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Estimation of ubiquitin and ubiquitin mRNA content in dark senescing wheat leaves

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Abstract

Wheat leaves (*Triticum aestivum* L. cv. San Agustin INTA) were detached at the moment they had reached maximum expansion, put in tubes containing water and left in darkness. Under these conditions, leaf protein content decreased mainly as consequence of an increased rate of breakdown. In the range of 0 to 72 h after detachment, western blot analysis of leaf protein extracts displayed both similar proportions of total protein and quality of ubiquitin conjugates. Northern blot analysis of leaf RNA extracts revealed a 1.6 kb ubiquitin mRNA transcript which increased 3.5-fold after 48 h of treatment. Thus wheat leaves maintain both their ability for the ubiquitination of proteins and the transcription of ubiquitin mRNA at stages of senescence in which rates of protein breakdown are increased. The results suggest that the ubiquitin-dependent proteolytic pathway contributes to leaf protein breakdown during senescence.

Additional key words: *in vitro* senescence, protein turnover, *Triticum aestivum* L.

Introduction

Studies on leaf senescence are often performed by incubating detached leaves in the dark (Thimann 1980). The most characterized changes observed during leaf senescence in either whole plants or detached leaves are loss of chlorophyll, nucleic acids and proteins (Wollgiehn 1967). During dark induced senescence, loss of both leaf rRNA and total protein are mainly due to increased rates of breakdown

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Abbreviations: IgG - immunoglobulin G; SSPE - sodium chloride-sodium phosphate-EDTA; TBST - Tris-buffered saline-Tween.

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(Lamattina *et al.* 1985, 1988). To date, the proteolytic pathways involved in the breakdown of leaf proteins during senescence are not generally known. Ubiquitin (a 76 amino acids peptide) plays a pivotal role in an ATP-dependent proteolytic pathway which degrades abnormal and short-lived proteins (Ciechanover *et al.* 1978, Finley and Chau 1991, Hershko and Ciechanover 1992). Ubiquitin is encoded by a multigene family of two classes of natural gene fusions. The first class consists of a single ubiquitin-coding unit. The second class consists of ubiquitin repeats fused together in a contiguous head to tail fashion (Ozkaynac *et al.* 1987, Pieterse *et al.* 1991).

Although investigations on the role of the ubiquitin-dependent proteolytic pathway in plant protein turnover have been conducted (Callis and Vierstra 1989, Ziegenhagen and Jennissen 1990, Ferguson *et al.* 1990, Beers *et al.* 1992, Gindin and Borochov 1992, Schulz *et al.* 1993a,b), little is known about its participation in protein metabolism of leaves during dark-induced senescence. In the present report, we used polyclonal antibodies against ubiquitin and Northern blot analysis with a ^{32}P-labeled ubiquitin DNA probe to estimate both the amount and characteristics of ubiquitin-conjugates and ubiquitin-mRNA transcripts in senescing wheat leaves. This has led us to suggest that the ubiquitin proteolytic pathway is involved in protein metabolism at advanced stages of dark-induced leaf senescence.

Materials and methods

Plant material: Wheat (*Triticum aestivum* L. cv. San Agustin, INTA) was generously supplied by O. Bariffi from the Instituto Nacional de Tecnologia Agropecuaria, Balcarce, Argentina. Plants were grown in a chamber with 14:10 light-dark cycle at 25 °C and an irradiance of 250 μmol(PAR) m^{-2} s^{-1}. The substrate was vermiculite soaked in Hoagland's nutrient solution. Thirteen days after sowing, fully expanded first leaves were detached and processed (zero time) or kept in darkness at 25 °C in tubes containing distilled water for 24 to 72 h.

Gel electrophoresis and immunoblotting: Leaf proteins were extracted as described by Ferguson *et al.* (1990) and the extracts were stored at -70 °C. Protein separation was carried out by SDS-PAGEs in 12 % gels (Laemmli 1970). Protein extracts were run simultaneously with high and low prestained molecular mass markers. Gels were stained with *Coomassie Brilliant Blue R-250* 0.2 % (m/v). Immunoblot analyses were performed as described by Turner (1986) with the modifications introduced by Swerdlow *et al.* (1986). Bovine ubiquitin, rabbit antibovine ubiquitin serum (U-5379) and alkaline phosphatase conjugated goat antirabbit IgG-whole molecule antibodies were from *Sigma* (St. Louis, USA).

Extraction and analysis of total RNA: Total RNA from leaves obtained after 0, 24, 48 and 72 h of treatment was extracted as described by Greenberg and Bender (1991). RNA was analyzed by electrophoresis on formaldehyde-agarose gel and blotted on to nylon filters (*Hybond-N 0.45*, Amersham, U.K.). Ubiquitin (Binet *et al.* 1991) and rDNA probes (Choumane and Heizmann 1988) were labelled with a random primer

labelling kit (*Dupont*, Wilmington, USA) according to the instructions supplied by the manufacturers using [^{32}P] dCTP. The ubiquitin probe was a friendly gift of Dr. Luc Henri Tessier (Institut de Biologie Moleculaire des Plantes, Strasbourg, France). It is cloned in a Bluescript vector and contains a *Bam* H I fragment of 450 bp from the coding region of the sunflower ubiquitin gene. Probes were hybridized to filters at 42 °C in 50 % formamide, 5 × SSPE, 5 × Denhardt's, 0.5 % SDS for 20 h. Stringency washes were performed at 65 °C in 0.1 × SSPE and 0.1 % SDS for 60 min. Densitometric scanning of autoradiograms was performed using a *Shimadzu Dual-Wavelength Scanner CS-910* (*Shimadzu Corporation*, Japan).

Protein determination: After elimination of mercaptoethanol (Ferguson *et al.* 1990), the protein content of samples was measured by the micro-biuret method with bovine serum albumin as standard (Goa 1953).

Results and discussion

Occurrence of ubiquitin-protein conjugates in senescing wheat leaves: The extraction of leaf proteins for analysis of free ubiquitin and ubiquitin-protein conjugates was performed by a procedure designed to allow both the prevention of proteolysis (Vierstra *et al.* 1985, Rose 1988) and adequate solubilization (Ferguson *et al.* 1990). The SDS-PAGE protein analysis of extracts obtained 0, 24, 48 and 72 h after detachment did not show significant changes (Fig. 1*A*). The western blot analysis of these extracts with anti-ubiquitin polyclonal antibodies revealed some ubiquitin-protein conjugates which remained near the origin and well discernible bands in the 73, 64, 53, 43, 25 and 14 kDa ranges (Fig. 1*B*). The 64, 25 and 14 kDa conjugates (indicated by *arrows* at the right of the frame) exhibited a strong immunoresponse. Neither the size nor the amount of conjugates related to total protein changed significantly throughout the 72 h after detachment. In spite of the use of an antiserum with specificity for both free and conjugated ubiquitin, the free polypeptide was practically not detected (Fig. 1*B*). Since the extracts were obtained in conditions in which proteolysis was prevented, this result could not be attributed to the incapacity of polyclonal antibodies for the recognition of partially digested free ubiquitin (Vierstra *et al.* 1985); it probably reflects a low ratio between free and conjugated forms as has been reported by Gindin and Borochov (1992).

Control experiments did not detect unspecific reactions, either when the transblotted membranes were probed with the pre-immune serum or when 200 µg of BSA were probed with the specific antiserum to ubiquitin (data not shown). Because the antibodies used were raised against bovine ubiquitin, it is unlikely that they would recognize plant proteins other than those containing the ubiquitin sequence. Furthermore, the distribution of ubiquitin-immunoreactive proteins in the leaf extracts did not coincide with major protein detected by staining with *Coomassie Blue*. On the other hand, is improbable that the observed ubiquitin-protein conjugates were those produced before detachment. In support of this are the high rate of protein breakdown displayed by leaves (Lamattina *et al.* 1985) and the rapid turnover of

these conjugates (Hershko and Ciechanover 1992, Vierstra 1993). Hence, data in Fig. 1 provide evidence that senescent leaves produce ubiquitin-protein conjugates. Preliminary work on protein extracts obtained by procedures different from that of Ferguson *et al.* (1990) displayed blots consistent with those depicted in Fig. 1*B*. Moreover, either by dot blotting or radioimmuno-assay, an average ubiquitin content of 0.90 μg g^{-1}(fresh mass) was found (data not shown). This value is in the range of those reported for other tissues (Wilkinson 1988).

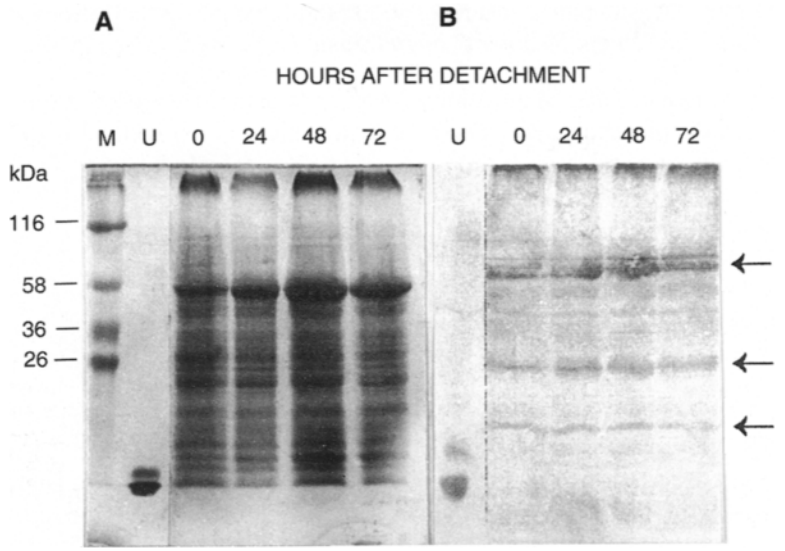

Fig. 1. *A*: SDS-PAGE of protein extracts (80 μg samples) obtained from whole leaves at 0, 24, 48 and 72 h after detachment. *Lane M* - molecular mass markers, *lane U* - bovine ubiquitin (5 μg). *B*: immunoblot analysis with ubiquitin-protein antibodies of protein extracts obtained from whole leaves at 0, 24, 48 and 72 h after detachment and run in SDS-PAGE as in *A*. *Lane U* - bovine ubiquitin. *Arrows* at the right of the membrane indicate strong immunostainings.

The Rubisco large subunit is recognizable in leaf extracts by its molecular size (Ragster and Chrispeels 1981) and proportion of leaf soluble proteins (Dalling 1987). It displayed a size of 53 kDa in PAGE gels (Fig. 1*A*) and the ubiquitin antibodies reacted with a 53 kDa band (Fig. 1*B*). Because a ubiquitin-Rubisco conjugate should exhibit a molecular mass higher than 53 kDa, this result could be explained by a nonspecific reaction of antibodies with Rubisco, as has been postulated by Beers *et al.* (1992). Since unspecific reactions have been avoided, the presence of an unknown 53 kDa ubiquitinated protein appears as a more feasible explanation. On the other hand, since Veierskov and Ferguson (1991) have reported the *in vitro* ubiquitin dependent breakdown of ^{125}I-Rubisco and the presence of the ubiquitin binding system in isolated oat chloroplasts, the presence of ubiquitin-Rubisco conjugates in leaf extracts cannot be discarded. To date, it is well established that phytochrome (Shanklin *et al.* 1987), calmodulin (Ziegenhagen and Jennissen 1990), and part of phosphoenol pyruvate carboxylase (Schulz *et al.* 1993a) are degraded *in*

vivo via the ubiquitin-dependent proteolytic system. The identity of most leaf ubiquitin-conjugates observed in this and other works remains to be investigated.

Estimation of ubiquitin mRNA content: Because significant amounts of ubiquitin-conjugates were observed 48 and 72 h after detachment, it was of interest to obtain information about ubiquitin-mRNA transcription. The level of ubiquitin-mRNA in detached leaves incubated in the dark for 0, 24, 48, and 72 h was studied by Northern blot analysis. The ubiquitin probe hybridized predominantly to an unique size class of RNA of 1.6 kb (Fig. 2). This transcript might correspond to 5 or 6 ubiquitin

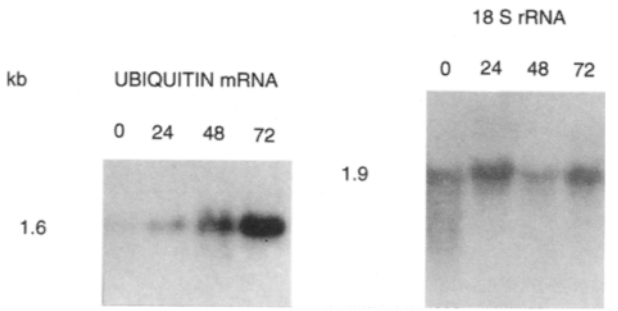

Fig. 2. Northern blot analysis of wheat leaf total RNA. RNA isolated from wheat leaves undergoing 0, 24, 48 and 72 h of darkness were size-fractionated on a 1 % agarose denaturing gel containing formaldehyde, transferred to nylon, probed with a ^{32}P-labeled fragment of sunflower ubiquitin gene and autoradiographed. The blots were washed and re-probed with a ^{32}P-labeled 18S rDNA (1.9 kb). Shown is a figure representative of three different experiments.

coding units as has been reported in potato tubers (Garbarino *et al.* 1992), aleurone layers of *Avena fatua* (Reynolds and Hooley 1992) and *Nicotiana* protoplasts (Genschik *et al.* 1992). To correct RNA loading errors, the same blot was reprobed with rDNA fragment (Fig. 2). The ubiquitin mRNA/18 S rRNA ratio was determined from the scanning densitometry of corresponding autoradiographs and subsequently corrected for 18 S rRNA loss throughout the studied period (Lamattina *et al.* 1988). Ubiquitin mRNA content relative to initial rRNA increased 1.2-, 3.8- and 2.5-fold after 24, 48, and 72 h of detachment, respectively (Fig. 3). That increase did not correlate with a possible ubiquitin accretion with respect to total protein (Fig. 1). This could be explained if an increase in ubiquitin breakdown complements an increase in ubiquitin synthesis. In fact, leaf proteins are degraded at the highest rate 48 - 72 h after detachment (Lamattina *et al.* 1985). It is also possible that an increased level of ubiquitin mRNA is required for maintaining a constant rate of ubiquitin synthesis and, therefore, to preserve its content. In support of this it has been reported that senescent ribosomes read mRNAs at a slow rate (Gallie 1993), but leaves display rates of protein synthesis similar to those of controls (Lamattina *et al.* 1985). It may be noted that results in which mRNA accretion do not lead to protein accumulation have been already described (Della Penna *et al.* 1989, La Rosa *et al.* 1992). Experiments must be carried out in order to obtain a better understanding of the mechanisms involved in these situations.

In conclusion, whereas exposure of detached fully expanded wheat leaves to darkness results in high rates of protein breakdown 48 - 72 h after detachment (Lamattina *et al.* 1985), ubiquitin-protein conjugates are produced. At the same time,

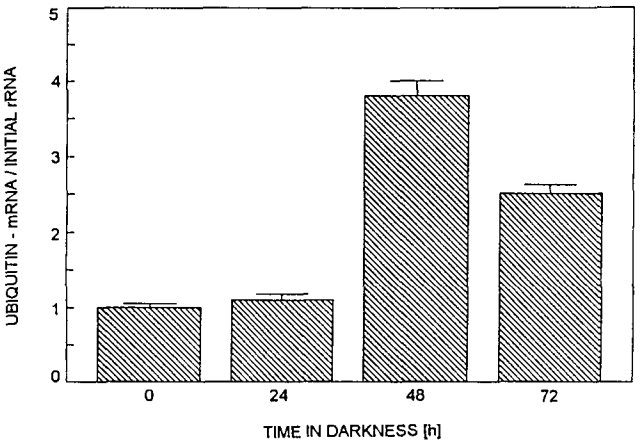

Fig. 3. Relative amounts of ubiquitin mRNA to initial rRNA content of leaves undergoing different times of darkness. The ratios ubiquitin mRNA/18 S rRNA were calculated from the densitometries of the northern blots shown in Fig. 2 and corrected for the daily loss in 18 S rRNA content (Lamattina *et. al.* 1988). Zero time ratio was taken as 1. Bars indicate SD for three different experiments.

ubiquitin mRNA was dramatically increased (Fig. 3). In other words, senescing leaves maintain both their capacity for ubiquitin transcription and for its conjugation to proteins. This is consistent with results of Courtney *et al.* (1993) who detected ubiquitinated proteins during floral senescence. Since other roles of ubiquitination in addition to proteolysis remain to be demonstrated (Vierstra 1993), the results of the present study support the idea that the ubiquitin proteolytic pathway contributes to leaf protein breakdown during dark induced senescence. Further experiments must be performed in order to quantify this contribution. These studies should be performed under the premise that, even under stress, a close relationship exists between the different cellular proteolytic pathways (Ciechanover *et al.* 1991, Mayer *et al.* 1991, Fagan *et al.* 1992, Vierstra 1993).

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