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Ribulose Bisphosphate Carboxylase and Proteolytic Activity in Wheat Leaves from Anthesis through Senescence[1]

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ABSTRACT

Changes in ribulose bisphosphate carboxylase (RuBPCase) and proteolytic activity were followed in the flag leaf and second leaf of field-grown winter wheat (cv. Arthur). These changes were followed in relation to changes in leaf chlorophyll, protein, and photosynthesis, and seed development. Levels of RuBPCase were determined by rocket immunoelectrophoresis as described previously (Wittenbach 1978 Plant Physiol 62: 604-608). RuBPCase constituted 40 to 45% of the total soluble protein in the flag leaf and an even higher percentage of the soluble protein in the second leaf. This ratio remained unchanged until senescence when RuBPCase protein was apparently lost at a faster rate than total soluble protein. No change in the specific activity of RuBPCase on either a milligram protein or RuBPCase basis was observed until senescence. A close correlation existed among the various indices of senescence in the field, namely, the decline in chlorophyll, protein, photosynthesis, and RuBPCase activity. In addition, proteinase activity increased with the onset of senescence. These enzymes readily degraded RuBPCase, exhibiting a pH optimum of 4.8 to 5.0 and a temperature optimum of 50 C. Proteinase activity was modified by sulfydryl reagents suggesting the presence of sulfydryl groups at or near the active sites.

RuBPCase[2] is both the key enzyme in photosynthesis and the major leaf protein. Prior to senescence, RuPBCase functions to provide photosynthate to the growing regions. During senescence, the breakdown of RuBPCase provides additional nitrogen and carbon for mobilization to the seed. Thus, the breakdown of this enzyme by proteolytic enzymes may play an important role in controlling plant senescence.

The initial loss of soluble protein during senescence in detached (14) and attached (16) primary leaves in the dark has been shown to result almost entirely from the loss of RuBPCase. In the latter study with wheat seedlings, the specific activity of RuBPCase, based on actual levels of RuBPCase, did not change until the onset of the irreversible stage of senescence, after over 50% of the RuBPCase had been degraded. Recent work by Hall *et al.* (7) indicates that RuBPCase in the flag leaf of wheat does not decline until late in senescence and undergoes marked changes in specific activity during seed development. This change in RuBPCase activity prior to senescence is contrary to earlier results from this lab (16).

The present study extends previous work on wheat seedlings (16) to field-grown plants. Changes in the level and activity of RuBPCase in the flag leaf and the leaf immediately below it have been followed from full leaf expansion through senescence. These changes have been followed with respect to changes in leaf Chl, protein, and photosynthesis. In addition, proteolytic activity in these leaves was followed, since these enzymes are responsible for the breakdown of RuBPCase.

MATERIALS AND METHODS

Plant Material. Samples of flag leaves, second leaves (leaves immediately below the flag leaves), and seed heads were collected from field-grown winter wheat (*Triticum aestivum* L. cv. Arthur) beginning 1 week prior to anthesis and ending at harvest during the 1977 and 1978 seasons. All collections and photosynthesis measurements were made between 2 and 4 PM. Samples were immediately frozen in liquid N_2 and transported to the lab on dry ice. They were then stored in liquid N_2 until assayed. Freezing in liquid N_2 had no significant effect on RuBPCase activity, and no activity was lost following storage for at least 1 month.

Photosynthesis and RuBPCase Assays. Photosynthetic measurements were made using a $^{14}CO_2$-pulsing apparatus similar to that of Naylor and Teare (11). All determinations were made on clear days in full sunlight (~1,800 $\mu E/m^2 \cdot s$) with the temperature between 22 and 30 C. Leaves were pulsed for 15 s with air (364 $\mu l/l$ CO_2) containing $^{14}CO_2$. Leaf discs were removed with a cork borer, placed in scintillation vials with 1 ml of tissue solubilizer, and left overnight on a shaker to digest. Counting solution was then added and the dpm determined by liquid scintillation spectroscopy.

RuBPCase activity was determined by following $^{14}CO_2$ incorporation into acid-stable products as described previously (16). Leaf samples of 300 mg were extracted in 5 ml of 25 mM Hepes (pH 7.5) containing 4 mM DTT, 1 mM Na_2EDTA, and 1% (w/v) PVP using a Polytron homogenizer. The extracts were centrifuged at 30,000*g* for 20 min, and the supernatant fractions were used to assay for RuBPCase activity.

The levels of RuBPCase were quantitated using the procedure of rocket immunoelectrophoresis (15). Leaf samples of 200 mg were extracted in 5 ml of 25 mM Hepes (pH 7.5). The extracts were centrifuged at 30,000*g* for 20 min, and an aliquot of the supernatant fraction was diluted to about 200 μg protein/ml. The diluted extracts were then applied (10 μl/well) to 1% agarose gels containing antisera for immunoelectrophoresis as described (16). Purified wheat RuBPCase was prepared according to the procedure of Paulsen and Lane (12), as modified by Lorimer *et al.* (9). This RuBPCase was used to quantitate the level of carboxylase in the gels and to study the activity of the purified enzyme.

Proteinase Assay. Leaf samples of 300 mg were extracted in 5 ml of 25 mM Hepes (pH 7.5) containing 4 mM DTT and 1 mM EDTA. After centrifugation, the supernatant fractions were run through Sephadex G-25 columns, and the protein coming off in the void volume was used to assay for proteinase activity. Purified

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[2] Abbrevation: RuBPCase: ribulose bisphosphate carboxylase.

tobacco RuBPCase was used as the substrate (4), and the assay mixture routinely contained 0.4 ml of 200 mM citrate buffer (sodium citrate-citric acid), 0.2 ml substrate (generally 1–2 mg/ml final concentration), and 0.4 ml crude enzyme protein (from 15 to 40 mg tissue). Assays were run at 38 C for 1 to 2 h, and the reaction was stopped by adding 1 ml of 10% (w/v) trichloroacetic acid. Reaction tubes were placed at 4 C for 30 min, and the precipitate was removed by centrifugation. The free amino N in the supernatant fraction was determined by the ninhydrin procedure (18).

Chl, Protein, and N Analysis. Chl content was determined on an aliquot of the extract following the method of Arnon (1). Protein analysis of samples was made following centrifugation using the Bio-Rad procedure (2), which agreed with Kjeldahl nitrogen analysis data. Interference was observed with the Lowry method (10) of analysis, especially during senescence, and therefore its use was discontinued. Seed N was determined from Kjeldahl analysis (8).

Results from 1977 and 1978. The results for these two years have been combined for the quantitation of RuBPCase, Chl levels, and proteinase activity. Photosynthesis was only measured during 1978, and since the Lowry method of protein analysis was used in 1977, those data have not been combined for the 2 years.

RESULTS AND DISCUSSION

Changes in Specific Leaf Weight, Chl, and Protein. Both the flag leaf and second leaf changed little in specific leaf weight from 1 week prior to anthesis until the start of senescence (Fig. 1A). The increase in specific weight of the flag leaf between 1 and 2 weeks after anthesis may be due to an increase in starch reserves, since photosynthesis was increasing during this period (see Fig. 3). During senescence, specific leaf weight declined, which most likely indicates the remobilization of material from the senescing leaves.

The Chl content of both leaves remained nearly constant from full expansion until the onset of senescence (Fig. 1B). In the second leaf the rapid decline in Chl began about 1 week earlier than in the flag leaf and occurred over a longer period, possibly reflecting the older age of the second leaf and the effect of shading on the onset of senescence.

The soluble protein content of the flag leaf showed much larger fluctuations than Chl between full expansion and senescence (Fig. 1C). Protein increased just prior to anthesis, followed by a decline at anthesis, and another increase just after anthesis. The level then declined and appeared to stabilize just prior to the onset of senescence 3 weeks after anthesis. In the second leaf the soluble protein content showed a steady decline until just prior to the onset of senescence when the level appeared to stabilize or slightly increase. The onset of senescence, as indicated by the rapid decline in protein, occurred 4 to 5 days earlier in the second leaf than in the flag leaf. No large increases in amino N were associated with the loss of protein from the flag leaf (data not shown), indicating that the breakdown products were being rapidly translocated out of the senescing leaves to the growing regions. Since the major sinks at this time are the developing seeds, which are rapidly accumulating N (Fig. 2), it is reasonable to assume that this protein N is being translocated to the seeds.

Photosynthesis and RuBPCase. There was an apparent, although insignificant, rise in photosynthesis in the flag leaf following anthesis (Fig. 3). The rate then leveled off and began to decline rapidly about 3 weeks after anthesis. Removing the seed heads in this variety at anthesis or later had no apparent effect on the onset of senescence. However, removing the seed heads did result in a 50% decline in flag leaf photosynthetic rates. Rates of photosynthesis in the second leaf began to decline about 5 days prior to those in the flag leaf, and paralleled the decline observed in the flag leaf. A good correlation existed between the decline in photosynthesis associated with senescence and the decline in RuBPCase activity for both leaves (Fig. 4). Moreover, the change in carboxylase activity very closely followed the change in protein levels on an area or dry weight basis.

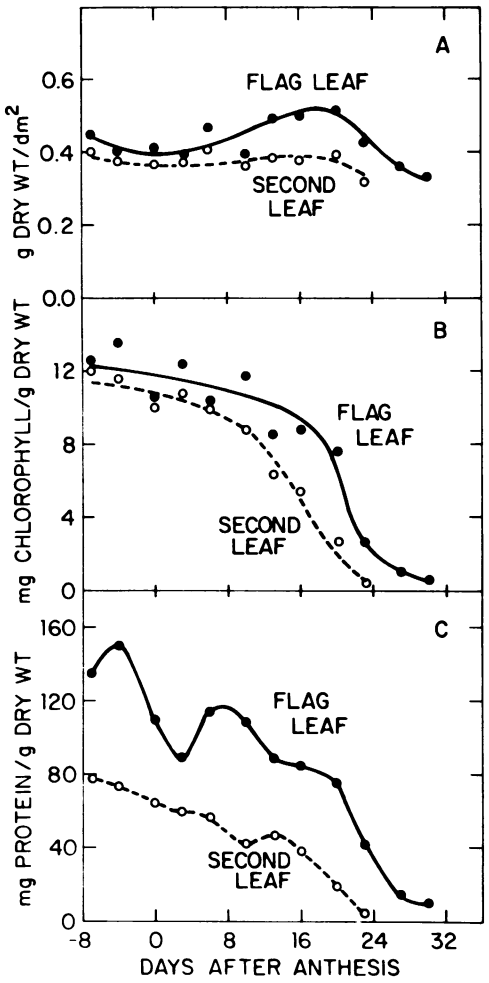

FIG. 1. Changes in (A) specific leaf weight, (B) Chl, and (C) total soluble protein in the flag leaf (●) and second leaf (○) of field-grown winter wheat from 1 week prior to anthesis through senescence. The SE for Chl values ranged from ± 0.2 to 1.1 mg/g dry weight and for protein from ± 5.7 to ± 15.9 mg/g dry weight.

The change in RuBPCase as a per cent of the total soluble protein is shown in Table I. This per cent, for either leaf, did not change significantly until the onset of senescence. In primary wheat leaves during dark-induced senescence there was a reversible stage of senescence when the total loss of protein could be accounted for by the loss of carboxylase (16). There appeared to be a similar stage at the onset of senescence in the field in 1977; however, this was not evident in 1978 and consequently is not evident in the combined data in Table I. As observed in the seedlings, carboxylase was apparently lost at a faster rate than other soluble protein during senescence, as indicated by the decline in carboxylase as a per cent of the total soluble protein. The capacity for protein synthesis declines in the chloroplasts following leaf expansion (17). If this occurs in wheat, little or no turnover of RuBPCase would be possible during protein degradation, since the large subunit would very shortly become limiting (3). Thus, if other soluble proteins were turning over during senescence, carboxylase would apparently be lost at a faster rate as shown in Table I.

The specific activity of carboxylase per mg protein and per mg

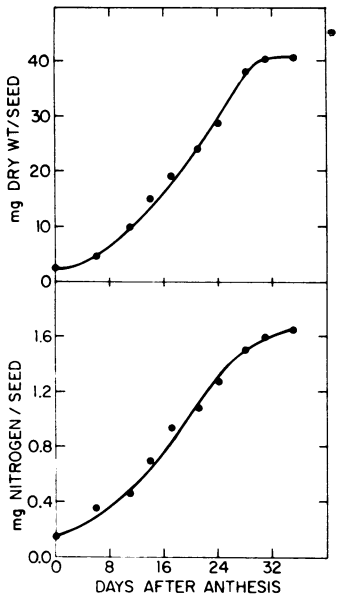

FIG. 2. Increase in dry weight and N during seed development in winter wheat. N content was determined by Kjeldahl analysis (7).

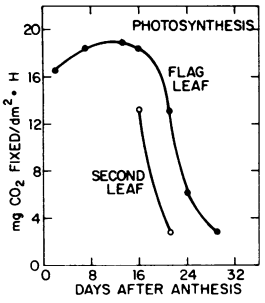

FIG. 3. Changes in photosynthesis in the flag leaf (●) from anthesis through senescence and in the second leaf (○) during senescence (see text for details of measurements). Values represent the mean of at least eight observations. The SD ranged from ± 1.9 to ± 3.5 mg CO_2 fixed/dm^2·h.

RuBPCase remained constant for both leaves until senescence (Table I). With the onset of the rapid loss of protein associated with senescence, a marked decline in specific activity on a mg protein basis occurred. A similar although less rapid decline in specific activity per mg RuBPCase also occurred, indicating a more rapid loss of active sites than of immunochemical recognition sites during senescence. This decline in specific activity appears the same as that observed during the irreversible stage of dark-induced seedling senescence (16). Apparently, senescence in the field was also an irreversible process in this variety, since no delay in the onset of senescence was observed by removing the seed heads at several stages.

The reason for the discrepancy between our data and those of Hall *et al.* (7) is unknown. However, from their data it would appear that their plants were already undergoing an irreversible senescence by the time of anthesis, since Chl, photosynthesis, and RuBPCase activity were already rapidly declining. This would resolve the differences in our data with respect to changes in specific activity by carboxylase; however, it would not resolve our differences with respect to the stability of RuBPCase during senescence.

The properties of purified wheat RuBPCase were determined on the same protein which was isolated for quantitation of the peak heights in rocket immunoelectrophoresis. This RuBPCase was homogeneous as judged by disc gel electrophoresis of 100 μg of protein on 7.5% gels stained with Coomassie blue R-250, and had an absorbancy ratio A_{280}/A_{260} of 1.91. The activity of the enzyme as isolated was 707 ± 27 nmol CO_2 fixed/min·mg protein at 25 C. After heat activation at 50 C for 20 min, the activity of the purified enzymes was 1,148 ± 70 nmol CO_2 fixed/min·mg protein, which is close to the activity per mg RuBPCase observed in the crude extracts (Table I).

Proteinase Activity. Proteolytic activity assayed with RuBPCase as the substrate showed a gradual increase following anthesis in both leaves (Fig. 5). The activity then appeared to stabilize or slightly decline beginning about 6 days prior to the onset of senescence. Then, just prior to the decline in photosynthesis, protein, and Chl which indicated the onset of senescence, the proteolytic activity began to rise, rapidly reaching a peak about midway through senescence—during the most rapid decline in soluble protein. This change in proteolytic activity is very similar to that reported by Dalling *et al.* (5). Whether this increase in activity is due to increased synthesis or activation of existing proteinases cannot be determined from these data. It seems unlikely that this magnitude of an increase in activity would be sufficient to initiate such marked changes as occur during senescence. It seems more reasonable that the physiological changes during senescence result from an alteration in compartmentation and/or a change in form (13) of the proteinases, which would not be reflected in measurements of total activity. Preliminary evidence does suggest a change in enzyme form with the onset of senescence.

The proteolytic enzymes in the flag and second leaf during senescence appeared similar to those in the primary leaf of seed-

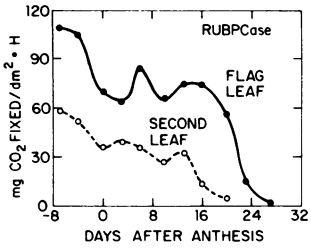

FIG. 4. Changes in RuBPCase activity on an area basis in the flag leaf (●) and second leaf (○) of wheat. Carboxylase activity in crude extracts was determined by $^{14}CO_2$ incorporation into acid-stable products. The SE ranged from ± 3.4 to ± 13.1 mg CO_2 fixed/dm^2·h.

Table I. *Changes in the Level and Specific Activity of RuBPCase in the Flag Leaf and Second Leaf of Wheat during Maturation and Senescence*

The level of RuBPCase was determined by rocket immunoelectrophoresis. Activity was determined from crude extracts by $^{14}CO_2$ incorporation at 25 C in the presence of $NaH^{14}CO_3$ and ribulose diphosphate. Total soluble protein was assayed using the Bio-Rad procedure.

Time after anthesis	Flag Leaf			Second Leaf		
	RuBPCase[a]	CO_2 Fixed		RuBPCase[a]	CO_2 Fixed	
days	*% soluble protein*	*nmol/min·mg protein*[b]	*nmol/min·mg Ru-BPCase*	*% soluble protein*	*μmol/min·mg protein*	*nmol/min·mg Ru-BPCase*
−7	45.5	759	1668	54.4	778	1429
−4	47.6	743	1561	57.9	788	1362
0	42.2	653	1548	55.3	624	1128
3	43.3	706	1630	57.2	694	1213
6	41.8	676	1617	49.1	643	1309
10	41.7	635	1522	46.6	710	1524
13	39.8	691	1737	49.6	748	1508
16	41.5	726	1750	38.6	321	832
20	39.8	608	1526	29.2	232	795
23	34.1	321	942			
27	21.7	43	199			

[a] The SE ranged from ± 0.9 to ± 3.9%.
[b] The SE ranged from ± 17 to ± 73.

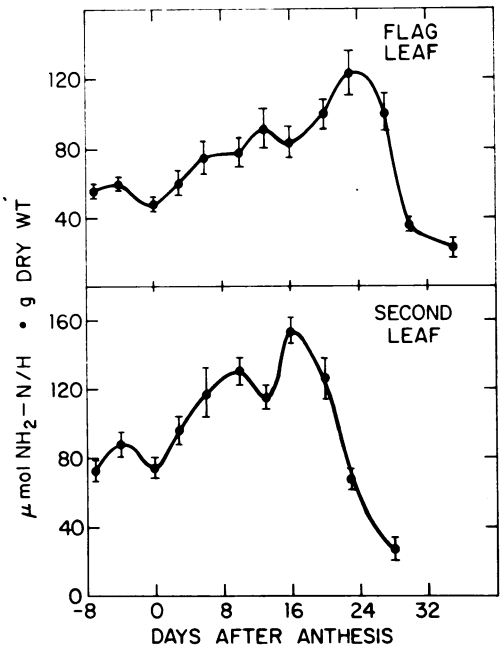

FIG. 5. Changes in proteolytic activity in the flag leaf and second leaf of wheat from 1 week prior to anthesis through senescence. Proteinase activity was assayed using purified tobacco RuBPCase as the substrate. Vertical lines indicate ± SE.

lings during dark-induced senescence (16). Most of the activity on RuBPCase was due to endopeptidases, since the ratio of total N to α-amino N released was 5 rather than the expected 1 to 2 for exopeptidases. The optimum pH for activity was 4.8 to 5.0, and activity on RuBPCase showed a temperature optimum of 50 C, as was also shown by Frith *et al.* (6) for activity of wheat proteases on hemoglobin. Moreover, these enzymes were strongly inhibited by ρ-chloromercuribenzoic acid and showed activation by DDT, suggesting the presence of sulfydryl groups at or near the active site.

CONCLUSION

These results show that the specific activity of RuBPCase does not change until the onset of senescence. During senescence RuBPCase protein appears to be lost at a faster rate than soluble protein in general, but this may simply reflect the loss of capacity to synthesize RuBPCase at this late stage in plant development. A good correlation was observed among the various indices of senescence, namely, the decline in Chl, protein, photosynthesis, and RuBPCase activity. In addition, proteinase activity increased with the onset of senescence, indicating that these enzymes may have a regulatory role in plant senescence.

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