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Cytokinin Inhibits the Proteasome-Mediated Degradation of Carbonylated Proteins in *Arabidopsis* Leaves

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Under normal conditions, plants contain numerous carbonylated proteins, which are thought to be indicative of oxidative stress damage. Conditions that promote formation of reactive oxygen species (ROS) enhance protein carbonylation, and protein degradation is required to reverse the damage. However, it is not clear how the degradation of carbonylated proteins is controlled in planta. In this report, we show that detached *Arabidopsis* leaves rapidly and selectively degrade carbonylated proteins when kept in the dark. The loss of carbonylated proteins corresponded to a loss of soluble protein and accumulation of free amino acids. Degradation of carbonylated proteins and the loss of soluble protein was blocked by MG132 but not 3-methyladenine, suggesting that the 26S proteasome pathway rather than the autophagic pathway was involved. Consistent with this, *rpn10* and *rpn12* mutants, which are defective in proteasome function, had increased (rather than decreased) levels of carbonylated proteins when detached in the dark. Feeding metabolites (amino acids and sucrose) to detached leaves of wild-type *Arabidopsis* in the dark had little or no effect on the loss of carbonylated proteins, whereas providing soybean xylem sap via the transpiration stream effectively prevented degradation. The effect of xylem sap was mimicked by feeding 10 μM kinetin. We postulate that disruption of cytokinin flux to detached leaves triggers the selective degradation of carbonylated proteins via the proteasome pathway. The results may have implications for the control of protein mobilization in response to changes in N availability.

Keywords: *Arabidopsis* — Autophagy — Cytokinin — Protein carbonylation — Protein degradation — Proteasomes.

Abbreviations: AEBSF, 4-(aminoethyl)benzenesulfonyl fluoride hydrochloride; BHT, butylated hydroxytoluene; CHX, cycloheximide; DNP, dinitrophenyl; DNPH, dinitrophenylhydrazone; DPTA, diethylenetriamine penta acetate; 3-MA, 3-methyladenine; MCLR, microcystin-LR; ROS, reactive oxygen species; RbcL, Rubisco large subunit.

Introduction

Reactive oxygen species (ROS) are produced continuously in chloroplasts, peroxisomes and mitochondria as by-products of respiration and photosynthesis, and can also be produced as the dedicated end-product of certain enzymes such as NADPH oxidase. The production of ROS is enhanced under abiotic stress, but even under non-stress conditions, plants contain a significant level of H_2O_2 (Mittler 2002, Cheeseman 2006). An emerging notion is that ROS, in particular H_2O_2, function not only as toxic by-products but also as signaling molecules providing information about the plant's environment and allowing adaptation to changes in conditions. In the presence of metals, even mild oxidants such as H_2O_2 can result in modification of amino acid side chains to form reactive carbonyls via the metal-catalyzed protein oxidation pathway (Adams et al. 2001). There are also non-oxidative pathways for production of reactive carbonyls in proteins, but protein carbonylation is generally recognized as a marker of oxidative stress and damage (Adams et al. 2001, Dalle-Donne et al. 2003, Ghezzi and Bonetto 2003). In plants, protein carbonylation is readily observed even under normal, non-stress conditions, indicating that some proteins are very susceptible to oxidative modification in vivo (Johansson et al. 2004, Kristensen et al. 2004, Job et al. 2005). The widely accepted assumption is that protein carbonylation leads to loss of function, and hence those proteins must ultimately be degraded, as there are no repair mechanisms known to replace damaged side chains.

The pathway involved in degradation of carbonylated proteins in vivo can potentially involve either the proteasome or autophagy pathway, and there are multiple distinct routes within each of these major pathways. For example, the proteasome pathway can be ATP and ubiquitin dependent (involving the 26S proteasome) or independent (involving the 20S proteasome) (Vierstra 1996). Likewise, the transfer of cytoplasmic components to the vacuole for degradation can involve either microautophagy or

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macroautophagy pathways (Thompson and Vierstra 2005). Which pathways participate in the degradation of carbonylated proteins in plants under different conditions is not entirely clear. That carbonylated proteins can be degraded in vivo was inferred from the developmental studies with *Arabidopsis*, where it was noted that protein carbonyl content decreased dramatically as plants began reproductive development (Johansson et al. 2004). Because total protein content remained constant, the removal of oxidatively modified proteins can be inferred. However, the pathway involved was not elucidated. In a more recent study, evidence for the removal of carbonylated proteins via the autophagy pathway was provided for *Arabidopsis* plants subjected to severe oxidative stress induced by exogenous H_2O_2 or methyl viologen (Xiong et al. 2007). However, the pathway of degradation under conditions of low oxidative stress (i.e. normal growth conditions) has not been explored, nor are there any suggestions as to the physiological mechanisms that control the degradation process.

During the course of our studies on protein carbonylation in *Arabidopsis* leaves, we initiated experiments with detached leaves to test the effect of feeding leaves with various exogenous compounds. An initial surprising result, which forms the basis of the present study, was that leaves selectively and rapidly lost protein carbonyl content when detached and kept in the dark for 6–12 h. We demonstrate that the proteasome is involved, and that a disruption of cytokinin flow to the leaf (when detached) may be the trigger. The results may have relevance to the control of protein turnover in intact plants in response to changes in N nutrition.

Results

Degradation of carbonylated proteins in detached leaves

The initial observation that served as the foundation of the present study is shown in Fig. 1A. When expanded leaves of 5-week-old *Arabidopsis* plants were detached and incubated in the light or dark (with cut petioles in water), we observed that the loss of protein carbonyl content was much greater in the dark than in the light. In the experiment presented in Fig. 1, equal amounts of soluble protein (10 μg) were loaded in each lane and, as shown, about 20% of the protein carbonyl content was lost after 12 h of incubation of leaves in the light, whereas about 80% was lost in the dark-incubated leaves. Interestingly, carbonylated proteins were not lost in a similar manner from attached leaves (i.e. intact plants) that were darkened even for extended periods (up to 4 d; Fig. 1B). These results suggested that carbonylated proteins can be rapidly and selectively degraded under some conditions, and are consistent with the notion that something provided from the root may control the metabolism of carbonylated proteins in leaves. Subsequent experiments were designed to determine the basis for the loss of carbonylated proteins and the nature of the signal that may be transmitted from the root.

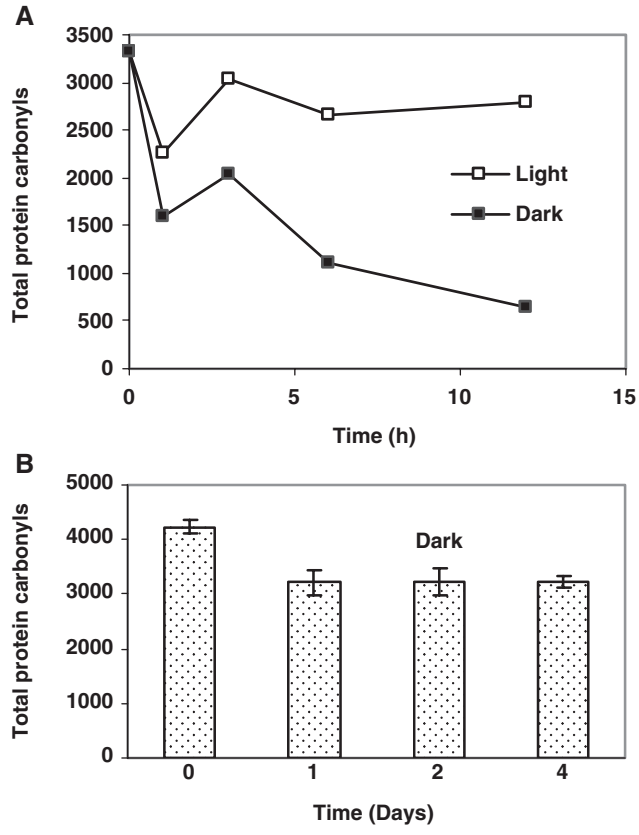

Fig. 1 Loss of *Arabidopsis* leaf protein carbonyl content in (A) detached and (B) attached leaves. In (A), the cut petioles of leaves were placed in water and incubated in the light (same intensity as for growth, 70 μmol m^{-2} s^{-1}) or dark for up to 12 h. In (B), leaves were harvested at daily intervals for plants that were in the dark. In these and subsequent experiments, the oldest leaves from 5-week-old plants were used. Leaf soluble protein was extracted, derivatized with DNPH, and proteins (10 μg) were resolved by SDS–PAGE. Following electrophoretic transfer to PVDF, the membranes were probed with anti-DNP antibodies. Immunoblot signals were quantitated by densitometry for each entire gel lane to give the total protein carbonyl content for 10 μg of protein, and are expressed as relative units.

Role of proteasomes

We wanted to determine whether the loss of protein carbonyls in darkened, detached leaves was indeed the result of protein degradation and, if so, which proteolytic system was involved. As one approach to address these issues, we provided detached leaves (via the transpiration stream) with 3-methyladenine (3-MA), an inhibitor of autophagy in mammalian (Seglen and Gordon 1982) and plant (Takatsuka et al. 2004) systems, or MG132, a well recognized proteasome inhibitor (Vierstra 2003). Shown in

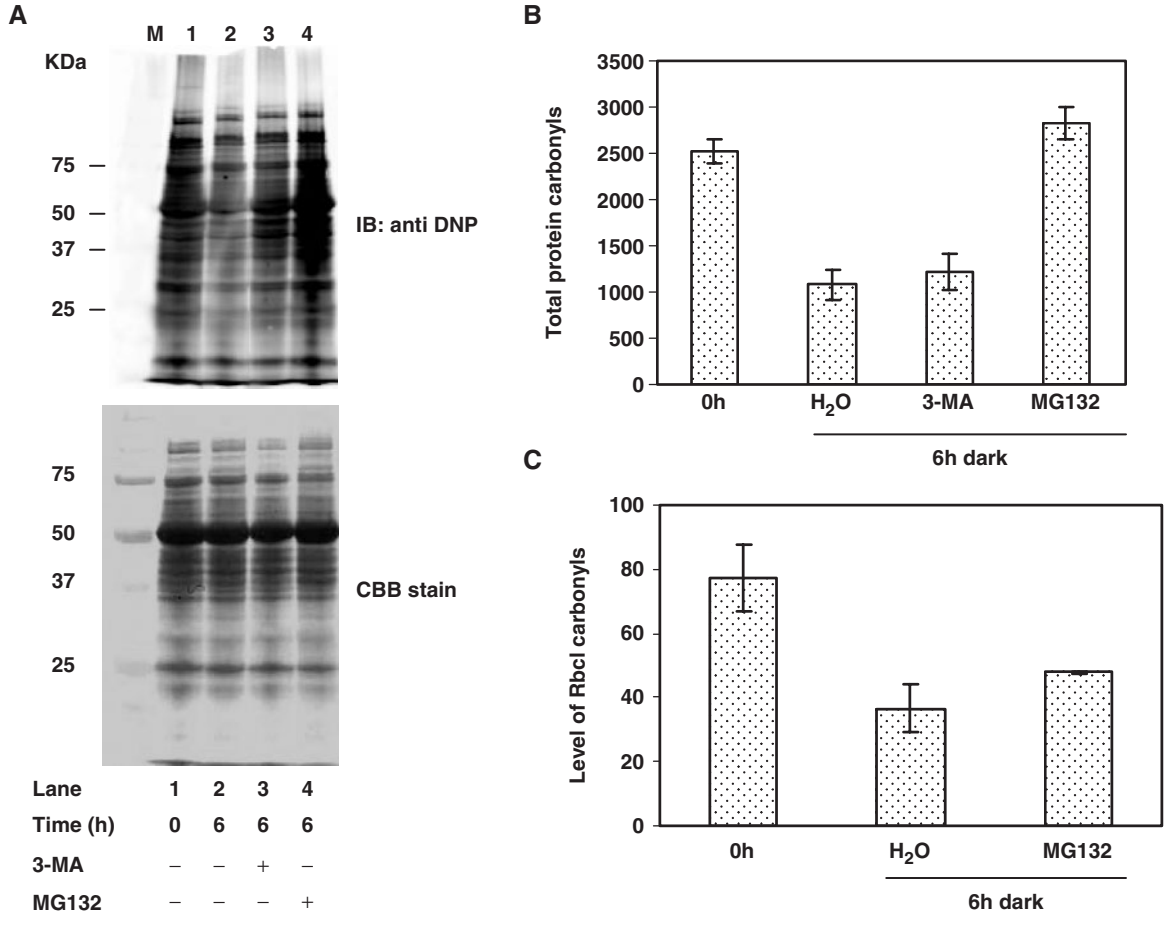

Fig. 2 The proteasome inhibitor, MG132, prevents the loss of protein carbonyl content in dark detached *Arabidopsis* leaves. (A) Representative Coomassie Brilliant Blue-stained gel and immunoblot analysis with anti-DNP antibodies of soluble proteins derivatized with DNPH. Time in the dark and application of 3-MA (1 mM) and MG132 (50 μM) were as indicated. Note that in the immunoblot, the molecular weight standard proteins were not detected by the anti-DNP antibodies but their positions are indicated by hatch marks. (B) Quantitation of the immunoblot results in (A) by densitometry using the Li-Cor Odyssey for each entire gel lane. (C) Quantitation of the immunoblot band corresponding to the ~50 kDa RbcL protein. In (B) and (C), values are means ± SEs ($n = 4$).

Fig. 2A are anti-DNP (dinitrophenyl) immunoblots of proteins extracted from detached leaves in the dark for 6 h provided with water (control), 3-MA or MG132. It is visually apparent that 3-MA had little effect on the loss of protein carbonyl content, whereas MG132 effectively prevented the loss. It is also apparent from the immunoblots that reactivity of the leaf proteins with the anti-DNP antibodies was specific, as the underivatized molecular weight standard proteins were not detected by the antibodies and the immunoblot pattern did not simply reflect protein abundance. Numerous proteins were carbonylated, in particular the prominent ~50 kDa Rubisco large subunit (RbcL) protein, which has also been observed in previous studies (Johansson et al. 2004, Xiong et al. 2007). We used densitometry to quantitate the carbonylated proteins in the entire gel lanes for each sample, and results from several experiments are presented in Fig. 2B. The densitometry analysis confirmed that 3-MA had little effect on the loss of protein carbonyl content, while the presence of MG132 not only prevented the decrease but resulted in a net increase of protein carbonyl content of about 10% over the 6 h period. Clearly, the proteasome is the major pathway affecting the loss of protein carbonyl content in dark, detached leaves. However, because both the 20S and 26S proteasomes are inhibited by MG132 (Braun et al. 2005), we cannot distinguish which components of the proteasome-dependent proteolytic system are involved. As mentioned above, one of the prominent carbonylated proteins in *Arabidopsis* leaf extracts was RbcL and, interestingly, the loss of RbcL carbonyl content was not appreciably inhibited by MG132 (Fig. 2C). Collectively, these results indicate that loss of protein carbonyl content involved protein degradation and suggested that different enzymes and pathways may

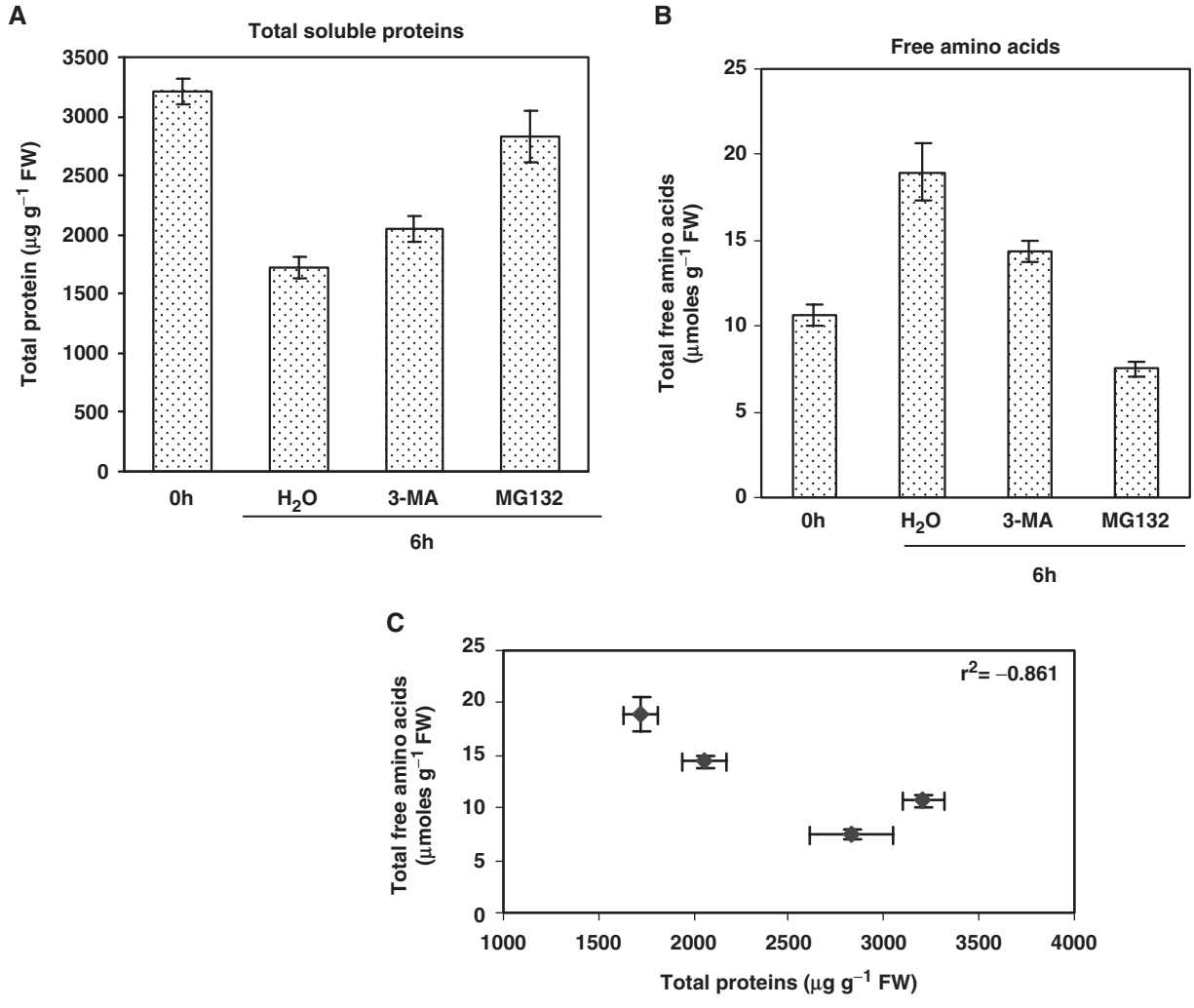

Fig. 3 Loss of soluble protein and accumulation of free amino acids in dark detached leaves of *Arabidopsis*. Detached leaves were incubated in water (control) containing 1 mM 3-MA or 50 μM MG132, as indicated, for 6 h in the dark prior to measurement of (A) soluble protein and (B) free amino acids (sum of the 17 most abundant amino acids). (C) Negative correlation between leaf content of free amino acids and total soluble protein. Values are means ± SEs ($n = 3$).

be involved depending on the cellular compartment in which the proteins reside.

In a related series of experiments, we wanted to determine whether increased protein carbonyl content in leaves would affect the extent of carbonyl loss when leaves were detached in the dark. To test this idea, intact plants were kept in normal light (70 μmol m^{-2} s^{-1}) or exposed to high light (250 μmol m^{-2} s^{-1}) for 3 h prior to detachment of leaves in the dark. The brief high light treatment increased protein carbonyl content about 25%, and the increased protein carbonyl content was completely lost in an MG132-sensitive manner over the next 6 h when leaves were detached in the dark (data not shown). Thus, increased protein carbonylation resulted in greater protein degradation following leaf detachment. These results are consistent with the notion that carbonylated proteins are selectively degraded in darkened, detached leaves.

Concurrent with the loss of protein carbonyl content in darkened detached leaves was a loss of soluble protein. The presence of 3-MA caused a partial inhibition of protein loss, while MG132 had a much larger effect, but did not completely prevent the loss of protein (Fig. 3A). The effects of 3-MA and MG132 on soluble protein loss basically paralleled the changes in protein carbonyl content, confirming that the mechanism involved protein degradation. Also consistent with this notion was the observation that loss of protein carbonyl content was associated with increased levels of free amino acids. In the darkened detached leaves, the total pool of free amino acids roughly doubled during the 6 h treatment in water, and this increase

Table 1 Changes in free amino acids in detached *Arabidopsis* leaves incubated in the dark for 6 h in the presence or absence of 3-MA or MG132

Amino acid	Amino acid content (nmol g^{-1} FW)			
	0 h	Dark 6 h		
		Control	3-MA	MG 132
Glu	3772 ± 265.7	**5,188** ± 698.3	4,359 ± 466.7	**1,117** ± 121.2
Gln	1880 ± 160.8	1,962 ± 233.2	**1,124** ± 154.3	**723** ± 59.5
Asp	1684 ± 102.6	**5,072** ± 973.1	**3,559** ± 597.8	2,092 ± 634.6
Ala	829 ± 66.9	**1,640** ± 208.1	1,326 ± 323.5	722 ± 66.0
Ser	738 ± 67.2	**984** ± 159.6	**948** ± 102.5	**430** ± 106.1
Thr	670 ± 44.9	**1,119** ± 169.2	740 ± 59.8	751 ± 41.6
Gly	452 ± 166.6	398 ± 215.9	522 ± 39.2	661 ± 73.9
Asn	429 ± 56.0	**1,120** ± 256.1	**1,041** ± 113.3	791 ± 82.7
Val	170 ± 28.2	**485** ± 104.5	209 ± 36.2	167 ± 27.7
Lys	19 ± 2.5	**230** ± 40.4	**62** ± 10.8	18 ± 4.5
Phe	17 ± 2.4	**127** ± 35.8	**101** ± 20.8	ND
Arg	11.0 ± 2.0	18 ± 6.6	15 ± 0.9	ND
Tyr	7.6 ± 3.0	**112** ± 35.9	**65** ± 6.1	8 ± 0.4
Ile	ND	**205** ± 36.8	**134** ± 6.4	8 ± 0.9
Leu	ND	**245** ± 56.7	**158** ± 62.4	12 ± 2.0
His	ND	**20** ± 11.9	**29** ± 5.3	ND
NH_3	569 ± 66.4	**718** ± 38.2	583 ± 14.9	490 ± 89.2

ND, not detected.
Mean values (±SE) from three replicates are shown. Bold values indicate statistically significant differences from the time zero sample ($P < 0.01$).

was somewhat reduced by 3-MA but completely inhibited by MG132 (Fig. 3B). Total leaf free amino acid content was correlated negatively with leaf soluble protein content (Fig. 3C), consistent with the notion that free amino acids were formed from the breakdown of protein as opposed to de novo synthesis. Table 1 details changes in specific amino acids in darkened leaves in the presence and absence of inhibitors. Two amino acids, aspartate and glutamate, accounted for >60% of the total amino acid pool at time zero, and both increased substantially during the 6 h treatment in water. Indeed, most amino acids increased in content during the treatment, but the relative increase was greatest for some of the minor amino acids such as the hydrophobic (isoleucine, leucine and methionine), aromatic (tyrosine and phenylalanine) and basic (lysine and histidine) amino acids. In the case of lysine, which is synthesized through the aspartate family biosynthesis pathway, the free pool is normally maintained at low concentrations by strong feedback inhibition mechanisms (Galili 1995). The fact that lysine and the other minor amino acids increased dramatically in detached leaves would be consistent with their production by protein degradation rather than biosynthesis. In the presence of 3-MA, there was substantial accumulation of free amino acids as in the control; however, overall the increases were somewhat reduced (Table 1). In the presence of MG132, none of the amino acids was increased relative to the time zero control. For the three amino acids that showed a statistically significant difference compared with time zero, all three decreased rather than increased in concentration (Table 1). Continued catabolism or interconversion of serine, glutamate and glutamine may be responsible for the observed decrease in content.

To test further the pathway involved in loss of protein carbonyl content, we examined plants containing mutations in the proteasome, *rpn10* (Smalle et al. 2003) and *rpn12a-1* (Smalle et al. 2002), or the autophagy pathway, *atg7-1* (Doelling et al. 2002) and *atg5-1* (Thompson et al. 2005). Expanded leaves of the wild type (Col-0) and mutants were detached and incubated in water in the dark for 6 h, and changes in protein carbonyl content were monitored. Interestingly, leaves of the wild-type plant and also of both of the autophagy mutants lost 50–60% of the carbonylated proteins present at time zero, whereas both of the proteasome mutants actually increased in carbonyl content during the 6 h period (Fig. 4B). These results are consistent with the notion that the proteasome pathway is the major mechanism for turnover of carbonylated proteins in darkened detached leaves. Secondly, the results also suggest that 26S proteasomes, rather than 20S proteasomes, are involved in the process, because RPN10 and RPN12 are both components of the 19S regulatory particle (lid and base) and not the 20S core particle (Smalle et al. 2002,

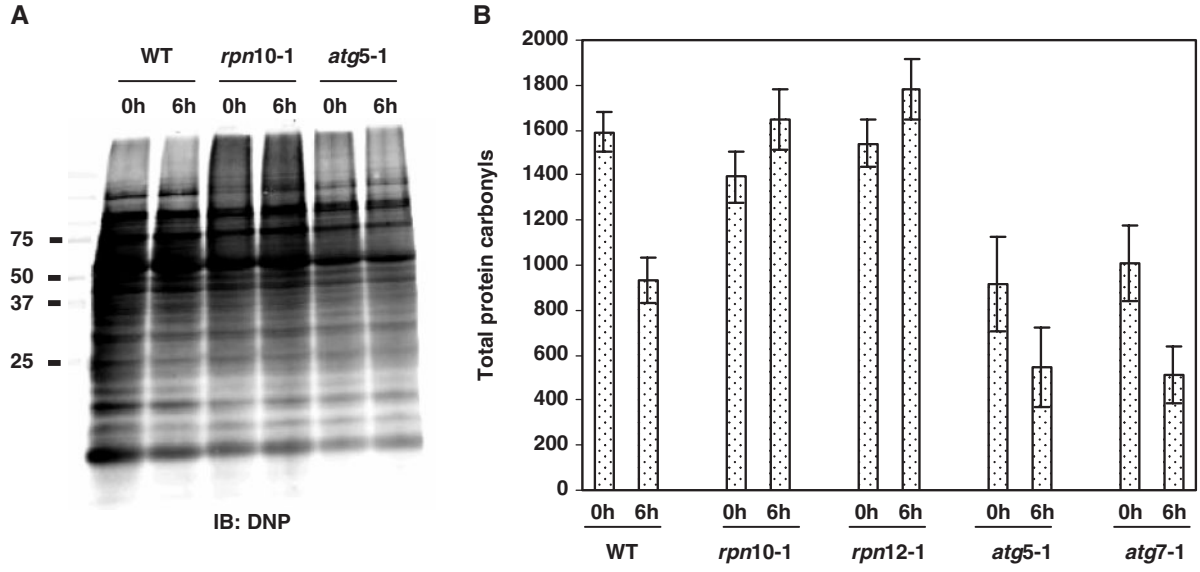

Fig. 4 Degradation of carbonylated proteins in mutants impaired in the proteasome or autophagy pathways of protein degradation. (A) Representative immunoblot showing carbonylated proteins in leaves of wild type (WT) and the *rpn10-1* and *atg5-1* mutants before and after detachment and incubation in the dark for 6 h. The positions of molecular weight standard proteins, which did not strongly cross-react with the anti-DNP antibodies, are indicated with hatch marks. (B) Quantitation of immunoblots similar to those in (A) for wild type and various mutants impaired in the proteasome (*rpn10-1* and *rpn12-1*) or autophagy (*atg5-1* and *atg7-1*) pathways. Values are means ± SEs ($n = 4$).

Smalle et al. 2003). In the experiment presented in Fig. 4, the wild type and mutants are in the Col-0 background, with the exception of *atg7-1*, which is in the WS ecotype. We verified in other experiments (data not shown) that wild-type plants in the WS ecotype behaved similarly to the Col-0 plants shown in Fig. 4. It is interesting to note that the steady-state level of carbonylated proteins in leaves differed among the genotypes tested. Both autophagy mutants had significantly lower levels of protein carbonyls compared with the wild type and 26S proteasome mutants (compare time-zero samples in Fig. 4B). The basis for this difference is not apparent at the present time, but will be interesting to explore in the future.

Kinetin prevents loss of carbonylated proteins

The observation that detachment of leaves was required for the rapid loss of carbonylated proteins (Fig. 1) suggested that some factor provided by the roots to the leaves via the transpiration stream may inhibit the degradation of oxidized proteins. Prior to testing possible factors such as metabolites, ions and hormones, an initial experiment was conducted to determine whether any cytoplasmic protein synthesis was required to prime or initiate the observed protein degradation following leaf detachment. This experiment was conducted because some genes, such as *CEVI-1*, which encodes an anionic peroxidase, are specifically induced in detached leaves independent of the wound response (Mayda et al. 2000).

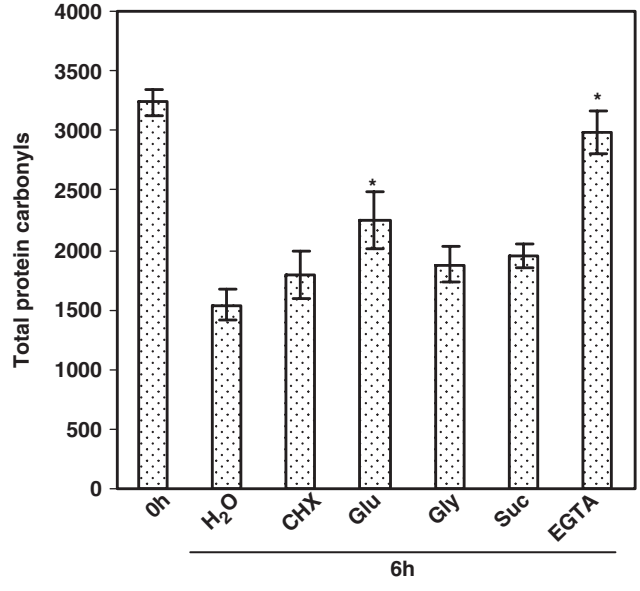

Fig. 5 Effect of exogenous metabolites and compounds on the loss of protein carbonyl content in detached leaves of wild-type *Arabidopsis* incubated in the dark for 6 h. Values are means ± SEs ($n = 4$) ($^*P < 0.001$).

However, as shown in Fig. 5, feeding leaves with cycloheximide (CHX) did not prevent the loss of protein carbonyl content, indicating that transcription and translation were not required for the response. Moreover, we also confirmed that detachment of leaves did not alter

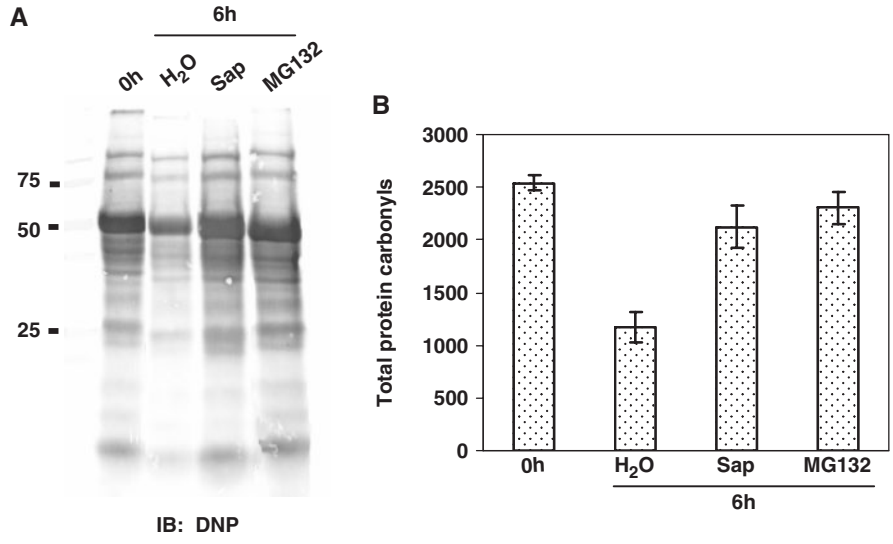

Fig. 6 Soybean xylem sap prevents the loss of protein carbonyls in dark detached *Arabidopsis* leaves. Detached leaves were provided with water (control), 50 μM MG132 or soybean xylem sap (collected from vegetative soybean plants growing in the field) as indicated via the transpiration stream. (A) Representative immunoblot showing carbonylated proteins before and after incubation of leaves in the dark for 6 h. The positions of molecular weight standard proteins, which did not strongly cross-react with the anti-DNP antibodies, are indicated with hatch marks. (B) Quantitation of immunoblots similar to those in (A); values are means ± SEs ($n = 4$).

extractable 26S proteasome activity (data not shown) using the assay described previously (Hardin et al. 2003). Hence, we focused subsequent experiments on possible metabolites such as amino acids (glycine or glutamate) and sugars (sucrose) that might be delivered to leaves via the transpiration stream in intact plants. Providing leaves with glycine or sucrose (Fig. 5) had no effect on the loss of protein carbonyl content, although there was a partial inhibition caused by glutamate. The treatment that had the greatest effect was feeding EGTA, which suggests that influx of apoplastic Ca^{2+} may be required for the response, although we cannot rule out inhibition of Ca^{2+}-dependent processes in the apoplast. Because individual metabolites had relatively little effect, we next considered whether hormones might be involved, and tested xylem sap obtained from field-grown soybeans. As shown in Fig. 6, feeding xylem sap almost completely inhibited the loss of protein carbonyls and was as effective as treatment with MG132. Xylem sap contains a mixture of ions, metabolites and hormones that includes the cytokinins, which are prominent hormones produced in roots and transported to leaves in an N-dependent manner (Takei et al. 2001). Hence, we tested the effect of kinetin, a synthetic cytokinin, on the loss of protein carbonyl content. As shown in Fig. 7, 10 μM kinetin completely prevented the loss of protein carbonyl content (Fig. 7A) and also prevented the loss of soluble protein (Fig. 7B). These results collectively suggest that disruption of the flux of cytokinin in detached leaves may be the signal that triggers the selective degradation of carbonylated proteins in detached leaves.

Discussion

It is well documented that under normal conditions, plants contain numerous proteins that are carbonylated, presumably as a result of oxidative damage (Johansson et al. 2004, Xiong et al. 2007). It is generally assumed that carbonylation results in loss of function and that oxidatively damaged protein must be degraded. In animal systems, carbonylated proteins can be degraded by the 20S proteasome (Grune et al. 2003) or by chaperone-mediated autophagy (Kiffin et al. 2004). In plants, developmental regulation of carbonylated protein degradation was inferred because there was a dramatic reduction in leaf protein carbonyl content when *Arabidopsis* plants began reproductive development (Johansson et al. 2004); because the soluble protein content remained constant, degradation of oxidized proteins followed by resynthesis was postulated but the pathway of degradation was not elucidated. However, two plant studies have examined the pathway involved in degradation of oxidatively modified proteins. In maize, Basset et al. (2002) reported that dark treatment of maize plants for 24 h stimulated 20S proteasome activity possibly as a result of oxidative modification of the proteasome itself, and the authors suggested that carbonylated 20S proteasomes may function to degrade oxidatively modified proteins during carbon starvation. More recently, the pathway of carbonylated protein degradation was carefully explored in *Arabidopsis* under severe oxidative stress imposed by application of H_2O_2 or methyl viologen (Xiong et al. 2007). Under these conditions, oxidized

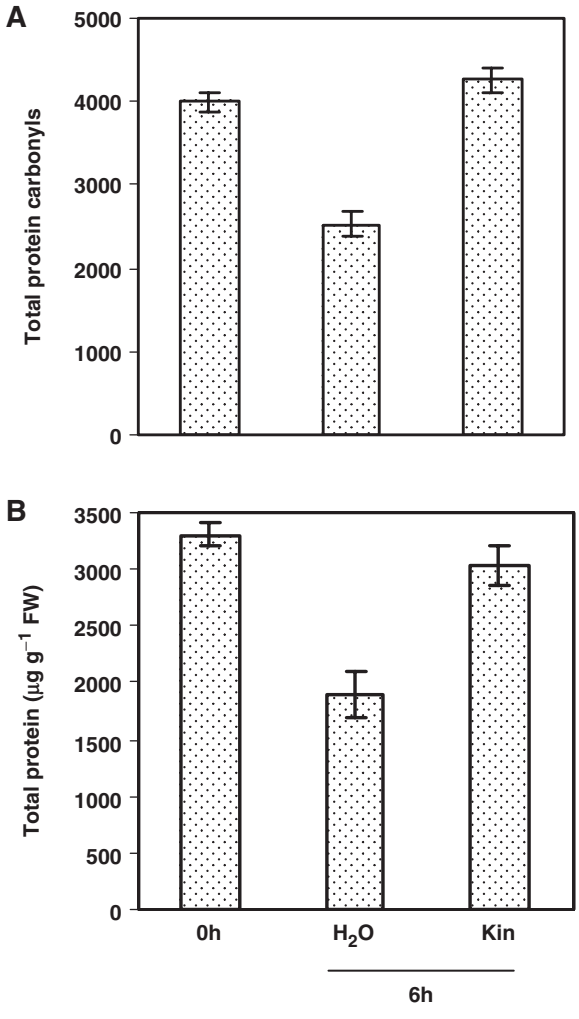

Fig. 7 Kinetin (10 μM) mimics soybean xylem sap and prevents the loss of (A) protein carbonyls and (B) soluble protein in dark detached *Arabidopsis* leaves. Values are means ± SEs ($n = 4$).

proteins were degraded by the autophagy pathway. During normal senescence, plants also use autophagy pathways to degrade the organellar and cytoplasmic proteins indiscriminately (Doelling et al. 2002). In the present study, involving proteins oxidatively modified during normal growth, we found that the 26S proteasome was involved rather than the autophagy pathway. Thus, it appears that different pathways may be involved under different conditions and in response to varying levels of protein oxidation.

Our results also suggest that the trigger for degradation of carbonylated proteins in darkened detached leaves may be disrupted cytokinin flux to the leaf. This is based primarily on the observation that exogenous kinetin (or soybean xylem sap) almost completely prevented the loss of protein carbonyl content as well as the loss of soluble protein (Fig. 6). When leaves are attached, xylem flux of hormones (and other constituents) is maintained and protein carbonyl content remains high and stable even for extended dark periods (up to 4 d; Fig. 1B). This could explain why leaf detachment was required for rapid degradation of carbonylated proteins, but it is not immediately clear why light prevents the degradation of carbonylated proteins in detached leaves (Fig. 1A). It is possible that light influences the metabolism of endogenous cytokinins (such that a deficiency does not occur within 6–12 h), or that light signals somehow attenuate the impact of restricted cytokinin flux to the leaf. Darkened leaves also experience carbon starvation (Brouquisse et al. 1998), which could be another important factor; however, sucrose feeding to darkened, detached leaves had no effect (Fig. 5) despite the fact that sucrose is readily taken up into darkened detached leaves (Fujiki et al. 2001).

Another unresolved but important question is how carbonylated proteins are selectively targeted for degradation by the 26S proteasome. Whatever mechanism is involved apparently does not depend on de novo protein synthesis as CHX feeding had no effect on the loss of protein carbonyl content (Fig. 5). It is possible that existing targeting mechanisms become activated, perhaps by post-translational modification. Clearly much remains to be done to elucidate details of the mechanism, but it is apparent that carbonylated proteins can be selectively and rapidly degraded under certain conditions.

While the conditions tested in the present study are completely artificial (detached leaves maintained in darkness for 6–12 h), the apparent linkage identified between cytokinins and carbonylated protein degradation may have relevance to protein homeostasis in responses to changes in N nutrition. It is well known that N availability is a major factor controlling cytokinin biosynthesis in roots for transport to shoots (Takei et al. 2001, Takei et al. 2004, Sakakibara et al. 2006). Thus, cytokinins, along with organic and inorganic forms of N, are signals to the shoot regarding soil N status. One response to a decrease in N availability is increased protein mobilization from old leaves to sustain life and support new growth such as root elongation to explore new soil for resources. Indeed, careful studies of wheat leaf senescence in response to a rapid removal of nitrate identified a rapid decrease in the cytokinin, isopentenyl adenosine, and suggested that this was the trigger to induce protein degradation in response to N deficiency (Criado et al. 2007). Our results suggest that carbonylated proteins may be specifically targeted for degradation under these conditions. How this selectivity is achieved, and the mechanism(s) by which cytokinins control this process, are important questions for future study. However, while many questions remain, our results add a new dimension to the previously recognized link between the 26S proteasome and cytokinin responses (Smalle et al. 2002).

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana plants (Col-0) were grown in sterile soil in growth chambers (light intensity of 70 μmol m^{-2} s^{-1}, day/night temperature of 22°C/18°C and relative humidity of 60%/80%) with a short day photoperiod (10 h). The wild-type plants were fertilized biweekly with Hoagland solution. The proteasome mutants, *rpn 10-1* and *rpn12-1* (Smalle et al. 2002, Smalle et al. 2003), and autophagy mutants, *atg5-1* and *atg7-1* (Thompson et al. 2005), were grown under similar conditions. The proteasomal and autophagy mutants were grown on nutrient solid half-strength MS medium for 7 d at 22°C under long day conditions. *Arabidopsis* seeds were surface sterilized in 100% ethanol (10 min), 30% sodium hypochlorite and 0.1% Tween-20 for 20 min followed by 3–4 rinses with sterile water and followed by vernalization for 2 d. The 7-day-old seedlings were transplanted from the half-strength MS medium to the soil and fertilized with quarter-strength Hoagland solution regularly.

Detached leaf treatments

The detached leaves from the 5-week-old *Arabidopsis* plants (oldest leaves of the same age only) were detached 3 h after the start of the photoperiod and then exposed to light or dark as indicated. Leaves were cut under degassed water and incubated in degassed water alone (control) or containing 3-MA (1 mM), MG132 (50 μM), CHX (50 μM), glutamate (10 mM), glycine (10 mM), sucrose (10 mM), EGTA (0.5 mM) or kinetin (10 μM) as indicated in the text. In the experiment presented in Fig. 6, cut leaves were put into undiluted xylem sap collected from *Glycine max*.

Collection of xylem sap

Well-watered field-grown soybean plants were cut 10–15 mm above the root/shoot junction, starting at 10:30 [central standard time (CST)]. The cut stem was gently washed with distilled water, the stump was carefully dried with Kimwipe™ tissues, and the sap extruded from the stem over the next 15 min was collected with the help of micropipets. Sap was collected daily from several plants over the course of 1 week.

Extraction of the total proteins and their derivitization with DNPH

Powdered leaf tissue was ground in a chilled mortar (0.5 g FW ml^{-1}) in protein extraction buffer (PEB) containing 100 mM Tris–HCl, pH 8.0, 2% (v/v) β-mercaptoethanol, 5 mM EGTA, 10 mM EDTA, 1 mM 4-(aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μg ml^{-1} leupeptin, 1 mM *p*-amino-benzamidine, 5 mM 6-aminohexanoic acid, 2 μM E64, 10 μM MG132, 0.2 μM microcystin-LR (MCLR), 10 mM NaF, 1 mM diethylenetriamine penta acetate (DPTA) and 1 mM butylated hydroxytoluene (BHT). The leaf soluble protein extracts were mixed with 2 vols. of 10 mM dinitrophenylhydrazone (DNPH) in 2 M HCl at room temperature for 30 min with gentle agitation. A control sample was mixed with 2 vols. of 2 M HCl. Five volumes of ice-cold phenol (Tris-buffered, pH 7.9) was added to each tube. After vortexing for 1 min, the mixture was centrifuged for 10 min at 10,000×*g*. The upper phase was removed and discarded, leaving the interface intact, and the phenol phase was re-extracted twice with ice-cold Tris–HCl buffer (50 mM, pH 8.0). Five volumes of cold 0.1 M ammonium acetate in methanol were added to the lower phase and incubated at –20°C overnight. The following day, the mixture was centrifuged at 10,000×*g* for 20 min, and the resulting pellets were washed three times with 1 ml of 0.1 M ammonium acetate in methanol and once with 1 ml of cold ethanol. The pellets were resuspended in sample buffer and subjected to SDS–PAGE. Resolved proteins were electrophoretically transferred to Immobilon-P (PVDF, Millipore) membranes, and oxidized proteins were detected using anti-DNP antibodies (DakoCytomation, Denmark).

Amino acid measurement

Frozen leaf tissue was sequentially extracted with 80% ethanol, 50% ethanol and finally distilled water. Debris was pelleted by centrifugation and the supernatant taken to dryness under vacuum. Amino acids were measured with an automated amino acid analyzer (Biochrom 20 plus; Biochrome Ltd, Cambridge, UK).

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