues because protein content markedly decreased during senescence. Flowers were harvested at three different developmental stages: Young flowers were those from when the bud had begun to open, with petals already yellow, until just before the flowers were fully opened (between stages 2 and 3; as described by Barry

Role of RNase LX in Abscession and Senescence
et al., 1996); mature flowers were only those that were fully opened (stage 3; Barry et al., 1996); and senescing flowers were those in which senescence had begun, as indicated by initiation of petal closure, and those in which the color had started to fade (stage 4 and later; Barry et al., 1996).

Protein extracts were mixed with sample buffer and boiled for 3 min before being separated on 12.5% or 15% SDS-PAGE gels (Laemmli, 1970). Separated proteins were either visualized with Coomasie Blue staining or blotted onto nitrocellulose membranes with a gel blotter (Bio-Rad). Membranes were blocked with a solution containing 5% (v/v) nonfat milk and 0.1% (v/v) Tween 20 in Tris-buffered saline buffer for 60 min, with one change of the blocking solution. The anti-LX serum was diluted 1:2,000 into the blocking solution and incubated with the membrane for 12 to 16 h at 4°C. The membrane was washed for 30 min with Tris-buffered saline buffer containing 0.1% (v/v) Tween 20, with the washing buffer being changed several times. The secondary antibody used was goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad), which was diluted 1:50,000 in blocking solution and incubated with the membrane for 1 h at room temperature. For signal detection, the SuperSignal West Pico Chemiluminescent kit (Pierce) was used. The presented immunoblots are representative of multiple experiments.

For tissue printing, the joints, including part of the primary stems and the relevant petioles, were halved longitudinally and the exposed tissue was pressed gently on a dry nitrocellulose membrane. The membrane was then used for LX protein detection by immunoblot analysis as described above.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Induction of LX protein level during flower senescence and in response to ethylene in different organs.

Supplemental Figure S2. Localization of LX protein following tissue printing.

ACKNOWLEDGMENTS

We are grateful to Dr. Jay De Rocher and Beth Kasiborski for the construction of plasmid p9-11.

Received March 8, 2006; accepted August 10, 2006; published August 18, 2006.

LITERATURE CITED

Brummell DA, Hall BD, Bennett AB (1999) Antisense suppression of tomato endo-1,4-β-glucanase Cel2 mRNA accumulation increases the force required to break fruit abscission zones but does not affect fruit softening. Plant Mol Biol 40: 615–622


Downloaded from on July 20, 2017 - Published by www.plantphysiol.org
Copyright © 2006 American Society of Plant Biologists. All rights reserved.
in tomato (Lycopersicon esculentum cv. Lukullus). Phytochemistry 65: 1343–1350
Young TE, Gallie DR (2000) Programmed cell death during endosperm development. Plant Mol Biol 44: 283–301

Role of RNase LX in Abscission and Senescence