Leaf Senescence Induced by Mild Water Deficit Follows the Same Sequence of Macroscopic, Biochemical, and Molecular Events as Monocarpic Senescence in Pea

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We have compared the time course of leaf senescence in pea (Pisum sativum L. cv Messire) plants subjected to a mild water deficit to that of monocarpic senescence in leaves of three different ages in well-watered plants and to that of plants in which leaf senescence was delayed by flower excision. The mild water deficit (with photosynthesis rate maintained at appreciable levels) sped up senescence by 15 d (20°Cd), whereas flower excision delayed it by 17 d (27°Cd) compared with leaves of the same age in well-watered plants. The range of life spans in leaves of different ages in control plants was 25 d (34°Cd).

In all cases, the first detected event was an increase in the mRNA encoding a cysteine-proteinase homologous to Arabidopsis SAG2. This happened while the photosynthesis rate and the chlorophyll and protein contents were still high. The 2-fold variability in life span of the studied leaves was closely linked to the duration from leaf unfolding to the beginning of accumulation of this mRNA. In contrast, the duration of the subsequent phases was essentially conserved in all studied cases, except in plants with excised flowers, where the degradation processes were slower. These results suggest that senescence in water-deficient plants was triggered by an early signal occurring while leaf photosynthesis was still active, followed by a program similar to that of monocarpic senescence. They also suggest that reproductive development plays a crucial role in the triggering of senescence.

Senescence is the final phase of leaf development, during which a large part of leaf nitrogen, carbon, and minerals is recycled to other organs of the plant (Noodén, 1988a). It consists of an ordered sequence of physiological, biochemical, and ultrastructural changes, involving a decline in functions associated to carbon assimilation and a massive degradation of macromolecules (RNAs, proteins, and lipids) and chlorophylls (Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Noodén et al., 1997). This process relies on the execution of a specific genetic program, which is under the control of a high and complex regulation by various endogenous and environmental factors (Smart, 1994; Gan and Amasino, 1997). It is sped up by water or nitrogen deficits (Merrien et al., 1981; Wolfe et al., 1988a, 1988b) and delayed when reproductive sinks are removed (Lindo and Noodén, 1976; Noodén, 1988b; Wolfe et al., 1988a).

Acceleration of leaf senescence is thought to be adaptive in plants subjected to water shortage (a) because it reduces the water demand cumulated over the whole plant cycle, thereby avoiding water deficit during seed filling, and (b) because it allows recycling of scarce resources to the reproductive sinks. However, in crops species, early leaf senescence usually correlates with lower yield because cumulative photosynthesis is reduced (Fischer and Kohn, 1966; Merrien et al., 1981; Gifford and Jenkins, 1982; Wolfe et al., 1988a). Selection based on delayed leaf senescence (“stay-green” plants) under drought conditions allowed to obtain sorgho hybrids with improved yields under water deficit (Borrell et al., 2000). Nevertheless, stay-green plants do not necessarily produce higher yields, especially when chlorophyll catabolism and nutrient remobilization are disabled (for a review, see Thomas and Howarth, 2000). Prediction and manipulation of leaf senescence is therefore crucial to optimize crop management and plant response to water deficit.

This prediction is made difficult because the first events that trigger senescence may occur long before chlorophyll degradation and associated leaf yellow-
ing, which seems to be one of the latest events of leaf senescence (Hensel et al., 1993; Bernhard and Matile, 1994; Humbeck et al., 1996). The senescence program may therefore be triggered by an environmental constraint occurring before any detectable symptom, making difficult to ascribe it to a particular environmental event. Decreases in net photosynthesis and stomatal conductance are not good indicators of senescence initiation because they exhibit rapid fluctuations unrelated to the senescence program. Monitoring changes in gene expression might give a more reliable indication.

The aim of this work was therefore to identify early events in the sequence of macroscopic, biochemical, and molecular events associated with leaf senescence of plants experiencing water deficiency. We tested whether this sequence of events differed in water-deficient plants and in “normal” monocarpic senescence. To avoid confusion of effects, this sequence of events in well-watered plants was established in leaves inserted at three positions on the stem, which had markedly different life spans. We also compared it with that observed in plants in which leaf senescence was delayed by flower excision. The water deficit imposed in this study corresponded to the mild stresses commonly observed in field conditions, i.e. which affect plant architecture and water flux without appreciably affecting leaf water status (Lecoeur et al., 1995; Tardieu and Simonneau, 1998). In this respect, it differed in intensity and duration from those imposed in other studies by dehydrating detached leaves or by withholding irrigation (Becker and Apel, 1993; Weaver et al., 1998; He et al., 2001).

We simultaneously analyzed changes with time in net photosynthesis, chlorophyll and protein contents, and relative abundances of several mRNAs in pea (Pisum sativum L. cv Messire) leaves. The relative abundance of a mRNA encoding a chlorophyll a/b (CAB) protein was measured because the corresponding gene was shown to be down-regulated during leaf senescence (Hensel et al., 1993; Lohman et al., 1994). We also monitored the relative abundances of mRNAs encoding a Cys-proteinase and a ferritin, which correspond to senescence up-regulated genes (Hensel et al., 1993; Smart et al., 1995; Drake et al., 1996; Buchanan-Wollaston and Ainsworth, 1997). Measuring the abundance of the proteinase mRNA necessitated the cloning of a cDNA that we named PsELSA (GenBank accession no. AJ278699). The mode of expression of time as it is sensed by plants is essential in studies of the time course of leaf development. We have expressed it in two ways: (a) physical time, which is the most intuitive, and (b) thermal time, which allows more precise comparisons when temperature undergoes fluctuations and is a necessary expression for modeling (Turc and Lecoeur, 1997; Granier and Tardieu, 1998).

RESULTS

A Mild Water Deficit, Compatible with an Appreciable Photosynthesis Rate, Caused an Acceleration of Senescence by 13 d

In the treatment with water deficit, soil water potential decreased from −10 to −80 kPa during the first 24 d (336°Cd) of the experiment, and was then maintained at that level until the end of the experiment. The stabilization of soil water potential at about −80 kPa, therefore, occurred 22 d (270°Cd) before unfolding of leaf 14 (Fig. 1). In the other two treatments, soil water potential remained above −15 kPa. Predawn leaf water potential decreased to −0.5 Mpa in the water deficit treatment, whereas it remained above −0.25 Mpa in the other two treatments (data not shown). Net photosynthesis in leaf 14 (Table I) was halved in water-deficient plants compared with well-watered plants at leaf unfolding, but the value of maximum net photosynthesis was nearly unaffected by the water deficit. These results show that the water deficit experienced by plants was very mild. However, the decrease in net photosynthesis was much faster (net photosynthesis was close to zero 27 d [390°Cd] after leaf unfolding in water-deficient plants, whereas it was still close to its maximum in the other two treatments [Table I], and leaf yellowing occurred 13 d (180°Cd) earlier compared with controls (Table II).

Figure 1. Change with time in soil water potential in the treatment with excised flowers, control treatment, and treatment with water deficit. Soil water potential was measured daily at depths of 0.20 (○) and 0.30 m (●). For better legibility, means and sds (12 measurements) are given every 4 d.

Three Independent Sources of Variations Caused Large Differences in Leaf Duration

In leaf 14, phytomere initiation and leaf unfolding occurred synchronously in the three treatments (Table II). In contrast, the duration from leaf unfolding to yellowing largely differed between treatments, and was 27 (390 °Cd), 42 (600 °Cd), and 59 d (870 °Cd), respectively in water-deficient plants, control plants, and plants with excised flowers (Table II). These differences are consistent with the measurements of photosynthesis, which decreased before yellowing in all studied cases (Fig. 2). The decline in chlorophyll content always began shortly before visible yellowing. It lasted 59 d (870 °Cd) in all cases until chlorophyll content reached values close to zero (lower than 0.2 μg mm⁻²), except in leaf 14 of plants with excised flowers where chlorophyll content was still 35% of its maximum level at the end of the experiment (Table II).

Declines in Chlorophyll and Protein Contents Began Slightly before Yellowing in All Studied Cases

Chlorophyll content per unit leaf area was similar in the five studied cases at leaf unfolding (except in leaf 10 of control plants, in which it was halved). It remained roughly constant afterward and declined at a time that greatly varied across leaf positions and treatments, from 20 d (290 °Cd) after leaf unfolding in leaf 14 of plants with water deficit to at least 50 d (730 °Cd) in leaf 14 of plants with excised flowers (Fig. 2). The decline in chlorophyll content always began shortly before visible yellowing. It lasted about 11 d (170 °Cd) in all cases until chlorophyll content reached values close to zero (lower than 0.2 μg mm⁻²), except in leaf 14 of plants with excised flowers where chlorophyll content was still 35% of its maximum level at the end of the experiment.

Protein content closely paralleled chlorophyll content in all studied cases (Fig. 2). It first decreased slowly, and then dropped rapidly at the same date as chlorophyll content. It reached values close to zero (lower than 2 μg mm⁻²), again with the exception of leaf 14 of plants with excised flowers) at the same date as chlorophyll content.

Table II. Initiation, unfolding, visible yellowing, life span, and desiccation of the studied leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phytomere Position</th>
<th>Phytomere Initiation</th>
<th>Leaf Unfolding</th>
<th>Leaf Visible Yellowing</th>
<th>Leaf Life Span</th>
<th>Leaf Desiccation</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>°Cd (d)</td>
<td>°Cd (d)</td>
<td>°Cd (d)</td>
<td>°Cd (d)</td>
<td>°Cd (d)</td>
<td>°Cd (d)</td>
</tr>
<tr>
<td>Excised flowers</td>
<td>14</td>
<td>151 (10)</td>
<td>580 (44)</td>
<td>1,450 (103)</td>
<td>870 (59)</td>
<td>–¹</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>46 (3)</td>
<td>436 (33)</td>
<td>1,165 (85)</td>
<td>729 (52)</td>
<td>1,311 (94)</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>151 (10)</td>
<td>583 (44)</td>
<td>1,180 (86)</td>
<td>597 (42)</td>
<td>1,311 (94)</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>344 (25)</td>
<td>804 (60)</td>
<td>1,196 (87)</td>
<td>392 (27)</td>
<td>1,311 (94)</td>
</tr>
<tr>
<td>Water deficit</td>
<td>14</td>
<td>149 (10)</td>
<td>604 (46)</td>
<td>996 (73)</td>
<td>392 (27)</td>
<td>1,094 (80)</td>
</tr>
</tbody>
</table>

¹ —. Dessication had not yet occurred in leaf 14 of plants with excised flowers at the last sampling date (1,643 °Cd [115 d] after emergence).
Rapid Changes with Time in Relative Abundances of mRNAs Encoding a CAB Protein and PsELSA Cys-Proteinase Were Early Events in the Time Course of Senescence

RNA-blot analysis of changes with time in relative abundance of mRNAs coding for a CAB protein, the

PsELSA Cys-proteinase and a ferritin are presented in Figure 3. The curves present the results obtained with the CAB and the proteinase probes after quantification of the signals and normalization relative to the most intense signal. The last lane of the blots (first date of measurement after leaf yellowing) was not taken into account for quantifications because ethidium bromide staining of the electrophoresis gels showed that RNA deposition was much lower in this lane (data not shown).

Maximum accumulation of the CAB mRNA occurred at unfolding in all cases, except in leaf 14 of water-deficient plants in which it was delayed (Fig. 3, second or third sampling date according to the considered blot). The subsequent decrease was the slowest in leaves having the longest life spans: 30% of the initial level was reached about 50 d (715°Cd) after leaf unfolding in leaf 14 of plants with excised flowers and in leaf 10 of control plants (Fig. 3). It was the most rapid in leaves having the shortest life spans: 30% of the initial level was reached, respectively, 23 (330°Cd) and 20 d (290°Cd) after leaf unfolding in leaf 20 of control plants and in leaf 14 of water-deficient plants (Fig. 3).

The mRNA coding for the PsELSA Cys-proteinase first accumulated slowly and began to accumulate rapidly 11 to 21 d (150°Cd–330°Cd) before leaf yellowing according to the studied case (Fig. 3). The onset of rapid accumulation occurred the latest in leaf 14 of plants with excised flowers and in leaf 10 of control plants (38 and 41 d, 540°Cd and 570°Cd, after leaf unfolding, respectively). It occurred the soonest in leaf 20 of control plants and in leaf 14 of water-deficient plants (about 14 d, 200°Cd, after leaf unfolding in both cases). In contrast, the ferritin mRNA was detected at low levels at leaf unfolding, nearly undetectable afterward, and accumulated markedly after leaf yellowing in all studied cases (Fig. 3).

DISCUSSION

Synchrony of the Progression of Senescence in the Five Studied Cases

The time course of senescence-associated events in water-deficient plants displayed striking similarities with those observed in leaves of control plants, whatever the ages of these leaves, and with that observed in plants with excised flowers. This is illustrated in Figure 4. The beginning of the decline in chlorophyll content, which occurred at different leaf ages between treatments and leaf positions, was taken as a time reference. Certain events were simultaneous in all cases and marked the beginning of the degradation of the photosynthetic apparatus. These were the onset of the rapid declines in chlorophyll and protein contents, and the time when the relative abundance of the CAB mRNA was reduced to 30% of its initial level. The decreases in chlorophyll and protein contents and in the abundance of the CAB mRNA were also simul-
Figure 3. Change with time in relative abundance of mRNAs coding for a CAB protein, the PsELSA Cys-proteinase and a ferritin in leaf 14 of plants with excised flowers, in leaves 10, 14, and 20 of control plants, and in leaf 14 of water-deficient plants. The blots (10 μg of total RNA per lane) were successively hybridized with 32P-labeled probes corresponding to the PEACAB66 CAB protein, the PsELSA Cys-proteinase, and the PSFERRI ferritin cDNAs (for GenBank accession nos., see “Materials and Methods”). These results were obtained with two independent RNA extracts and at least three blots for each extract. For each blot, the intensity of the signals obtained with the CAB and the Cys-proteinase probes was normalized relatively to the most intense signal among those that were quantified (see text). The curves present means and SDs of all the results obtained for each leaf with the CAB and the Cys-proteinase probes (black and white symbols, respectively). Dotted lines represent the dates of leaf visible yellowing.
taneous in senescing leaves of Arabidopsis in the analysis of Lohman et al. (1994), whereas the decline in chlorophyll content occurred slightly later than the other two events in the study of Hensel et al. (1993).

Leaf yellowing shortly followed these events at the three studied positions on the stem in control plants and was slightly delayed in plants subjected to water deficit. Leaf desiccation occurred approximately 1 week after yellowing in these four cases (Fig. 4). The delay before leaf yellowing was longer in plants with excised flowers, in which chlorophyll and protein contents were still at appreciable levels 12 d (190°Cd)

Figure 4. Chronology of events in leaf 14 of plants with excised flowers, leaves 10, 14, and 20 of control plants, and leaf 14 of plants with water deficit. 1, Leaf unfolding and low accumulation of the ferritin mRNA. 2, Beginning of rapid accumulation of the mRNA coding for the PsELSA Cys-proteinase. 3, End of the period with stable chlorophyll content. 4, End of the period with stable protein content. 5, Abundance of the CAB mRNA reduced to 30% of its initial level. 6, Leaf visible yellowing. 7, Strong accumulation of the ferritin mRNA. 8, Chlorophyll content close to zero. 9, Protein content close to zero. 10, Leaf desiccation. Graphs are positioned to align event 3 (end of the period with stable chlorophyll content) in the five studied cases, irrespective of leaf age at that time.
after leaf yellowing, consistent with observations on soybeans (Wittenbach, 1983).

The beginning of rapid accumulation of the mRNA encoding the PsELSA Cys-proteinase preceded the beginning of chlorophyll and protein degradation by a constant delay of about 9 d (125°Cd), regardless of the source of variation of senescence, either environmental or due to leaf position on the stem (Fig. 4). In all studied cases, this first event of senescence was observed while the photosynthesis rate and the chlorophyll and protein contents were still high. It was not related to leaf age, which ranged from 14 to 41 d (200°Cd–575°Cd) after unfolding at that time. The ferritin mRNA exhibited a contrasting behavior, as it accumulated only at leaf unfolding and, more markedly, at late stages of senescence; in all cases, that late accumulation occurred after leaf yellowing, at a time when chlorophyll and protein contents were close to zero and leaf desiccation was under way (Fig. 4). These results are consistent with those obtained by Buchanan-Wollaston and Ainsworth (1997).

The sequence of events was therefore common to the five studied cases. The 2-fold variability in the life span of the leaves was essentially linked to the duration from unfolding to the beginning of rapid accumulation of the Cys-proteinase mRNA (Fig. 4). In contrast, the duration of the subsequent phases until complete desiccation was essentially conserved in all studied cases, except in plants with excised flowers, where the degradation of chlorophylls and proteins and leaf desiccation were delayed, probably due to the lack of sinks. This suggests that the life span variability was determined by early events, whereas the time course of events occurring during protein depletion was largely insensitive to environmental conditions. The synchrony of the progression of senescence once it was triggered at different dates by different promoting factors is consistent with the concept of regulatory network (Gan and Amasino, 1997; He et al., 2001), in which multiple pathways responding to various autonomous and environmental factors are interconnected to control senescence.

Accumulation of the PsELSA Cys-Proteinase mRNA Marked an Early Step of Senescence

Accumulation of the PsELSA Cys-proteinase mRNA during pea leaf senescence is consistent with the results obtained in other plant species (Hensel et al., 1993; Smart et al., 1995; Drake et al., 1996; Buchanan-Wollaston and Ainsworth, 1997; Weaver et al., 1998). The originality of this work was to show (a) that this mRNA began to accumulate rapidly at a time when the degradation processes (especially protein degradation) had not yet begun, and (b) that the kinetics of the subsequent sequence of events was conserved through various leaf positions and experimental conditions (Fig. 4).

High accumulation of mRNAs encoding the SAG2 and SAG12 Cys-proteinases occurred later in Arabidopsis than that of PsELSA mRNA in our study at a time when yellowing and protein degradation were under way and CAB mRNA had dropped down (Hensel et al., 1993; Lohman et al., 1994; Weaver et al., 1998). Our results do not necessarily contradict these observations. The most marked difference is that the duration from leaf unfolding to yellowing was considerably longer in pea (up to 59 d in plants with excised flowers) compared with Arabidopsis (5–6 d). This gives a better time definition to analyze sequence of events, and makes it easier to distinguish short-term events after a stress from longer term developmental processes. Another consequence is that two events may appear concomitant in Arabidopsis (within 1 or 2 d) and distinct in pea (by several days). It is also possible that kinetics slightly differ in Arabidopsis and in pea. The PsELSA cDNA is highly homologous to that of barley (Hordeum vulgare) aleurain (Rogers et al., 1985). Our observation of an early accumulation of the corresponding mRNA, before the drop in leaf protein content, rises again the question of whether this class of Cys-proteinases plays a direct role in protein dismantling or rather activates enzymes involved in massive protein degradation (Holwerda and Rogers, 1992).

What Triggered Senescence?

In the water-deficit treatment, the beginning of the senescence program was probably not a direct consequence of a water stress sensed at the cellular level, in opposition with cases where water deficit was imposed by dehydrating detached leaves or withholding irrigation (Becker and Apel, 1993; Oh et al., 1996; Weaver et al., 1998). More than 1 month elapsed from the beginning of water deficit to the increase in the Cys-proteinase mRNA, and photosynthesis rate was still high when this increase occurred. Furthermore, a mild water deficit does not appreciably alter the day-time leaf water status in plants such as maize (Zea mays) or pea, in which a combination of abscisic acid and hydraulic signalings allows stomatal control to avoid any leaf dehydration by fine-tuning transpiration (Tardieu and Davies, 1993; Tardieu and Simonneau, 1998). Finally, the life span and the time course of events in leaf 14 of water-deficient plants were very similar to those observed in leaf 20 of control plants. These three arguments suggest that the early senescence observed under mild water deficit followed a program similar to that of monocarpic senescence, after it was triggered by a signal, which was linked to soil water deficit rather than to a stress-induced leaf water status.

The onset of monocarpic senescence was probably not caused in our case by an age-related decline in photosynthetic processes, as proposed by Hensel et al. (1993). First, the rapid increase in the Cys-proteinase mRNA began at least 1 week before the declines in photosynthesis and in chlorophyll and
protein contents in all studied cases. It is therefore difficult to consider that the decline in photosynthesis could have a causal effect. Second, the life span of lately initiated leaves was much shorter than that of early initiated ones, and all leaves of a plant senesced within a short time (Table II). This suggests that the onset of senescence was linked to a developmental control at the whole plant level, rather than to the age of individual leaves. Third, excision of flowers greatly delayed senescence. Taken together, these data suggest that source-sink relationships and relationships between reproductive and vegetative development may have a crucial role in the onset of senescence. This is consistent with analyses on soybean (Lindoo and Nooden, 1976) and maize (Wolfe et al., 1988a), in opposition to the case of Arabidopsis where mutants with sterile flowers senesced at the same rate as wild-type plants (Hensel et al., 1993).

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Environmental Measurements

Pea (Pisum sativum L. cv Messire) plants were grown in a greenhouse in Montpellier (France). Seeds were sown in 36 pots (0.35-m diameter and 0.35-m height) filled with a 1:1 (v/v) mixture of loamy soil:organic compost complemented with 130 g m\(^{-3}\) of P\(_2\)O\(_5\)K\(_2\)O (10:25, w/w). Twenty seeds were sown at 25-mm depth in each pot. All the pots were fully irrigated after sowing and allowed to drain freely for 24 h. Plants were thinned to 12 per pot at emergence (8 d after sowing) and to eight per pot at the beginning of flowering. Lateral branches were removed as soon as they became visible, so each studied plant consisted of one main stem only.

Air temperature and relative humidity were measured with a capacitive hygrometer (HMP35A Vaisala Oy, Helsinki) protected from direct radiation. Photosynthetic photon flux density (PPFD) was measured using silicon cells calibrated in situ using a PPFD sensor (LI-190SB, LI COR, Inc., Lincoln, NE). Air temperature, relative humidity, and PPFD were measured at 1.5 m from the soil. Temperature of the apical bud was measured with a fine copper-constantan thermocouple (0.2 mm) inserted in the apical bud of two plants per treatment. Data were collected every 20 s, and means were stored every 1,800 s in a data logger (Campbell Scientific, LTD-CR10 Wiring Panel, Shepshed, Leicestershire, UK). Air temperature was regulated to maintain a day/night amplitude of 20°C/10°C, and was never allowed to exceed 25°C. Natural light was supplemented with sodium lamps (250 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) to obtain a constant photoperiod of 16 h. Soil water potential was measured daily with two tensiometers (DTE 1000, Nardex, Saint-Avertin, France) per pot placed at depths of 0.20 and 0.30 m.

Experimental Treatments

Three treatments were imposed to 12 randomly chosen pots each. Control plants were watered manually every day either with water or, once a month, with a 1:10 (v/v) Hoagland nutritive solution corrected for minor elements. In the treatment with flower excision, watered as control, newly opening flowers were excised as soon as they appeared on successive phytomeres. In the water deficit treatment, water supply was withheld after sowing until soil water potential reached approximately –70 kPa at the two depths of measurement. The pots were then watered daily to maintain soil water potential between –70 and –80 kPa at the two depths.

Follow-Up of Leaf Development and Leaf Sampling

The studied leaves were those inserted on phytomeres 10, 14, and 20 in control plants and that on phytomere 14 in the other two treatments. Phytomere 14 was the first reproductive phytomere in all sampled plants (the few plants in which this was not the case were excluded from sampling). The number of initiated phytomeres was determined once a week on six to eight plants per treatment as described in Turc and Lecoeur (1997). The number of fully unfolded leaves was determined on the same plants plus, two or three times a week, on 12 marked plants per treatment (one per pot) followed non-destructively during the whole experiment. The dates of phytomere initiation and leaf unfolding (Table II) were deduced from those countings as described in Turc and Lecoeur (1997). A leaf was considered to reach visible yellowing when its leaflets exhibited visual symptoms equivalent to those that characterize stage 52 of Arabidopsis leaves in the study of Lohman et al. (1994). Leaf complete desiccation was also recorded visually. The progressions of leaf yellowing and desiccation from the bottom to the top of the plant were followed twice or three times a week in each treatment to determine the dates of yellowing and desiccation of the studied leaves (Table II).

The final area of each leaf was measured after completion of leaf expansion with an image analyzer (Bioscan-Optimas V 4.10, Edmonds, WA) on six to 14 plants. On the first sampling date (leaf unfolding), at which leaf area was still increasing, leaf area was calculated from the developmental stage of the leaf and from its final area (Turc and Lecoeur, 1997).

Time courses were expressed in thermal time calculated by daily integration of temperature minus a base temperature of 3°C. However, day-to-day variations in temperature were not very large, so physical time was still acceptable and was also provided.

Leaves were sampled every week (twice a week as soon as photosynthesis began to decrease) from unfolding to complete desiccation, i.e. on six to 11 sampling dates depending on treatment and leaf position. Ten whole leaves (stipules + leaflets + tendrils) were collected on randomly chosen plants and immediately frozen in liquid nitrogen. They were pooled in a calibrated plastic flask, and total fresh matter was weighed. They were then kept at –50°C. Specific leaf area was calculated at each sampling date as the ratio between mean leaf area and mean fresh weight per leaf at that date.
Photosynthesis Measurements and Determination of Chlorophyll and Protein Contents

Photosynthesis of leaf 14 was measured on leaflets with a Photosynthesis Portable System LI-6200 (LI-COR, Inc., Lincoln, NE) according to Leuning and Sands (1989) and Peary et al. (1991). Only measurements with saturating PPFD greater than 700 μmol m⁻² s⁻¹ were taken into account.

For the determination of chlorophyll and protein contents, the samples (250 mg of fresh matter) were homogenized (Ultra-Turrax T25, shaft type N-10G, IKA Labortechnik, Staufen, Germany) at 15,000 rpm in cold 80% (v/v) NaOH, and the tubes were placed under shaking overnight (1960). The pellet was resuspended in 10 mL of 0.1 M NaOH, and the tubes were placed under shaking overnight at 4°C. After centrifugation at 5,000 rpm for 5 min at 4°C, the supernatant was collected, and acetone extraction was repeated once on the pellet. The second supernatant was used to determine the first one, and chlorophyll (a + b) content was determined by spectrophotometry according to Vernon (1960). The pellet was resuspended in 10 mL of 0.1 M NaOH, and the tubes were placed under shaking overnight at 4°C. After centrifugation at 6,000g for 15 min at 4°C, the supernatant was used for protein content determination according to Bradford (1976), using bovine serum albumin supernatant as a standard. Titration was performed in microtitration plates (POLYSORB, Flat-Bottom, Nunc, Naperville, IL). In each well, proteins (0–1.5 μg) were solubilized in 50 μL of 0.02 M NaOH, and 200 μL of Bradford reagent was then added. Absorbance was measured at 620 nm with a Titer-tek Multiskan apparatus (MCC/340, Flow Laboratories, Irving, UK).

Chlorophyll and protein contents were expressed on a fresh weight basis, or on a leaf area basis by dividing the former by the specific leaf area. In leaf 14 of plants with excised roots (in particular carbohydrates, as the main sink for plant carbon was removed in these plants), which diluted chlorophyll and accumulated other compounds in the leaves (in particular carbohydrates, as the main sink for plant carbon was removed in these plants), which diluted chlorophyll and protein contents expressed per unit fresh weight. We therefore expressed all concentrations per unit leaf area in further analyses.

DNA Probes Used for RNA Hybridization

The degenerate primers THPS', 5'-THTTCARADIT-GYTCNGCNC-3', and THPS', 5'-THTTINAYIIGGT-AGGANGRCRC-3' (IUPAC ambiguity code “I” stands for inosine) were made from the alignment of the deduced amino acid sequences of five Cys-proteinases (Tournaire et al., 1996). These primers were used to reverse transcriptase-PCR amplify a partial cDNA from pea leaf RNA. This partial cDNA was further used to screen a pea leaf cDNA library provided by D. Macherey (Commissariat à l’Energie Atomique, Grenoble). The insert of the longest polyadenylated clone was finally sequenced on both strands and named PsELSA (GenBank accession no. AJ278699). Database search revealed that this cDNA is nearly 100% identical to that of the PSRNACP Cys-proteinase isolated from germinating seeds of pea by Jones et al. (1996). It shares a high degree of derived amino acid sequence similarity with Arabidopsis SAG2 (Hensel et al., 1993), petunia (Petunia hybrida) PeTh3 (Tournaire et al., 1996), barley aleurain (Rogers et al., 1985), and rice (Oryza sativa) oryzain-γ (Watanabe et al., 1991). The probe used for RNA hybridization corresponded to a 636-bp internal fragment of the PsELSA cDNA obtained by PCR amplification with the primers PsELSA 1, 5'-CCGATGCTAATCTTCCTGACGAGA-3', and PsELSA 2, 5'-CAACACCGCAGATTTCTCCCATT-3'.

The CAB protein probe was the PsI1 insert of the pAB96 plasmid (Broglie et al., 1981), which corresponds to the PEACAB66 cDNA (GenBank accession no. M64619). The ferritin probe was a 636-bp internal fragment of the PSFERRI cDNA (GenBank accession no. X73369) generated by Sau 3A I digestion of the EcoRI insert fragment from the cDNA clone PeSd1 (Lobréaux et al., 1992).

RNA Purification and Hybridization

Total RNA extract was prepared from leaf tissue using a modified phenol-SDS method (Teyssendier de la Serve and Jouanen, 1979) and further purified by centrifugation through a 5.7 M CsCl cushion (Chirgwin et al., 1979). RNA concentration of the extract was estimated assuming that A260 = 1 corresponds to 40 ng μL⁻¹ RNA. For the northern-blot analyses, RNA (10 μg) was separated by electrophoresis (1.25% [w/v] agarose) under denaturing conditions according to Sambrook et al. (1989a). Equivalent RNA deposition in all the lanes of the electrophoresis gel was checked by ethidium bromide staining and observation of the gel under UV light. Transfer to Hybond N membrane was carried out as described in Sambrook et al. (1989b). Probes were ³²P-labeled by the random primer method (T7 Quick Prime Kit, Pharmacia Biotech, Piscataway, NJ) and purified on Sephadex columns (NICK Column, Pharmacia Biotech). Hybridization was performed overnight at 42°C in the following buffer: 750 mM NaCl, 50 mM Na₂HPO₄ (pH 7), 5 mM Na₂EDTA, 50% (v/v) formamide, 1% (w/v) sarkosyl, and 10% (w/v) dextran sulfate. Final wash was carried out in 0.1× SSC, 0.1% (w/v) SDS, at temperatures depending on the probe, i.e. 48°C, 45°C, and 42°C for the CAB, the Cys-proteinase, and the ferritin probes, respectively. Intensity of the signals was measured using an optical scanner (STORM, Molecular Dynamics, Sunnyvale, CA) and quantified with Image QuaNT software (Molecular Dynamics). Autoradiographs (X-OMAT AR films, Kodak, Rochester, NY) were then exposed in the presence of a fluorescent screen (Cronex, DuPont, Wilmington, DE) at −80°C, for 3 h in the case of hybridization with the CAB probe, 8 h for the Cys-proteinase probe, and 2 weeks for the ferritin probe.

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