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The Anticyclic Timing of Leaf Senescence in the Parasitic Plant *Viscum album* Is Closely Correlated with the Selective Degradation of Sulfur-Rich Viscotoxins[1]

Gesine Schrader-Fischer and Klaus Apel*

Swiss Federal Institute of Technology (ETH), Institut für Pflanzenwissenschaften, Abteiling Pflanzengenetik, Universitätsstrasse 2, 8092 Zurich, Switzerland

Leaf senescence and abscission have been studied in the semiparasitic plant mistletoe (*Viscum album*). Leaf senescence and abscission occur in the summer, when the metabolic activity of the host has reached its maximum. In contrast with their hosts, mistletoes selectively degrade only one major leaf protein during leaf senescence, the sulfur-rich viscotoxin, whereas most of the remaining leaf proteins are lost during abscission. The changes in viscotoxin content are paralleled by changes in the concentration of the corresponding mRNA. Shortly before the onset of leaf senescence, the mRNA for viscotoxin has disappeared from the leaves. The anticyclic timing of leaf senescence and the degradation of only one major leaf protein seems to reflect an adaptation of the parasite to its habitat.

Leaf senescence in higher plants leads to cellular disassembly and the mobilization of released material that eventually results in the death of the organ. Nearly all of the cellular nitrogen and other minerals are remobilized and transported to the sinks of the plant (Peoples and Dalling, 1988). The apparent physiological advantages of this recycling process lead one to view senescence as an endogenously controlled developmental process rather than a passive degeneration (Leopold, 1961). In many higher plant species that are adapted to drastic seasonal climate changes, the onset of leaf senescence is triggered by external stimuli such as a shortening of the photoperiod or a drop in the average daily temperature. Leaf abscission and the state of dormancy may allow plants to cope with unfavorable climatic conditions (Leopold, 1961; Stoddart and Thomas, 1982).

In contrast with free-living plants, parasitic plants depend to varying degrees on the metabolic state of their host and thus are placed under constraints that may force them to regulate leaf senescence and abscission differently. We have studied the control of leaf senescence in the mistletoe (*Viscum album*), a semiparasitic plant that contains Chl and is photosynthetically active but receives water and minerals from its hosts (Becker, 1986). Leaf senescence in the parasite occurs at a time when the metabolic activity of the host has reached its maximum. The formation of new leaves on mistletoe is completed long before the host plant initiates its own leaf senescence. In contrast with their hosts, mistletoes selectively degrade and apparently relocate only one major fraction of leaf proteins, the sulfur-rich viscotoxins, whereas most of the remaining leaf proteins appear to be lost during abscission. The timing of leaf senescence seems to reflect an adaptation of mistletoes to their semiparasitic mode of life.

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* Corresponding author; fax 41–1–252–08–29.

MATERIALS AND METHODS

Plant Material

Leaves of European mistletoe (*Viscum album* L.) were harvested from plants growing on apple trees (*Malus domestica* Barkh.) in the Botanical Garden of the University of Kiel, Federal Republic of Germany.

Isolation of Proteins

Protein was extracted from a leaf homogenate in 50 mM sodium phosphate buffer, pH 7.0, by stirring it for 90 min on ice. Proteins were separated electrophoretically on an SDS-15% polyacrylamide gel according to Laemmli (1970).

Immunological Detection of Proteins

Proteins were separated electrophoretically on an SDS-15% polyacrylamide gel and then transferred electrophoretically onto nitrocellulose sheets according to Towbin et al. (1979). Viscotoxins were detected immunologically on nitrocellulose sheets as described by Dehesh and Ryberg (1985) using an antiserum against the carboxymethylated viscotoxin.

RNA Analysis

Total RNA was isolated from mistletoe leaves according to Chirgwin et al. (1979) with adaptations to plant material as described by Melzer et al. (1990). For the northern blot analysis, equal amounts of RNA were dotted directly onto nitrocellulose (5 μg of each sample) and probed with a ^{32}P-labeled 26S-rRNA-specific cDNA from barley (Forde et al., 1981; Sambrook et al., 1989). Alternatively, RNA (30 μg/sample) was separated electrophoretically on a 1% agarose gel containing formamide, transferred onto nitrocellulose, and hybridized with the ^{32}P-labeled cDNA for viscotoxin A3 (Sambrook et al., 1989; Schrader and Apel, 1991).

Immunogold Labeling

Segments of leaves were fixed with 5% glutarparaformaldehyde, rinsed with 0.1 M phosphate buffer, pH 7.2, dehydrated in a graded ethanol series, and embedded in Spurr's medium (Spurr, 1969).

Ultrathin sections were prepared and incubated with the antiserum against carboxymethylated viscotoxin diluted 1:100 with PBS, pH 7.2, containing 0.05% (v/v) Tween 20 (Casper and Meyer, 1981). After rinsing in PBS/Tween 20, the sections were incubated with protein A-gold (10 nm; Janssen Life Sciences Products, Beerse, Belgium), rinsed again, and finally stained with 2% uranylacetate (van Cleve et al., 1988).

RESULTS

Mistletoe leaves were harvested at monthly intervals throughout the year. In June, the Chl content began to decline in leaves that had been formed in the previous year. This change in Chl content marked the beginning of leaf senescence in mistletoes. At the same time, the next generation of leaves was formed (Fig. 1). Leaves of the host plant had emerged in May and began to senesce in September. By this time, the newly formed mistletoe leaves had reached their full size and were maintained throughout the following winter and spring seasons.

Proteins were extracted from the mistletoe leaf material and were separated electrophoretically on an SDS-polyacrylamide gel. Through the year, the total protein content of mistletoe leaves remained constant except for the last 2 months before leaf abscission, when the protein content declined slightly, by less than 30% (data not shown). At the same time, the polypeptide composition did not change drastically, with the exception of one major polypeptide species of an apparent molecular mass of 5000 D. This polypeptide was highly abundant in leaves collected from August to May. In samples taken in June and July, however, this protein had completely vanished from the old leaves, whereas the concentration and the composition of the remaining proteins apparently had not been greatly affected (Fig. 2). When the young leaves of the next generation formed in June were analyzed, large amounts of this low molecular mass polypeptide were present together with most of the other polypeptides that were also found in the older leaves (Fig. 2).

The size of this low molecular mass polypeptide was indistinguishable from that of purified viscotoxin, one of the most abundant leaf proteins of mistletoe (Schrader and Apel, 1991). Thus, it seemed likely that the low molecular mass polypeptide that had disappeared from older leaves of mistletoe in June and July represented viscotoxin. To test this suggestion, total leaf proteins separated electrophoretically on a polyacrylamide gel were transferred onto nitrocellulose and probed with an antiserum raised against viscotoxin (De-

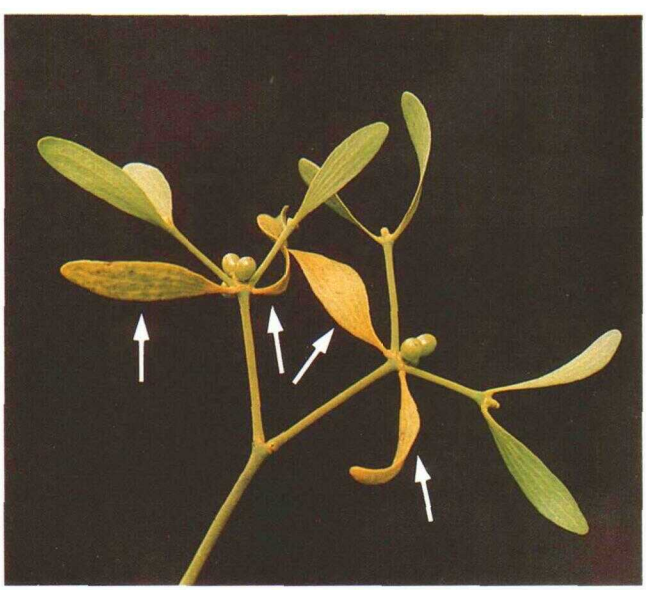

Figure 1. Twig of a mistletoe in August with leaves formed in the previous year (arrows) and new leaves.

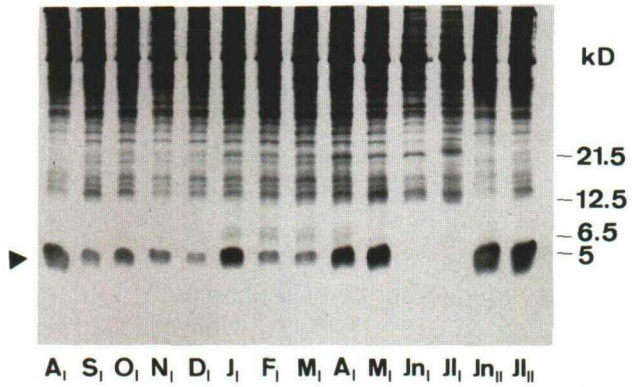

Figure 2. Seasonal changes in the polypeptide composition of leaf proteins of mistletoe. Leaves were harvested at monthly intervals and total leaf protein was extracted. Equal amounts of protein (50 μg/lane) were separated by SDS-PAGE and stained with Coomassie blue. A-Jl, August to July. I, Old leaves; II, new leaves. The arrowhead indicates the position of viscotoxin.

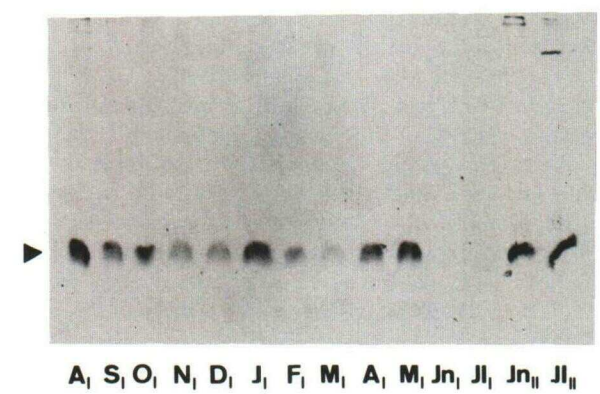

Figure 3. Seasonal changes in the concentration of viscotoxins. A protein gel similar to that shown in Figure 2 was used for a western blot analysis with an antiserum against carboxymethylated viscotoxin. A-Jl, August to July. I, Old leaves; II, new leaves. The arrowhead indicates the position of viscotoxin.

hesh and Ryberg, 1985; Schrader and Apel, 1991). The antiserum cross-reacted specifically with the low molecular mass leaf protein (Fig. 3). Thus, viscotoxin seems to be selectively removed from mistletoe leaves that undergo senescence, whereas in leaves of the next generation it reappears as one of the most abundant leaf polypeptides.

The subcellular localization of viscotoxin in the mistletoe leaf was determined by immunogold labeling. Ultrathin sections were taken from leaves collected in August. After addition of viscotoxin antiserum and processing of the samples with protein A-gold particles, almost all of the antigens were found to be stored in vacuoles (Fig. 4).

The rapid decline of viscotoxin in older leaves and the concomitant accumulation of this polypeptide in young leaves could be due to a developmentally regulated change in the stability and/or the synthesis of viscotoxins. We have tested this possibility by analyzing changes in the concentration of viscotoxin-specific mRNAs throughout the year. Total RNA was isolated from mistletoe leaves at monthly intervals starting in August. Equal amounts of RNA were either blotted directly onto nitrocellulose and probed with a ^{32}P-labeled rRNA-specific cDNA (Fig. 5A) or were separated electrophoretically on a 1.0% agarose gel, transferred onto nitrocellulose, and hybridized with a radioactively labeled cDNA encoding the higher molecular mass precursor of viscotoxin (Fig. 5B) (Sambrook et al., 1989; Schrader and Apel, 1991).

In August, a high level of viscotoxin mRNA was present in leaves. The amount of this mRNA declined continuously in September and October and dropped beyond the level of detection in November. In January, this transcript began to reappear, reaching a high level in spring and vanishing from the leaves in June at the same time that the polypeptide disappeared. In contrast, in young leaves of the next generation, which started to emerge in June, transcripts encoding viscotoxins reached their highest level throughout the year (Fig. 5B).

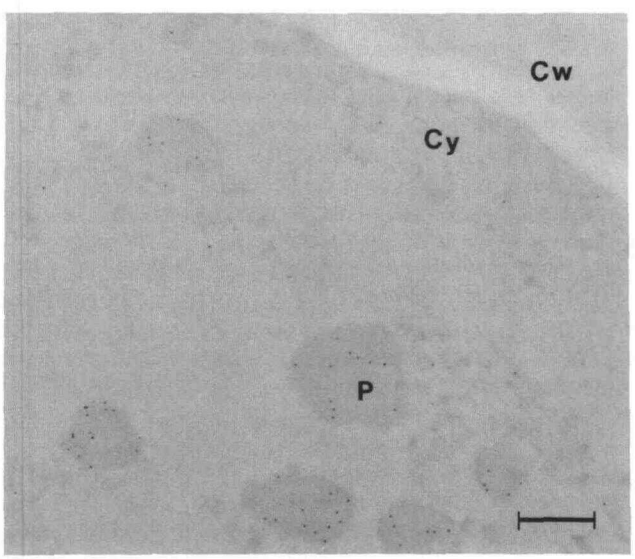

Figure 4. Ultrathin sections of young mistletoe leaves harvested in August subjected to immunogold labeling using an antiserum against carboxymethylated viscotoxin. Cw, Cell wall; Cy, cytoplasm; P, protein body. Bar = 0.5 μm.

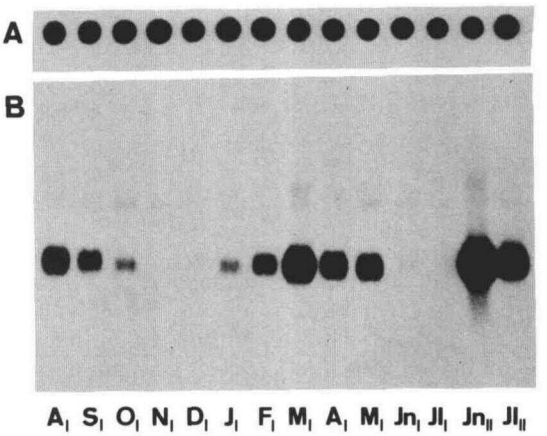

Figure 5. Seasonal changes in the relative concentration of viscotoxin mRNA. Total RNA was extracted from mistletoe leaves at monthly intervals. Equal amounts of RNA were either dot blotted onto nitrocellulose and hybridized with a ^{32}P-labeled rRNA-specific cDNA probe (Forde et al., 1981) (A) or were separated electrophoretically, transferred onto nitrocellulose, and hybridized with a ^{32}P-labeled viscotoxin cDNA (Schrader and Apel, 1991) (B). A-Jl, August to July. I, Old leaves; II, new leaves.

Changes in the relative concentration of viscotoxin mRNA seem not to be the consequence of a general seasonal change in the RNA content of mistletoe leaf cells but rather to reflect a specific regulation of the viscotoxin mRNA. Roughly similar amounts of RNA were extracted from mistletoe leaves throughout the year. When the RNA dot blot was hybridized with the rRNA-specific probe, the hybridization intensity of each RNA sample remained constant throughout the year (Fig. 5A), indicating that similar quantities of total RNA had been applied to the blot.

DISCUSSION

Leaf senescence in higher plants is accompanied by a loss of Chl and the degradation of protein (Hendry et al., 1987). Most of the breakdown products are mobilized and relocated to young leaves, seeds, or specialized storage tissues in roots or stems (Peoples and Dalling, 1988). In the semiparasitic plant mistletoe, new leaves are formed in early summer that remain active for 1 year before they abscise and are replaced by the next generation of young leaves. Leaf abscission is preceded by the loss of Chl. However, in contrast with senescence in leaf cells of free-living plants, loss of Chl in mistletoe is not accompanied by a massive and general degradation and relocation of leaf proteins. Even though we cannot exclude the possibility that during protein analysis of leaf samples changes in some minor polypeptides that are degraded during senescence are not detected because these polypeptides are concealed on the polyacrylamide gel by other polypeptides, most of the leaf proteins remain unaffected within the senescing leaves of mistletoe and are lost upon abscission. The only detectable exception is the highly abundant viscotoxin. In contrast with most other proteins,

viscotoxins are highly enriched in Cys residues. Cys's comprise more than 10% of the amino acids of viscotoxins (Samuelsson, 1961). It is tempting to speculate that the high sulfur content of viscotoxins is related to their selective degradation in senescing mistletoe leaves.

Mistletoes are semiparasitic plants that contain Chl and are photosynthetically active, but receive water and minerals from their hosts (Becker, 1985). Even though the transport of nutrients from the host to the parasite is not well understood, our results suggest that the amounts of sulfur offered by the host may not be sufficient for the growth of the parasitic plant. Next to nitrogen, sulfur is the most important mineral for many plants. It often is not available in sufficient quantities in the soil and thus must be supplemented by the farmer (Schnug, 1991). Selective degradation of viscotoxins in senescing leaves may provide the parasite with sulfur and nitrogen, both of which are needed at a time when new leaves emerge from the plant. Precedents for the selective degradation of a particular protein are known from other organisms. For instance, in *Lemna gibba* that are starved for sulfur, a preferential degradation of Rubisco has been described (Ferreira and Teixeira, 1992). In cyanobacteria that have been starved for nitrogen, biliproteins are selectively degraded. Normally, these proteins make up almost half of the cellular protein and serve as part of light-harvesting antenna pigment-protein complexes during photosynthesis. These proteins can be used as an internal source of nitrogen that allows the cells to survive the unfavorable conditions of nitrogen starvation (Allen and Smith, 1969; Yamanaka and Glazer, 1980).

Viscotoxins belong to the group of thionins (Garcia-Olmedo et al., 1989). Thus far, little is known about the biological function of these proteins. Like other thionins, viscotoxins could play a role as part of the defense mechanism against pathogens (Bohlmann et al., 1988; Schrader and Apel, 1991). In contrast with many other plant species, mistletoes are susceptible to only a very small number of pathogens (Brandenburger, 1985). This protection may be due to the large number of biologically active constituents that have been found in mistletoe extracts (Franz, 1985). Among these compounds, viscotoxins are extremely abundant.

Regardless of their possible function in plant defense, viscotoxins—as indicated by our work—appear to serve as a storage form of sulfur and nitrogen that can be utilized by the parasitic plant during leaf formation.

One intriguing aspect of our present study is the timing of senescence in the mistletoe. In contrast with the host leaf senescence, in the parasite it is closely coupled to the formation of new leaves. Whereas the host plant initiates senescence of its own leaves in early fall, senescence in the parasite starts during a period in which the metabolic activity of the host has reached its maximum. This shift in the timing of senescence may explain why the parasite does not have to degrade all of the leaf proteins but utilizes only the viscotoxins as a source for sulfur and nitrogen.

The parasite's dependence on the metabolic state of the host plant is also reflected in the seasonal changes of viscotoxin mRNA concentrations. When the host plant is metabolically highly active, the viscotoxin mRNA reaches its highest level in young mistletoe leaves. Similar high levels of this transcript accumulate transiently in mistletoe leaves of the first generation shortly before and after the host plant breaks the state of dormancy. On the other hand, viscotoxin mRNA levels drop beyond the level of detection during the dormant state of the host plant. Leaf senescence in trees is triggered by a combination of shortening of the daily light period and a drop in temperature. From our results, it is evident that leaf senescence in mistletoes is not affected by these same environmental factors but that the parasite must respond to different signals. At present, the identity of these signals is not known.

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