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RESEARCH PAPER

Endogenous ascorbate restrains apoplastic peroxidase activity during sunflower leaf development

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ABSTRACT

Several apoplastic enzymes have been implicated in the control of elongation growth of plant cells. Among them, peroxidases contribute to both loosening and stiffening of the cell wall. They appear to be regulated by various mechanisms, including the action of extracellular inhibitors. To obtain evidence of the role of the enzyme–inhibitor interaction during leaf development, the intercellular washing fluids from *Helianthus annuus* leaves of different ages were isolated using standard methods of vacuum infiltration and centrifugation. Peroxidase activities, assessed using tetramethylbenzidine as substrate, increased during leaf development, reaching a maximum value after the leaves were fully expanded. An inhibitor, chemically characterised as ascorbate, co-localised with the enzyme in the apoplast. Moreover, there was a strong negative correlation between the action of peroxidase and the micromolar concentration of ascorbate in the apoplastic fluid. The results show that in growing leaves, the *in planta* ascorbate concentration is able to restrain peroxidase enzyme activity. Then, at the time of growth cessation, the loss of extracellular ascorbate relieves the inhibition on this enzyme that contributes to wall fixation.

INTRODUCTION

Plant growth, defined as a permanent increase in size, involves coordinated molecular reactions that control the division of the cell, as well as its elongation. Plant cells grow under the stimulus of water uptake in a process restrained by the cell wall, thus several apoplastic proteins, *e.g.* expansins, proteases, xyloglucan endotransglucosylase/hydrolase and peroxidases, modulate the loosening of the wall and also allow this growth restriction to be overcome (Cosgrove 2005; O'Looney & Fry 2005).

Class III peroxidases are the extracellular subgroup of a large family of enzymes that catalyse the reduction of H_2O_2 with electrons transferred from a variety of molecules. They are proposed to perform two opposite processes: they can stiffen or loosen the cell walls, depending on their involvement in two related reactions, known as peroxidative and hydroxylic cycles, respectively (Liszkay *et al.* 2004; Passardi *et al.* 2004). The former cycle allows electron donors, such as lignin precursors and phenolic derivatives, to polymerise cell wall components. Therefore, stiffened walls and reduced elongation rates appear as a consequence of isodityrosine bond and/or diferulate bridge formation between proteins and polysaccharides, respectively (Fry 1987). The increase in cell wall peroxidases at the end of leaf expansion (MacAdam *et al.* 1992; de Souza & MacAdam 1998), as well as the increasing gradient of activity from the elongation zone to the highly lignified tissues in pine hypocotyls (Sánchez *et al.* 1995), etiolated pea shoots (de Pinto & de Gara 2004) and maize roots and leaves (Dragišić Maksimović *et al.* 2008) point to these enzymes having a role in growth cessation. On the other hand, ·OOH and ·OH radicals produced in the hydroxylic cycle are able to cleave cell wall polysaccharides and could contribute to cell elongation (Fry *et al.* 2001; Liszkay *et al.* 2004; Passardi *et al.* 2004). Thus, the occurrence of ·OH *in vivo* during the elongation of maize coleoptiles appears as a contribution of peroxidases to cell wall loosening, although enzymatic activity itself has not been evaluated (Müller *et al.* 2009). Additionally, ·OH might also be produced in the cell walls through a non-enzymatic, copper-dependent reaction (Fry *et al.* 2002).

The extracellular peroxidases have been classified as having wall-bound and soluble isoforms. Synthetic molecules such as guaiacol, *o*-dianisidine and tetramethylbenzidine are frequently used and assumed to represent the broad range of substrates of the many physiological processes in which peroxidases are involved. However, peroxidases have also been grouped according to their specificity toward natural substrates like ferulic acid (Lin & Kao 2001), ascorbate (Welinder & Gajhede 1993) and flavonols (Takahama 2004). Together with the cluster of peroxidase-encoding genes already reported (73 and 138 in *Arabidopsis* and *Oryza sativa*, respectively; Passardi *et al.* 2004), this complexity supports the idea that fine-tuning is needed to give functional specificity to members of this gene family.

The multiplicity of peroxidase isoforms able to react with a variety of potential ligands makes it difficult to obtain a

clear picture of their regulation, even in a restrained location like the apoplast. Theoretically, the extracellular activities can be controlled through protein synthesis, secretion and degradation, as well as through their access to substrates and/or inhibitors. Regarding inhibitors, a still uncharacterised molecule and ascorbate isolated from extracellular fractions have been proved to reduce peroxidase activity. The uncharacterised molecule is a small, heat labile, hydrophilic molecule obtained from the medium of cultured maize cells (Encina & Fry 2005). Ascorbate, however, is a well-known antioxidant that is assumed to be the extracellular scavenger of H_2O_2 and a promoter of the enzymatic and non-enzymatic generation of wall-loosening hydroxyl radicals (Takahama & Oniki 1992; Fry 1998; Foyer & Noctor 2005; Pignocchi *et al.* 2006). It inhibits *in vitro* apoplastic peroxidases of spinach leaves (Luwe *et al.* 1993) and *Vigna angularis* epicotyls (Takahama & Oniki 1992; Takahama 1993). However, with the exception of studies showing that ascorbate contributes to onion root elongation through the inhibition of peroxidases in meristematic as well as in elongation zones (Cordoba-Pedregosa *et al.* 1996, 2003, 2005), the *in planta* interaction of these molecules remains scarcely analysed. Interestingly, in white spruce germinating embryos, it was shown that treatments that enhance ascorbate *in vivo* reduce the activity of isolated guaicol and ferulic peroxidases (Stasolla & Yeung 2007); therefore, this molecule can not only inhibit the enzyme but also control its content. Thus, the physiological significance of the relationship between peroxidases and their inhibitors in the extracellular matrix emerges as an unexplored subject to be evaluated in the context of growth regulation. The sequential development of each developing leaf at different positions on the stem of cultivated sunflower follows a conserved pattern of events, irrespective of environmental conditions and genotype (Pereyra-Irujo *et al.* 2008). Thus, to obtain a better understanding of the role of the peroxidase–inhibitor interaction in leaf growth, we assessed in parallel the net enzymatic and inhibitory activities, as well as the ascorbate content, in intercellular washing fluids of different sunflower leaves throughout their development.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of the *Helianthus annuus* L. inbred line HAR2 were sown in cylindrical pots (35 cm high, 10 cm diameter), one per pot, filled with Typic Argiudoll horizon A soil. Soil water content was maintained at water-holding capacity throughout the experiment. Pots were randomly accommodated in a growth chamber under controlled environmental conditions as described in Pereyra-Irujo *et al.* (2008). Photosynthetic photon flux density (PPFD), air temperature and relative humidity in the growth chamber were measured continuously at plant level and recorded with a PPFD sensor, a thermistor and a humidity sensor (Cavadevices, Buenos Aires, Argentina), respectively. Thermal time was calculated as the daily integral of the difference between temperature and base temperature (4.8 °C) to allow the time courses of different traits (*e.g.* leaf area, peroxidase activity in this paper) to be unique under a large range of conditions, even at naturally fluctuating temperatures (Granier & Tardieu 1998). The starting point for even leaf growth measurements began when the fourth leaf of each plant was fully expanded.

Growth measurements

Non-destructive measurements of length (L) and width (W) of leaf numbers 6, 8, 10, 12 and 14 were done every 2 days until the end of their expansion. A highly significant linear relationship ($R^2 > 0.99$, $n = 1755$, $P < 0.0001$), applicable to any leaf, regardless of stem position and age, was previously established between $L \times W$ and leaf area (Pereyra-Irujo *et al.* 2008). Leaf area (A) was calculated from the equation $A = L \times W \times 0.65$, and the leaves were considered fully expanded when three consecutive measurements with equal or decreasing A were obtained. Relative leaf area was calculated considering a fully expanded leaf was equal to 1. Relative leaf area was described from a sigmoid curve using the following equation: $y = A/(1 + \exp\{-[(t - t_0)/b]\})$ (Pereyra-Irujo *et al.* 2008), where t is the thermal time elapsed since the initiation of each leaf. A leaf was assumed to be initiated when its primordium was visible (about 40 μm long) on the apical meristem under a microscope (Leica stereomicroscope, Wild F8Z, Wetzlar, Germany) at a magnification ×80.

Isolation of apoplastic fluids

To obtain apoplastic fluids (AFs), at least four leaves were immersed in buffer A (50 mM Na phosphate, pH 7.25, 0.1% 2-mercaptoethanol, 0.15 M NaCl, 0.18% Tween-20) for 1 h before vacuum infiltration for six 20 s periods following Pinedo *et al.* (1993) at a pressure of 45 kPa. Afterwards, leaves were surface-dried with filter paper and placed in glass funnels located inside centrifugation tubes. The tubes were centrifuged at 400 *g* for 50 min and the AFs recovered at the bottom of the tube. The collected AF volume was recorded and immediately used for enzyme determinations.

Peroxidase and inhibitor activity assays

Total peroxidase activity was measured using a spectrophotometric assay according to Liem *et al.* (1979). The reaction mixture, prepared immediately before use, contained 3 mg ml^{-1} 3,3′,5,5′-tetramethylbenzidine (dissolved in dimethylsulphoxide), 100 mM H_2O_2 and 50 mM Na acetate buffer, pH 5.2. The reaction was followed at room temperature over 1 min at 640 nm with a Beckman DU 500 spectrometer (Beckman Coulter, Buenos Aires, Argentina). OD was recorded every 5 s after the addition of 1–5 μl AFs to 800 μl reaction mixture. The reaction was linear during 1 min and the slope determined ($R^2 > 0.98$). One unit was defined as the amount of enzyme that produced a change in OD of 0.4 units min^{-1}.

Peroxidase inhibitory activity was assayed as described above for peroxidase activity but with the addition of 1–10 μl 10 mM ascorbate to the reaction mixture, or AFs were filtered through Centricon YM-3 filters (the non-retained fraction was used as source of inhibitor) (Millipore, Billerica, MA, USA). The enzymatic source was a volume of AF obtained from leaf 6 that was previously checked for peroxidase activity. One unit of inhibitor was defined as the amount of sample that reduced peroxidase activity by 50%.

To evaluate the thermal stability of the inhibitor, the AF from leaf 14 was heated at 100 °C for 5 min or frozen at −20 °C and thawed three times before analysing inhibitor activity as described above. Different aliquots were incubated with 36 mg ml^{-1} trypsin (Sigma T1426; Sigma-Aldrich, St. Louis, MO, USA) or ascorbate oxidase (Sigma AO157) for 0 or 60 min in order to assay the polypeptide or ascorbate-related nature of the inhibitor.

Gel electrophoresis and zymograms

The AFs were obtained as described above and their proteins concentrated three-fold using a Centricon YM-3 filter (Beckman Coulter). The concentrated AFs were added to sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol) and immediately loaded in a 12% polyacrylamide SDS–PAGE (Laemmli 1970). After electrophoretic separation, the gels were washed twice, for 15 min each time, with 2 mM H_2O_2 in 80 mM sodium acetate buffer, pH 5.2, and a zymogram for peroxidase was developed by incubating the gel in 2 mM H_2O_2 and 2 mg ml^{-1} 4-chloro-1-naphthol, as described in Hadži-Tašković Šukalović *et al.* (2007).

Ascorbate quantification

The AFs for ascorbic acid quantification were isolated as described above, but recovered on 20% metaphosphoric acid (w/v). Samples were frozen at −80 °C until measurement with reverse-phase HPLC and electrochemical detection. Samples, diluted to 10% with metaphosphoric acid (w/v), were assayed for total ascorbic acid according to the protocol described in Bode & Rose (1999).

Statistical analysis

Four independent experiments were done with at least 30 plants in each. AFs were obtained and analysed for peroxidase activity in triplicate in each independent experiment for all even-numbered leaves. Data on peroxidase activity for each AF were fitted to the following equation: $y = k * \exp(i * x)$, where k and i are 0.9559 and 0.0061, respectively. These parameters were obtained with SigmaPlot 11.0 software (Systat Software Inc., San Jose, CA, USA).

RESULTS

The even-numbered leaves of sunflower plants were vacuum infiltrated and centrifuged to isolate the apoplastic fluid using a standard method. The obtained AFs were checked as previously reported (Pinedo *et al.* 2012), and those with significant cytosolic contamination were discarded.

Leaf area and extracellular peroxidase activity were measured throughout leaf development, from the emergence of each even-numbered leaf (area ≤ 3.5 ± 0.57 cm^2, at 180 degree-days after leaf initiation). The peroxidase activity, assessed with the broad range substrate tetramethylbenzidine, only increased slightly until the leaves reached 400 degree-days old and then increased drastically. Peroxidase activity was highest after the organs reached their final area, around 500 degree-days (Fig. 1). To investigate if the basis of the low activity detected in leaves during early developmental stages could be related to the occurrence of an enzymatic inhibitor, AFs were isolated from young leaves at 250 degree-days (YAF). Increasing volumes of YAF were added to the reaction mix immediately before the addition of a constant volume of AFs obtained from a fully expanded leaf at 600 degree-days (EAF). Figure 2 shows that peroxidase activity in EAF was reduced in the presence of YAF, in agreement with the occurrence of an extracellular inhibitor. The same curve was obtained irrespective of leaf position along the stem from which AFs were obtained (data not shown).

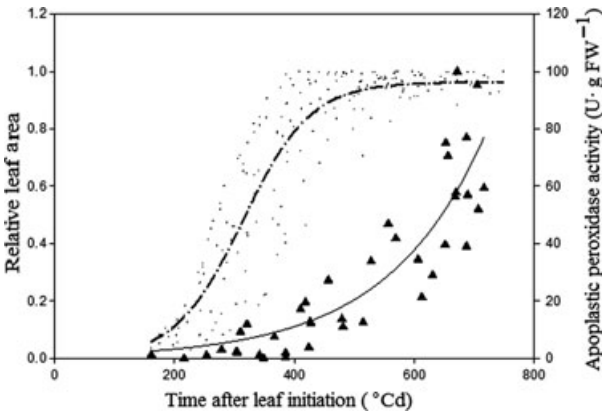

Fig. 1. Analysis of soluble apoplastic peroxidase activities and relative leaf area throughout leaf development. The area of leaf numbers 4, 6, 8, 10, 12 and 14 from more than 30 plants was non-destructively measured during their development. At different times, at least four leaves, at the same position on the shoot, were detached and vacuum infiltrated to obtain AFs. Relative leaf area to final size (dots, dashed line) and total soluble peroxidase activity (triangles, solid line) are depicted against thermal time accumulated for each leaf after its initiation. Accumulated thermal time (°Cd) was calculated as the daily average temperature minus a base temperature (4.8 °C) so that the time courses of peroxidase activity were unique over a large range of temperature conditions. Peroxidase activity was assayed spectrophotometrically with 3,3′,5,5′-tetramethylbenzidine. The evolution of both parameters is represented with fitted curves (relative leaf area = a/(1+ exp(–(–°Cd–c)/b)) with a = 0.9631, b = 56.31 and c = 314.26; peroxidase activity = a*exp(b*°Cd) with a = 0.9559, b = 0.0061 and P > 0.05). One enzyme unit was defined as the amount of enzyme that produced a change in $OD_{640\ nm}$ of 0.4 units in 1 min. The data are from three independent experiments.

The molecular nature of this inhibitor was studied using classical methods: heating and freezing stability, resistance to protease digestion and selective molecular weight filtration (Encina & Fry 2005). The characterisation showed that the inhibitor had a molecular weight of <3 kDa since it was not retained with a YM-3 membrane. Furthermore, as it was stable after trypsin digestion or freezing and heating cycles, it was concluded that it was not a protein (Fig. 3A). These characteristics suggest it might be ascorbate, a molecule whose stability depends on time, temperature and media pH (Davey *et al.* 2000). To confirm the identification, experiments were performed by heating a solution of 40 μM ascorbic acid dissolved in 50 mM Na acetate, pH 5.2, in order to mimic pH conditions in the apoplast. The inhibitory activity and concentration of this solution remained unchanged after heating for 5 min at 100 °C (Table S1); therefore, the lack of an effect of boiling on inhibitor potency (Fig. 3A) could

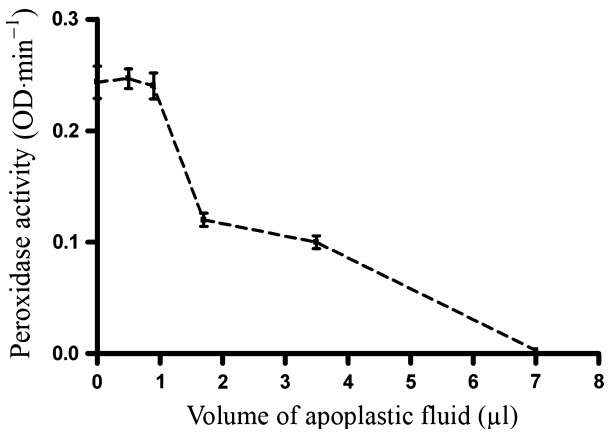

Fig. 2. Peroxidase inhibition by apoplastic fluids obtained from leaves at 250 degree-days. AF from leaves of 600 degree-days (leaf 6) was used as the source of peroxidase activity. The volume of this fraction able to produce a change in OD of 0.25 min^{-1} in spectrophotometric analysis performed with 3,3′,5,5′-tetramethylbenzidine was determined. Subsequently, the reaction mixture was supplemented with 0.5, 1.0, 1.5, 3.5 or 7 µL of AF from leaves of 250 degree-days and the reaction was started by addition of the previously determined volume of AF from leaf 6. Each point corresponds to the mean ± SD of three replicates.

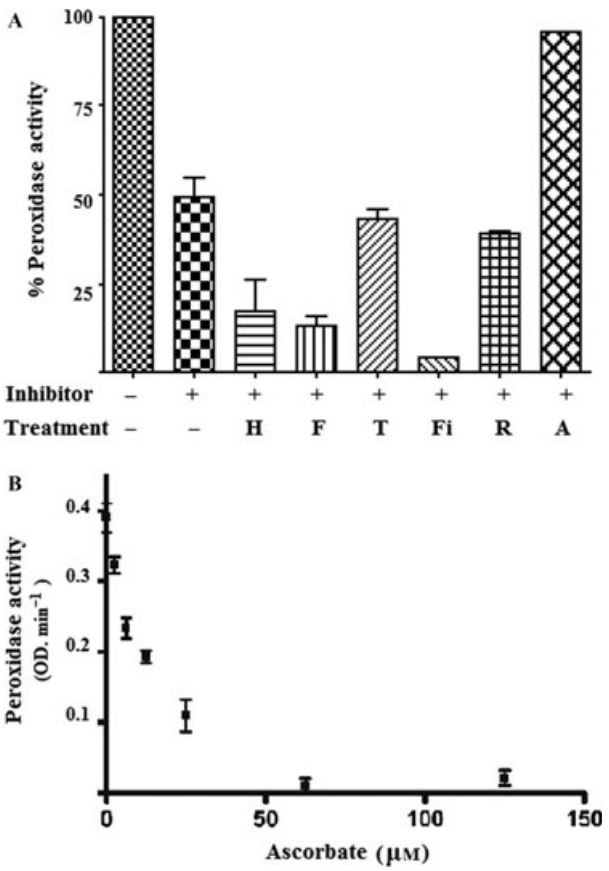

Fig. 3. Peroxidase inhibitor characterisation. A: Soluble peroxidase activity in AF from leaf 6 was determined in incubation mixtures containing AF from leaf 14 with or without treatment. Aliquots of this sample containing one unit of inhibitory activity were boiled (H), frozen and thawed (F), incubated with trypsin (T), filtered through Centricon YM-3 to select the non-retained fraction (Fi), filtered through Centricon YM-3 to select the retained fraction (R), or incubated with ascorbate oxidase (A). Values of peroxidase activity are expressed as percentages of the total activity obtained without the addition of AF from leaf 14. B: The dose for 50% inhibition was calculated by adding 0–125 µM commercial ascorbate to the reaction mixture before addition of the volume of AF from leaf 6 able to give OD 0.4 min^{-1} in spectrophotometric analysis with 3,3′,5,5′-tetramethylbenzidine. Data are means of three replicates from two independent experiments.

suggest that the inhibitor is ascorbate. The molecule was further checked by pre-incubation of YAF with pure ascorbate oxidase. The treatment destroyed apoplastic inhibitory activity and confirmed the identification as ascorbate (Fig. 3A). In addition, it could be concluded that the product of the reaction, *i.e.* dehydroascorbic acid, lacked inhibitory capacity. A dose–response curve produced with commercial ascorbate to assess the sensitivity of the apoplastic peroxidase to the inhibitor showed that 20 µM ascorbate reduced the activity by 50% (Fig. 3B).

To further understand the contribution of ascorbate to control of apoplastic peroxidase activity, both ascorbate content and the inhibitory activity were simultaneously measured in AFs during leaf development. Ascorbate, quantified by electrochemical detection after HPLC separation, reached 1.5 nmol g^{-1} FW in AFs obtained early during leaf development (near 250 degree-days) and decreased to 0.15 nmol g^{-1} FW in 500 degree-day leaves, close to the detection limit of the method (Fig. 4A). The inhibitor was isolated from the enzyme by filtration of AF aliquots through a 3 kDa membrane before quantification of its activity. Interestingly, apoplastic peroxidase activity decreased with increasing leaf age, with a similar pattern to ascorbate content and opposed to the peroxidase activity curve (Fig. 4A). In addition, the values for extracellular ascorbate (Fig. 4A) were in agreement with the inhibitory micromolar concentrations of pure ascorbate (Fig. 3).

The isoforms of peroxidase from the same fractions were assessed with zymograms using non-denaturing SDS-PAGE and 4-chloro-1-naphthol as substrate. A major band was evident in fluids from 250–600 degree-day leaves and a second appeared in fully expanded leaves older than 600 degree-days (Fig. 4B). The occurrence of only one abundant band was confirmed with a zymogram performed after the separation of AFs from 500 degree-day leaves in an isoelectrofocusing gel (Fig. S1). Given that the relative intensity of the signal at 400 degree-days was higher than the relative spectrophotometric value of activity observed in Fig. 1 (dotted line in Fig. 4A), the inhibitor appeared to have separated from the enzyme during the electrophoresis. Accordingly, no bands were found in zymograms if the developing reaction was supplemented with 5 mM ascorbate (data not shown), providing evidence that the ascorbate action was mediated through a reversible mechanism.

DISCUSSION

As leaves grow, cells proliferate and elongate through molecular reactions that are usually intimately connected. Ascorbate, characterised as the major apoplastic antioxidant and

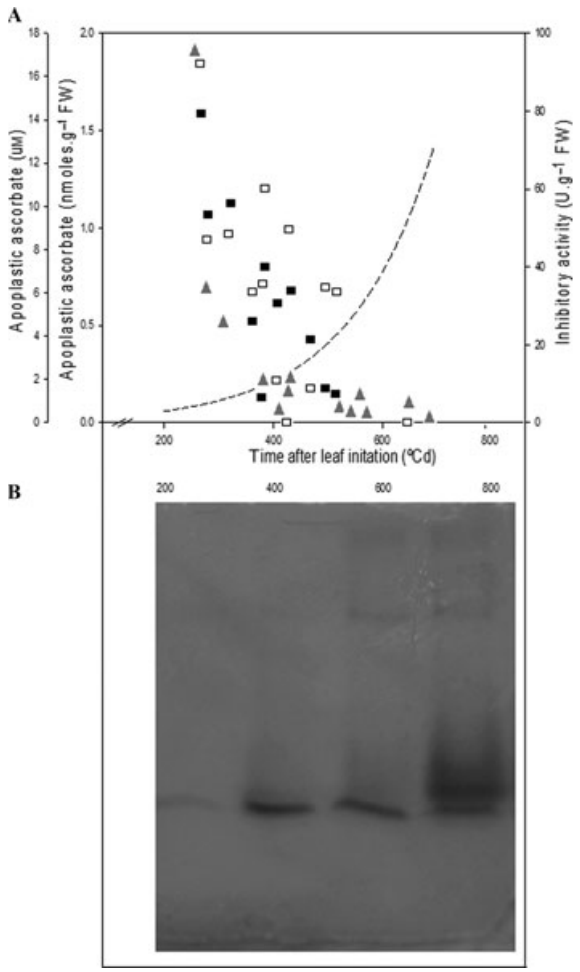

Fig. 4. Apoplastic ascorbate content and peroxidase inhibitory activity throughout leaf development. A: Peroxidase inhibitory activity (white triangles) and ascorbate content in nanomole per gram^{-1} FW (black squares) or micromolar (white squares). For inhibitory activity, AF obtained from even-numbered leaves 200–800 degree-days old and the fractions not retained on a 3 kDa membrane were analysed to determine the volume of these fractions able to produce 50% inhibition of the reaction of AF from leaf 6 and 3,3′,5,5′-tetrametylbenzidine. The non-inhibited reaction produced a change in OD of 0.4 min^{-1}. One unit of inhibitor was defined as the amount of sample that reduced peroxidase activity by 50%. Ascorbate content was measured in AFs recovered on an equal volume of 20% metaphosphoric acid and quantified using reverse phase HPLC with electrochemical detection. Dashed line corresponds to the fitted curve for peroxidase activity shown in Fig. 2. Data are means of three replicates from three independent experiments. B: Peroxidases zymograms. AFs obtained from leaves at 200, 250, 400, 600 and 800 degree-days were analysed in 12% polyacrylamide SDS gel electrophoresis. After electrophoresis, the gel was washed with 2 mM H_2O_2, 80 mM sodium acetate buffer, pH 5.2, for 15 min, and the activity was developed by incubation in 2 mM H_2O_2 and 2 mg ml^{-1} 4-chloro-1-naphthol. The profiles are representative of three independent experiments.

the first barrier in ozone resistance (Luwe *et al.* 1993; Conklin & Barth 2004; Foyer & Noctor 2005), is assumed to be a metabolic promoter that sustains cell growth since it is related to several aspects of growth (Potters *et al.* 2000; Smirnoff 2000; Pastori *et al.* 2003). Ascorbate induces cell division (De Tullio *et al.* 1999), promotes wall loosening through ·OH production (Fry *et al.* 2002) and protects auxins from oxidation (Gazaryan *et al.* 1996). Thus ascorbate appears as a central metabolite in several reactions directly or indirectly related to the control of growth. Interestingly, when the extracellular content of this multi-faceted molecule was decreased through overexpression of an ascorbate oxidase gene, plant growth and defence were altered through the regulation of signal transduction cascades and gene expression (Pignocchi *et al.* 2006).

In this work, a strong negative correlation was evidenced in the extracellular fluids of sunflower leaves between peroxidase activity and ascorbate content, as well as growth rate during leaf development (Figs 1 and 4). The antioxidant acts as an inhibitor that reduces the activity of peroxidases at stages in which the leaves show the strongest growth (Figs 1, 2 and 4). The highest extracellular concentrations of ascorbate were observed at the earliest stages of leaf development and dropped abruptly close to the detection limit in 500 degree-day leaves (Fig. 4A), similar to values observed in the extracellular fluids from pea shoots (de Pinto & de Gara 2004). Since commercial ascorbate could cause a 50% reduction in sunflower peroxidase activity at micromolar concentrations (Fig. 3B), which coincides with values measured in isolated AFs (Fig. 4A), it is proposed that apoplastic ascorbate could act as an indirect regulator of growth. In agreement with the occurrence of the peroxidase and its inhibitor in sunflower AFs, methods that isolate or denature the enzyme could increase availability of the inhibitor, possibly explaining why some treatments, *e.g.* boiling, actually increased peroxidase inhibition (Fig. 3A). Similarly, AFs obtained from leaves at the end of their expansion had negligible peroxidase activity according to spectrophotometric assays (Fig. 1), which contrasted with the very high signal detected in the zymogram (Fig. 4B) as a result of separation of the inhibitor during electrophoresis. Therefore, the *in planta* inhibitor concentration in the apoplast appears to be sufficient to inhibit peroxidase present in the same compartment (Figs 3 and 4), supporting the contribution of this enzyme to the stiffening of the cell wall at the end of leaf expansion through the loss of extracellular ascorbate. However, the content of endogenous apoplastic substrates, *i.e.* H_2O_2 and phenolic compounds, should be assessed to obtain a more detailed picture of leaf growth control. In this regard, Ranieri *et al.* (2003) proposed that natural substrates, such as coniferyl and ferulic acids, are fine-tuned during the development of sunflower leaves and require micro-localised control, which would make construction of a model representing the *in vivo* conditions a complex task.

Ascorbate has previously been proposed as the natural substrate for extracellular peroxidases (Mehlhorn *et al.* 1996; de Pinto & de Gara 2004), despite ascorbate peroxidases being grouped in the intracellular type I subgroup of isoenzymes (Cosio & Dunand 2009). The *in vitro* inhibitory effect of ascorbic acid on extracellular peroxidases has also been characterised in samples obtained from epicotyls of *Vigna angularis* (Takahama 1993) and needles of Norway spruce (Otter & Polle 1994). In agreement with this role, it was shown that the level of ascorbic acid in the apoplast of tobacco leaves decreased during aging, while the activity of peroxidases simultaneously increased (Takahama *et al.* 1999). The occurrence

of apoplastic inhibitors of peroxidases, other than ascorbate, has also been reported. Burr & Fry (2009) reported a dramatic increase in the crosslinking of feruloylated arabinoxylans to the cell walls of maize after 8 days of cell culture, and proposed that an inhibitor would most likely be the regulator of the reaction, although the molecule was not identified. This proposal was based on the fact that the reaction depended on the activity of an extracellular peroxidase that was present at all cell culture ages. On the other hand, evidence of the *in vivo* ascorbate–peroxidase relationship in the apoplast has only been obtained in onion roots, where peroxidase activity and ascorbate were higher in the meristem and elongation zone than in the base of the root, in which there are no actively growing cells. Moreover, *in situ* detection of the enzyme showed that the meristem and elongation zone were not stained, and the intensity of the signal gradually recovered toward the base of the root (Cordoba-Pedregosa *et al.* 1996, 2005). Incubation with ascorbate or a precursor of its synthesis increased the cell elongation rate while reducing guaiacol and ferulic peroxidase activity (Cordoba-Pedregosa *et al.* 2005). Therefore, the present work is relevant in showing for the first time that *in planta* ascorbate concentrations in the apoplast of growing young leaves restrains the peroxidase co-localised in the same compartment. Subsequently, in organs with reduced growth rates, ascorbate level is reduced and relieves inhibition on the enzyme and contributes to wall fixation at the time of growth cessation.

In summary, *in vivo* and *in vitro* approaches support a role for ascorbate as an inhibitor of extracellular peroxidases in actively growing zones. Moreover, the reversible peroxidase–inhibitor interaction appears to be common in the control of both leaf and root growth. Our observations emphasise the significance of the post-translational regulation of specific soluble apoplastic peroxidase and complement novel approaches using microarray technology in peroxidase characterisation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Zymogram of apoplastic peroxidases.

Table S1. Effect of boiling on ascorbate stability and peroxidase inhibitory capacity.

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REFERENCES

Bode A.M., Rose R.C. (1999) Analysis of water-soluble antioxidants by high-performance liquid chromatography with electrochemical detection. *Methods in Enzymology*, **299**, 77–83.

Burr S.J., Fry S.C. (2009) Feruloylated arabinoxylans are oxidatively cross-linked by extracellular maize peroxidase but not by horseradish peroxidase. *Molecular Plant*, **2**, 883–892.

Conklin P., Barth C. (2004) Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant, Cell and Environment*, **27**, 959–970.

Cordoba-Pedregosa M.C., Gonzalez-Reyes J.A., Cañadillas M.S., Navas P., Cordoba F. (1996) Role of apoplastic and cell-wall peroxidase on the stimulation of root elongation by ascorbate. *Plant Physiology*, **112**, 1119–1125.

Cordoba-Pedregosa M.C., Cordoba F., Villalba J.M., Gonzalez-Reyes J.A. (2003) Zonal changes in ascorbate and hydrogen peroxide content, peroxidase, and ascorbate-related enzyme activities in onion roots. *Plant Physiology*, **131**, 697–706.

Cordoba-Pedregosa M.C., Villalba J.M., Cordoba F., Gonzales-Reyes J.A. (2005) Changes in intracellular and apoplastic peroxidase activity, ascorbate redox status, and root elongation induced by enhanced ascorbate content in *Allium cepa* L. *Journal of Experimental Botany*, **56**, 685–694.

Cosgrove D. (2005) Growth of the plant cell wall. *Nature Reviews*, **6**, 850–861.

Cosio C., Dunand C. (2009) Specific functions of individual class III peroxidase genes. *Journal of Experimental Botany*, **60**, 391–408.

Davey M.W., Van Montagum M., Inzé D., Sanmartin M., Kanellis A., Smirnoff N., Benzie I.J.J., Strain J.J., Favell D., Fletcher J. (2000) Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing. *Journal of the Science of Food and Agriculture*, **80**, 825–860.

De Tullio M.C., Paciolla C., Dalla Vechia F., Rascio N., D'Emerico S., de Gara L., Liso R., Arrigoni O. (1999) Changes in onion root development induced by the inhibition of peptidyl-prolyl hydroxylase and influence of the ascorbate system on cell division and elongation. *Planta*, **209**, 424–434.

Dragišić Maksimović J., Maksimović V., Živanović B., Hadži-Tašković Šukalović V., Vuletić M. (2008) Peroxidase activity and phenolic compound content in maize root and leaf apoplast, and their association with growth. *Plant Science*, **175**, 656–662.

Encina F., Fry S. (2005) Oxidative coupling of a feruloyl-arabinoxylan trisaccharide (FAXX) in the walls of living maize cells requires endogenous hydrogen peroxide and is controlled by a low-M_r apoplastic inhibitor. *Planta*, **223**, 77–89.

Foyer C.H., Noctor G. (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell*, **17**, 1866–1875.

Fry S.C. (1987) Formation of isodityrosine by peroxidase isozymes. *Journal of Experimental Botany*, **38**, 853–862.

Fry S.C. (1998) Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemical Journal*, **332**, 507–515.

Fry S.C., Dumville J.C., Miller J.G. (2001) Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit. *Biochemical Journal*, **357**, 729–737.

Fry S.C., Miller J.G., Dumville J.C. (2002) A proposed role for copper ions in cell wall loosening. *Plant and Soil*, **247**, 57–67.

Gazaryan I.G., Lagrimini L.M., Ashby G., Thorneley A.R.F. (1996) Mechanism of indole-3-acetic acid oxidation by plant peroxidases: anaerobic stopped-flow spectrophotometric studies on horseradish and tobacco peroxidases. *Biochemical Journal*, **313**, 841–847.

Granier C., Tardieu F. (1998) Is thermal time adequate for expressing the effects of temperature on sunflower leaf development? *Plant, Cell and Environment*, **21**, 695–703.

Hadži-Tašković Šukalović V., Kukavica B., Vuletić M. (2007) Hydroquinone peroxidase activity of maize root mitochondria. *Protoplasma*, **231**, 137–144.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Liem H.H., Cardenas F., Tavassoli M., Poh-Fitzpatrick M.G., Muller-Eberhard U. (1979) Quantitative determination of hemoglobin and cytochemical staining for peroxidase using 3, 3′, 5, 5′ tetramethylbenzidine dihydrochloride, a safe substitute for benzidene. *Analytical Biochemistry*, **98**, 388–393.

Lin C.C., Kao C.H. (2001) Cell wall peroxidase against ferulic acid, lignin, and NaCl-reduced root growth of rice seedlings. *Journal of Plant Physiology*, **158**, 667–671.

Liszkay A., Zalm E., Schopfer P. (2004) Production of reactive oxygen intermediates ($O_2^{\cdot-}$, H_2O_2 and OH) by maize roots and their role in wall loosening and elongation growth. *Plant Physiology*, **136**, 3114–3123.

Luwe M.W.F., Takahama U., Heber U. (1993) Role of ascorbate in detoxifying ozone in the apoplast of

spinach (*Spinacia oleracea*) leaves. *Plant Physiology*, **101**, 969–976.

MacAdam J.W., Nelson C.J., Sharp R.E. (1992) Peroxidase activity in the leaf elongation zone of tall fescue. *Plant Physiology*, **99**, 294–878.

Mehlhorn H., Lelandais M., Korth H.G., Foyer C.H. (1996) Ascorbate is the natural substrate for plant peroxidases. *FEBS Letters*, **378**, 203–206.

Müller K., Linkies A., Vreeburg R.A.M., Fry S.C., Krieger-Liszkay A., Leubner-Metzger G. (2009) *In vivo* cell wall loosening by hydroxyl radicals during cress (*Lepidium sativum* L.) seed germination and elongation growth. *Plant Physiology*, **150**, 1855–1865.

O'Looney N., Fry S.C. (2005) Oxaziclomefone, a new herbicide, inhibits wall expansion in maize cell-cultures without affecting polysaccharide biosynthesis, xyloglucan transglycosylation, peroxidase action or apoplastic ascorbate oxidation. *Annals of Botany*, **96**, 1097–1107.

Otter T., Polle A. (1994) The influence of apoplastic ascorbate on the activities of cell-wall associated peroxidase and NADH oxidase in needles of Norway spruce (*Picea abies* L.). *Plant and Cell Physiology*, **35**, 1231–1238.

Passardi F., Penel C., Dunand C. (2004) Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends in Plant Science*, **9**, 534–540.

Pastori G.M., Kiddle G., Antoniw J., Bernard S., Veljovic-Jovanovic S., Verrier P.J., Noctor G., Foyer C.H. (2003) Leaf vitamin C content modulates plant defense transcripts and regulates genes that control development through hormone signaling. *The Plant Cell*, **15**, 939–951.

Pereyra-Irujo G.A., Velazquez L., Lechner L., Aguirrezábal L.A.N. (2008) Genetic variability for leaf growth rate and duration under water deficit in sunflower: analysis of responses at cell, organ, and plant level. *Journal of Experimental Botany*, **59**, 2221–2232.

Pignocchi C., Kiddle G., Hernández I., Foster S.J., Asensi A., Taybi T., Barnes J., Foyer C.H. (2006) Ascorbate-oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. *Plant Physiology*, **141**, 423–435.

Pinedo M., Segarra C., Conde R. (1993) Occurrence of two endoproteinases in wheat leaf intercellular washing fluid. *Physiologia Plantarum*, **88**, 287–293.

Pinedo M., Regente M., Elizalde M., Quiroga I.Y., Pagnussat L.A., Jorrín-Novo J., Maldonado A., de la Canal L. (2012) Extracellular sunflower proteins: evidence on non-classical secretion of a jacalin related lectin. *Protein and Peptide letters*, **19**, 270–276.

de Pinto M.C., de Gara L. (2004) Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation. *Journal of Experimental Botany*, **55**, 2559–2569.

Potters G., Horemans N., Caubergs R.J., Asard H. (2000) Ascorbate and dehydroascorbate influence cell cycle progression in a tobacco cell suspension. *Plant Physiology*, **124**, 17–20.

Ranieri A., Castagna J., Pacini B., Baldan A., Mensuali S., Soldatini G.F. (2003) Early production and scavenging of hydrogen peroxide in the apoplast of sunflower plants exposed to ozone. *Journal of Experimental Botany*, **54**, 2529–2540.

Sánchez M., Revilla G., Zarra I. (1995) Changes in peroxidase activity associated with cell walls during pine hypocotyl growth. *Annals of Botany*, **75**, 415–419.

Smirnoff N. (2000) Ascorbic acid: metabolism and functions of a multi-faceted molecule. *Current Opinion in Plant Biology*, **3**, 229–235.

de Souza I., MacAdam J. (1998) A transient increase in apoplastic peroxidase activity precedes decrease in elongation rate of B73 maize (*Zea mays*) leaf blades. *Physiologia Plantarum*, **104**, 556–562.

Stasolla C., Yeung E.C. (2007) Cellular ascorbic acid regulates the activity of major peroxidases in the apical poles of germinating white spruce (*Picea glauca*) somatic embryos. *Plant Physiology and Biochemistry*, **45**, 188–198.

Takahama U. (1993) Regulation of peroxidase-dependent oxidation of phenolics by ascorbic acid: different effects of ascorbic acid on the oxidation of coniferyl alcohol by the apoplastic soluble and cell wall-bounded peroxidases from epicotyls of *Vigna angularis*. *Plant and Cell Physiology*, **34**, 809–817.

Takahama U. (2004) Oxidation of vacuolar and apoplastic phenolic substrates by peroxidase: physiological significance of the oxidation reactions. *Phytochemistry Reviews*, **3**, 207–219.

Takahama U., Oniki T. (1992) Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. *Plant and Cell Physiology*, **33**, 379–387.

Takahama U., Hirotsu M., Oniki T. (1999) Age-dependent changes in levels of ascorbic acid and chlorogenic acid, and activities of peroxidase and superoxide dismutase in the apoplast of tobacco leaves: mechanism of the oxidation of chlorogenic acid in the apoplast. *Plant and Cell Physiology*, **40**, 716–724.

Welinder K.G., Gajhede M. (1993) Structure and evolution of peroxidases In: Welinder K.G., Rasmussen S.K., Penel C., Greppin H. (Eds), *Plant peroxidases: biochemistry and physiology*. University of Geneva, Geneva, pp 35–42.