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Ethylene regulates ascorbic acid content during dark-induced leaf senescence

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ABSTRACT

The aim of this work was to determine if changes in antioxidant contents are directly controlled by ethylene or are indirect effects associated with modulation of leaf senescence. Detached spinach (*Spinacia oleracea* L. cv Bison) leaves were treated with ethephon to induce ethylene production, placed in polyethylene bags and stored under darkness at 23 °C for 3 days. The ethephon treatment produced a rapid decrease on ascorbic acid (AA) content and an increase in its oxidised state before any leaf senescence parameters changed. Both AA synthesis and recovery from oxidised forms were affected by ethylene. In addition, leaves from Arabidopsis (*Arabidopsis thaliana* (L.) Heynh) ethylene insensitive mutants (*ein2-1*, *ein3-1* and *ein4*) presented higher AA content than leaves from wt plants before dark storage. Furthermore, *ein3-1* and *ein4* but not *ein2-1*, showed slower leaf AA content declining than wt plants under dark-induced leaf senescence. The Arabidopsis mutant with constitutive triple response (*ctr1-1*) showed lower leaf AA content than wt plants but similar AA degradation rate with a higher oxidised state after dark storage for 3 days. These results demonstrate that ethylene is an important factor controlling AA content in mature leaves.

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1. Introduction

Senescence is the last stage of leaf development that involves the degradation of cell constituents and their remobilization to growing organs [1]. Chlorophyll lost has been widely used for the characterization of the senescence syndrome [2]. In addition, other changes like protein degradation, photosynthetic impairment and antioxidant modifications are observed during leaf yellowing. When leaves are detached from the plant and placed in the dark several changes that characterize their senescence are triggered [3]. It is well known that the initiation of plant senescence is under hormonal control but the mechanisms triggered by hormones are not fully understood [4]. Leaf detachment provokes an imbalance of plant hormones like lower availability of cytokinins synthesized in the roots or higher ethylene evolution produced by cut petioles, both accelerating leaf senescence.

One of the most important changes observed during plant senescence is the decline in antioxidant contents and the increase in the steady state of reactive oxygen species. Reduced ascorbic acid (AA) is considered the central component of the plant antioxidant defense [5]. Modifications on its content and redox state affect gene expression participating in the acclimation of the plant to the environment [6,7]. Gibberellin and cytokinins treatments promote plant growth and increase the production or keep high reduced/oxidised ratio of AA [8,9]. Ethylene stimulates plant senescence and consequently the decrease in AA content [10]. This antioxidant modification may be directly regulated by hormones or may be just a secondary senescence associated process. Hodges and Forney [11] observed a decrease in leaf antioxidant contents when ethylene was applied. The decreases on the accumulation of antioxidants may be the consequence of the hormone effect on lowering their biosynthesis or on increasing their degradation through reactions with reactive oxygen species. Although the changes on antioxidant contents are well characterized during leaf senescence, the physiological processes involved in the hormonal control of their accumulation remain unknown [11].

This work studied the effect of ethylene on AA metabolism during dark-induced leaf senescence. Here are presented experimental evidences demonstrating that ethylene regulates the AA content without modification of the oxidative stress status of leaf tissues during leaf senescence.

2. Materials and methods

2.1. Plant material and ethylene treatment

Spinach plants (*Spinacia oleracea* L. cv Bison) were supplied by a local producer and mature leaves were used for the experiments.

Abbreviations: AA, ascorbic acid; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; Fv/Fm, photosystem II potential quantum yield; GSH, reduced glutathione; L-GalLDH, L-galactone-1,4-lactone dehydrogenase; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; wt, wild type.

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Ethylene treatment was performed by immersing leaves in a solution containing 200 ppm ethephon including 0.1% dimethyl sulfoxide for 20 min. Untreated samples were immersed in a similar solution without ethephon. Then leaves were carefully blotted with paper towels, stored at 23 °C in low density polyethylene bags and samples were taken daily. Other experiments were performed with *Arabidopsis thaliana* (L.) Heynh ethylene insensitive mutants (*ein2-1*, *ein3-1* and *ein4*) and a triple constitutive response mutant (*ctr1-1*) obtained from the Arabidopsis Biological Resource Center. Plants were grown in a mixture of soil with vermiculite (1:1) under 100 μmol m^{-2} s^{-1} of photon flux density with a 10-h photoperiod at 23 °C. Leaves from 1-month-old plants were detached and stored at 23 °C in low density polyethylene bags. Then samples were taken before and after 72 h of dark storage.

2.2. Leaf senescence parameters

Chlorophyll was extracted from leaf tissues with dimethylformamide and measured spectrophotometrically (Shimadzu Corporation, UV-160A) according to Inskeep and Bloom [12]. Photosystem II potential quantum yield (Fv/Fm) was measured with a portable modulated chlorophyll fluorometer (FMSII, Hansatech, UK) using dark-adapted leaves [13]. Solute leakage was determined as previously described [14] immersing leaf disks in deionised water and its conductivity measured after 3 h of incubation.

2.3. H_2O_2 production

In vivo H_2O_2 production was measured as described in Gómez et al. [14] using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes®).

2.4. Malondialdehyde (MDA) measurements

Leaves were ground in 1 mL of 50 mM potassium phosphate buffer pH 7.0 and 100 μL of a 0.2% (w/v) ethanol solution of butylated hydroxytoluene. Homogenates were centrifuged at 16,000 × *g* for 10 min. Supernatants were mixed with 1 M HClO (1:1), shacked vigorously and centrifuged again at 16,000 × *g*. Then one volume of 0.8% thiobarbituric acid was added to two volumes of the supernatant and incubated at 90 °C for 1 h. Samples were filtered and injected onto a HPLC system (Shimatzu LC-10Atvp solvent delivery module) equipped with a C-18 column (Varian Chromsep 100 mm × 4.6 mm) and detected at 590 nm (Shimatzu UV-Vis SPD-10Avp detector). The HPLC conditions consisted in a running buffer of 70% 100 mM potassium phosphate buffer pH 7.0 and 30% methanol (v/v) as described by Templar et al. [15].

2.5. Antioxidant determinations

Contents of AA and its oxidised form, dehydroascorbate, were determined as described in Bartoli et al. [16]. The redox state of AA was calculated as: oxidised form % = [(oxidised form) (reduced + oxidised)$^{-1}$] × 100.

2.6. Enzyme assays

About 200 mg of leaves were ground in a medium containing 100 mM bicine (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol and 0.6% (v/v) cocktail of protease inhibitors (Sigma–Aldrich Co.). The homogenates were centrifuged at 16,000 × *g* for 10 min and the supernatants used for the enzymatic determinations. Catalase (EC 1.11.1.6) was measured according to Aebi [17] and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) were measured as described by De Gara et al. [18] with minor modifications. Ascorbate peroxidase (APX, EC 1.11.1.11) was extracted and measured according to Amako et al. [19]. L-Galactone-1,4-lactone dehydrogenase (L-GalLDH, EC 1.3.2.3) was extracted and measured according to Bartoli et al. [20].

2.7. Statistical analysis

Data are presented as the average of at least four independent experiments and analyzed by means of one-way ANOVA. The means were compared by the LSD test at a significance level of 0.05 or 0.01 as indicated.

3. Results

3.1. Effect of ethylene treatment on spinach senescence

The senescence of detached leaves was induced by dark storage and followed measuring chlorophyll content, solute leakage and Fv/Fm (Fig. 1A–C). Ethylene-induced symptoms were not developed until the third day after ethephon treatment (Fig. 1A–C). It was observed a decrease in chlorophyll content and an increase in solute leakage (Fig. 1B) but there were no modifications in Fv/Fm (Fig. 1C).

3.2. Effect of ethylene treatment on oxidative stress metabolism

The *in vivo* production of H_2O_2 increased in leaves after 24 h of storage in darkness and then decreased during the following days (Table 1). The MDA leaf content did not change during the period of dark-induced senescence studied (Table 1). Both ethephon treated and untreated samples showed similar H_2O_2 production and MDA contents (Table 1). Catalase and APX activities were neither modified by senescence nor by ethephon application (Table 1).

3.3. Effects of ethylene treatment on AA

The AA content decreased in untreated leaves at 48 h of dark storage and ethylene exacerbated this decrease. AA contents in ethephon treated samples were only 21 and 25% of those values measured in untreated samples after 48 and 72 h of dark storage, respectively (Fig. 2A). Meanwhile ethylene decreased AA content, the proportion of the oxidised form increased (Fig. 2B).

Table 1
Effect of ethylene on oxidative stress metabolism. Ethephon treated or untreated leaves were stored in the darkness at 23 °C. Samples were taken for the measurement of H_2O_2 production, oxidative damage to lipids (MDA), catalase (CAT) activity and ascorbate peroxidase (APX) activity at the indicated times.

	0 h	24 h		48 h		72 h	
	Untreated	Untreated	Ethephon	Untreated	Ethephon	Untreated	Ethephon
H_2O_2 (μmol g^{-1} Fw h^{-1})	193 ± 32	301 ± 14	321 ± 38	293 ± 23	312 ± 40	299 ± 50	268 ± 43
MDA (nmol g^{-1} Fw)	2.6 ± 0.8	2.9 ± 0.9	2.88 ± 0.6	2.1 ± 0.5	2.3 ± 0.4	2.2 ± 0.4	3.2 ± 0.7
CAT (μmol g^{-1} Fw min^{-1})	0.8 ± 0.1	1.6 ± 0.12	1.2 ± 0.27	1.5 ± 0.11	1.7 ± 0.26	1.3 ± 0.21	1.6 ± 0.2
APX (μmol g^{-1} Fw min^{-1})	105 ± 19	151 ± 19	126 ± 15	82 ± 18	111 ± 10	64 ± 12	69 ± 10

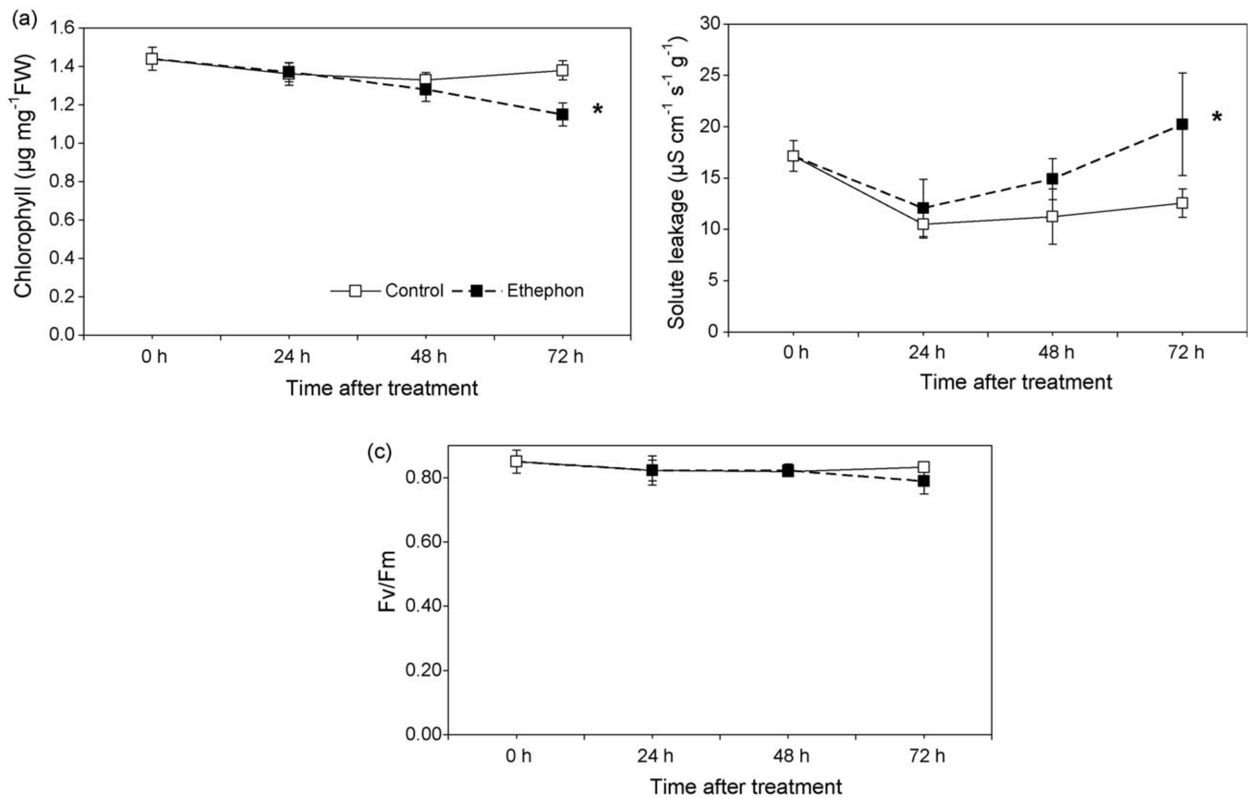

Fig. 1. Effect of ethylene on spinach leaf senescence. Ethephon treated or untreated leaves were stored in the darkness at 23 °C. Samples were taken for the measurement of chlorophyll content (A), solute leakage (B) and Fv/Fm (C) at the indicated times. Asterisks denote statistical differences with untreated samples (ANOVA, $P \leq 0.05$).

3.4. Effect of ethylene treatment on the leaf capacity to increase AA content

On the other hand, ethephon treatment avoided the increase in L-GalLDH activity observed in untreated samples during dark storage (Fig. 3A). DHAR and MDHAR activities decreased with senescence and ethephon treatment induced a rapid decrease of these enzyme activities after storage for 24 h (Fig. 3B and C).

3.5. Oxidative stress metabolism and AA accumulation in ethylene signalling mutants

Arabidopsis mutants for the ethylene signalling pathway were used in the next experiments to confirm the effects of this hormone on AA accumulation. Three ethylene insensitive mutants were selected with alterations in the expression of the following proteins: EIN2 is a positive regulator of ethylene responses, EIN3 is a transcriptional factor that accumulates in ethylene treated tissues and EIN4 is a membrane localised ethylene receptor. Finally, *ctr1-1* is a mutant with a loss of function of a negatively ethylene regulated kinase displaying constitutive triple responses [21].

AA accumulation was analyzed in leaves of ethylene signalling mutants before and after 72 h of dark storage. AA content was expressed on the basis of chlorophyll content to demonstrate that changes in the antioxidant accumulation depend on the leaf ethylene sensitivity and not in the progress of leaf senescence. Plants of *ein3-1*, *ein2-1* and *ein4* presented 38, 86 and 125% higher leaf AA content than wt and plants of *ctr1-1* showed 34% lower leaf AA content than wt before dark storage (Table 2). When leaves

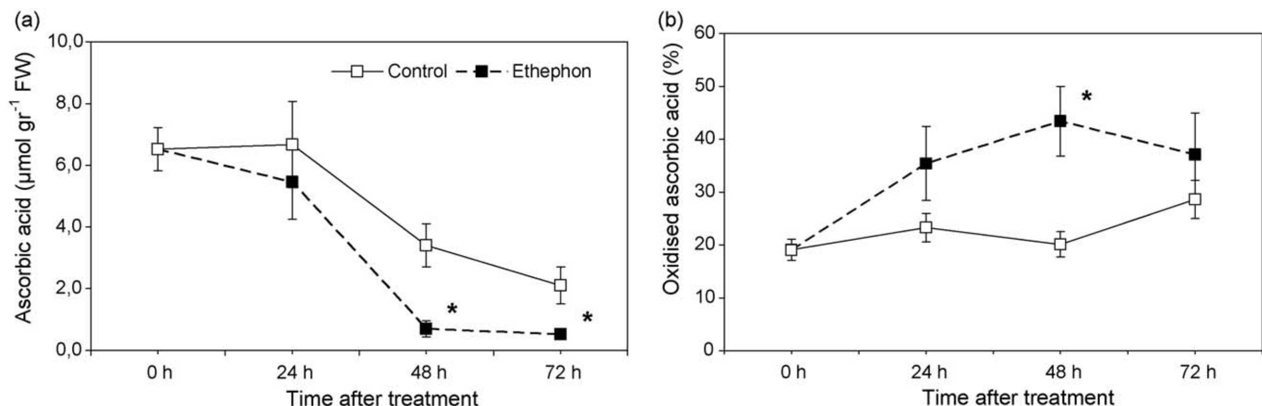

Fig. 2. Effect of ethylene on AA content and redox state. Ethephon treated or untreated leaves were stored in the darkness at 23 °C. Samples were taken for the measurement of AA content (A) and redox state (B) at the indicated times. Asterisks denote statistical differences with untreated samples (ANOVA, $P \leq 0.05$).

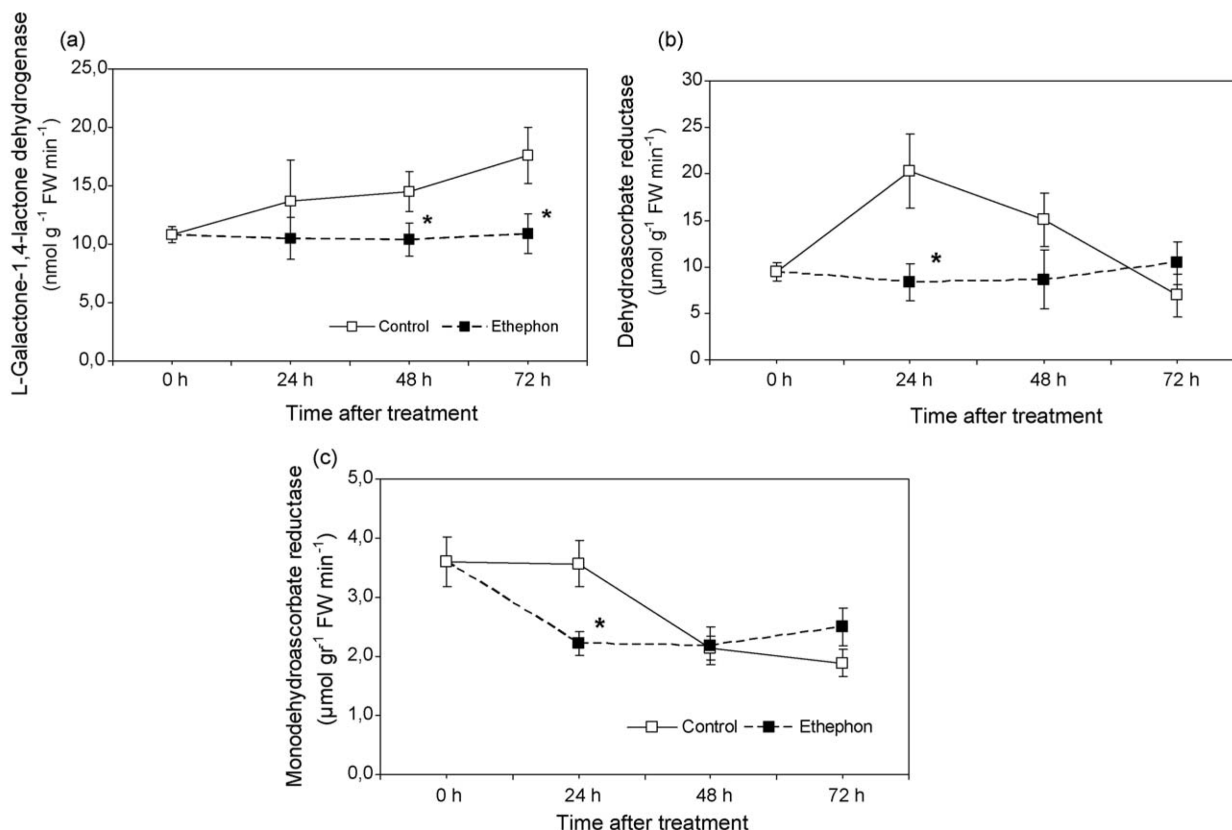

Fig. 3. Effect of ethylene on L-GalLDH, DHAR and MDHAR activities. Ethephon treated or untreated leaves were stored in the darkness at 23 °C. Samples were taken for the measurement of L-GalLDH (A), DHAR (B) and MDHAR (C) activities at the indicated times. Asterisks denote statistical differences with untreated samples (ANOVA, $P \le 0.05$).

Table 2
AA accumulation in ethylene signalling mutants leaves.

Arabidopsis lines	AA content (µmol mg^{-1} chlorophyl)	
	Before storage	After 72 h storage
wt	4.4 ± 0.11	2.4 ± 0.08 (56)
ein2-1	8.2 ± 0.15*	4.0 ± 0.21* (49)
ein3-1	6.1 ± 0.01*	5.3 ± 0.47* (86)
ein4	9.9 ± 0.7*	8.5 ± 0.36* (86)
ctr1-1	2.9 ± 0.37*	1.8 ± 0.03* (63)

Mature leaves were detached from the plants, placed in polyethylene bags and stored in darkness at 23 °C. The numbers between brackets indicate the % of AA content with respect to those before storage.
* Statistical differences with wt (ANOVA, $P \le 0.01$).

were detached and exposed for 3 days to dark-induced senescence, *ein3-1* and *ein4*, but not *ein2-1*, retained higher amounts of AA than wt (Table 2) with no differences in the senescence indicators (Supplementary material 1). Unexpectedly, *ctr1-1* leaves presented a similar decrease in AA content compared with the wt (Table 2) with a higher senescence rate (Supplementary material 1). The oxidised/reduced forms ratio was similar for all Arabidopsis lines before dark storage, but was higher in the *ctr1-1* than in wt leaves at the end of the experiments (Supplementary material 2). L-GalLDH activity was higher in *ein3-1* and *ein4* than in wt but intermediate in *ein2-1* before storage (Table 3). Only *ein4* plants kept a higher L-GalLDH activity than wt with intermediate activities measured in *ein2-1* and *ein3-1* plants after dark storage. DHAR activity was higher in *ein3-1, ein2-1* and *ein4* than in wt, and lower in *ctr-1* before dark storage. This activity was similar in wt, *ein2-1* and *ctr-1* but was kept higher in *ein3-1* and *ein4* after 72 h under dark conditions. MDHAR activity was only higher in *ein3-1* at the beginning of the experiments and was higher in *ein3-1* and *ein4* and lower in *ein2-1* and *ctr1-1* than in wt leaves after dark storage.

4. Discussion

Dark storage and ethylene treatments accelerate leaf senescence [22]. When leaves are induced to senesce several deteriorative processes are triggered, such as degradation of chlorophyll and proteins, modifications in membrane permeability and in the antioxidant contents. These changes have been well characterized but not the sequence of the events occurring during leaf

Table 3
Capacity of Arabidopsis leaves to increase AA accumulation. Mature leaves were detached from the plants, placed in polyethylene bags and stored in darkness at 23 °C.

Arabidopsis lines	L-GalLDH activity (nmol g^{-1} FW min^{-1})		DHAR activity (µmol g^{-1} FW min^{-1})		MDHAR activity (µmol g^{-1} FW min^{-1})	
	Before storage	After 72 h storage	Before storage	After 72 h storage	Before storage	After 72 h storage
wt	67.0 ± 9.0**a**	61.0 ± 4.9**a**	1.50 ± 0.06**a**	2.10 ± 0.06**a**	0.51 ± 0.02**a**	0.67 ± 0.01**a**
ein2-1	77.7 ± 9.8**ab**	75.7 ± 10.4**ab**	1.83 ± 0.09**b**	2.03 ± 0.07**a**	0.54 ± 0.06**a**	0.45 ± 0.06**b**
ein3-1	101.0 ± 9.3**b**	68.6 ± 7.5**ab**	1.83 ± 0.11**b**	2.60 ± 0.06**b**	0.77 ± 0.11**b**	0.76 ± 0.02**c**
ein4	96.0 ± 3.2**b**	86.8 ± 8.9**b**	2.62 ± 0.07**c**	5.50 ± 0.70**c**	0.47 ± 0.05**a**	0.74 ± 0.02**c**
ctr1-1	59.0 ± 5.8**a**	44.9 ± 12.8**a**	0.67 ± 0.09**d**	2.30 ± 0.2**ab**	0.52 ± 0.04**a**	0.36 ± 0.04**b**

Data with similar letter represent a statistically homogenous group (ANOVA, $P \le 0.05$).

senescence. This work focuses on the effects of ethylene on antioxidant contents during the early stages of leaf senescence.

4.1. Ethylene stimulates leaf senescence but does not alter oxidative metabolism

Leaf senescence-induced modifications may be related to increments on the oxidative load of the cells [23 and references there in]. The H_2O_2 *in vivo* generation increased during dark-induced spinach leaf senescence but was not altered by ethylene treatment, and the oxidative damage to lipids measured as MDA, did not change by any treatment. Furthermore, catalase and APX activities, the main H_2O_2 detoxifying enzymes in plants, were not affected by ethylene. Previous works found that this hormone did not have any effect on the development of oxidative stress but it might be a further consequence of an acceleration of senescence induced by this hormone [24]. Together these results indicate that oxidative stress is not involved in the initial events of ethylene induced leaf senescence.

4.2. AA content is modified by ethylene

The decrease in antioxidant contents observed in leaves during dark-induced senescence may be directly controlled by ethylene or may be indirectly associated with leaf senescence. The results presented here show that ethylene promoted the decline in AA content and the increase in its oxidised redox state before any change in the typical senescence parameters were observed in spinach mature leaves. To further demonstrate the regulation exerted by ethylene on AA content, Arabidopsis mutants with altered hormone signalling were analyzed. The higher AA contents observed in mature leaves of *ein2-1*, *ein3-1* and *ein4* mutants and the lower AA content observed in mature leaves of the *ctr1-1* mutant than those of the wt suggest that the accumulation of this antioxidant is regulated by ethylene. In addition, the maintenance of a higher proportion of AA per chlorophyll after dark storage for 72 h demonstrate that the change in the antioxidant is not strictly connected to dark-induced senescence but highly coupled to ethylene signalling through EIN 3 and EIN 4 proteins, but not EIN 2. This different response of *ein2-1* mutant may be a consequence of its pleiotropic phenotype since it interacts with other hormone signalling pathways. In contrast, wt and *ctr1-1* plants showed similar AA decreases during dark storage suggesting that ethylene signalling pathway is saturated in detached leaves of Arabidopsis wt plants and no further effect can be observed in the *ctr1-1* mutant.

Previous studies showed that exogenous applications of AA have successfully delayed leaf senescence [25,26]. All these evidences suggest that AA degradation might be an early event specifically triggered by ethylene as part of the senescence syndrome. Considering that there were no evidences that oxidative stress was involved during the period of dark storage studied, AA declination might affect other processes like gibberellins biosynthesis or other reactions [27] that accelerate leaf senescence.

4.3. Ethylene controls AA formation in mature leaves

The synthesis of AA is catalysed by L-GalLDH, an enzyme localised in the inner mitochondrial membrane that strongly interacts with respiration [8–20]. Hodges and Forney [28] have observed that L-GalLDH activity is increased in spinach leaves during dark storage. The present work presents evidences showing that mature leaves treated with ethylene lack the increment of L-GalLDH activity observed during their dark storage. Ethylene insensitive mutants display similar or higher L-GalLDH activities than wt leaves. Since AA synthesis depends in both L-GalLDH content and mitochondria electron transport chain [29], further studies will be performed to study the effect of ethylene in respiration (e.g. cytochrome *c* oxidised availability) that might influence the leaf AA synthesis capacity.

Ethylene quickly induced decreases in both MDHAR and DHAR activities in spinach leaves after 24 h of ethephon application leading to a lower capacity of AA recovery from oxidised forms and could have produced a reduction of AA content during the following days. In fact, the ratio of oxidised/reduced forms was higher in ethylene treated samples. Plants overexpressing DHAR showed elevated amounts of AA in leaves demonstrating its important contribution to the reduced pool [30]. Similarly, the recovery capacity of AA from oxidised forms was higher in ethylene insensitive mutants with high AA content and lower in leaves of *ctr1-1* plants. While both AA recovery capacity and AA content decreased in *ein2-1* leaves, *ein 3-1* and *ein4* leaves increased their AA recovery activity and kept high AA content after dark storage.

All these results suggest that ethylene constitute an important signal regulating AA accumulation in mature leaves by both lowering its *de novo* synthesis and recovery from oxidised forms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2009.12.003.

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