Changes in the Number and Composition of Chloroplasts during Senescence of Mesophyll Cells of Attached and Detached Primary Leaves of Wheat (*Triticum aestivum* L.)

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ABSTRACT

Changes in the number and composition of chloroplasts of mesophyll cells were followed during senescence of the primary leaf of wheat (*Triticum aestivum* L.). Senescence was due to the natural pattern of leaf ontogeny or was either induced by leaf detachment and incubation in darkness, or incubation of attached leaves in the dark. In each case discrete sections (1 centimeter) of the leaf, representing mesophyll cells of the basal, middle, and tip regions, were examined. For all treatments, senescence was characterized by a loss of chlorophyll and the protein ribulose 1,5-bisphosphate carboxylase (RuBPCase). Chloroplast number per mesophyll cell remained essentially constant during senescence. It was not until more than 80% of the plastid chlorophyll and RuBPCase was degraded that some reduction (22%) in chloroplast number per mesophyll cell was recorded and this was invariably in the mesophyll cells of the leaf tip. We conclude that these data are consistent with the idea that degradation occurs within the chloroplast and that all chloroplasts in a mesophyll cell senesce with a high degree of synchrony rather than each chloroplast senescing sequentially.

In spite of the importance of chloroplasts, and in contrast to our extensive knowledge of the processes associated with photosynthesis, we know little of the mechanisms which are responsible for the senescence of this organelle. Two hypotheses can be advanced to describe chloroplast senescence. According to the first hypothesis chloroplasts senesce through a lysosomal interaction with the vacuole (19) so that senescence would be characterized by a decline in chloroplast number per mesophyll cell. This view is supported by the studies of Wittenbach et al. (25) and Lampma et al. (14) who observed a marked decline in chloroplast number per mesophyll cell during senescence of wheat and pea leaves, respectively. The alternate view is that the chloroplast is autonomous and the Chl, RuBPCase, and other constituents are degraded within the chloroplast. According to this view, chloroplast number per cell would remain constant, at least until general cellular autolysis occurs. There is both direct and indirect support for this hypothesis. Indirect evidence is of two forms. First, ultrastructural studies of senescing chloroplasts are in general agreement that the internal features are degraded well before any comprehensive disruption to the chloroplast envelope (3, 7, 8, 12, 20, 22). Second, there is evidence that the chloroplast contains a broad spectrum of hydrolytic enzymes, including peptide hydrolases (9, 11, 24), oxidative and peroxidative Chl-degrading enzymes (17), galactolipase (1), and β-galactosidase (P. L. Bhalla and M. J. Dalling, unpublished data). Direct evidence is presented in the recent report of Martinoia et al. (18) who observed no change in chloroplast number per mesophyll cell of senescing barley leaves in spite of the loss of over 80% of Chl and RuBPCase. In addition, these authors measured the Chl content of individual intact chloroplasts by computer-assisted cytofluorimetry and observed a 50% reduction in Chl content per chloroplast after 10 d of induced senescence. Gradual degradation of all chloroplasts is also evident in the studies of Jenkins and Woolhouse (13).

The aim of the experiments described in this paper was to determine whether chloroplasts senesce as a coordinated group, or whether they senesce sequentially. To minimize variability, all observations were made on discrete 1-cm segments of the wheat primary leaf. This ensured that each sample of mesophyll cells examined was of uniform age. Primary leaves were chosen for two reasons. First, because it is possible to produce large numbers of very uniform leaves (cf. other leaves) with negligible variability between batches. Second, we had previously characterized senescence of this leaf in terms of the gross changes in Chl, soluble protein, and RuBPCase protein (21).

MATERIALS AND METHODS

Plant Material. Wheat seeds (*Triticum aestivum* L. cv. Eger) were planted in a composted soil mixture and grown in a naturally illuminated phytotron at 20°C. The plants were watered daily but received no additional nutrients.

Analyses. Chlrophyll and Soluble Protein. Leaf segments were ground at 4°C with a mortar and pestle in 25 mM sodium phosphate buffer, pH 7.0. Before centrifugation, Chl content of the extract was determined by the procedure of Arnon (2). Following centrifugation, total soluble protein was measured by the method of Bradford (6).

RuBPCase Protein. Changes in the level of RuBPCase protein were by the procedure of rocket immunoelectrophoresis (15). Details have been outlined previously (22).

Determination of Mesophyll Cell Number. Mesophyll cell number in each leaf segment was determined by the procedure of Dean and Leech (10). At each harvest, 30 1-cm segments were incubated individually in 5% (w/v) chromium trioxide at 4°C for 7 d. After separating the cells by passage through a Pasteur pipette, the mesophyll cells were counted with a hemocytometer.

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2 Abbreviation: RuBPCase, ribulose 1,5-bisphosphate carboxylase.
Fig. 1. A comparison of two methods for estimating chloroplast numbers of mesophyll cells at various ages. The Y-axis represents counts of chloroplasts of glutaraldehyde-fixed mesophyll cells released following incubation in EDTA. The X-axis represents counts of chloroplasts of equivalent mesophyll protoplasts. \( r^2 = 0.97 \).

Fig. 2. Light micrograph of chloroplasts in an isolated glutaraldehyde-fixed mesophyll cell viewed by Nomarski microscopy. All chloroplasts (C) and the nucleus (N) are clearly defined within the cell.

Determination of Chloroplast Number/Mesophyll Cell. The procedure followed was essentially that described by Possingham and Smith (23). Each leaf segment was first fixed for 2 h in 3.5% (v/v) glutaraldehyde. The segments were then rinsed with distilled H\(_2\)O and incubated with vigorous shaking (300 oscillations/min) for 3 h at 60°C in 0.05 M EDTA, pH 9.0. Tissue that was visibly senesced was incubated in EDTA for only 1 h. Chloroplasts in the separated mesophyll cells were counted with a Zeiss photomicroscope fitted with Nomarski differential interference optics.

Determination of Mesophyll Cell Age. The age of cells in each leaf segment was determined according to the procedure of Boffey et al. (4).

Fig. 3. The course of natural senescence of the attached primary leaf. Changes in Chl (○) and RuBPCase (□) per chloroplast and chloroplast number per mesophyll cell (■) from the basal (B), middle (M), and tip (T) regions. The vertical bars represent ±SE. Number of mesophyll cells per leaf segment (□) and changes in total soluble protein (△) and Chl (Δ) per g fresh weight. The SE was always less than 15% of the mean.

RESULTS

Assessment of Methodology for Estimating Chloroplast Numbers. It appears from studies already published that the methodology for estimating chloroplast number/mesophyll cell is of two forms. The first approach is that described in this paper and previously by Lamppa et al. (14) and Martinolli et al. (18). This involves a direct counting of chloroplasts contained in isolated protoplasts or glutaraldehyde-fixed mesophyll cells. Assuming that all the chloroplasts/cell can be counted, a major potential limitation of this method is the isolation of cells which are representative of the leaf mesophyll cell population. However, the results of Martinolli et al. (18) and Wittenbach et al. (25) have indicated that this is not a serious problem. The second method is to isolate protoplasts, lyse them, and count the released chloroplasts (25). This method, in our view, is the least reliable since it is based entirely on the assumption that lysis of the protoplasts releases all chloroplasts and that senescence itself does not affect the stability of the chloroplasts during lysis. It was our experience during assessment of these methods that chloroplasts became increasingly fragile with the progress of senescence; an observation consistent with our marked reduction in density and size during senescence (18). We chose to use glutaraldehyde-fixed mesophyll cells for three reasons. First, direct counting of
Characteristics of Chloroplast Senescence. Natural Senescence. Figure 3 shows the changes in composition of 1-cm sections of the primary leaf during natural senescence. Full leaf expansion occurred by day 9 and all measurements commenced 12 d after sowing. On day 12 the age of the mesophyll cells of the basal, middle, and tip regions was 6, 8, and 9 d, respectively. Chl and RuBPCase/mesophyll cell declined in each section so that within 14 d of the commencement of the experiment each section had lost more than 80% of its Chl and RuBPCase. Mesophyll cell number/section did not change during this period, good testimony to the reliability of the method of Dean and Leech (10). The number of chloroplasts/mesophyll cell did not change appreciably during the experimental period, although late in senescence there was a small decline. This observation was particularly marked in the mesophyll cells of the leaf tip where, at the start of the experiment, there were 185 ± 12 chloroplasts/cell and after 14 d, 140 ± 25 chloroplasts/cell, a decline of 22%. There was only a 14% decline in chloroplasts/cell during senescence of the basal and middle leaf sections. In marked contrast to chloroplasts/cell there was a dramatic decline in RuBPCase and Chl/chloroplast during the experimental period. In each section, more than 90% of these chloroplast constituents were lost.

Induced Senescence. Senescence of the primary leaf was induced by either placing the whole plant in darkness (Fig. 4) or detaching the primary leaf and incubating in the dark (Fig. 5). In either case, senescence was greatly accelerated. There was, however, always a consistent difference between these treatments in the timing of the initiation of senescence. Detached, darkened leaves begin to senesce immediately whereas attached, but darkened, leaves do not begin senescence for about 2 d after darkening. After that time senescence is more rapid in the attached, darkened leaves.

Inducing senescence by either of the two treatments described
does not change the characteristics of chloroplast senescence. In both treatments and in each segment, the decline in RuBPCase and Chl assumes major proportions in comparison to any change in chloroplast number. In a similar manner to natural senescence (Fig. 3), it was noticeable that any reduction in chloroplasts/cell was invariably associated with the segments closer to the leaf tip, i.e. the oldest mesophyll cells.

DISCUSSION

Our observations are in accord with those reported recently for senescing barley leaves (18). In both wheat and barley it would seem that the senescence of mesophyll cells is not characterized by a marked reduction in chloroplasts/cell. Instead, we conclude that the constituents of the chloroplast are most likely degraded in situ. Any reduction in chloroplasts/cell may come later in senescence and be a consequence of general cellular autolysis. It follows from these data that two phases of senescence are identifiable. The first phase is orderly and cellular compartmentation is maintained (22). It is during this phase that the major portion of the cytoplasm and contents of organelles, especially the chloroplast, are degraded. The second phase commences slowly as the integrity of the different compartments of the cell begin to decline, simply because of the effects of continuing senescence. Later in this second phase general cellular autolysis occurs and the last ration of mobilizable material is removed from the cell. It is during this second phase that the number of chloroplasts/cell may decline (Figs. 3–5).

The source of the necessary hydrolytic enzymes is open to speculation. The vacuole is an obvious candidate, simply because many of the enzymes with the necessary capacity for degradation of the constituents of the chloroplast have been detected in this organelle (5, 11, 16, 24). The opposing view, and the one we favor, is that the chloroplast is largely autonomous and contains the necessary hydrolytic enzymes. As stated previously (see "Introduction"), recent evidence goes some way to supporting this view. However, like the vacuolar hydrolases it cannot yet be concluded that these enzymes, although in the correct location, function to achieve chloroplast senescence. This distinction is currently under study.

LITERATURE CITED