Iron triggers a rapid induction of ascorbate peroxidase gene expression in *Brassica napus*

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Abstract In plants, only ferritin gene expression has been reported to be iron-dependent. Here it is demonstrated that an iron overload of *Brassica napus* seedlings causes a large and rapid accumulation of ascorbate peroxidase transcripts, a plant-specific hydrogen peroxide-scavenging enzyme. This result documents a novel link between iron metabolism and oxidative stress. The ascorbate peroxidase mRNA abundance was not modified by reducing agents like *N*-acetyl cysteine, glutathione and ascorbate or by pro-oxidants such as hydrogen peroxide or diamide. Furthermore, the iron-induced ascorbate peroxidase mRNA accumulation was not antagonized by *N*-acetyl cysteine. Abscisic acid had no effect on the ascorbate peroxidase gene expression. Taken together these results suggest that iron-mediated expression of ascorbate peroxidase gene occurs through a signal transduction pathway apparently different from those already described for plant genes responsive to oxidative stress.

Key words: Iron; Ascorbate peroxidase; Oxidative stress; *Brassica napus*

1. Introduction

Plants, like all aerobic organisms, are exposed to reactive oxygen species which are by-products of oxygen consumption. Reactive oxygen species are responsible for protein, lipid and nucleic acid degradation and are thought to play a major role in aging, cell death [1–3], and possibly also in cancer [4]. To avoid the accumulation of these compounds to toxic level, animals and plants possess several detoxifying enzymatic systems. While superoxide dismutases and catalases are found in animals, plants and microorganisms, ascorbate peroxidase (APX, EC 1.11.1.11) is a plant-specific hydrogen peroxide (H_2O_2)-scavenging enzyme [5]. APX isoforms are encoded by a nuclear multigene family and are found in various subcellular compartments. Ascorbate peroxidase has been detected in both the cytosol and chloroplasts [5]. More recently a pumpkin peroxisomal [6] and a cotton glyoxysomal [7] APX have been isolated.

The activities of antioxidant enzymes such as ascorbate peroxidase, catalases and superoxide dismutases are up-regulated in response to several abiotic stresses such as drought [8], low temperature [9], high light intensities [10], ozone, SO_2 and UV-B [11] and salinity [12]. All these adverse environmental conditions have a common consequence, i.e., the generation of oxidative stress in plants. Another potential source of oxidative stress is free iron because it can react with reduced forms of oxygen leading to the formation of highly reactive oxygen species such as the hydroxyl radical produced in the Fenton reaction.

Indeed increasing attention has focussed recently on the integration of oxygen and iron metabolisms [13]. If iron is a true inducer of oxidative stress, it would be advantageous for cells to adjust their antioxidant defenses with the level of free iron. Indirect evidence for such a regulatory system have been obtained in bacteria, yeast and mammals. For example, in *E. coli*, the genes coding for Fe uptake and two superoxide dismutases (MnSOD and FeSOD) are regulated by the same transcription factors [14,15]; in the yeast *Saccharomyces cerevisiae*, the transcription factor MAC1 is required for both the expression of a Fe^{3+} reductase and a cytosolic catalase [16]; in mammalian cells H_2O_2 is able to activate the regulator IRP1, responsible for the post-transcriptional control of the synthesis of proteins involved in iron homeostasis [17]. In plants, recent evidence suggest that iron can lead to oxidative stress responses. In *Nicotiana plumbaginifolia*, an iron excess resulted in the conversion of antioxidant molecules such as glutathione and ascorbate to their oxidized forms [18]. In addition, iron loading of *Nicotiana plumbaginifolia* leaves led to an increase in catalase and ascorbate peroxidase activities [18]. However, the only documented example of plant gene expression activated by iron through a major transcriptional control is ferritin, an iron-storage protein localized within the plastids (for a review, see Ref. [19]). In maize, ferritin mRNA accumulation is induced by iron [20] as a result of an oxidative stress [21], and this has been confirmed recently in *A. thaliana* [22].

The aim of this work was to find out if an iron overload could result in an increased expression of genes that participate in the antioxidant responses of plants. Ascorbate peroxidase was selected as a putative candidate for the following reasons. First, APX is an iron-containing protein [5]; therefore iron availability could regulate the expression of *apx* gene(s). Indeed, an iron-deficiency in *Euglena gracilis* has been shown to result in the complete loss of ascorbate peroxidase activity [23]. Second, iron and ascorbate metabolisms are intimately linked because an iron-mediated oxidative stress triggers both ferritin accumulation [21] and ascorbate oxidation to dehydroascorbate [18], a condition favoring iron uptake by plant ferritin [24,25]. Third, a protein sharing 71% homology in its N-terminal sequence with the cytosolic form of ascorbate peroxidase from *Arabidopsis thaliana,* is accumulated in the tomato *chloronerva* mutant as shown by 2D electrophoresis analysis [26]. This mutant is defective in nicotian-

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Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; DTT, dithiothreitol; NAC, *N*-acetyl cysteine; SOD, superoxide dismutase

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amine synthesis and accumulates abnormally high levels of copper and iron, enhancing free radical production [26,27].

2. Materials and methods

2.1. Plant growth conditions

Rapeseed (*Brassica napus*, cv Drakkar) were purchased from Ringot S.A. (La Chapelle d'Armentières, France). They were surface-sterilized with 1% sodium hypochlorite. Hydroponic culture was carried out in a growth chamber set at 25/20°C day/night temperature and a 14 h photoperiod with light (600 mmol m^{-2} s^{-1} PAR) according to the following schedule. Seeds were germinated for 72 h in water; then the plantlets were supplied with Hoagland's solution lacking iron for 48 h. The roots were cut at about 1 cm from the bottom of hypocotyls and the de-rooted seedlings were subjected to various treatments as described in the text. Exposure to Fe(III)-citrate started 2 h after the onset of light which means that with the exception of the 24 h treatment, iron was supplied only during the light period. For each experiment about 50–100 seedlings were used and at least three independent extractions were carried out. Fe(III)-citrate solution was prepared as described previously [21].

2.2. RNA extraction and analysis

RNA was extracted from cotyledons (2–5 g) of young *Brassica napus* seedlings. For Northern blot analysis total RNA was prepared using the guanidinium-isothiocyanate procedure [28] with modifications described previously [29]. Equal amounts (10 μg) of RNA were denatured in 20 mM Tris-acetate (pH 7.0) containing 50% (v/v) dimethyl sulfoxide and 1 M glyoxal for 20 min at 50°C. The RNA samples were then fractionated on 1.2% agarose gels and transferred to nylon membranes (Positive; Appligene, Illkirch, France) according to the recommendations of the manufacturer. Equal RNA loading and transfer were checked by short-wave (254 nm) UV examination of the blots. Pre-hybridization and hybridization conditions were as described previously [29]. A *Brassica napus* cytosolic *apx* cDNA was isolated by screening a rapeseed cDNA library with a *Raphanus sativus apx* cDNA previously characterized in our laboratory [12]. This *B. napus* ascorbate peroxidase cDNA (EMBL accession number Y11461) exhibited a strong homology with both the radish [12] and the *A. thaliana* [30] cytosolic *apx* cDNAs. Fluoresceine-labelled probes were generated by PCR reactions using the cloned ascorbate peroxidase *B. napus* cDNA or the radish *rbcS* cDNA [31] as templates, with the appropriate oligonucleotide primers. Blots were then washed in 2×SSC (1×SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% (w/v) SDS at 65°C for 2 times 15 min and in 0.2×SSC, 0.1% SDS at 60°C for 2 times 15 min. Blots were incubated with anti-fluoresceine antibodies coupled to alkaline phosphatase and finally with chemiluminescent substrate (CDP-star, Tropix, Bedford, USA). When appropriate, for positive controls of iron and ABA-induced gene expression, the blots were probed with a *A. thaliana* ferritin cDNA (*AtFer1*)[22] or the *A. thaliana rab 18* cDNA, respectively, labelled as described above [32].

3. Results

3.1. Iron-induced apx mRNA accumulation in rapeseed cotyledons

The effect of iron excess on the accumulation of ascorbate peroxidase transcripts in rapeseed cotyledons was analysed. Removing the roots is a convenient way to increase the Fe uptake in plants [18,21]. When 5-day-old de-rooted rapeseed plantlets were supplied with 500 μM Fe(III)-citrate (the main form of iron in the xylem sap [33]) for periods up to 24 h, a rapid accumulation of *apx* transcripts was observed (Fig. 1a). The *apx* mRNA level was very low in cotyledons of control seedlings grown in the absence of iron. The accumulation could be detected within 1–3 h after the exposure to Fe(III)-citrate. The induction was maximal after 6 h of iron loading and remained elevated for at least 24 h. As a control, the RNA blots were also probed with a cDNA coding for the small subunit of radish RubisCO [31]. The level of *rbcS* mRNA remained unchanged during the period investigated (Fig. 1a). The excision itself had no effect on *apx* mRNA abundance during the first 6 h and a slight increase in the *apx* mRNA level in the control plants was observed 24 h after removing of the roots (Fig. 1a). Thus *apx* gene expression is not induced by wounding, at least not systemically. Fe(III)-EDTA treatment was also able to induce an increase in *apx* mRNA abundance, as observed with Fe(III)-citrate, whereas citrate or EDTA alone had no effect, demonstrating that the inducer was iron itself and not the chelators (data not shown). No toxicity symptoms were observed following the Fe(III)-citrate treatments. Moreover when excised plantlets exposed to 500 μM Fe(III)-citrate for 6 h were then transferred to standard Hoagland media they were able to regenerate roots and grow like control plants (data not shown). In contrast, a 24 h Fe(III)-EDTA treatment resulted in the appearance of leaf necrotic spots (data not shown), as already reported for *Nicotiana plumbaginifolia* [18].

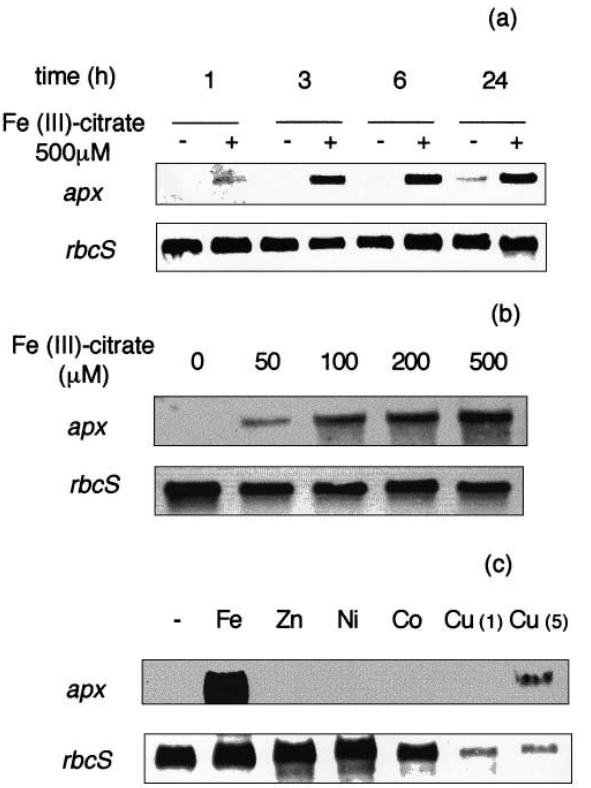

Fig. 1. Iron-induced *apx* mRNA accumulation in *Brassica napus* cotyledons. *B. napus* seedlings were grown in the absence of iron; the roots were excised and the plantlets were transferred to water containing iron or other metals at the indicated concentrations. RNA was extracted and subjected to Northern blot analysis with either the *apx* or *rbcS* probes. a: Time course of iron induction of *apx* mRNA accumulation. b: Effect of treatments with Fe(III)-citrate at indicated concentrations for 3 h. c: Effect of different metals on *apx* mRNA abundance. De-rooted plantlets were exposed to water (−), Fe(III)-citrate 500 μM (Fe), $ZnSO_4$ 5 mM (Zn), $NiCl_2$ 5 mM (Ni), $CoCl_2$, 5 mM (Co), $CuSO_4$ 1 mM (1) or 5 mM (5) for 6 h. Northern presented are representative of at least 3 independent experiments.

The accumulation of *apx* transcripts was clearly dependent on the iron-citrate concentration in the range of 0–500 μM Fe(III)-citrate (Fig. 1b). Moreover, the induction of *apx* ex-

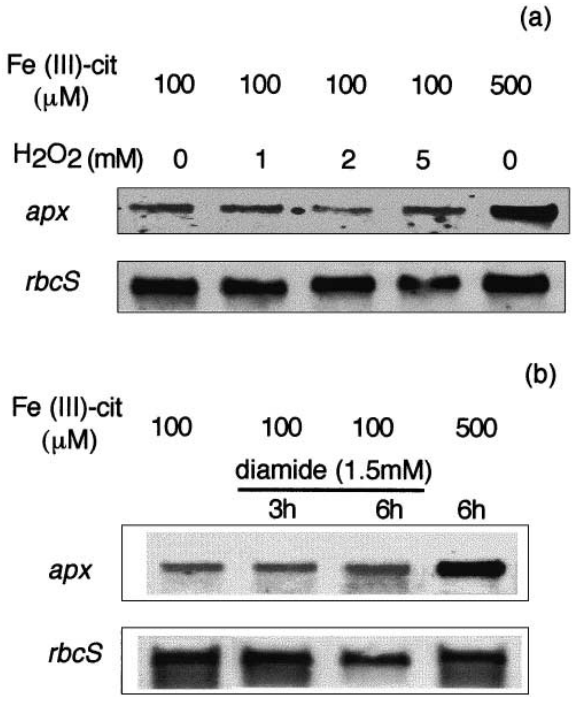

Fig. 2. Effect of H_2O_2 on *apx* mRNA abundance. a: De-rooted *B. napus* seedlings were exposed to 100 μM Fe(III)-citrate and increasing concentrations of H_2O_2 for 6 h. A positive control (500 μM Fe(III)-citrate, 6 h) is presented on the right. b: De-rooted *B. napus* seedlings were exposed to 1.5 mM diamide for 3 or 6 h in presence of 100 μM Fe(III)-citrate; on the left: control minus diamide and a positive control (500 μM Fe(III)-citrate, 6 h) is presented on the right.

pression seems to be iron-specific since other transition or heavy metals such as zinc, nickel and cobalt had no effect (Fig. 1c); the *rbcS* mRNA level was also unaffected by these treatments. The exposure of de-rooted seedlings with copper ($CuSO_4$) at the concentration of 1 mM had no effect, but at 5 mM a slight increase of *apx* mRNA accumulation was observed. However, this effect was relatively weak in comparison with that obtained with a 10-fold lower concentration of iron (500 μM) whereas a clear drop in the *rbcS* mRNA occurred in the $CuSO_4$-treated seedlings (Fig. 1c). The down-regulation of the *rbcs* gene by copper is a good indication that this element has been translocated to the leaves (see Section 4).

3.2. *Is apx expression responsive to oxidative stress?*

In order to analyse whether the observed iron-induced *apx* mRNA accumulation involves the participation of H_2O_2, de-rooted *B. napus* seedlings were treated with H_2O_2 for 6 h in the presence of a non-saturating Fe(III)-citrate concentration (100 μM). Increasing concentrations of H_2O_2 from 1 to 5 mM did not increase the abundance of *apx* mRNA and *rbcS* mRNA levels were not affected by this treatment (Fig. 2). The effect of another pro-oxidant molecule, diamide, a thiol-oxidizing drug [34] on *apx* expression was also investigated. Fig. 2b shows that exposure of *B. napus* seedlings to 1.5 mM diamide for 3–6 h, in the presence of 100 μM Fe(III)-citrate, also had no effect on the *apx* mRNA abundance.

The induction of ferritin gene expression by iron can be blocked by antioxidants such as *N*-acetyl cysteine (NAC) or glutathione [21,22]. In contrast, cytosolic *Cu/Zn superoxide dismutase* gene is activated by thiol compounds [35]. It was therefore of interest to examine the effect of several reducing agents on *apx* expression in presence or absence of an excess of iron. In a first set of experiments, rapeseed seedlings were de-rooted, exposed to various NAC concentrations for 3 h and finally, Fe(III)-citrate (500 μM) was added for a further 3 h period. The level of *apx* mRNA was not decreased by the NAC pre-treatment (Fig. 3a) and the iron-induced *apx* expression occurred as observed previously (see Fig. 1). The pre-treatment of de-rooted seedlings with reduced glutathione (1–2 mM) or ascorbate (1–10 mM) gave the same results (data not shown). As a positive control the blots were also probed with *AtFer1*, a cDNA encoding an *A. thaliana* ferritin. As expected ferritin expression was induced by iron in *B. napus* and this iron-induced expression was abolished by the NAC pre-treatment (Fig. 3a) as observed in maize [21] and *A. thaliana* [22]. Also, this control indicates that the antioxidant NAC has been translocated to the leaves (see Section 4).

The effect of several antioxidants alone, i.e., without iron loading, was also investigated. The result is depicted in Fig. 3b: NAC, reduced or oxidized glutathione (1–2 mM), reduced or oxidized ascorbate (1–10 mM) were unable to promote an accumulation of *apx* transcripts.

3.3. *apx expression is not mediated by ABA*

It has been suggested that antioxidant gene expression

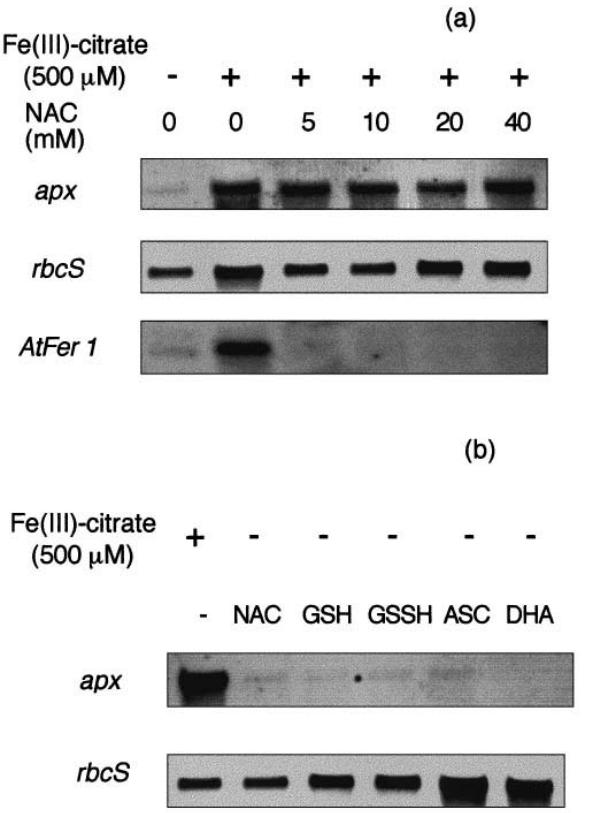

Fig. 3. Effects of antioxidants on the accumulation of *apx* mRNA. a: De-rooted *B. napus* seedlings were pre-treated with increasing concentrations of NAC for 3 h then Fe(III)-citrate was added (500 μM, final concentration) for a further 3 h; on the left: non-induced control seedlings. *AtFer1*: Blot probed with the *A. thaliana* ferritin cDNA (see text). b: NAC (10 mM), glutathione (2 mM) reduced (GSH) or oxidized (GSSH), ascorbate (2 mM) reduced (ASC) or oxidized (DHA) were given to de-rooted seedlings for 6 h; on the left: control seedlings induced with 500 μM Fe(III)-citrate for 3 h.

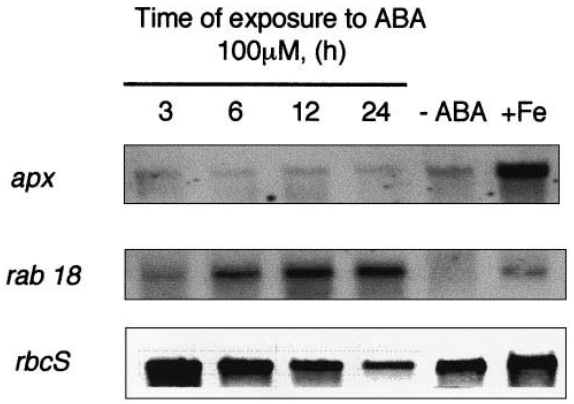

Fig. 4. Effect of ABA on *apx* mRNA levels. De-rooted *B. napus* seedings were subjected to 100 μM ABA for periods varying from 3 to 24 h; −ABA: control without ABA; +Fe: positive control (500 μM Fe(III)-citrate, 6 h). *rab 18*: Northern blot hybridized with a probe to the *A. thaliana* ABA-responsive gene.

could be mediated by ABA [36]. In addition, one of the maize ferritin genes, namely *ZmFer2*, is activated in response to iron through a transduction pathway involving ABA as a cellular relay [37,38]. It was therefore of interest to investigate whether ABA promoted *apx* expression. ABA (100 μM) was supplied to de-rooted *B. napus* seedlings for 3–24 h. The RNA blots were hybridized with *apx*, *rbcs* and *rab18* probes. Fig. 4a shows that ABA had no effect on the *apx* mRNA level while it caused a decline in *rbcS* transcripts and stimulated the accumulation of *rab 18* mRNA. Furthermore the effect of various ABA concentrations was investigated. No induction of *apx* expression was observed when a range of ABA concentrations (10–100 μM) were supplied for 6 h to *B. napus* seedlings (data not shown).

4. Discussion

Iron plays a dual role in cellular metabolism. As a constituent of metalloproteins (e.g., cytochromes), it is essential for enzymatic reactions involving electron transfer. In contrast, iron can catalyse the production of the highly reactive hydroxyl radicals from H_2O_2 through the Fenton reaction. Consequently, an excess of free iron can lead to the accumulation of hydroxyl radicals to a lethal level. To avoid the occurrence of a large pool of free iron, animal and plant cells sequester iron in specialized structures such as ferritins. The expression of ferritin genes is controlled by iron in both animals and plants; however, while the main level of control is translational in animals [39], it is transcriptional in plants [40], resulting in the accumulation of ferritin mRNA in maize and *Arabidopsis thaliana* in response to an excess of iron [21,22]. This study was undertaken to investigate whether an iron excess could regulate the expression of an antioxidant enzyme, namely ascorbate peroxidase.

For the investigation of the effect of several potential inducers of *apx* gene expression a system with a low level of expression was advantageous. The abundance of *apx* transcripts is very low in the cotyledons of young *B. napus* seedlings in comparison to that in mature leaves (Lopez et al., unpublished data), so this system was chosen for further studies. In dicots the uptake of iron implies several steps involving the reduction of Fe^{3+} to Fe^{2+}. A convenient way to allow a rapid iron uptake is to cut off most of the root system so that the iron loading of leaves can occur through the transpiration flux [18,21]. No visible symptoms of injury (necrotic spots) were detectable in *Brassica napus* seedlings treated in this way during the time course of experiments, i.e., up to 24 h. Moreover, when treated plants were returned to Hoagland culture medium, their roots regenerated. In addition, the phenotypes of iron-treated or untreated plants were identical at all the developmental stages. In contrast, in *Nicotiana plumbaginifolia* leaves, necrotic spots developed rapidly when excised-root plantlets were infiltrated with Fe(III)-EDTA [18]. Indeed, when *B. napus* seedlings were exposed to Fe-EDTA, we also observed necrotic spots which appeared after 24 h of treatment. Chelation of iron with citrate appears, therefore, to be less toxic for plants.

Apart from ferritin mRNA in soybean [40], maize [20] or *A. thaliana* [22], no other iron-induced increase in mRNA abundance has been described in plants to date. In the iron-starved control seedlings the *apx* transcripts were hardly detectable but when de-rooted *Brassica napus* seedlings were exposed to a solution of Fe(III)-citrate (500 μM), a rapid and large increase of the *apx* mRNA level occurred. It was not possible to quantify the level of induction because the *apx* transcript abundance in untreated plants was below detection limits.

The triggering of *apx* expression by iron was also clearly demonstrated by using increasing amounts of Fe(III)-citrate. A progressive accumulation of *apx* mRNA was recorded when the iron concentration was raised from 50 to 500 μM. Consistent with our results are the following observations. First, the activities of ascorbate peroxidase and catalase are doubled in the leaves of de-rooted *Nicotiana plumbaginifolia* incubated in 100 μM Fe-EDTA for 12 h [18]. Secondly, ascorbate peroxidase is one of the overexpressed protein in the tomato mutant *chloronerva* which accumulates abnormally high iron and copper levels [26].

Furthermore the *apx* mRNA accumulation was shown to be iron-specific. Transition or heavy metals such as Zn, Ni or Co had no effect on the *apx* gene expression. Although there is no direct evidence for leaf loading of these metals, it is reasonable to assume that they are transported via the transpiration stream, since the salts used in this study are water soluble. Copper induced a slight increase in *apx* transcripts at the relatively high concentration of 5 mM, i.e., 10-fold higher than that of Fe(III)-citrate which induces a massive accumulation of *apx* mRNA. Interestingly, the *rbcS* mRNA level was significantly decreased in the Cu-treated seedlings. This is consistent with the fact that Cu has been shown to affect several components of the chloroplast machinery [41]. The observed up- and down-regulation of *apx* and *rbcs* transcript levels, respectively, implies that copper has been efficiently translocated to the leaves through the transpiration stream.

We did not observe any stimulation of *apx* mRNA accumulation in *B. napus* seedlings treated with antioxidants such as NAC, glutathione, or ascorbate. Using a transient expression system in tobacco protoplasts, it has been shown that the promoter of the cytosolic Cu/Zn *sod* was inducible by reduced glutathione, DTT and NAC above 1 mM [35]. Our data shows that under our experimental conditions *apx* expression is not activated by reducing agents suggesting that the *apx* promoter possesses control elements different to those present in the *sod* promoter. In addition, antioxidants such as NAC failed to inhibit the iron activation of *apx* gene(s). Thus, the signalling pathway of *apx* expression triggered by iron differs

from that of ferritin since the iron-induction of ferritin synthesis is abolished by NAC in *B. napus* seedlings (Fig. 3a) as well as in maize [21] or *A. thaliana* [22]. The lack of effect on *apx* transcript levels in leaves, by the antioxidants tested, could be a result of their inefficient loading. In the case of NAC, however, an inhibition of the iron-induced ferritin synthesis was observed indicating that at least this compound was efficiently loaded (Fig. 3a); in addition, it has previously been shown that NAC does not alter iron loading in *A. thaliana* [22].

The best understood cause for iron toxicity is its ability to catalyse the formation of the very toxic hydroxyl radical from superoxide and H_2O_2. It has been proposed that an iron-mediated oxidative stress triggers the activation of some ferritin genes [21,22]. Since it is important for the cells to scavenge H_2O_2, one could hypothesize that H_2O_2 would induce the expression of ascorbate peroxidase. However we found that exogenous H_2O_2 did not stimulate the *apx* expression. A similar result has been obtained in *A. thaliana* infiltrated with H_2O_2 up to high concentrations (40 mM) [42]. The treatment of *B. napus* seedlings with the thiol-oxidising compound diamide, used at 1.5 mM, also had no effect on *apx* expression. Although both H_2O_2 and diamide are not inducers of *apx* mRNA accumulation, one cannot exclude that other pro-oxidants might regulate the *apx* gene expression. Such a situation has already been observed in yeast: the expression of *yap1* and *trx2* are strongly activated by H_2O_2 and diamide, but not by t-BOOH or paraquat [34]. Alternatively, the iron-induced *apx* gene expression might be unrelated to oxidative stress as iron treatments were performed using iron-starved plantlets. Since this element is required for APX holoenzyme synthesis the effect of iron on *apx* transcript levels could be due to this synthetic requirement rather than to oxidative stress.

Exogenous ABA did not promote *apx* mRNA accumulation, although the ABA treatment resulted in the induction of *rab 18* expression, a gene known to be ABA responsive [32]. Moreover the *rbcS* mRNA level declined after a 6 h exposure to ABA. This latter observation is in agreement with previously reported ABA-mediated down-regulation of *rbcS* gene expression in bean [43], tomato [44], and *Lemna gibba* [45]. Our data is in apparent contradiction with a previous report where ABA was shown to induce *apx* mRNA accumulation in *Pisum sativum* [46]; however, this effect was obtained with a high ABA concentration (1 mM) which is 10-fold that usually employed in physiological studies. Also the plant systems are different (cotyledons vs. mature leaves) and therefore might not respond to ABA to the same extent.

The fact that ascorbate peroxidase is encoded by a multigene family raises the question of which *apx* genes are regulated by iron. In the present work a probe corresponding to a putative cytosolic form of APX has been used but currently it cannot be ruled out that this probe may hybridize with other *apx* transcripts. The recent isolation of a chloroplastic *apx* cDNAs from spinach [47], and a glyoxysomal *apx* cDNA from cotton [7] is promising for the acquisition of molecular tools that will allow the exact nature of the iron-induced ascorbate peroxidase mRNA accumulation to be determined.

In conclusion, in this work we have shown that ascorbate peroxidase gene expression can be controlled by iron at the level of mRNA accumulation. Until now, ferritin has been the only known plant gene with an iron-mediated expression. The fact that the expression of ascorbate peroxidase, an enzyme participating in the scavenging of reactive oxygen species, is iron-dependent provides an attractive model with which to decipher the molecular links between transition metal metabolism and oxidative stress in plants. Another novel aspect of this work is that the accumulation of *apx* mRNA, although sharing some kinetics and dose-responsive iron-induced gene expression with ferritin, appears to be regulated by a specific pathway which remains to be elucidated.

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