Progression and Specificity of Protein Oxidation in the Life Cycle of Arabidopsis thaliana*

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Protein carbonylation is an irreversible oxidative process leading to a loss of function of the modified proteins, and in a variety of model systems, including worms, flies, and mammals, carbonyl levels gradually increase with age. In contrast, we report here that in Arabidopsis thaliana an initial increase in protein oxidation during the first 20 days of the life cycle of the plant is followed by a drastic reduction in protein carbonyls prior to bolting and flower development. Protein carbonylation prior to the transition to flowering targets specific proteins such as Hsp70, ATP synthases, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and proteins involved in light harvesting/energy transfer and the C2 oxidative photosynthetic carbon cycle. The precipitous fall in protein carbonyl levels is due to the specific reduction in the levels of oxidized proteins rather than an overall loss of carbonyl levels is due to the specific reduction in the levels of oxidized proteins rather than an overall loss of carbonyl levels. Oxidations at specific sites result in the enzyme. Oxidations at specific sites result in the enzyme. Oxidations at specific sites result in the enzyme. Oxidations at specific sites result in the enzyme.

Endogenously generated reactive oxygen species, produced by the electron transport chains, react with different macro-molecules in the cell and cause oxidative damage. Such oxidative damage accumulates over time during the life cycle of many organisms and has been suggested to be one possible cause of aging (1, 2). Oxidative modification of proteins may be detected by a variety of specific markers, and sensitive methods for its detection have been developed by Stadtman and Levine (3). Direct oxidative attack on Lys, Arg, Pro, and Thr or by secondary reactions via reactive carbohydrates and lipids on Cys, His, and Lys residues can lead to the formation of protein carbonyl derivatives (4, 5). Carbonylation of proteins inhibits or alters the activities of the proteins and increases their susceptibility to proteolytic attack (5, 6). In rats, aged flies, and senescing yeast and bacteria, this oxidation has been shown to be specific and targets enzymes of the Krebs cycle and other proteins, including stress proteins, Hsp70 chaperones, and translation elongation factors (7–10). In contrast, little is known about the occurrence of and targets for protein oxidation during the life cycle of annual plants.

The annual model plant Arabidopsis thaliana has a life cycle from seed to seed of about 6 weeks under optimal growth conditions. After germination, the above ground portion of the Arabidopsis plant initially develops two cotyledons and later a compressed rosette of leaves. All structures originate from the apical shoot meristem (11, 12). Later the shoot apical meristem will undergo a transition to an inflorescence meristem. Under long day conditions this occurs when the plant has six to ten leaves, although this varies with the genetic background and the environmental conditions. Somewhat later, after the main stem has elongated and produced a complex of higher order branches, a transition to a lower meristem occurs, from which the reproductive organs develop. After fertilization and as the seeds mature, the somatic tissues of the whole plant become increasingly senescent.

Leaf senescence is the final stage of leaf development that ultimately results in the death of the plant (reviewed in Ref. 13). During this final stage, the leaf cells undergo programmed cell death process, referred to as the senescence syndrome (14). The most widely used biomarker for the senescence syndrome is the loss of chlorophyll associated with the degeneration of chloroplast internal structures (15). Leaf senescence is an intrinsic age-dependent process and its onset appears to be regulated by the age of individual leaves (16).

We demonstrate here that the quantitative and qualitative pattern of carbonylation during progression through the life cycle of the plant A. thaliana is distinct from that of the yeast, fly, nematode, and mammalian model systems. Carbonylation first increases with the age of the plant, similar to animals and microbes, but drops abruptly prior to the vegetative to reproductive transition and does not coincide with leaf senescence. Proteomic identification of the major targets for protein carbonylation in the plant leaves indicates that proteins directly and indirectly linked to chloroplast activities exhibit special problems with respect to oxidative damage. The data highlight that there is no inevitable, stochastic, increase in protein oxidation in aging tissues of the plant A. thaliana.

EXPERIMENTAL PROCEDURES

Culturing Conditions—Wild-type A. thaliana plants (ecotype Col-0) were grown on a mixture of 2:1:1 of soil:vermiculite:perlite in a greenhouse in a day/night temperature regime of 22/18 °C, an 18-h photoperiod, a photon flux density of 125 μmol m⁻² s⁻¹, and a relative humidity of 70%. Plants were watered twice a week with a nutrition solution as described (17). Isolation of Cellular Proteins—Protein extractions and chlorophyll measurements were performed from leaf number 5 and 6, unless the extractions were from very young plants in which case the whole seedlings were used. Rosette leaves from different life stages were grinded in liquid N₂ using a precooled mortar and pestle. The powder was resuspended in a buffer containing 250 mM Tris-Cl, pH 8.5, 0.5 mM EDTA, 2% lithium dodecyl sulfate, 10% glycerol, and 2 mM phenol (protease inhibitor, Roche Applied Science). The sample was heated for 5 min at 75 °C. Tubes were cooled and centrifuged at 20,000 × g at 4 °C for 10 min. The supernatant was transferred to a fresh tube and the protein was acetone-extracted by addition of acetone to a final concentration of 80% (v/v) at –20 °C for 30 min and then centrifuged for 20,000 × g for 5 min at room temperature. After centrifugation and vacuum drying, the protein pellet was resuspended in the same buffer as above and stored.

* This work was supported by a grant from the Swedish Natural Science Research Council (to T. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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at -20 °C after the protein concentration had been determined. The concentration of proteins was estimated using Pierce BCA protein quantification kit according to the manufacturer’s instructions. The relative abundance of Rubisco1 was determined using specific antibodies (from Agrisera, Vännäs, Sweden) and Western blotting. As a secondary antibody, anti-chicken IgG from Sigma (product number A9046) was used.

Carbonylation Assays—Detection of carbonylated proteins was performed using the chemical and immunological reagents of the OxyBlotTM Oxidized Protein Detection Kit (Intergen). The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazine; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid. The band was collected by aspiration with a 1-ml pipetteman and diluted with wash buffer in a chilled 15-ml correx tube. This tube was prepared and run on two-dimensional polyacrylamide gels by the methods of Farrell (18) with modifications (7). Ampholines with a pH range of 3.5–10 from Amersham Biosciences AB were used. Gel electrophoresis was carried out using 11.5% SDS-polyacrylamide gels, and the gels were either stained with Coomassie Blue or immunoblotted according to standard procedures. Signal intensities in gels and blots were analyzed by the computer program PDQuest (Bio-Rad). This program detects the chemiluminescence signal contributed by each separate protein spot and divides it by the total chemiluminescence signal of the whole blot. The program also detects the intensity of the Coomassie spot contributed by each protein spot and divides it with the intensity of the total Coomassie intensity of the whole gel. By making a ratio of these parameters a relative measurement of carbonylation/amount of protein can be calculated (the oxidation index).

Mass Spectrometry—Matrix-assisted laser desorption ionization time of flight data were acquired on an M@LDI-LR (Micromass, Manchester, UK) in reflection mode and ESI data in mass spectrometry and mass spectrometry-mass spectrometry mode on a Q-Tof Ultima (Micromass, Manchester, UK). Protein identifications were done using MASCOT (available at www.matrixscience.com). Proteins were identified against the nr protein data base at NCBI, where a score of 74 corresponds to a 95% confidence level. All proteins were identified with scores above 95.

RESULTS

Protein Oxidation Is Age-dependent—A. thaliana plants (ecotype Col-0) were grown in a photoperiod of 18 h of light and 6 h of darkness, and protein extractions were made from different life cycle stages, ranging from before bolting to 25% leaf senescence. As shown in Fig. 1, A and B, protein carbonyl levels per total protein first increases as the rosette leaves becomes chronologically older. However, this increase is followed by an abrupt decrease in oxidation levels around 20 days after planting. The carbonylation levels in the last stages of the life cycle of the plant did not change (not shown). To evaluate whether there was any difference in carbonylation status during the photoperiod (day) and the period of active mitochondrial respiration (night), total cellular protein was extracted from plants, either 4 h after the light was switched on or 5 h after the light

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The abbreviations used are: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; DNP, 2,4-dinitrophenylhydrazine; DNFH, 2,4-di-nitrophenylhydrazine; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)-ethylamino]ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
Chloroplast Proteins Are Major Targets for Protein Carbonylation—Protein carbonylation does not occur at random and is targeted to specific proteins in senescing and oxidatively stressed organisms (7, 19). To investigate whether protein oxidation is specific also in A. thaliana, we utilized a proteomic immunochemical approach to identify possible targets for protein carbonylation in aging leaves. We used the method described by O’Farrell (18) with the modifications described under “Experimental Procedures.” We analyzed the spectrum of carbonylated proteins in samples taken from plants during the day (light) period and night (dark) period. The most prominent carbonylated proteins of the aged (20 days) rosette leaves, e.g. Rubisco large subunit, Rubisco-activase, the β-subunit of ATP synthase, OEC33, and chlorophyll a/b-binding protein, are all residing in the chloroplast (Fig. 2A and Table I). Among these, all, except the chlorophyll a/b-binding protein, were carbonylated both during the day and night (Table I). It is possible that the carbonylated Hsp70 chaperone identified also resides in the chloroplast, but the mass spectrometry data did not allow us to firmly distinguish between the different Hsp70s. A. thaliana has 14 members of the Hsp70 family (20), targeted to different subcellular locations. They all share a very high degree of conservation, and therefore it was not clear which specific Hsp70 protein of the family was carbonylated. The identified carbonylated proteins of the chloroplasts are abundant plant proteins, but it is clear that many equally abundant proteins, such as the α-subunit of the ATP synthase or the small subunit of Rubisco, did not show any signs of being carbonylated. Thus, like in other model systems, carbonylation appear to be specific in the plant A. thaliana.

Since Rubisco is a very abundant protein in the plant cell, it is possible that the total carbonylation signal obtained (Fig. 1B) simply follows age-dependent alterations in the abundance of Rubisco itself. However, this does not seem to be the case. Western blot analysis, using a specific antibody against Rubisco (Fig. 1C), demonstrated that Rubisco concentrations stay more or less constant during the time when carbonylation first increased gradually and subsequently dropped abruptly. In other words, the precipitous fall in protein carbonyl levels must be due to a specific reduction in the levels of oxidized proteins rather than an overall loss of Rubisco, for example, associated with the senescence syndrome.

Carbonylation of Mitochondrial Proteins Is Specific—The relative abundance of some carbonylated proteins of the chloroplasts makes it difficult to detect carbonyl signals from other radical oxygen species-generating organelles, such as the mitochondria, in the crude cell extracts. Thus, to identify the most carbonylated proteins in the mitochondria, we isolated pure mitochondria from plants after 21 days of growth and applied immunochemical proteomics as described above. We found that the major targets for carbonylation in the mitochondria are glycine dehydrogenase (glycine cleavage system P-protein), a P-protein-like protein, the β-subunit of the ATP synthase, a glycine hydroxymethyltransferase-like protein, and aminomethyltransferase (glycine cleavage system T-protein) (Table II). Like in chloroplasts, some very abundant mitochondrial proteins, e.g. the mitochondrial F₄₅ ATP synthase α-subunit, citrate synthase, and malate dehydrogenase, did not show any signs of carbonylation suggesting that carbonylation sensitivity is specific also in this organelle.

### DISCUSSION

Since targets for carbonylation have not been thoroughly examined in plants, many of the carbonylated proteins identified here denote new proteins on the list of oxidation-sensitive proteins. However, heat shock Hsp70 chaperones and F-type

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**Table I**

| Protein carbonylated        | Condition | Source, NCBI gi no. |
|-----------------------------|-----------|---------------------|
| Hsp70                       | Yes       | Several hits        |
| Rubisco-large subunit       | Yes       | 7525041             |
| β-Subunit of chloroplast ATPase | Yes | 755040             |
| Rubisco-activase splice form 1 | Yes  | 7438143           |
| Rubisco-activase splice form 2 | Yes   | 7438144           |
| OEC, 33 kDa                 | Yes       | 15230324            |
| Chlorophyll a/b-binding protein | No     | Several hits        |

**Chloroplast Proteins Are Major Targets for Protein Carbonylation**

Protein from total cell extract was derivatized and run on two-dimensional gels. The gels were blotted to membranes, and the most carbonylated proteins were detected with antibodies. The corresponding Coomassie spots were cut out and subjected to mass spectrometry.
ATP synthases have been shown previously to be sensitive to oxidative attack both in *Escherichia coli* and *Saccharomyces cerevisiae* (7, 8). The identity of the other oxidized proteins of the chloroplast plastids key processes that appear to be targets for oxidative modification: specifically (i) photosynthetic CO\textsubscript{2} assimilation and photorecorporation carbon oxidation (Rubisco, rubisco-activase), (ii) light-induced water oxidation at photosystem II (OEC33), and (iii) light harvesting and energy transfer at photochemical reaction centers (chlorophyll *a/b*-binding protein).

Rubisco catalyzes the first step in net photosynthetic CO\textsubscript{2} assimilation and photorecorporation carbon oxidation. The active site of Rubisco assumes a closed conformation with certain phosphorylated ligands, and in the absence of catalysis, conversion of Rubisco from the closed to the open conformation is extremely slow. Rubisco-activase facilitates this process by releasing the inhibitory sugar phosphates (reviewed in Refs. 21 and 22). Rubisco-activase is an ATP-dependent AAA+ protein (ATPases associated with a variety of cellular activities), which includes a variety of proteins with chaperone-like functions (23). Interestingly, Rubisco-activase is present in two isoforms (24), differing only at the carboxyl terminus, which arise by alternative splicing of the gene transcript (25). The longer form is subjected to redox regulation via thioredoxin-f-mediated reduction of a pair of cysteine residues in the COOH-terminal extension (26). Based on pf, molecular mass, abundance, and mass spectrometry data, we concluded that both isoforms of the activase are carbonylated, but the longer form (activase I; Fig. 2A) is significantly more oxidized. This is also supported by the oxidation index calculated by the computer program PDQuest (Fig. 2B).

The OEC33 protein, together with OEC24 and OEC18, bind to the core of photosystem II at the luminal surface to regulate the water oxidation process. Photosystem II is susceptible to damage caused by excess light, and the D1 protein of this system is a specific target for such photodamage. When damaged, D1 is rapidly degraded, and it has been suggested that OEC33 stimulates this degradation (27). It would be interesting to elucidate whether the carbonylation of OEC33 affects D1 proteolysis and whether oxidation of OEC33 is, itself, a direct consequence of photodamage.

Light-harvesting chlorophyll *a/b*-binding (Lhc) proteins are associated with the photosystems where they absorb energy through chlorophyll excitation and transfer the energy to photochemical reaction centers. In the case of higher plants, several reports have shown that the mRNA levels of *cab* transcripts fluctuate diurnally (reviewed in Ref. 28) with *cab* mRNA levels peaking in the late morning and reaching a minimum in the late evening. Interestingly, the carbonylation of this protein also fluctuated such that oxidation was mainly observed during the night (Table I).

All of the proteins identified as carbonylated from the mitochondrial fraction (except for the *β*-subunit of the ATP synthase) play roles in the C\textsubscript{2} oxidative photosynthetic carbon cycle. Besides its carboxylase activity Rubisco has oxygenase activity, and therefore some ribulose-bisphosphate is converted to 2-phosphoglycolate, a compound that cannot be utilized by the Calvin cycle. 2-Phosphoglycolate is therefore salvaged by the C\textsubscript{2} oxidative photosynthetic carbon cycle. In this process, two molecules of glyoxylate are joined to form glycine. Glycine moves into the mitochondrion and becomes oxidatively decarboxylated by the glycine decarboxylase complex. As shown here, several components of this complex are modified by carbonylation. This is in line with the data of Taylor *et al.* (29) demonstrating that glycine decarboxylase activities in peas are exquisitely sensitive to oxidative stress.

The "stochastic" as opposed to "programmed" view of aging states that aging results from random deleterious events, and oxidative damage has been suggested to be a major contributor to such stochastic degeneration of cells and organisms. It has been demonstrated that the levels of carbonylated proteins increase with age in a large variety of species, including mammals, nematodes, and flies, and that such oxidative modification plays havoc with the catalytic activity and structural integrity of the target proteins (2, 3, 6, 7). The hypothesis is supported by experimental data demonstrating that the life span of fruitflies, for example, can be prolonged by overproducing antioxidants and that alleles that retard aging in yeast, flies, worms, and mice often boost the defense of the organism against oxidative damage (30, 31).

In plants, the concept of an individual, and therefore aging, is ambiguous. Nevertheless, the mechanisms that determine the life span of whole plants are obviously amenable to scientific examination and hypotheses aimed to explain plant longevity states that plant aging may be linked to resource allocation and competition between old and young tissues (e.g. Ref. 32). Specifically, it has been argued that older leaves are out-competed by young tissues for "anti-aging" hormones (33), such as cytokinins (34). A variation on this theme is that young tissues seek to satisfy their demand for resources by exporting "death hormones" to older cells/tissues (35), and ethylene exhibits some characteristics of such hypothetical senescence-promoting substances (36). These theories are, in part, analogous to some evolutionary models of senescence in animals, which propose that there is a trade-off, or antagonism, between reproduction activities and cellular maintenance and repair (37, 38). As an inevitable consequence, an optimal life history entails an imperfect ability of the soma to resist intrinsic stress, such as oxidative damage.

With the above-mentioned theories in mind, we argued that old tissues, *e.g.* leaves, of an annual plant might loose their ability to defend themselves against intrinsically generated oxidative damage during transition to reproductive development. The results obtained did not support this notion. Instead, we found that old leaves rid themselves of oxidized proteins prior to bolting and flower development. At first glance, this suggests that the progression of oxidative damage in the life cycle of animals and plants is fundamentally different. Indeed, it clearly demonstrates that increased protein oxidation is not universally and inevitably related to the age of a biological
tissue. However, one aspect of protein oxidation is similar in animals and plants, that is, production of offspring occurs at a time at which the overall oxidative damage in the organism is low. In animals this coincides with the early to middle stages of the life cycle of the organism, whereas in Arabidopsis thaliana it signifies the end point of its developmental progression. This begs the question of the physiological significance of the drastic drop in protein oxidation preceding flower development and to what extent this may affect the fitness of the offspring. In addition, it would be of interest to identify the temporal and molecular nature of the signals triggering the removal of damaged proteins and whether the elimination of such damage is a prerequisite for the transition to flowering.

Acknowledgments—We thank Professor Elzbieta Glaser for all the help concerning isolation of mitochondria and Åsa Fredriksson for the artwork.

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