Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
COLTURE ARBOREE ED AGROSISTEMI FORESTALI,
ORNAMENTALI E PAESAGGISTICI

Ciclo XXII

Settore scientifico-disciplinare di afferenza: AGR/03-Colture Arboree

ROLE OF REACTIVE OXYGEN AND NITROGEN SPECIES IN THE RESPONSE OF *Prunus spp.* TO BICARBONATE

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Esame finale anno 2010

INDEX

1 INTRODUCTION

Plant stress can be defined from a physiological point of view, as any factor altering the homeostatic equilibrium of the plant; on the agricultural side, it is measured as the economic loss due to the same factor. Based on the nature of its causes, a major distinction is made between biotic and abiotic stress. The first case includes the interactions with living organisms and viruses, which use the plant as a source of food or a means for their reproduction. The abiotic stresses are nonoptimal growth conditions, such as drought, salinity, chilling temperatures, nutritional deficiency or pollutants, which prevent the plant to express its full genetic potential, in terms of carbon fixation or production. The severity of a stressing agent depends on the evolutionary background of the plant species or variety.

Homeostasis is achieved by means of an electron flux, through tightly controlled redox-exchange pathways. Since plants use sunlight as the primary energy source to activate the redox cascade, stressing factors will limit the efficiency of the process, causing, as a final result, an increased electron leakage towards acceptors which do not take part in the cascade. Among such acceptors, oxygen and nitrogen compounds emerge for several reasons: (1) their formation cannot be completely avoided; (2) their high reactivity would compromise the plant's metabolic apparatuses, in case of deregulated production; (3) their controlled formation has been adopted, during plant evolution, as a protective or signalling mechanism in stress responses.

1.1 Reactive oxygen species in abiotic stress

Abiotic stress is considered the main limiting factor in crops yield, which can be reduced by more than 50% worldwide [*144*]. In contrast with the resistance to pathogens, the tolerance to abiotic stresses is essentially a quantitative trait; yet, from the understanding of its physiology, some pivot signals and genes emerge as possible targets for the genetic improvement of crops, or the remediation and prevention of a stress instance.

In field conditions, several abiotic stresses are often combined together, potentially leading to conflicting responses (heat and drought on stomata opening) or synergistic effects (drought and heavy metals). Adaptation to stressing conditions is an active process, which may be impaired by nutritional deprivation or low availability of key elements or cofactors.

A transcriptomic and metabolite analysis in *Arabidopsis* evidences only a partial overlapping between heat stress, drought stress, and a combination of the two. Carbohydrates catabolism is likely to have an outstanding role in the process, since photosynthesis slows down and starch degradation is required for the production of energy and protective compounds [*155*; *190*].

Abscisic acid (ABA) is known to be implicated in many stress responses, leading to a regulation of the hydric balance, and inducing specific gene sets. In some cases, distinct ABA-dependent and ABA-independent signalling pathways can be distinguished in the response to the same stress [*227*; *244*]. A coarse classification can be made between early and late induced genes. Early genes respond within minutes and include transcription factors and intermediates of the signalling cascade, while late genes lead to the accumulation of protective products to cope with a chronic stress [*144*].

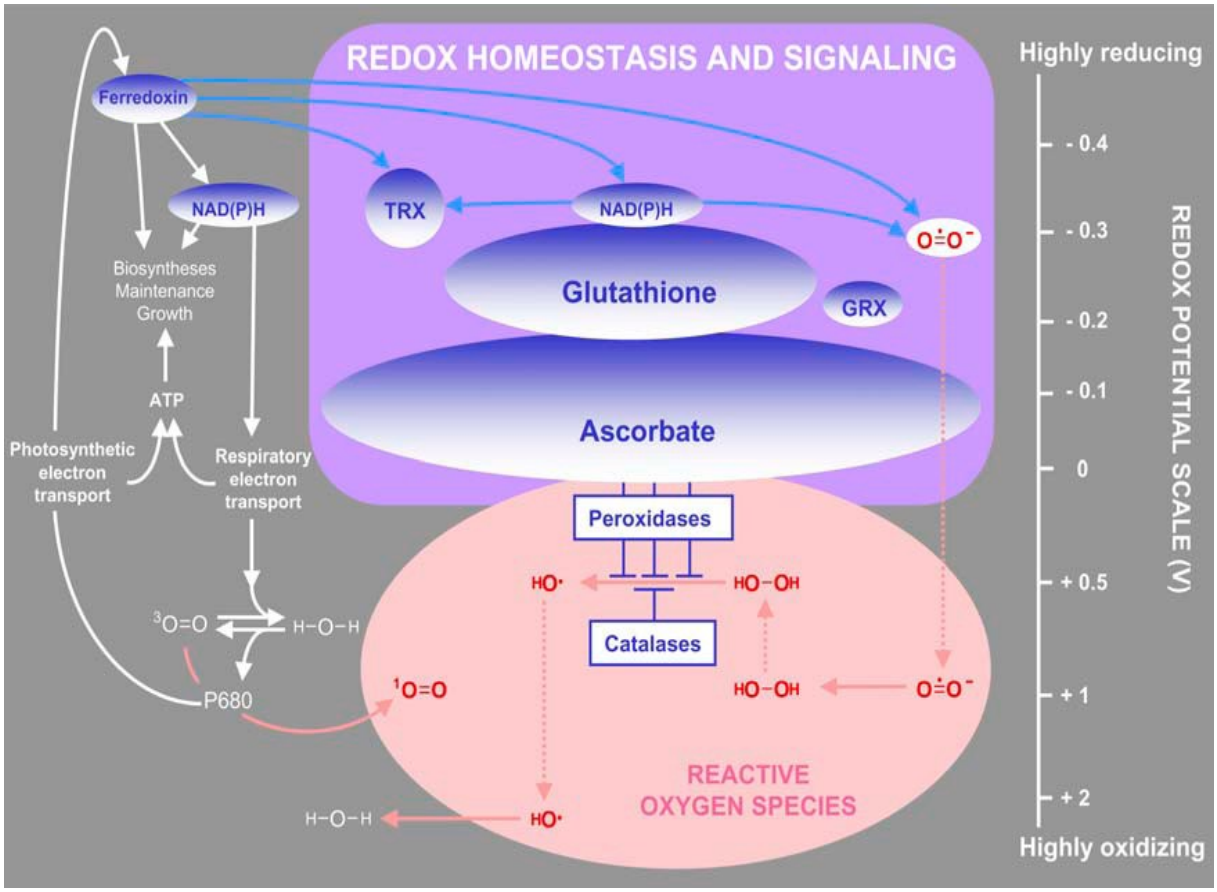

Figure 1.1: Resume of the interactions among oxidative species and redox buffers [75].

1.1.1 ROS production

Abiotic stresses are associated with an unbalance in the electron flux from excited chlorophylls or reduced compounds to physiological electron acceptors, such as ferredoxin, NAD^+ and $NADP^+$, through electron transport chains, most notably in mithocondria, peroxysomes, plasma membrane, and, above all, in chloroplasts, where O_2 production and the greatest part of electron transport take place. As a result, reactive oxygen species (ROS) deriving from O_2 would deteriorate the cellular components; notably, the photosynthetic apparatus would lose its electron exchange efficiency (photoinhibition).

The excited chlorophyll can be stabilized by electron spin inversion; in this state, named excited triplet, it is blocked in an inactive form and can transfer its energy to a triplet O_2 molecule, converting it to singlet ($^1O_2^*$). This chemical species is highly reactive, having a half-life of a few ns. To prevent photoinhibition, the PSII complex is surrounded by carotenoids in the antenna systems, and is subject to a rapid turnover, as a complete protection from the reactive oxygen species formed in the process can't be achieved.

When the reduction of the acceptors is outcompeted by that of molecular oxygen, the formation of superoxide ($O_2^{-}\cdot$) takes place. This may occur when $NADP^+$ is not readily available during photosynthesis (the overall metabolism is not consuming NADPH efficiently), or when the stomata are closed (a common feature of plant stress, inducing a rise in oxygen concentration in the cell). This reaction is most frequently occurring either at the [4Fe-4S] cluster in the excited PSI cluster (where the radical form is stabilized by the absence of H^+ in the membrane) or by ferredoxin oxidation.

Other metabolic pathways are minor sources of ROS. Photorespiration regenerates $NADP^+$ evolving H_2O_2 in peroxisomes. Purines catabolism involves a reaction in which xanthine is converted to uric acid with evolution of $O_2^{-}\cdot$, then uric acid produces allantoin, H_2O_2 and CO_2. An oxidative burst, driven by a NAD(P)H-dependent membrane oxidase (Respiratory Burst Oxidase Homologue, Rboh) is the main source of ROS during an incompatible pathogenic reaction and hypersensitive reaction, and occurs during several abiotic stresses as well, in response to ABA; specific isoforms are required for stomata closure, root hair development, and wound response. All isoforms are regulated by Ca^{2+}, Rop (Rho-like small G-proteins from plants) and N-term phosphorilation, possibly CDPK-dependent [*115*; *223*]. Electron leakage to O_2 can occur during reactions catalyzed by

cytochromes, in particular cytochrome P450. Upon alkalinization of the apoplast, several peroxidases and oxidases (amine oxidase, oxalate oxidase) are activated and evolve H_2O_2 in presence of a reductant, resulting in a pathogen-targeted oxidative burst and driving lignification and cell wall strengthening [*237*].

1.1.2 ROS detoxification

The toxic effects of ROS include DNA damage, lipid peroxidation and inactivation of proteins.

DNA damage (strand breakage, deoxiribose fragmentation or base modification) is due to OH· but not $O_2^{-}\cdot$, requiring a Fenton-active transition metal; the damage level can be extimated by the level of 8-hydroxyguanine [*10*].

Removing a hydrogen atom from a polyunsaturated fatty acid, OH· can trigger the lipid peroxidation chain reaction, thus disrupting the structure and functionality of membranes, which can be measured by electron leakage or thiobarbituric acid reactive substances (TBARS) like malondialdehyde [*68*]. A reversion of the lipid peroxidation level after the recovery from stress can be explained assuming that the plant is producing new tissues, rather than rescuing old ones; indeed, such a reversion has been observed in roots [*31*].

Proteins can suffer from carbonilation (mainly Lys, Arg and Pro residues), redox inactivation (for Cys-rich proteins), or metal cluster disruption [*111*]; the latter case, often leading to ubiquitin-depending degradation, can provide Fenton-reactive metals, thus feeding the oxidative stress.

1.1.2.1 Electron dissipation

$O_2^{-}\cdot$ can behave both as a reductant and as a prooxidant, and can reduce either cytochrome *b6f* or plastocyanin, feeding a cyclic electron transport around PSI on the thylakoid lumen side. Alternatively, it tends to dismutate spontaneously or is enzymatically converted by superoxide oxidase (SOD) to hydrogen peroxide (H_2O_2), which is relatively stable, but can react with transition metals such as Fe(II) or Cu(I) to form the extremely reactive hydroxyl radical (OH·), according to the Fenton reaction (fig. 1.2). Since the hydroxyl radical cannot be controlled, the antioxidant apparatus is centered on the removal of its precursors superoxide and hydrogen peroxide, by the activities of SOD, catalase (CAT)

and peroxidases. The whole process is named pseudocyclic electron transport or water-water cycle, to underline electrons cross the thylakoid membrane through the photosynthetical electron transport chain, although the end product is the same as the precursor; it yields a proton gradient between thylakoid and stroma, but no net reduction of ferredoxin or NADPH occurs, so that the ATP/reducing equivalents ratio can be adjusted; moreover, it is required to dissipate electrons during photoinhibition, in order to keep the carbon fixation provided with ATP [*7*]. The rate of H_2O_2 formation in chloroplasts under optimal conditions is estimated to be about 100 mM s^{-1}, but it can suffer an increase of several orders of magnitude under photo-oxidative stress, and a H_2O_2 level as low as 10 mM inhibits CO_2 fixation by 50% because of the oxidation of thiol-modulated enzymes [*103*].

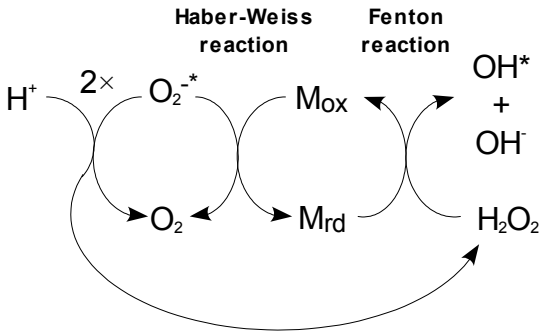

Figure 1.2: Haber-Weiss and Fenton reactions. M – transition metal.

In mithocondria, the ubiquinone pool can feed either the cytochrome pathway (comprehending the complex III, the cytochrome c and the cytochrome oxidase, with O_2 as final electron acceptor) or the alternative oxidase (AOX), which is an electron transport chain uncoupled to proton movement, ubiquitous in plants, acting as a dissipator of electrons. Mutants lacking the AOX display an increased ROS content and an upregulation of antioxidant activities; notably, heme inhibitors as N_3 and NO block the complex IV and induce the programmed cell death (PCD), while AOX is resistant to heme inhibitors and confer higher resistance to PCD. In the thylakoid membrane, the plastoquinol oxidase (PTOX) acts similarly, reducing O_2 and keeping the photosynthetic electron transport effective [*3*].

Plasma membrane and tonoplast house an ascorbate-dependent cytochrome *b*561, allowing the reducing equivalents to cross the membrane. In fact, apoplast is less buffered than cytosol, being poor in NADPH and GSH, and rich in peroxidase activity. Thylakoid lumen could be another critical compartment, since it can be reached by ascorbate only by diffusion, in spite of being one major site of ROS production; an accumulation of ROS in the lumen could be important as a signal for PCD [*75*].

1.1.2.2 Non-enzymatic antioxidants

The interaction between ROS and the antioxidant apparatus works as an interface between stressing conditions and metabolism, allowing to maintain proteins and other components in an active state, and, at the same time, to elicit a response against stress.

Ascorbic acid (AsA) is one of the main antioxidant compounds, and shows its maximal efficiency when coupled with glutathione in the ascorbate-glutathione (Halliwell-Asada) cycle. It is used as the electron donor by the ascorbate peroxidase to reduce H_2O_2 to water, forming monodehydroascorbate, either disproportionated to ascorbate and dehydroascorbate, or reduced to ascorbate by a specific, NADPH-dependent reductase. Reduced glutathione (GSH), besides acting as a thiol buffer, provides electrons for dehydroascorbate reduction, and is in turn reducted by the NADPH-dependent glutathione reductase. Ascorbate biosynthesis can be achieved through different pathways, the main being the D-man/L-gal pathway (D-glucose 6-P →→ D-mannose 1-P →→ L-galactose → L-galactono-1,1-lactone → ascorbic acid), occurring in mitochondria; other pathways can proceed from myo-inositol or pectins through, respectively, glucuronate or galacturonate intermediates [*103*].

Tocopherols are among the most important antioxidant factors in membranes, and are considered the main scavengers for singlet oxygen and lipid peroxides; their regeneration depends on ascorbate. They are augmented after several abiotic stresses such as light excess, drought, high temperature and heavy metal toxicity, probably in order to protect polyunsaturated fatty acid-enriched membranes from oxidative stress, and their absence induces phytoalexins, jasmonic acid, phytodienoic acid (OPDA) and other stress-related compounds [*143*]. Carotenoids can quench both the excited triplet state of chlorophylls and the singlet oxygen through the xanthophyll cycle, consisting in the enzymatic removal of epoxy groups from violaxanthin to yield zeaxanthin; the intermediate antheraxanthin is also a lipophylic antioxidant.

Typical stress products are heat-shock proteins, chaperonins, LEA proteins (for which is postulated a role in maintaining the structure and functionality of proteins), stress-associated metabolites such as aminoacids (mainly proline), amines (glycine-betaine and polyamines), sugars and polyalcohols, which can act as osmoprotectants, N/C sources or antioxidants, either directly or in their synthesis [*233*]. Yet, only in a few cases an overproduction of one of these compounds succeeded in improving plant fitness. In particular, proline is known to accumulate under stress, but in no case reaches concentrations which could support an

osmotic function; rather, since its synthesis proceeds from glutammate through two reductive steps, it may help controlling the ratio $NADP^+$/NADPH when carbon fixation slows down [*93; 231*].

1.1.2.3 Antioxidant enzymes

Some enzymatic activities overtake a main role in ROS scavenging, being induced under stress and located in proximity of the evolving site of their substrate. Nevertheless, part of their physiological function is debated, and various reports evidence different responses under stressing conditions, possibly reflecting different tolerance strategies and mechanisms among species. Peroxidases both remove H_2O_2 and contribute to lignification, which might be an adaptation to abiotic stress by limiting growth. H_2O_2 is a stress marker, a toxic by-product against pathogens and a signal for its own detoxification. Being mainly located in peroxisomes, catalase could be involved in removing the photorespiration-derived H_2O_2; in this case, ascorbate peroxidase or peroxiredoxins would undertake the main role of H_2O_2 scavengers.

Peroxidase family

Peroxidases are a large class of heme-binding enzymes catalyzing the oxidation of an electron donor, in order to convert H_2O_2 to water. According to their interspecific homology, they are divided in three superfamilies: animal peroxidases, catalases and plant peroxidases.

The first group is represented in plants only by the glutathione peroxidases (EC 1.11.1.9). While the mammalian enzymes are dependent on selenocysteine for their catalytic mechanism, this rare aminoacid is replaced by cysteine in plant isoforms, whose activities result therefore far lower. A particular kind of glutathione peroxidase has been identified, which uses phospholipid hydroperoxide as electron donor rather than H_2O_2, thus potentially blocking the Fenton chemistry in membranes. In plants, this enzyme has been shown to be induced by several abiotic and biotic stressing agents, and to be located in mitochondria and possibly other organules, linking it to the major sites for ROS production [*249*].

Catalase (CAT; EC 1.11.1.6) is a specialized type of peroxidase for H_2O_2 dismutation (H_2O_2 acts both as electron donor and acceptor). Catalase isoforms display a reaction mechanism in which one hydrogen peroxide mole reacts with the heme-Fe(III) giving the compound I, heme–Fe(IV)=O + H_2O. A second H_2O_2 mole reacts with the compound I

yielding back heme–Fe(III) + H_2O + O_2. A large number of plant isoforms exist, belonging to the small (<60 kDa) or large (>75 kDa) type, and showing different kinetic properties, quaternary structure and thermal stability [*35*].

The plant peroxidases, found in bacteria and fungi as well, include ascorbate peroxidase (APX; EC 1.11.1.11) and the so-called class III plant peroxidases (EC 1.11.1.7). The members of the latter class have a vacuolar or apoplastic localization, and display a low electron donor specificity (unlike APX and others); they are often assayed with guaiacol as the electron donor, and therefore named GPX. They are proposed for a great number of roles, such as H_2O_2 removal, cell wall strengthening by lignification, suberization after wounds, toxicity toward pathogens (in this case, evolving H_2O_2), auxin catabolism, and even transcription factor activity (for isoforms featuring AP2/EREBP, MYB or WRKY domains). Peroxidases have three strictly conserved domains (two heme-binding, each containing a histidine residue, and a domain of unknown function included between them); 8 invariable cysteine residues form a disulphide bridge network, and the region comprised between the sixth and the seventh is proposed to play a role in electron donor specificity. Nevertheless, due to the great number of possible substrates, the physiological function of each isoform should be probably found in a combination of characteristics, such as the expression pattern, localization and *in-vitro* properties [*98*].

APXs are classified into three groups according to the subcellular localization (chloroplast, either in a soluble or thylakoid membrane-bound form; cytosol; microbodies). APXs are unstable at low ascorbate concentration, and rapidly lose their activity [*103*]. Chloroplastic membrane-bound and soluble isoforms, active as monomers, derive from alternative splicing of the sampe transcript, with a C-terminal hydrophobic domain in the membrane-bound enzyme, which is found in stoichiometric ratio with PSI. Cytosolic isoforms are active as homodimers and display a lower substrate specificity [*7*].

Peroxiredoxins

Peroxiredoxins (Prx; EC 1.11.1.15) have recently been characterized as a class of heme-free thiol peroxidases, sharing their base reaction mechanism towards hydroperoxides and peroxynitrites. A conserved cysteine residue carries out a nucleophilic attack to the substrate, turning into a sulphenic acid (–SOH) which can be regenerated by direct reduction, intra- or intermolecular disulphide bridge formation. The disulphide is then reduced by an electron

donor such as thioredoxin or glutaredoxin. The regeneration pathway allows a classification among peroxiredoxins, into 1-Cys Prx, type II Prx (both having the sulphenic acid reduced directly by an external donor), 2-Cys Prx (where the sulphenic acid is reduced by an intermolecular disulphide bond), or Prx Q (forming a intramolecular disulphide bond). Glutathione peroxidase can also be ascribed to thiol peroxidases [*199*].

Superoxide dismutases

The superoxide dismutases (SOD; EC 1.15.1.1) are considered the first line of defence against ROS formation. They usually locate near the centres of $O_2^{-}\cdot$ production, since $O_2^{-}\cdot$, as a charged molecule, cannot cross membranes and must be removed *in loco*. Chloroplasts, mitochondria and peroxisomes are thought to be the main sites for ROS evolution. A reversible activation of SOD was found in roots, but not in shoots of salt-stressed cowpea plants [*30*; *31*]; therefore, SODs may be crucial in the tissues which directly sense the stressing agent, and not necessarily depend on chloroplast activity. With regard to their metal cofactor, SODs are distinguished as iron-, manganese- or copper-zinc-containing isoforms. Iron SODs are found in chloroplasts, and can be active as homodimers (20 kDa per subunit) or homotetramers (about 90 kDa per subunit). Manganese SODs might have evolved from iron-containing isoforms when the level of O_2 in the environment rose, making soluble Fe(II) less available; they are localized in mitochondria and peroxisomes, and work as homodimers or homotetramers. Copper-zinc SODs, the most recently evolved type, are very different from the other isoforms, because of the different electrical properties of the metal cofactors. This group is further divided into homodimeric forms, present in the cytoplasm and in the periplasm, and homotetrameric, extracellular or chloroplastic forms. In the chloroplast, they have been found to be attached to the stromal side of the thylakoidal membrane, where PSI is located. The metal cofactor dependence can be discriminated since Fe SODs are inactivated by H_2O_2, Cu-Zn SODs by both H_2O_2 and KCN, and Mn SODs by neither of these inhibitors. Different isoforms might have some specificity towards stressing agents [*2*].

1.1.3 ROS signalling

In contrast with biotic stresses, resistance to abiotic stress is mainly multigenic, and is associated to morphological or phenological avoidance. The known stress receptors are few,

consistently with the idea of a broadly overlapping, partly aspecific response to stresses. ROS signalling is probably based on thiol chemistry in target proteins, and is affected by thermodinamic (redox potentials) and kinetic (competition with antioxidants) factors. Thiols are subjected to a variety of modifications, such as disulfide bond formation, glutathionylation, sulphenic acid formation, or nitrosilation. Variations in the redox state of the glutathione pool could be as affecting as variations in the ROS content [*75*].

Hydrogen peroxide is a key signal, inducing detoxification responses at sublethal levels, PCD at higher concentrations, and potentially necrosis. As a relatively stable, uncharged molecule, it is able to cross lipidic membranes and to act at some distance from the evolving site. Its concentration can increase either when the antioxidant apparatus is weakened, or after elicited synthesis. Hydrogen peroxide is a mediator in hormonal signalling, including ABA-induced stomatal closure (by activation of the Rboh NAD(P)H oxidase) and auxin-depending gravitropism. PCD is controlled in interaction with nitric oxide (NO). Controlled H_2O_2 generation requires and induces ion fluxes (by opening plasmalemma calcium channels and closing potassium channels) and protein phosphorilation, in an autocatalytical way. Its effect is probably mediated by disulfide bridge reduction in specific target proteins and sites. Several genes are modulated by H_2O_2, including antioxidant enzymes, calmodulin, PCD proteins, protein kinases and transcription factors. H_2O_2-responsive promoters are known, although their working mechanism is not clear; WRKY proteins, Zatl1 (containing a C2H2 Zn-finger domain) and NAM proteins are reported as H_2O_2-activated transcription factors [*79*; *169*].

A specificity in the signalling properties of different ROS can be hypothesized, as studied in the *flu* mutant of Arabidopsis: these plants accumulate protochlorophillide in the dark, which evolves singlet oxygen when exposed to light, leading to a gene expression pattern differing from the one induced by paraquat (which causes the overproduction of $O_2^{-\cdot}$ and H_2O_2); furthermore, the double mutant *flu*/*tAPX* (overexpressing a thylakoidal APX, thus containing increased $^1O_2$ and less H_2O_2 than the wild type) shows an exasperated *flu* phenotype. This could be explained with an antagonistic role of H_2O_2 towards $^1O_2$ in the chloroplast, where H_2O_2 would compete against excess GSH in keeping PSII electron acceptor Q_A oxidized, thus making the electron flux more efficient and the $^1O_2$ production less probable [*123*].

1.1.3.1 Calcium

Calcium ion is present in millimolar concentrations mainly in vacuoles and endoplasmic reticulum, but also in chloroplasts and mitochondria, while its cytosolic concentration is about 200 nM. Various stressing conditions, including cold, drought and heat, induce a release of Ca^{2+} into the cytoplasm. In *Arabidopsis* guard cells, a hyperpolarization-dependent, Ca^{2+} permeable channel is activated by H_2O_2. Ryanodine and IP_3 receptors are Ca^{2+} channels characterized by a high number of cysteines, potential targets of redox regulation. ATP-depending Ca^{2+} pumps and Na^+:Ca^{2+} exchangers, which decrease the cytosolic concentration of calcium, can be inactivated by ROS. On one side, Ca^{2+} increases the production of ROS, as a promoter of pathways increasing the mitochondrial activity (mainly the Krebs cycle), of the Rboh NAD(P)H oxidase; on the other hand, several antioxidant enzymes like catalase, GSH reductase, SOD, among a great number of other proteins, are regulated by Ca^{2+} [*248*]. Among the calcium sensors, calmodulin, protein kinases like CDPK and SOS, annexins, calnexins and several transcription factors have been demonstrated [*227*].

1.1.3.2 Signalling network

Responses, aimed to the restoration of homeostasis, need a signalling network composed by G-proteins, mitogen-activated protein kinases (MAP-K), tyrosine phosphatases, salt overly sensitive kinases (SOS-K), phospholipases, transcription factors (heat-shock factors, C-repeat binding factors, ABA-responsive element factors) [*233*].

Some genes from *Arabidopsis thaliana*, induced after osmotic stress, can complement yeast mutants lacking specific osmosensors, consisting in a two-component system, including a histidine kinase and a response regulator.

Calcium ion serves as a second messenger in many responses, and is thereby a main player in the cross-talk; Ca^{2+} channels must exist, being regulated by mechanical or membrane-deriving signals such as IP_3 (possibly elicited by ABA or ROS), cyclic nucleotides (induced by NO), or factors affecting membrane fluidity and citoskeletal reorganization [*227*]. The SOS pathway for sodium stress tolerance consists in a signal transmission from the membrane-associated SOS3 protein, containing 3 low-affinity EF-hand motifs, thus reacting to great variations in Ca^{2+} concentration. In the Ca^{2+}-binding form, it can activate the SOS2 protein, a Ser-Thr protein kinase, which in turn activates its target SOS1 by ATP-dependent phosphorilation. SOS1 is a Na^+:H^+ membrane exchanger; other targets may be Na^+ channels

or vacuolar Na^+:H^+ antiport systems. Although the SOS pathway appears to be specific for saline stress, other SOS-like genes are involved in response to ABA. Another class of regulators, the calcium-dependent protein kinases (CDPK), allows the response to drought, wound or cold, probably by activation of transcription factors [*38*].

1.2 Nitric oxide and related compounds (RNS)

Besides ROS, the role of other reactive species has been pointed out. Since early 1990s, nitrogen monoxide, more commonly called nitric oxide (NO), and related compounds, collectively named reactive nitrogen species (RNS, in analogy with ROS), have been characterized as actively produced physiological regulators in plants, despite their unusual features. Indeed, in contrast to other signalling molecules, NO is a small, ubiquitous and unstable radical, freely diffusible both in aqueous and lipidic media [*247*].

1.2.1 Chemistry of RNS

The NO molecule is a lipophilic radical gas, with the unpaired electron delocalized between the N and O atoms. For these properties, it results uncommonly stable; its half-life inversely depends on its concentration, spanning from minutes or hours at < 1 μM to seconds at higher concentrations, and, moreover, on the presence of targets such as: proteins, transition metals, thiols, O_2 and ROS [*213*]. A maximum range of 200-500 μm is estimated for free NO diffusion [*101*; *225*; *247*].

The redox status of NO can vary among three different forms:

$$NO^+ \text{ (nitrosonium)} \underset{-e^-}{\overset{+e^-}{\rightleftarrows}} NO\cdot \underset{-e^-}{\overset{+e^-}{\rightleftarrows}} HNO \text{ (nitroxyl)}$$

Each of these species has a specific chemistry, but their interconversion is slow, and their effects appear to be distinct, possibly depending on the pahway NO derives from [*73*; *101*].

The reactivity of NO towards O_2 is increased in a lipophilic medium, and yields NO_2. This compound can further react with NO, according to the following reactions:

$NO_2 + NO \rightarrow N_2O_3$

$N_2O_3 + H_2O \rightarrow 2\ HNO_2$

The transfer of a NO^+ group to a nucleophilic target, named nitrosation or nitrosylation, may be driven by NO_2 or N_2O_3; else, NO· can be activated to nitrosonium by reducing metals, or react with thiyl moieties; in no case can NO directly substitute a thiol group. On the contrary, HNO can react with thiols [*101*; *134*]. Protein *S*-nitrosothiol formation seems to occur in specific consensus sequences, or might be mediated by low molecular weight nitrosothiols (like nitrosoglutathione) in a transnitrosilation reaction. Nitrosothiols tend to be labile in presence of reducing agents (AsA, GSH), transition metals or light, with half-lives of minutes or seconds. Moreover, nitrosothiol decomposition can also be catalyzed by thioredoxins, nitrosoglutathione reductase, Cu-Zn SOD and other enzymes [*134*].

NO has a strong affinity to octahedral metal centres, and can induce the release of metal ions (including Cd) from their storage and chelation sites, or prevent iron to accumulate in ferritins. Iron forms mono- or dinitrosyl iron complexes $[(R\text{-}S^-)_2Fe^+(NO^+)_2]^+$ with *S*-nitrosothiols; NO· specifically binds Fe(II), while HNO seems to favour Fe(III). Hence, another difference between the radical and the reduced forms could emerge [*101*; *186*].

NO reacts with O_2^{-}· at a nearly diffusion-limited rate, yielding $ONOO^-$ (peroxynitrite). This reaction depends on the relative concentrations of NO and SOD, which controls O_2^{-}· availability; moreover, peroxynitrite itself is highly reactive toward both of its precursors, which can therefore act as $ONOO^-$ scavengers when present in excess. $ONOO^-$ can also be formed by HNO and O_2 [*101*] and act as a *S*-nitrosylating compound [*134*]. Despite being less toxic than other peroxides, $ONOO^-$ is a powerful oxidant for aromatic moieties; in particular, tyrosine modification (nitration) yields nitrotyrosine. In physiological conditions, very little O_2^{-}· is produced and not removed by SOD; therefore, the formation of nitrotyrosine is proposed as a stress marker.

1.2.2 RNS metabolism

1.2.2.1 GSNO and nitrosothiols (RSNO)

Nitrosothiols (RSNOs) are considered important intermediates of NO biochemistry, since the half-life of NO *in vivo* is very short, whereas RSNOs can participate to NO storage, transport and delivery, due to their higher stability.

In particular, nitrosoglutathione (GSNO) has been recognized as a physiologically relevant compound since the characterization of the previously called class III alcohol dehydrogenase (synonimous: glutathione-dependent formaldehyde dehydrogenase; EC 1.2.1.1) as nitrosoglutathione reductase (GSNOR), catalyzing the reaction GSNO + NADH → GSSG + NH_3 + NAD^+ [*203*]. GSNOR-coding genes are evolutionarily conserved, ubiquitously expressed in the plant, and present in one single copy in the *Arabidopsis thaliana* genome [*129*].

GSNO is generally considered a NO storage and translocation form [*9*; *72*], and can transnitrosylate other thiols, including proteic cysteine residues; thus, GSNOR activity indirectly affects RSNOs. GSNO and nitrosothiols vary in concentration and distribution during stress conditions, notably in or near the vascular tissues, and GSNOR expression is affected by the same stressors, as well as by JA and SA [*9*; *45*; *61*; *229*]. Nevertheless, both induction and repression occur in the reported works. An increase in GSNO concentration, obtained through an antisense GSNOR RNA, promoted pathogen resistance and systemic acquired resistance [*201*]. *Arabidopsis GSNOR* mutants result more sensitive to heat stress, while GSNOR-lacking plants display pleiotropic phenotypes (reduced fertility and germinability, sensitivity to the light period); their NO content is higher and less stress-inducible in comparison to the wild type, and nitrosothiols correlate to an increased nitrate concentration [*129*]. Taken together, these results could mean GSNOR activity, rather than being a first-line actor in stress sensing or adaptation, depends on other factors like NO production or the rate of RSNO formation.

1.2.2.2 Protein nitrosylation

Protein *S*-nitrosothiols can be obtained by direct interaction of several RNS species, including other nitrosothiols such as GSNO. The process is reversible and no enzymatic mechanism appears to be required. In presence of reductants like ascorbate or reduced

glutathione, or metal ions, nitrosothiol half-life can be estimated in seconds or minutes, making *S*-nitrosylation a suitable fine-tuning mechanism. Indeed, a mechanism in NO-mediated regulation of proteins consists in the reversible nitrosylation of specific Cys residues; the consensus motif is probably a 3D environment featuring an acid-base pair, an aromatic ring, possible docking sites for GSNO or interaction with NOS, an enhanced acidity for the thiol group, and an hydrophobic environment. From a bioinformatic approach, the degenerate nitrosylation motif [GSTCYNQ]-[KRHDE]-C-[DE] was found in about one hundred *Arabidopsis* proteins, involved in signalling, metabolism, cell cycle and transport [*92*; *134*; *238*].

Proteomic screens of potential targets for nitrosylation [*132*; *133*] and of *S*-nitrosylated proteins during hypersensitive response [*197*] in *Arabidopsis* led to the identification of transcription factors, ion channels, primary metabolism enzymes (PSII; Rubisco and Rubisco activase; glyceraldehyde 3-phosphate dehydrogenase; glycolisis, oxidative phosphorilation and pentose phosphate pathway enzymes), possibly driving to the balancing of the redox status (thioredoxins and glutaredoxins are candidate targets too). Allene oxide synthase (JA pathway), methionine synthetase, *S*-adenosyl homocysteinase, and methionine adenosyltransferase (MAT) were also spotted.

Besides the proteomic approach, only few proteins were demonstrated to undergo regulation in plants. A MAT isoform loses its activity after nitrosylation, consistently with the reported inhibition of ethylene by NO [*133*]. During the hypersensitive response, peroxiredoxin IIE undergoes nitrosylation; since its activity includes the thioredoxin- or glutaredoxin-dependent peroxynitrite reduction, and is inhibited by nitrosylation, the observed increase in tyrosine nitration, mediated by peroxynitrite, can be explained [*196*]. Metacaspases are plant proteases, related to metazoan caspases (essential for the apoptosis process and known to be regulated by nitrosylation), which are kept in a inactive form while the catalytic Cys residue is nitrosylated. After the nitrosyl moiety is lost, the protein is converted to the mature, active form by autocleavage; in this state, the enzyme is not prone to inhibition, because of a second, nitrosylation-insensitive cysteine residue which can undertake the catalytic function if the first is blocked [*13*]. NO is also known to inhibit glyceraldehyde-3-phosphate dehydrogenase activity and AtMYB2 binding to DNA [*45*]. Rubisco undergoes nitrosylation in both subunits, and consequently reduces its carboxilation activity, in *Brassica*

juncea subjected to cold stress; other affected enzymes belong to photosynthesis, glycolysis, antioxidant (APX, SOD, peroxiredoxin) and defensive metabolisms [*1*].

1.2.2.3 Nitration and nitrosative stress

The potential toxicity of RNS led to the consideration of markers for cellular damage deriving from an unregulated radical production, defined nitrosative stress.

Superoxide and NO react in an extremely fast reaction, to yield peroxynitrite ($ONOO^-$), a powerful oxidant which can modify a number of biological molecules, including proteins, lipids and nucleic acids; in particular, tyrosine and other aromatic molecules undergo nitration in the ortho position. Since very little $O_2^{-}\cdot$ is thought to be evolved in healthy tissues, due to the SOD activity, nitrotyrosine is proposed as a biomarker of nitrosative stress.

Actually, both $ONOO^-$ and nitrotyrosine were found to increase in salt-stressed olive plant, together with NOS-like activity, nitrosothiol content, superoxide production; furthermore, NO and $O_2^{-}\cdot$ are shown to colocalize [*229*]. Tobacco cells require both NO and superoxide to induce hypersensitive response after elicitin treatment, and nitration is demonstrated by Western blotting [*202*]. Despite these data, it must be observed that oxidative and nitrosative stress do not necessarily overlap. A Cd treatment in pea plant did cause oxidative stress, but also a decrease in NO and GSNO content, thus making the production of RNS unlikely [*9*].

Nevertheless, observations in animal systems suggested that some proteins are selectively nitrated and nitration could be reversible; a nitrotyrosine denitrase activity has been described. Moreover, the plant peroxiredoxin IIE, which has been characterized as a target for *S*-nitrosylation, displays a peroxinitrite reductase activity in the non-nitrosylated form [*196*]. Thus, a signalling role for nitration cannot be excluded [*44*]. In plants, the number of proteins undergoing nitration during a stress instance is limited [*45*; *202*]; in sunflower, 21 proteins were spotted and identified, concerning metabolic processes such as respiration, photosynthesis, protein folding and degradation [*33*].

1.2.2.4 Affinity to transition metals

Many of the biological effects of NO depend on its strong affinity to octahedral metal centres, which leads to a typical weakening of the coordination bond pattern, and possibly the

release of the metal atom (especially in FeS clusters, causing the degradation of the cluster itself) and/or the regulation of the protein activity.

The soluble guanylate cyclase (sGC, EC 4.6.1.2) is known as one of the main targets for NO action in animal systems. Its product, cyclic GMP (cGMP), has then been identified in plants as well, as a second messenger in the NO signalling. NO interacts with the heme centre loosening the bond between Fe(II) and a coordination histidine residue, boosting the enzyme activity. The release of NO, and the consequent enzyme inactivation, can be enhanced by oxyhemoglobin, Mg-GTP (the substrate of sGC), and other redox agents [*146*].

In all living organisms, hemoglobins are among the main targets for NO action. Plant hemoglobins are classified in symbiotic, non-symbiotic, and truncated; class I (stress-induced) non-symbiotic hemoglobins, often expressed in hypoxic environments, are subjected to *S*-nitrosylation, and can react with NO and O_2 to yield nitrate (and possibly to free NO and oxygen from nitrite). Hemoglobin overexpression reduces NO levels and nitrosative stress incidence [*48*]. Thus, their proposed primary function might be the modulation of NO bioactivity, by detoxifying it to nitrate in a NAD(P)H-dependent reaction, storing it as a *S*-nitrosyl group, or reducing GSNO to GSH and nitrate [*178*].

Bacterial and metazoan aconitase isoforms can switch to DNA-binding proteins involved in the regulation of iron uptake, named IRP. NO is known to turn the functional aconitase to IRP, by disrupting the FeS cluster. Although IRP-binding sites are not characterized in plants, they might be represented by a structure rather than a sequence, and aconitase binding is reported to superoxide dismutase mRNA [*6*; *157*].

Besides the cytochrome *c* oxidase (complex IV or COX, EC 1.9.3.1), plant mithocondria have a second terminal oxidase (alternative oxidase, AOX, EC 1.-.-.-), not coupled to oxidative phosphorylation, bypassing the complexes III and IV and releasing the ubiquinol electron potential as heat. While COX is (reversibly) inactivated by NO, AOX is not [*153*]; therefore, NO affects the production of energy and the likelihood of ROS formation in plant mitochondria.

1.2.3 NO synthesis

1.2.3.1 NO synthase

NO synthase (NOS, EC 1.14.13.39) has been characterized as the enzymatic source of NO in mammals. Two isoforms are constitutively expressed in neuronal and endothelial tissues, while an inducible one (iNOS) is expressed by the immune system cells. All isoforms share the structure and the reaction mechanism: the enzyme, active as a homodimer, includes NADPH-, FAD-, FMN-, and CaM-binding sites in the C-term domain, and tetrahydrobiopterin (BH_4)-, heme- and L-arginine-binding sites in the N-term domain. The reaction consists in the oxidation of arginine to citrulline + NO, requiring O_2 and 5 e^- from NADPH bound on the adjacent subunit. Ca^{2+}-CaM are the main regulators of the constitutive isoforms, while the inducible NOS binds them strongly, so that it results constantly active [*19*].

After the discovery of NO as a regulator in mammals, a plant homologue has been searched by different means, including molecular, immunological and radiochemical ones.

The occurrence of the typical NOS reaction has been demonstrated in a number of plant models, such as *Pisum sativum* development, *Lupinus albus* nodulation, *Nicotiana tabacum* infected with TMV [*43*; *50*; *53*; *65*]. In particular, a constitutive activity is found in xylem and epidermis during pea seedling growth [*43*]; it is proposed that the role of NO in those tissues may reflect their importance in environment sensing and transfer of nutrients and signals. Cofactor requirements and inhibitors are the same as in the mammalian NOS, although it must be noted that BH_4 is not present in plants, where it could be substituted by tetrahydrofolate [*229*].

Despite this, no homology to mammalian NOS genes could be found in Arabidopsis or other species, where plenty of molecular data is available. All the claims for the identification of a plant NOS [*34*; *89*] have been retracted [*49*; *114*]. Thus, the activity is referred to as NOS-like. It has been observed that BH_4-free NOS can release nitroxyl (HNO) from arginine, in a standard monooxygenase reaction involving 2 NADPH, 2 O and a 4-e^- transfer, contrasting with the unique stoichiometry of NOS (2 O, 1.5 NADPH, 5 e^-). NO· could then derive from HNO, possibly undergoing SOD-mediated reduction [*101*]. Thus, HNO might be the actual product of NOS-like plant activity, which could be ascribed to a monooxigenase enzyme rather than a homologue of the mammalian NOS.

1.2.3.2 Cytosolic nitrate reductase

Nitrate reductase (NR; EC 1.6.6.1) catalyzes the limiting step in the reduction of NO_3^- to NH_4^+. The first reaction of the pathway is the NADH-dependent reduction of NO_3^- to NO_2^-; nitrite is then reduced to NH_4^+ by nitrite reductase (NiR) and ferredoxin as the electron donor. The NADH consumption and the NH_4^+ production can be crucial in some cases, like in root anoxia, when excess reducing potential and acidification occur; the tolerance to submersion has been associated to high nitrate fertilization [*214*].

NR is a cytosolic homodimeric enzyme; each subunit houses a N-terminal MoCo domain, a heme cofactor and a C-terminal FAD-binding domain. It is inactivated by interaction with 14-3-3 proteins, which recognize the phosphorilation of a conserved Ser residue between the heme domain and the MoCo domain. Several protein kinases can overtake this role; among them the Ca^{2+}-depending CDPKs. Moreover, NR synthesis is induced by nitrate; this also makes an important regulating strategy, since NR half-life is short (hours). Finally, the NRI protein inhibits specifically and irreversibly NR, probably by blocking the FAD-binding site. As a general rule, a high energy status is linked to a high NR activity, although the mediator is not known. In the dark, NR phosphorilation is complete and no NR activity can be found, since the ferredoxin-dependent NiR could not remove the toxic nitrite [*109*; *152*]. NO itself can increase NR activity, either by a direct stimulation (on the heme cofactor, or by cysteine nitrosylation), or by controlling the protein kinases and phosphatases which regulate NR activity [*64*].

As a side reaction, NR can also reduce NO_2^- to NO. The K_m for nitrite is about 100 μM, thus making nitrate the preferred substrate and antagonist for NO production; nevertheless, NO synthesis follows the activation status of NR and the accumulation of nitrite, even if induced by anoxia or uncouplers of the oxidative phosphorilation. Normally, NO emission occurs specifically in light, with a peak, as soon as the lamp is switched off, before dropping to zero (fig. 1.3); this activity, ascribed to NR, is positively affected by NO_2^- and CO_2, and depressed by O_2 [*182*; *191*], which is a possible competitor substrate for NR, and the resulting superoxide would further subtract NO [*245*].

Experiments on a tobacco clone expressing a NiR-antisense RNA showed a 5-fold content of nitrite, but a 10 or 15-fold increase in NO emission compared to the wild type; no "light-off peak" occurred. These data can be explained assuming that the NO_2^- distribution in the cell are different in the mutant, so that NR experiences locally a higher nitrite

concentration; such saturation is consistent with the idea that only the enzyme circadian activation status, but not the substrate concentration affects the production of NO [*160*]. The *Arabidopsis thaliana* double mutant *nia1 nia2*, where NR is not expressed, still displays a NO_2^--dependent NO production when NO_2^- is exogenously supplied, but contains reduced concentrations of aminoacids, in particular arginine [*156*].

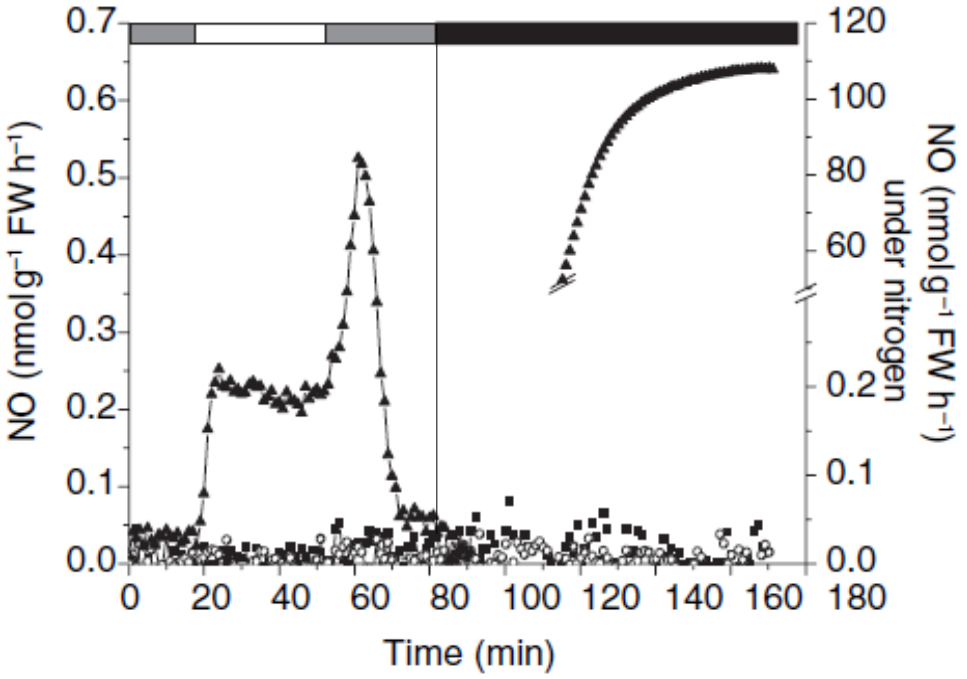

*Figure 1.3: NR-dependent NO production in detached tobacco leaves. Triangles – wild type; squares – tungstate-grown wild type; circles – NR-deficient mutant. Left: NO emission under air in dark and in light (grey-white in the top bar); right: NO emission in N_2 atmosphere and continuous dark [*182*].*

1.2.3.3 Nitrite:NO reductase

An enzymatic source of NO has been found in tobacco, on the apoplastic side of the root plasma membrane. Like the cytosolic NR, it requires NO_2^- as the substrate and was therefore named nitrite:NO reductase (NiNOR); yet, it does not require a molybdenum cofactor, and can use cytochrome *c* but not NADH as the electron donor. The enzyme appears to be membrane-bound, with an apparent molecular mass of about 300 kDa; its specific activity is estimated in 5 nmol mg^{-1} protein min^{-1}. Associated to the plasma membrane-bound nitrate reductase, it may be a sort of nitrate receptor, converting the reduction of NO_3^- into NO signal [*212*].

1.2.3.4 Other sources for NO production

Several non-enzymatic reactions can produce NO. Carotenoids can reduce NO_2 to NO in light; at low pH, NO_2^- can be reduced by ascorbate yielding NO and dehydroascorbate, or spontaneously dismutate to NO + NO_3^- [*213*]. Light-mediated reduction of NO_2 by carotenoids, and reaction of H_2O_2 with arginine are also proposed [*53*]. Some reports failed to link the production of NO to any of the known enzymes during cryptogein elicitation [*183*].

In barley aleurone apoplast, the non-enzymatic reaction

$$2\ NO_2^- + 2\ H^+ \rightarrow 2\ HNO_2 \rightarrow NO + NO_2 + H_2O \rightarrow 2\ NO + ½\ O_2 + H_2O$$

is suggested, since the pH is low enough (3-4) for nitrite protonation to occur, and antioxidants (proanthocyanidins) can subtract molecular oxygen. Indeed, NO synthesis from NO_2^- is confirmed, does not depend on proteins of >10 kDa, and may act as a signal or an antimicrobial compound [*16*].

Arginine-derivated polyamines (ornithine, putrescine, spermidine and spermine) accumulate under stress; among their roles, they act as protective compounds and also as signals for proliferation and differentiation. They have been found to promote NO evolution, with no lag phase and with the highest efficiency for spermine and the lowest for arginine: these observations would exclude a NOS-like-mediated effect. Non-physiological amines, like Tris and imidazole, give no NO rise. Polyamine oxidases are known to produce H_2O_2, but the existence of NO-evolving isoforms could be supposed [*78*; *225*]. In drought-stressed cucumber, polyamine-mediated NO production is abolished by tungstate and sensibly reduced by L-NAME, supporting the idea that polyamines act on NOS, NR or both [*4*].

Hydroxylamine and salicylhydroxamic acid can induce a rise in NO content in tobacco cell suspension in presence of oxygen. It can be noted that ROS promote the reaction ($O_2^{-}\cdot$ aspecifically, H_2O_2 with a catalyzer), and SOD can free NO from hydroxylamine in anaerobiosis (that is, with no precursor $O_2^{-}\cdot$ and no intermediate H_2O_2). Unknown monooxigenases might be proposed as hydroxylamine sources in plants [*200*]. *N*-hydroxyarginine and hydroxyurea, in presence of H_2O_2, can be substrates for NO production; horseradish peroxidase, and possibly other peroxidases, as well as heme-proteins like cytochrome P450, hemoglobin and catalase, have all been demonstrated to drive these reactions [*53*].

Xanthine oxidoreductase is a molybdenum cofactor-containing enzyme that can be found in the interconvertible forms xanthine oxidase and xanthine dehydrogenase. The former, according to O_2 availability, is reported to produce either $O_2^{-}\cdot$ or NO [*53*].

1.2.3.5 Subcellular localization of NO production

A dependence on electron transfer can be ruled out for the NO-evolving activities, since it has been associated to mitochondria, chloroplasts, peroxisomes.

Mitochondria

A link between NO metabolism and mitochondria exists in animal cells as well as in plants. NO was found to reversibly inhibit the mitochondrial respiration and to reduce the membrane potential, by targeting the cytochrome oxidase (COX) and also the cytochrome *c* [*25*]. In plants, the alternative oxidase (AOX, insensitive to all heme-blocking compounds, including NO) allows electron dissipation uncoupled to ATP production [*246*]. In the green alga *Chlorella sorokiniana*, NO synthesis is stimulated by light and nitrite [*222*]; nitrite is sufficient for NO emission in cultured tobacco cells or isolated mitochondria [*182*] and in the *Arabidopsis* double mutant *nia1 nia2*, lacking both NR genes [*156*]. In all these cases, NO_2^--dependent NO emission does not require NR, but is reduced or blocked by COX and AOX inhibitors. This holds true for tobacco root, but not leaves mitochondria, possibly because of the inability to reduce nitrite to ammonia in anoxic roots [*90*].

Chloroplasts

Despite not affecting the efficiency of PSII, NO causes a reduction in electron transfer rate and ATP synthesis in chloroplasts, maybe competing with the stimulator HCO_3^- for the same binding site; on the other hand, when photosynthesis is not able to efficiently reduce ferredoxin, a nitrite accumulation leads potentially to a higher NO production, mediated by cytosolic NR. Globally, an interplay between carbon and nitrogen metabolism could take place in chloroplasts, limiting carbon fixation when NO is produced as a consequence of a depressed nitrogen reduction [*217*].

Both a NOS-like and a NO_2^--depending activity are reported in soybean chloroplasts, the latter specifically located in thylakoids [*106*]. The *Arabidopsis* NOA1 protein, previously classified as a mitochondrial atypical NOS, has been demonstrated to be a functional GTPase

required for ribosome assembly located in plastids, with no NOS activity. Despite this, NOA1 mutants have a yet unexplained low NO production [*77*].

Peroxisomes

Peroxisomes are ubiquitous organules, delimited by a single membrane, and hosting oxidative metabolisms as their distinctive feature beyond a great metabolic plasticity (specialized forms, such as glyoxysomes or photorespirative leaf peroxisomes, can be found in different tissues or species). As a result of such metabolisms, H_2O_2 and other ROS are massively produced in peroxisomes. Oxidases (mainly flavin oxidases) and catalases are the most represented enzymes, along with SOD, ascorbate-glutathione cycle enzymes, glutathione peroxidase and peroxiredoxins. Several $NADP^+$ dehydrogenases allow the recycling of $NADP^+$ to NADPH [*52*; *54*].

A NOS activity in pea peroxisomes was identified according to several complementary approaches: [^{3}H]-citrulline production in purified peroxisomal fraction, sensitivity to typical NOS inhibitors, Western blotting, and electron microscopy immunocytochemical analysis of intact tissues [*8*]. Citrulline production is shown to co-localize with CAT activity, to be protein-mediated, to require Ca^{2+} and NADPH, and is extimated in about 5.5 nmol mg^{-1} protein min^{-1}. Two different antibodies, raised agains iNOS, were used for the Western blotting and the immunocitochemical analysis, giving a signal in peroxisomes and chloroplasts. Nevertheless, the specificity of antibodies raised against mammalian NOS is questioned in plants. In maize embryonic axes, 20 protein spots of a 2D gel reacted with the antibodies; 15 of them have been identified, having no relation to NOS [*28*].

Despite being a possible product for other enzymatic activites, citrulline is synthesized in a NADPH-depending reaction, which can be blocked by NOS inhibitors. Thus, although no definitive conclusions can be drawn about the identity of the enzyme, a NOS-like activity is likely to be present in peroxisomes. A role for NO could be proposed as a ROS scavenger, a signal in coordination with H_2O_2, or by forming peroxynitrite with $O_2^{-}\cdot$; peroxynitrite could regulate the activity or stability of proteins.

1.2.4 RNS signalling interactions

1.2.4.1 Cyclic GMP cascade

Cyclic GMP (cGMP) has been identified, firstly in animals, then in plants as well, as a second messenger in the NO signalling. NO induces the activation of the mammalian sGC by binding reversibly its Fe(II)-heme group. The concentration of cGMP is the result of the balance between its production, mediated by sGC, and its degradation, mediated by a phosphodiesterase. In turn, cGMP induces cADP-ribose (cADPR) and the release of Ca^{2+} in the cytosol, driving the regulation of Ca^{2+}-dependent proteins.

Despite neither a mammalian-like (heme-containing) sGC, nor the phosphodiesterase have ever been cloned in plants, cGMP induces several physiological responses, like the metabolism of phenolic compounds, the maturation of chloroplasts, auxin-induced adventitious rooting, and stomatal closure; specific inhibitors of sGC or phopshodiesterase, or cADPR antagonists, interfere with such responses [*119*; *124*; *170*; *174*].

1.2.4.2 Calcium

The variations in cytosolic calcium concentration must be perceived by sensors, such as calmodulin (CaM) or calcium-dependent protein-kinases (CDPK), and give rise to specific responses. NOS, but not NR, is activated by Ca^{2+}-CaM; in turn, calcium channels can be controlled by NO directly after nitrosylation, or indirectly through the cGMP/cADPR cascade [*126*; *174*]. Thus, Ca^{2+} can be required to intiate, but also amplify or maintain the NO signal [*47*]. The imposition of a thermic or osmotic stress in *Nicotiana plumbaginifolia* cell cultures increases the cytosolic Ca^{2+} concentration, according to a biphasic rise with an early, higher peak, and a second, lower peak a few minutes after the stress (fig. 1.4). The first peak is less affected, whereas the second phase appears to be delayed and lowered by the NO scavenger cPTIO; thus, NO could respond to the primary peak, and induce the second phase [*82*].

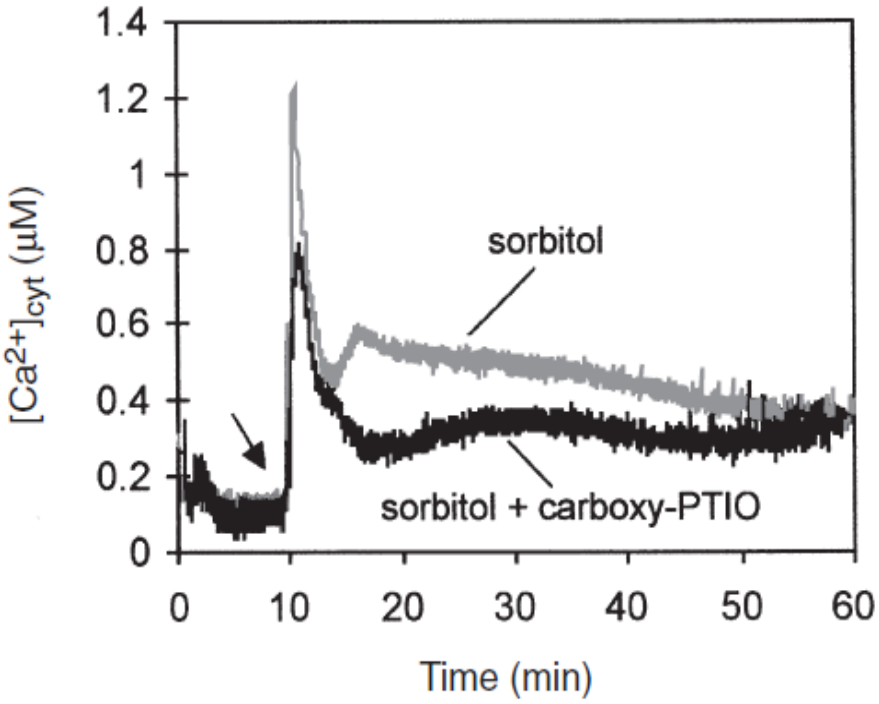

*Figure 1.4: Variation in cytosolic calcium concentration after osmotic (sorbitol) treatment (grey), and in presence of sorbitol plus the NO scavenger carboxy-PTIO (black) [*82*].*

The animal ryanodine receptor (RyR) is a class of Ca^{2+}-channel, activated by cADPR and cytosolic Ca^{2+}; a RyR-like channel in plants can be postulated, since a cADPR antagonist reduces significantly, although not completely, a NO-mediated calcium release. A different second messenger pathway could involve a phosphorilation cascade, since calcium release is limited by a protein-kinase inhibitor, and promoted by a tobacco NO-activated protein kinase (named NtOSAK), induced under osmotic stress [*124*].

Phosphatidic acid is also proposed as a mediator in the induction of hypersensitive response, after a rapid and prolonged production of NO. In this model, NO induces the phospholipase C to produce inositol triphosphate, provoking the release of the Ca^{2+} stores; phospholipase D and diacylglycerol kinase, combined, give rise to phosphatidic acid in response to the Ca^{2+} signal; other activation targets are the Rboh NAD(P)H oxidases, resulting in an increase in ROS [*128*].

1.2.4.3 Hormones

In some cases, NO mediates or alter the biological effects of hormones, for example by affecting tissue sensitivity, or being induced after elicitation by a long-distance signal. In these models, NO function is limited to its site of synthesis.

Citokinins

The perception of citokinins is carried out by the AHK receptors, activating the histidine phosphotransferase AHT to phosphorilate the nuclear response regulators ARR; other signalling pathways cannot be excluded.

NO was induced a few minutes after zeatin treatment in arabidopsis seedlings [*226*] and after benzyladenine (BA) treatment in arabidopsis cell cultures [*29*]. In the latter case, NO was shown to mediate the senescence programme and PCD activation, possibly by inhibiting the mitochondrial respiration [*29*]. In turn, senescence, due to NO deprivation, was partially reverted by BA [*154*].

Abscisic acid

ABA is mainly associated to water stress, because of its role in inducing stomatal closure through a signalling network involving second messengers (cADPR, Ca^{2+}), ion channels, protein phosphorilation. The NO/H_2O_2 ratio, and the identity of other RNS and ROS involved, are crucial for the induction of responses. Moreover, the ABA-induced stomatal closure and the ABA-induced stomatal opening inhibition are two distinct phenomena, with the first involving H_2O_2, MAP kinases and NO, whereas the second requires G proteins but probably no NO [*60*]. A simplified, general model (not accounting for other effects of each intermediate, autocatalysis or amplification) is proposed as follows [*24*]: ABA → H_2O_2 → NO → cGMP/cADPR → Ca^{2+} release → stomatal closure.

The stomatal closure occurs in two phases. The inactivation of the inwards K^+ channels is rapidly induced by ABA through a rise in the cytosolic calcium concentration, requiring the signal cascade of NO, cGMP and cADPR; on the other hand, the opening of the outwards K^+ channel is NO- and Ca^{2+}-insensitive, and depends on the alkalinization of the cytosol, which is still induced by ABA, but slower than the NO-dependent pathway. H_2O_2 is also able to induce a rise in cytosolic calcium levels, but since it affects both inwards and outwards K^+ channels, its signalling mechanism must be at least partly distinct from NO [*76*].

ABA-mediated induction of antioxidant enzymes is reported to require a NOS-like-derived NO production [*257*].

Phototropin is a blue light receptor with Ser/Thr kinase activity, inducing an increase in plasma membrane potential by plasmalemma H^+:ATPase activation, thus leading to K^+ intake and stomatal opening. NO, as a signal intermediate of ABA, counteracts the whole process by inhibiting the proton translocation, although no interactions with the H^+:ATPase or the protein kinase cascade are demonstrated [*252*].

In arabidopsis seeds, NO is an antagonist for ABA-induced dormancy: after a treatment with a NO donor, seeds lose sensitivity to ABA and germination takes place in spite of ABA concentration. Nitrite appears to be the source for NO production [*17*].

Auxins

In *Arabidopsis*, the root system architecture (that is, the balance between primary and lateral roots) is influenced by nutrient patches and osmotic stress, with indoleacetic acid (IAA) and indolebutyric acid (IBA) inducing adventitious rooting and lateral root emission [*117*; *118*; *126*; *174*]. The NO-cGMP cascade, and possibly the direct activation of phospholipases and calcium channels by NO, are proposed for the onset of the process. Other signals driving to the same radication effect (such as salicylic acid), or other effects of auxins, may require NO as a second messenger. Some differences arise in other species (for instance, pea), possibly due to modulating factors, like the intensity of an oxidative stress [*117*].

Ethylene and polyamines

Some of the key enzymes for ethylene biosynthesis are shown to be regulated by NO. Methionine adenosyltransferase and *S*-adenosyl homocysteine hydrolase, required for the synthesis of both ethylene and polyamines, lose their activity after *S*-nitrosylation and nitration, respectively [*33*; *133*]. ACC synthase transcription is promoted when NO is subtracted [*154*]. Supporting this view, NO is known to counteract the effects of ethylene by delaying senescence, flowering and maturation [*131*], and is proposed to act downstream to polyamines, possibly being a by-product of their metabolism [*4*].

In ozone-treated tobacco plants, NO and ethylene cooperate in promoting the transcription of the alternative oxidase, which is normally low in unstressed plants, since

treated leaf discs incubated with a NO scavenger fail to induce ethylene accumulation, ACC synthase mRNA transcription, and cell death [*67*].

Salicylic acid

NO and ROS, induced by pathogens or abiotic stresses, regulate the synthesis of salicylic acid. In turn, SA promotes the NO- and ROS-mediated redox signalling, in a positive feedback. SA activates a NOS-like activity in *Arabidopsis* and *Vicia faba*, which triggers Ca^{2+} release in the cytosol and protein-kinase activation [*260*] and depends on mitochondrial electron flux, through AOX [*78*]. Since nitration is shown to occur in the phloem, and GSNOR counteract the systemic acquired resistance (SAR) onset, NO or GSNO are proposed as mobile feedback messengers that spread the SA signal in the whole plant [*78*].

Jasmonates

Jasmonate (JA) and methyl-jasmonate (MeJA) are promoters of senescence, causing a rise in H_2O_2 (possibly through the Rboh oxidase), a decrease in ascorbate and glutathione, lipid peroxidation, protein and chlorophyll demolition, and an overall oxidative stress status. JA- and MeJA-induced senescence is contrasted by ROS scavengers; NO is shown to limit the lipid peroxidation and promote antioxidant enzymes [*102*]. Moreover, NO prevents wounding- and JA-induced H_2O_2 synthesis and expression of defence genes in tomato and sweet potato; on the other hand, wounding and JA induce NO production, and NO promotes the genes for JA biosynthesis, although not JA production [*86*; *100*]. This unclear relationship could include the negative regulation of JA effects by SA [*242*].

1.2.4.4 Interactions with ROS signalling

NO or RNS are requested in many ROS-mediated responses, such as cell death, SAR [*78*] and gene expression, specifically related to the metabolism of phenylpropanoid compounds, notably salicylic acid and flavonoids [*58*]. The relationship between NO (which can act both as a prooxidant and an antioxidant) and ROS is controversial; RNS appear to be in some cases required for ROS activity, counteracting in other cases.

Pathogen resistance is allowed by an oxidative burst, aimed to direct killing of the pathogen, cell wall strengthening, PCD and systemic resistance induction. Observing that, in the host-pathogen interaction, the oxidative burst is required but not sufficient to induce PCD,

along with SA and Ca^{2+} metabolism perturbations, the NO/H_2O_2 ratio was identified as a trigger for PCD [*56*; *57*]. $O_2^{-}\cdot$ is not directly implied, and $ONOO^-$ cannot elicit PCD (unlike mammalian apoptosis); therefore, a model is proposed in which excess $O_2^{-}\cdot$ or NO act as reciprocal scavengers, unless $O_2^{-}\cdot$ is efficiently dismuted to H_2O_2; the combined action of H_2O_2 and NO in inducing cell death could imply highly reactive oxygen species, possibly with trace metal ions.

With regard to abiotic stress, NO acts downstream to ABA-induced H_2O_2 to mediate stomata closure [*24*]. *Populus euphratica* can deal with excess Na^+ by excluding it or compartmentalizing it in vacuoles, and accumulating K^+ in the cytoplasm; these mechanisms rely on H^+:ATPase expression and activation promoted by H_2O_2, which in turn derives from NO-triggered NADPH oxidase [*251*].

While animal cells are susceptible to low doses of $ONOO^-$, plant cell viability is not affected, despite an increase in protein nitration. The rise in $O_2^{-}\cdot$ formation during abiotic stresses can also mask the basal NO level, which may directly control iron availability, and it is consistent with this model to postulate a system for the prevention of Fenton reactions. On the other side, ascorbate and cytochrome *c* oxidase can reduce peroxynitrite back to NO, helping NO conservation and/or ROS removal [*230*].

1.2.5 Physiological effects of RNS

1.2.5.1 Phenology and morphology

Acting downstream to auxins, NO mediates their effects on root architecture, promoting the formation of lateral roots against the elongation of the primary root [*46*], and inducing adventitious rooting [*174*] and root hair development [*136*]. By pharmacological treatments with NO donors and scavengers in maize roots, and by the analysis of NOS and NR mutant in arabidopsis, NO has been shown to respond to auxins, to overcome nitrate-induced growth inibition, to be actively produced under nitrate starvation, and to derive from NR activity [*117*; *255*].

The floral transition, in *Arabidopsis thaliana*, is controlled both by external (photoperiod, vernalization) and environment-independent (gibberellins and others) factors. NO acts as a mediator in photoperiod sensing, delaying the process; nevertheless, it also

regulates the expression of genes from the independent pathways, so that a possible role for NO emerges in integrating internal and environmental signals [*95*].

Seed dormancy is adversed by NO. Along with other light-dependent responses, such as de-etiolation and internodal growth, NO affects seed germination overcoming light requirement, similarly to gibberellic acid, and possibly sharing the same cGMP signalling pathway [*14*]. While ABA is essential for the establishment and maintenance of dormancy, the NO donor sodium nitroprusside (SNP) renders arabidopsis seeds insensitive to ABA [*17*].

According to its concentration, NO delays or promotes senescence, interplaying with ethylene, citokinins, and stress signals (ABA or JA) [*154*]. It is reported in a wide variety of species, both climateric and non climateric, that NO emission is higher in unripe fruits, whereas it falls along with the ethylene increase during ripening or senescence; nitrous oxide (N_2O) treatments can extend the postharvest life of crops [*131*]. Contrasting the lipid peroxidation and lipoxygenase activity in senescence, in dependence of treatment dose and timing, NO preserved unsaturated fatty acids in peach fruits [*258*].

In several angiosperms, the pollen tube emission is associated with a production of NO, required for the tube directioning. The unexpectedly high H_2O_2 concentrations in the stygma are shown to decrease after pollen interaction and with a NO donor treatment. Hence, a role for NO in pollen compatibility is proposed [*151*]. Moreover, the pollen tube elongation in *Pinus bungeana* requires a tip-based calcium gradient, achieved through an active, NO-dependent intake in the tip region [*239*].

1.2.5.2 Programmed cell death (PCD)

The programmed cell death (PCD) is an active pathway, supposedly derived from the host-endosymbiont interactions leading to cytoplasm shrinkage, chromatin condensation and DNA laddering. The process is normally kept suppressed in normal growth conditions, but gets active in developmental or defence processes, under the control of ROS and RNS. Mutants lacking catalase isoforms, and ascorbate-deficient plants display enhanced PCD, suggesting PCD to be initiated with high ROS production in compartments with (constitutively or induced) low redox buffering [*59*; *75*]. Mitochondrial functionality and a MAP-K cascade are involved in PCD activation.

Both H_2O_2 and NO appear to be required at the same time for PCD, none of them being able to induce cell death alone. Therefore, a main role is played by the superoxide dismutase:

$O_2^{-}\cdot$, as a NO scavenger, inhibits PCD onset; on the other side, SOD converts $O_2^{-}\cdot$ to the PCD-inducing H_2O_2. The direct reaction with NO is faster than superoxide dismutation; thus, SOD must be present in a relevant concentration and in a convenient localization to be efficient. A model is proposed, in which the balance between NO and $O_2^{-}\cdot$ is determinant: excess NO subtracts $O_2^{-}\cdot$, preventing it to be converted to H_2O_2; excess $O_2^{-}\cdot$ acts as a NO scavenger, so that even in presence of H_2O_2, PCD cannot take place [*57*].

While a prolonged H_2O_2 production induces necrosis, a single-pulse H_2O_2 treatment causes the onset of PCD through a rise in NO levels. Together with APX inhibition, decreased ascorbate and glutathione concentrations, rather than their redox state, seem to be linked to the PCD signal [*59*]. A low ascorbate content may lower the threshold for sensing stress, thus triggering PCD and SAR [*145*].

1.2.5.3 Genic regulation

Whole-genome analyses were carried out in *Arabidopsis thaliana*. Most of the NO-modulated genes respond to abiotic or biotic stress conditions, and are involved in signal transduction, cell death, defence, generation or detoxification of ROS, photosynthetic processes, intracellular trafficking, and basic metabolism; only a few among the classified ones can be directly linked to stress defence, but a dose-dependency to NO emerges for many genes, pointing to a form of signal specificity.

NO effect on transcription can consist either in the expression of specific factors, or in the regulation (probably by Cys-nitrosylation) of existing proteins. WRKY, EREBP and DREB1-2 transcriptor factor families appear to be induced by NO, together with transporters, ferritin and members of MAP-K cascades [*86*]. Members of WRKY, bZIP, MYB-MYC families are the most likely targets of NO-mediated posttranslational regulation. WRKY transcription factors are often involved in many responses, such as pathogen defence, senescence, wounding, drought, and morphological adaptation, often depending on SA. Members of the bZIP and related families mediate responses to pathogens or symbionts. MYB and MYC proteins work in the transcriptional regulation of genes associated with phenylpropanoid and anthocyanin metabolism and drought stress, and can be activated by environmental factors and hormones like ABA [*175*].

1.2.5.4 Abiotic stress protection

NO function in stress resistance can be carried out by a signal cascade, or by direct detoxification. Plants improve their health status in response to many kinds of environmental stress, when exposed to low NO concentrations. Thus, NO could be considered a generalized stress signal. To prove this, high temperature, salt stress, excess light and mechanical stress were imposed to *Nicotiana spp.* peels and cell cultures. Only heat and salt stresses resulted in enhanced NO production [*82*]. Low temperature and wounding increased NO content and NOS-like activity in pea plants, whereas high temperature, continuous light and continuous dark did not, and excess ligh enhanced NOS-like activity but no apparent NO content [*45*]. Wounding caused a NO burst also in arabidopsis, but the NOS inhibitor L-NMMA affected only slightly [*100*]. Hence, it must be concluded that increased NO content is not a generalized response to stress, although its occurrence varies among species, and possibly on the basis of tissue-specific mechanisms.

Cadmium

Cadmium is a heavy metal, not participating to Fenton reactions, and with no biological activity. Despite this, it is highly toxic, because of its rapid uptake, followed by oxidative stress. Nucleus activity, hormonal signalling, photosynthetic apparatus, and plasma membrane NADPH oxidases could be its primary targets.

The oxidative stress symptoms in Cd-stressed plants are either reverted by a NO treatment [*127*], or associated to a reduced NO content [*192*]. Reports about the formation of stable complexes between Cd and thiol groups, including glutathione and phytochelatins, support the protective role of NO and lower glutathione contents in sensitive plants [*127*].

Other metal ions, such as aluminium in acidic soils, are reported to have toxic effect against plants, and to inhibit the NOS-like activity as a direct effect [*221*].

Salinity and osmotic stress

Salinity involves both an osmotic stress and a ion toxicity. The resistance strategies consist in the regulation of transpiration and of Na^+ uptake, transport and redistribution. Proton concentration provides energy for Na^+ sequestration in the apoplast or in the vacuole; NO, along with ascorbate, GSH and Ca^{2+}, stimulate the H^+:ATPase and H^+:pyrophosphatase activities in maize seeds [*253*]. The rise in the intracellular $[Na^+]/[K^+]$ ratio is associated to

decreased NOS-like activity and NO contents in the salt-sensitive *Arabidopsis thaliana* [*256*], whereas the salt-tolerant *Populus euphratica* reacts inducing the NOS-like activity, the plasma membrane NADPH oxidase (through the NO signal), and the plasma membrane H^+:ATPase (through H_2O_2) [*251*]. With a proteomic approach, H_2O_2 and NO pretreatments were shown to prevent many of the proteome changes induced by salt stress in *Citrus spp.* (mostly regarding photosynthesis and defence or redox balance); moreover, H_2O_2 and NO effects are largely coincident [*218*].

Nevertheless, other experiments in maize show NO to actually cause a reduction in H_2O_2 content, together with an induction in antioxidant enzyme activities (GR, SOD and APX) [*204*]. Such differences in the responses may be due to the species, to the treatments (NaCl and PEG), and to different sites of NO and ROS production.

Hypoxia

Seeds and roots are frequently subjected to hypoxic conditions. Nitrite accumulation in hypoxia is often observed, and NO production is reported, inversely related to O_2 concentrations in the soil, apparently depending on NO_2^-. In turn, NO lowers O_2 consumption (for example, by repressing COX) and shifts the metabolism toward fermentative reactions, as a mechanism for hypoxic stress avoidance [*18*]. A class of plant hemoglobins, named stress-induced non-symbiotic hemoglobins, respond to environmental conditions leading to the inhibition of ATP synthesis in mitochondria (occurring when the electron transport is impaired), or a high sucrose concentration. Since hypoxia also promotes NO production from NR, several roles have been proposed for this hemoglobin class. They might contribute in NADH regeneration, associated to the oxidation of NO to NO_3^- or the de-repression of COX; NO scavenging would prevent PCD in the short term, allowing the development of adventitious root primordia [*63*]; reacting with nitrosothiols, as occurring also in bacteria, hemoglobins would control NO effect on proteins [*178*].

1.2.5.5 Pathogenetic and symbiotic relations

Plants adopt several strategies aimed to block pathogen proliferation. Non-host resistance is a default response, which can be avoided, tolerated or suppressed by host-specific pathogens. In this case, the plant adopt a specific recognition mechanism of avirulence traits, driving to the expression of *R* genes; beyond specific recognition, generic

pathogen-associated molecular patterns (PAMPs) stimulate the basal resistance [*72*; *99*]. NO was found to be required for the induction of phenylpropanoid metabolism genes, through the cGMP-cADPR pathway [*65*], and of hypersensitive response, together with H_2O_2 [*55*] after treatments with avirulent pathogens; furthermore, the production of peroxynitrite is suggested as a protective reaction, since plant cells can tolerate concentrations up to 1 mM with no or little effects on cell viability, whereas animal cells die at far lower concentrations [*56*]. Lipopolysaccharide PAMPs from a wide array of bacteria induced a NO burst and a set of defence genes in arabidopsis wild type, whereas the NO production-impaired mutant *AtNOA1* did not [*250*]. GSNOR is thought to affect pathogen resistance, but divergent results have been obtained: arabidopsis mutants were compromised in their response to pathogens at all levels (non-host, R and basal resistance) and in SA-induced gene expression [*72*]; arabidopsis antisense or overexpressive transgenic lines gave, respectively, increased and reduced SAR, according to nitrosothiols contents [*201*]. Differences may be explained considering that in the first study, GSNOR activity was probably absent, and SA level decreased, whereas in the second one the antisense plants had about half GNSOR activity, and SA content was similar, compared to the wild type [*99*].

Plant class 1 hemoglobins exhibit a high affinity to O_2 and NO and are involved in several NO-regulated processes, including the symbiosis between plants and host-specific symbiotic bacteria, such as legumes/rhizobia (leghemoglobins belong to class 2 hemoglobins) or *Alnus firma*/*Frankia spp*. During nodule formation, an early NO peak is rapidly quenched to allow the symbiosis to take place [*178*] and a class 1 hemoglobin is induced, whereas non-host rhizobia give neither of the responses, and pathogens provoke a high and continuous NO production and no hemoglobin induction [*167*; *205*]. Thus, a likely model for the infection process consists in the onset of a pathogen-directed response, started by the host plant with an increased NO production, but accompanied by the synthesis of a class 1 Hb (also responding to NO), which acts as a NO scavenger and allows the nodule formation. The expression profile of NO-regulated genes, analyzed in *Medicago truncatula* after infection with pathogen or symbiotic bacteria, offered significant data about the key regulation points which distinguish symbiosis from pathogenesis. 10 genes (some of them regarding lipid metabolism and signal transduction) were upregulated in both systems, whereas 47 showed an opposite regulation, controlling carbohydrate metabolism and redox balance [*73*].

1.3 Role of iron in plant systems

A great number of plant metabolic processes, such as: photosynthesis, respiration, nitrogen fixation, hormones biosynthesis, require iron, because of its capability to assume several oxidation states (Fe^0, Fe^{2+} or Fe^{3+}) at a physiological pH, with a plastic redox potential depending on the coordination ligands, thus taking part in reactions implying a redox exchange. Fe^{2+} and Fe^{3+} are small ions, easily forming hexacoordinated complexes with: O, N, S [*88*]. Such a paramount role of iron is reflected by the relatively high iron requirement in plants (2 μmol g^{-1} dry weight), and iron deficiency results in chlorosis in young actively growing tissues, severe yield losses and decreased nutritional quality of crops. Iron bioavailability is considered a major agricultural issue, because of the limited solubility of Fe^{3+} in alkaline conditions, as occurs in about 30% of cultivated land worldwide consisting in calcareous soils [*97*].

1.3.1 Iron metabolism

Despite its abundance, iron bioavailability is very low in alkaline calcareous soils. In fact, iron is found as insoluble Fe(III) hydroxides, with a solubility up to 10^{-17} M at pH 7.0; usually plants require an iron concentration between 10^{-8} and 10^{-4} M for proper growth [*97*]. On the other hand, not only iron insolubility, but also the potential dangers associated to iron when present in an oxidizing atmosphere must be tackled. In fact, the Fenton reaction ($H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$) is catalyzed by reduced transition metal ions, such as Fe^{2+} or iron complexes at a redox potential between -324 and +460 mV [*181*]. The hydroxyl radical ($OH\cdot$), a higly, unspecifically reactive oxygen species, can damage virtually any biological molecule, and cannot be detoxified. Thus, iron metabolism (fig. 1.5) must be tightly regulated, in order to achieve the balance between shortage and toxicity.

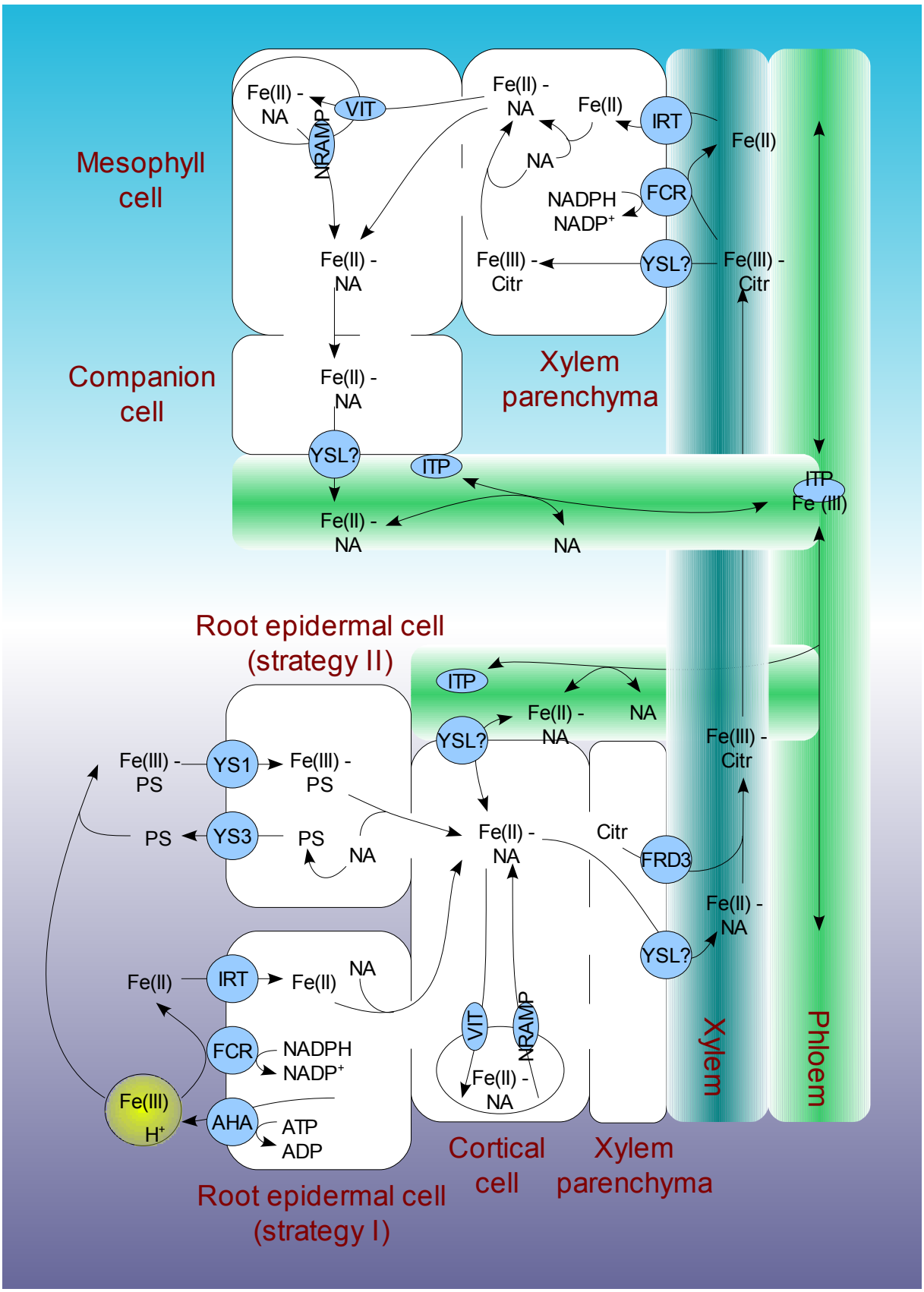

Figure 1.5: Overview of iron metabolism. PS – phytosiderophore; NA – nicotianamine; Citr – citrate.

1.3.1 Root uptake

The basic iron uptake model in plants is centered on a root plasma membrane Fe^{2+} transporter. Divalent iron is available in soils in reducing (for instance, anoxic) conditions; in oxidant soils, the insoluble, trivalent form is predominant, which can be solubilized by rhizosphere acidification or by chelating agents.

The remobilized Fe^{3+} is then reduced by a membrane-bound NAD(P)H-depending Fe(III)-chelate reductase (FCR; EC 1.16.1.7) of the FRO family. The main *Arabidopsis* member of the family, FRO2, is a protein composed by about 700 aminoacids arranged in 6 transmembrane helices, FAD and NAD(P)H binding sites, and a heme group. Its expression is maximal in peripheral tissues (epidermis, root hair) of iron-deficient roots, and is enhanced under iron deprivation [*41*]. Other members of the *FRO* gene family show different tissue expression, subcellular targeting, and respondence to iron or copper, addressing to the existence of stress-specific isoforms [*161*; *184*].

Fe^{3+} reduction is the limiting step in the whole iron metabolism [*41*]. The available form for uptake, Fe^{2+}, can be imported by means of transporters belonging to several families (ZIP, NRAMP), generally active towards several divalent metals and regulated by protein neosynthesis [*51*].

The ZIP family can be found in bacteria and eukaryotes, and is composed by wide-range transporters, including the IRT subclass. IRT1 is the main transporter, and is induced by iron deficiency, while IRT2, similar for sequence and structure, cannot complement IRT1 deletion, and its inactivation has no evident phenotype. IRT1 is regulated at transcriptional and post-transcriptional levels; in analogy with other members of the same family (ZRT in yeast), a regulation mechanism is proposed in which a specific Lys residue undergoes ubiquitinylation, leading to protein degradation in optimal growth conditions. The same transporter is permissive towards Zn and Cd, although with lower affinity [*40*].

NRAMP is a family of eukaryotic, membrane-bound metal transporters with a high affinity for iron [*97*]. Their regulation is transcriptional and post-transcriptional, and *AtNRAMP1*, *3* and *4* genes are shown to be induced under iron deficiency [*51*]. NRAMP1 and IRT2 are found in the vascular parenchyma, possibly indicating a role in long distance transport [*15*].

Iron deprivation induces an increased root uptake, according to two strategies. The widespread strategy I consists in the solubilization of Fe^{3+} by rhizosphere acidification,

mediated by plasma membrane P-type H^+:ATPase (AHA family in *Arabidopsis*), and in the induction of FCR and transporters. Besides that, root hair and transfer cell development and changes in the organic acid content occur [*97*; *112*].

The strategy II is limited to graminaceous plants, and is based on the release of phytosiderophores, non-proteinogenic aminoacids related to the mugineic acid (MA). These compounds are able to chelate Fe^{3+} even at high pH and in presence of bicarbonate, and their synthesis requires methionine and nicotianamine (NA) as precursors (3 methionine → 3 *S*-adenosyl methionine → nicotianamine → MA). NA can be found in all upper plants, but is converted to mugineic acid and related compounds (MAs) only in grasses. Phytosiderophores efficiency depends on their affinity to the plant uptake system, their resistance to microbial degradation, and their iron(III)-chelating power, determined by the degree of hydroxylation [*235*]. The secretion of phytosiderophores is controlled by *YS3* in maize [*51*], and follows a temperature-controlled circadian trend; it relies on H^+-ATPase activity and K^+ gradient, and is likely to occur by hexocytosis [*142*].

The uptake of Fe(III)-MAs involves the specific carrier YS1, belonging to the oligopeptide transporter (OPT) family. The level of YS1 specificity can vary among species, with barley isoform inactive towards Fe(II)-MA or Fe(III)-NA, and maize isoform permissive towards several metal complexes. The expression of YS1 is maximal in roots, and can be induced by iron deficiency; it works as an electrochemical potential-driven proton cotransporter, despite being insensitive to pH, and therefore efficient in calcareous soils as well [*162*; *206*]. Since NA is a metabolic precursor of phytosiderophores with iron chelating ability, and eight *Arabidopsis* genes (*YSL1-8*) share high homology towards *YS1*, such an iron-chelate transport can be proposed in strategy I plants too [*51*]. Indeed, phytosyderophores and grass consociation were shown to improve iron nutrition in several dicotiledonous crops, including *Citrus* species and peach [*32*; *150*]; interestingly, the *ys3* maize mutant, unable to secrete phytosiderophores, had no such effect on the consociated plant. Bacterial siderophores, although not directly metabolized by plants, can contribute to plant nutrition by improving metal solubility [*88*].

In barley, maize, and presumably most of strategy II species, the Fe(II) transporter IRT is not induced by iron deprivation. On the contrary, rice is adapted to water-logged soils, where Fe(II) is more abundant than Fe(III); thus, this species retains the ability to induce specific IRT and FRO isoforms. Despite a preference for Fe(III)-MAs, Fe(II)-MAs can be

uptaken as well by rice roots; yet, rice emits a relatively low amount of phytosiderophores, and Fe^{2+} could be the most nutritionally important iron form [*104*].

1.3.1.2 Long-distance transport

Xylem transports micro-nutrients from the root to mature transpirating organs, whereas forming organs rely essentially on phloematic transport.

The phloematic pH (between 7 and 8) induces iron precipitation. Nicotianamine (NA) shows a peculiar stability when complexed with Fe(II) in this pH range, and prevents the Fenton reaction; its actual presence in the phloem leads to propose a role as a Fe(II) chelator, and to postulate a membrane Fe(II)-NA transporter [*51*; *234*]. In absence of NA, iron remains in the xylem, as it cannot be uptaken in the symplast, particularly in growing tissues [*22*]. Eight genes, in *Arabidopsis thaliana*, show high homology towards *YS1*, which encodes for a root iron-phytosiderophore transporter in maize. Such genes, named *YS1*-like (*YSL1-8*), in a strategy I plant could encode for Fe(II)-NA carriers, involved in apoplast-symplast transloading [*22*]. Indeed, the expression of *YSL1* is verified under iron excess in shoot xylem, seeds and siliquae, allowing to store iron in ferritins and vacuoles [*130*]. The role of each YSL member is probably specified by its intracellular location. Thus, *AtYSL1-3* are expressed in the xylem parenchyma, whereas AtYSL4 and 6 were found in the vacuolar proteome, possibly taking part in the remobilization of stored iron [*22*].

The ITP protein was identified as a LEA homologue with a high affinity for Fe(III). Its presumed role consists in trasporting iron in the phloem, receiving it from NA in source tissues and delivering it back to NA in sink cells [*122*]. NA would therefore maintain a steady-state Fe^{2+} level in the symplast.

Iron loading in the xylem takes place as Fe(III)-chelate, complexed to citrate or other organic acids; the flow is driven by transpiration, and the complex is the substrate of plasma FCR in target cells [*51*]. The *Arabidopsis thaliana* FRD3 protein is a specific citrate transporter of the multidrug and toxin efflux (MATE) family, located in root pericycle membrane; *frd3* mutants constitutively express iron deficiency responses, since iron cannot be carried to leaves, which in turn signal their starved status to roots. In soybean, the FRD3 homologues expression and citrate contents in the xylem are shown to correlate with iron efficiency [*66*; *85*; *193*].

1.3.1.3 Subcellular localization, storage and detoxification

The subcellular iron buffering system is based on nicotianamine (NA). NA synthesis is stimulated by low iron concentrations; the tomato mutant *chloronerva* (*chln*), unable to produce NA, is constantly affected by iron deficiency symptoms even at high iron levels, because iron precipitates into insoluble salts. Thus, NA works as a chelator, keeping iron soluble and available, and possibly signalling its nutritional status. Interestingly, in *chln* mutants no ferritin induction takes place, suggesting that in the absence of NA, not even iron excess can be perceived [*11*].

Vacuoles, plastids and mitochondria are the main iron storage compartments in plants [*12*; *22*]. Iron phosphates or metal-phosphate phytate complexes are found in the apoplast, and could also have a physiological role [*11*]. In spite of the early observation of vacuolar iron inclusions under iron excess [*110*; *180*], vacuoles could be demonstrated to be the main iron storage site after the characterization of several iron transporters. The double *nramp3 nramp4* arabidopsis mutant is severely impaired in seed germination under low iron supply, and the seed iron stores remain associated to the forming vacuole; both NRAMP3 and NRAMP4 are vacuolar metal transporters, with a presumably redundant function, and promoted under iron deficiency [*125*]. VIT1, as its yeast orthologue, is required for vacuole loading [*113*].

Ferritin is a hollow protein complex, consisting in 24 identical, nuclear genome-encoded subunits, able to store up to 4500 Fe^{3+} ions. Ferritins can be found in plants, animals and bacteria; in animals, two different subunits, H and L, can be distinguished, specialized respectively in rapid uptake and in long-term stabilization; the main role of iron detoxification or storage is determined by the relative abundance of the two isoforms. Plant ferritins have some distinctive features: they are related to animal H subunit, but no specialization is detected in function, since both H and L characteristics (ferroxidase centre and nucleation-promoting aminoacid residues) are found on the same protein. They are synthesized as precursors containing a transit peptide, and are found in plastidia and possibly mitochondria. Because of this prokaryote-like environment, with a high P/Fe ratio, the iron core is amorphous, unlike crystalline structures in animal ferritins. A N-terminal extension in the mature protein regulates its stability [*23*]. The loading/unloading phase involves a ferroxidase activity, expressed by each protein subunit, according to the reaction:

$$2\ Fe^{2+} + O_2 + 4\ H_2O \rightleftarrows 2\ FeO(OH) + H_2O_2 + 4\ H^+$$

The release of iron causes the oxidation of the N-terminal extension peptide, leading to the degradation of the subunit. Ascorbate, which is reported to reduce ferric iron to ferrous, both provides the substrate for ferroxidase reaction, and promotes iron extraction from ferritin [*21*]. The main role of plant ferritins is probably in detoxification of excess iron rather than storage [*23*; *166*; *189*].

Frataxin is also a protein linked to iron metabolism. It is found both in prokaryotes and eukaryotes, where it is located in mitochondria. Among the proposed roles, it takes part in iron homeostasis, iron-sulphur cluster assembly, oxidative stress prevention. While frataxin knock-out mutants are lethal, a reduced frataxin expression is associated to lower activity of iron-sulphur enzymes, oxidative stress [*27*], increased iron accumulation, and NO production in roots, either as a Fe/ROS scavenger, or as a ferritin inducer [*148*].

1.3.1.4 Homeostasis

Iron metabolism homeostasis is maintained in plants mainly by transcriptional regulation of uptake and storage apparatuses. While it is possible to identify essential *cis* elements in the promoters of some [Fe]-responding genes (the IDRS element in *AtFer1* promoter [*179*], or the FRE element in soybean [*241*]), tissue-specific gene expression is supposedly determined by a *trans* element activation pattern. Among the transcription factors, the basic helix-loop-helix (bHLH) family includes about 160 proteins in *Arabidopsis*, characterized by a short N-term basic domain, and a HLH domain formed by 2 amphipatic α-helices, connected by a variable loop. The basic domain recognizes the target DNA E-box sequence (CANNTG), when dimerization takes place by protein-protein interaction at the HLH domain.

AtbHLH38 and *AtbHLH39* genes are the likely outcome of a recent duplication event. Their expression, mediated by SA and JA, drives to riboflavin accumulation and excretion under iron deficiency in several unrelated species (tobacco and sunflower, but not arabidopsis). Since tobacco and sunflower are considered iron-efficient plants, riboflavin could take part in iron metabolism, either as an electron donor for iron reduction, an enzymatic cofactor, or a modulator of rhizospheric microflora [*236*].

The tomato FER protein is also a bHLH transcription factor, essential for iron starvation responses, like the induction of FRO iron-chelate reductases and Fe(II) carrier (IRT) isoforms, and the morphological adaptation by means of lateral roots and root hairs. Despite the iron

deficiency response involves all tissues, the *FER* gene is specifically expressed in roots, addressing to a systemic messenger acting on FER protein [*135*]. Its expression is constant at low or sufficient iron levels, while it is reduced under iron excess [*26*]. The *FER* orthologue in arabidopsis, named *FIT1*, (synonyms: *AtbHLH29*, *FRU* [*39*; *105*]), is found in both root and leaves, is promoted under iron starvation, and possibly post-translationally removed by ubiquitin-dependent degradation. FER/FIT1 controls a large subset, but not the totality of iron-responding genes; its constitutive overexpression does not grant an increased inductions of *FRO* and *IRT* genes; IRT1 product stability (but not gene expression) is supposed to be only indirectly regulated by *FIT1* [*39*; *105*]. Taken together, these data suggest additional regulatory mechanisms, including post-translational ones. Nicotianamine is also required to trigger the induction of IRT1 and NRAMP1, possibly acting as a Fe sensor [*15*]. *YSL2* has the closest homology to maize *YS1*; its circadian expression parallels with FRO and IRT, and its product is located in the root pericycle and endoderm, addressing to a role in xylem loading under iron and zinc deficiency [*207*]. The genome of *Arabidopsis thaliana* includes 8 putative metal-chelate reductase (*fro*) genes. Their expression was studied according to tissue specificity and iron- or copper-deficiency respondence [*161*]; *fro2* appears to be the main FCR responding to iron shortage.

	Putative	Metal shortage		Tissue specificity		
Gene:	target	Fe	Cu	Root	Shoot	Silique
fro1	PM			-	-	+
fro2	PM	↑R		+	+	-
fro3	PM, mit	↑R↑S	↑R↑S	+	+	+
fro4	PM, mit			-	-	+
fro5	PM	↑R	↑R	+	-	-
fro6	PM, chlp	↓S	↓S	-	+	+
fro7	chlp	↑S	↓S	-	+	+
fro8	PM, mit	↓S	↓S	-	+	+

Table 1.1: Expression analysis of fro *genes in arabidopsis.* PM – *plasma membrane*; mit – *mitochondria*; chlp – *chloroplasts*; R – *root*; S – *shoot [*161*]*

In graminaceous plants, many of the genes induced under iron deficiency affect the synthesis and modification of phytosiderophores. A specific gene family encoding for putative bHLH proteins was found. The rice *iro2* gene is induced under iron shortage, mostly

in roots; its gene product specifically binds the CACGTGG sequence (G-box + G), which is found in promoters controlling the synthesis of phytosiderophores, energetic metabolisms, and other transcription factors [*173*]. Moreover, two synergistic sequences, named IDE1-2, were identified in iron deficiency-responding promoters in rice, and possibly in strategy I plants as well [*116*].

Split-root experiments allowed to show how the iron-starved part of the root induce deficiency responses also in the iron-sufficient part, thus suggesting a shoot-to-root phloematic signalling. This view is supported by evidences in the pea mutants *brz* and *dgl*, where the signal appears to be constitutive and the iron deficiency response cannot be turned off [*87*], and in the tomato mutant *chln*, where the synthesis of NA is impaired [*180*]. Thus, NA is proposed as a possible iron sensor and deficiency signal; it was found that NA biosynthesis is more active when iron availability is low [*12*; *51*]. Iron deficiency responses require a low, but significant iron content in the soil or growth medium, and are switched off at higher contents. Thus, a cross-regulation could be present, in which the root checks locally whether iron is available, while the shoot systemically transmits its nutritional status. A model involving two counteracting trends (external Fe-induced activation, internal Fe-induced suppression) would grant a fine-tune regulation of iron uptake [*232*].

Hormones are also thought to be involved in long distance signalling of iron status. Ethylene and auxins determine a morphologic adaptation, consisting in the development and growth of cluster roots, root hairs, or alternatively transfer cells [*240*]. However, it is not clear whether the same signal cascades triggered by hormones bring to physiological responses, besides morphological ones. The pea mutants *dgl* and *brz* can induce transfer cell differentiation according to the iron content, whereas the FCR is constitutively active [*208*], addressing to a different regulation of local and systemic responses. Ethylene is not autonomously able to induce FRO, IRT, FER, H^+-ATPase expression or activities in healthy plants, but inhibitors of its synthesis suppress such responses to iron deficiency; therefore, ethylene could be a secondary messenger depending on other, shoot-derived factors, such as auxins, and be repressed by phloematic iron content [*141*; *209*]. Shoot-derived auxins also activate the root H^+-ATPase, inducing the rhizosphere acidification and supporting the iron uptake strategy I. ABA, along with other signals linked to the redox status and iron excess, promotes ferritin transcription [*20*].

1.3.2 Responses to iron deficiency

More than 90% of total iron in leaves is found in chloroplasts, where it takes part in the construction of cytochromes, ferredoxin, thylakoydal electron transfer chain intermediates, chlorophyll precursors, and other enzymes containing single Fe atoms, heme moieties (such as catalase and peroxidases) or iron-sulphur clusters [*22*; *112*; *187*]. Iron deficiency causes a reduction in photosynthetic pigments, mainly affecting chlorophyll *b* and the antenna complexes, while xantophylls tend to be conserved, since their protecting action is required against the onset of photoinhibition and downregulation of photosystem II, due to the reduction of photosynthetic efficiency [*159*; *211*]. As a result, forming leaves turn chlorotic. Hydrogen peroxide content is reported to rise significantly, both because of the lack of catalase and peroxidase activity, and the expression of superoxide dismutase isoforms, possibly linked to superoxide formation after photosystem I disgregation. Ascorbate-glutathione cycle enzymes (monodehydroascorbate reductase, glutathione reductase) are promoted, but their competition for reducing equivalents, added to a limited NADPH regeneration with low photosynthetic activity, causes a drastic fall in carbon organication [*187*; *188*].

As a general response, nutrient deficiency promotes the stimulation and differentiation of biochemical and morphological adaptations, such as cluster roots, root hairs and transfer cells, along with an increased FCR activity in strategy I plants [*208*; *240*]. Iron deficiency symptoms appear more severe when the root energetic status is depressed, for example in hypoxia.

Microarray analyses in different species [*39*; *97*; *168*; *220*] evidenced significant variations in transcription in iron-deficient conditions. Among the upregulated genes, H^+-ATPases, enzymes required for methionine biosynthesis and Yang cycle (catalyzing 3 Met → NA → MA), 14-3-3 proteins, putative transcription factors, homologues of eIF and EF (initiation and elongation of mRNA translation; the regulation of iron metabolism is in many cases post-transcriptional), metal ion transporters (YS1 and ABC). Downregulated genes include ferritin and ferredoxin, while antioxidant enzymes such as catalase and lipoxygenase show no significant variations. Interestingly, an effect of pH was found on *FER* in tomato (fig. 1.6), with alkaline medium reducing its expression, but promoting *FRO*, *IRT1-2* and *NRAMP*, and confirming the uncomplete dependence of these genes from FER [*254*].

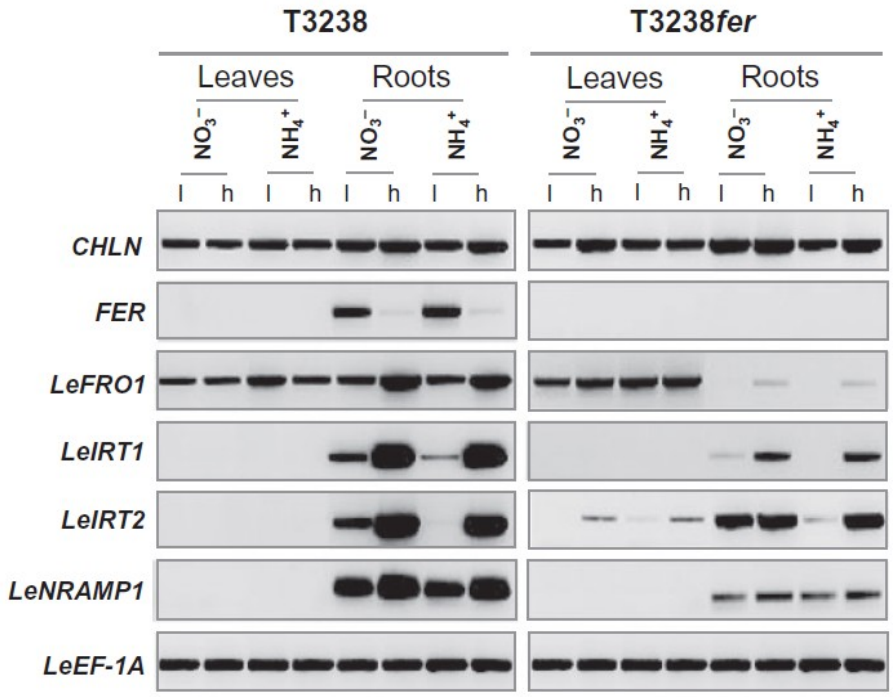

*Figure 1.6: Expression of iron uptake-related genes under low (l, 5.0) or high (h, 7.5) pH in wild type (T3238) tomato, and in the corresponding FER-deficient mutant [*254*].*

1.3.2.1 Iron deficiency in field systems

In perennial plants, chlorosis is complicated by the two-year fruit bearing cycle [*216*]. A high fruit load in one season will worsen the symptoms of chlorosis in the following year, since fruits are strong charbohydrate sinks, and resources left for the next season may be not sufficient to support root growth and function. Fruit trees are usually grafted, which allows a better management of iron nutrition by the use of resistant rootstocks; on the other hand, incompatibility, by limiting root-to-shoot translocation, and specific scion susceptibility, may lead to iron nutritional deficit.

Iron availability in soils is usually higher than expected from the theoretical equilibrium between soluble and adsorbed iron, because of rhizosphere conditioning by organic chelating agents, such as phytosiderophores, and pH control [*187*]. Despite this, the presence of bicarbonate in soils determines a buffering in a pH range from 6.5 (bicarbonate/carbonic acid) to 8.5 (carbonate/bicarbonate) [*140*]. In these conditions, since pH cannot be significantly lowered, the iron uptake strategy I is not efficient; thus, the amount of iron solubilized by chelants (such as bacterial or plant siderophores) or organic matter is crucial. The kind of

nitrogen source also affects the soil pH, since nitrate intake is coupled to H^+, whereas ammonium must lose one H^+ [*108*; *254*].

The linkage between soil bicarbonate and chlorosis is not completely established. While some authors believe chlorosis to be essentially caused by a reduced uptake [*171*; *172*; *198*], others find an increased apoplastic pH, supporting the inactivation (that is, precipitation) of the internal iron pool, mainly in the root apoplast [*121*; *138*; *194*]. In some cases, chlorotic leaves are reported to contain iron amounts comparable to green leaves, a phenomenon referred to as the 'chlorosis paradox'. In the first view, the high concentration is explained with a reduced leaf expansion (yielding an equivalent concentration), whereas in the second hypothesis iron results mostly unavailable.

Iron efficiency does not appear to be necessarily associated to an increased uptake, although root apoplast iron precipitates can be mobilized by acidification. Nevertheless, the transient induction of iron-chelate reductase activity has been proved to correlate to tolerance towards chlorosis, in Fe-deprived peach rootstocks after iron resupply [*80*; *81*]. Carbon fixation by means of the phosphoenolpyruvate carboxylase (PEPC) activity, on the other hand, helps contrasting chlorosis, by subtracting bicarbonate, controlling the internal pH, stimulate the Krebs cycle to feed the FCR activity with NAD(P)H, or producing chelants to support xylematic iron translocation [*137*; *139*; *216*], since stomata stay open and carbon compounds can follow the transpiratory stream [*195*]. A higher PEPC activity was actually found in roots of tolerant, but not sensitive kiwifruit cultivars [*194*]. A direct interaction of bicarbonate or CO_2-derived radicals (for example, by reaction with peroxynitrite [*101*]) with Fe(III) reduction or tissue growth cannot be excluded [*198*]. Iron deprivation increased FCR activity, whereas bicarbonate depressed it in roots of sensitive, but not resistant peach rootstocks; proton pumps, strongly active in the resistant genotype under nutritional starvation, failed to be induced with bicarbonate [*158*]. These data suggest that the two growth conditions represent distinct stress instances.

Chlorosis can be reverted by iron chelate treatments either in the soil, or on leaves. The first case is poorly effective, since iron precipitation, and possibly leaching, may occur; the foliar sprays are more effective, but only allow a nutritional integration in limited, critical phases; in both cases, the high cost of iron chelates must be considered. Several soil management practices allow to reduce the incidence of chlorosis, such as the control of organic matter, localized acidification, or the consociation with grasses. Acidic treatments on

leaves were also shown to induce the regreening of chlorotic leaves, suggesting the remobilization of apoplastic inactivated iron [*120*; *194*], but the efficiency was not up to the field practice [*216*].

1.3.3 Responses to iron excess

Iron excess seems to affect photosynthetic capacity, lowering the saturating CO_2 concentration and reducing potential. As a consequence, glycolysis and oxidative pentose phosphate pathway are stimulated, to provide respectively ATP and NADPH (also required for ROS scavenging). Vacuolar iron inclusions can be found, addressing to a detoxification mechanism relying on sequestration and precipitation, as found in yeast [*110*]. Induction of peroxidase is reported, to prevent H_2O_2 reaction with free transition metals [*70*].

Abscisic acid is generally involved in stress responses; one of its main effects is in stomata closure, and consequently the lowering of the $[CO_2]/[O_2]$ ratio. To balance the risks connected to increased O_2 levels, ABA can induce at least some ferritin isoforms, preventing ROS to react with iron. Pea mutants *brz* and *dgl*, accumulating excess iron in leaves, show a sensibly increased concentration of ferritin compared to the wild type [*12*]. Ferritin regulation in animals is post-transcriptional and depends on NO, since the ferritin mRNA possess a IRE sequence in 5' (blocking translation when iron is not available). Plant ferritins are transcriptionally and post-transcriptionally regulated, but no IRE sequences have ever been found; nevertheless, NO (specifically nitrosonium, NO^+) is required, along with other factors such as ABA [*163*; *164*; *165*]. Under standard iron nutrition, ferritin genes are kept repressed by uncharacterized factors interacting with promoter elements, such as IDRS in *Arabidopsis* [*179*] and FRE in soybean [*241*]. Under iron excess conditions, a signal cascade involving NO and protein phosphorilation leads to IDRS de-repression. *AtFer3* follows the same expression pattern of *AtFer1*, whereas the other ferritin genes, *AtFer2* and *4*, do not, despite all of them including the IDRS element in the promoter, and other regulating elements could be active [*219*]. The analysis of the expression of ferritins under photoinhibition [*163*] or other conditions associated with an increased ROS production allowed to demonstrate an induction of *AtFer1*; on the other hand, knock-out mutants display early senescence symptoms [*166*] and increased sensitivity to iron excess, but no seed germination or plant growth defects [*189*]. Taken together, these results suggest a role for ferritins in iron detoxification rather

than storage [*23*]. It must be noticed that in the chloroplast, both oxygen and reductants are produced, and Haber-Weiss and Fenton reactions must therefore be strictly avoided.

1.3.4 Roles of NO in iron metabolism

NO has been shown to affect iron metabolism in several ways. Considering the impact of NO on the antioxidant defences at different suboptimal iron concentrations in maize, hydrogen peroxide, superoxide and lipid peroxidation were kept as low as the control (optimal Fe supply) by NO, while APX, GPX and catalase were increased and SOD and GR decreased. In all cases, the effect of NO contrasted with the decreased iron concentration [*215*]. A NO donor improved the symptoms in iron-deficient maize plants, along with chlorophyll content and carbon fixation. Iron concentration in treated and untreated, iron-deficient plants was similar, despite the fresh weight was double in the first case; thus, NO acts supposedly by improving iron availability rather than iron uptake. Both tomato (a strategy I plant) and maize (strategy II) iron-inefficient mutants reverted after a NO treatment [*83*].

Some evidences exist for a role of NO in controlling the onset of the iron uptake strategy I. In iron deficient tomato plants, NO content and *FER*, *LeFRO1* and *LeIRT1* transcription in roots are increased. A good correlation emerges between the concentration of GSNO, used as a NO donor, and the expression of the same genes under iron starvation. None of these effects resulted in the *fer* mutant, suggesting NO and FER to act together in mediating the iron deficiency signal. Nevertheless, not even in this case, variations in iron content could be found between iron-starved plants, submitted or not submitted to GSNO treatment [*84*]. Supporting the view of an increased iron availability, the NO donor SNP was shown to cause both a rise in nitration, and a decrease in oxidative stress (protein carbonylation and membrane disruption), along with a sensible increase in the labile (that is, redox-active) iron pool. Labile iron is reported to be a minor fraction (3-5%) of total iron content; thus, NO

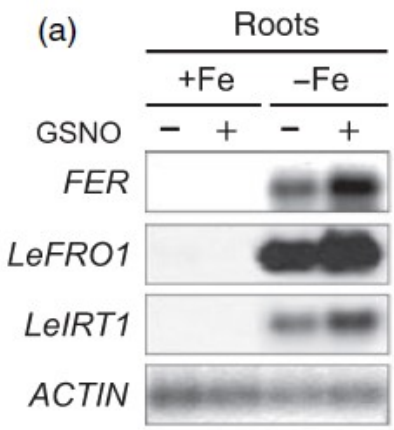

*Figure 1.7: Effect of the NO donor, GSNO, on iron-starved or iron-sufficient tomato roots [*84*].*

could extract iron from biological storage or immobilization forms, and in the same time subtract it to the Fenton chemistry [*107*].

In case of iron excess, NO improves the stability of ferritin mRNA, and its action spans several hours after the stress is imposed. These observations address to NO as a signal downstream to iron concentration, and exclude a role in oxidative stress protection [*58*]. The same kind of regulation is achieved in mammals through the Iron Regulating Element (IRE)/Iron Regulating Protein (IRP) interaction: specific elements in mRNAs are recognized by an aconitase isoform, after the NO-mediated release of its iron-sulfur cluster, either in the 3'-UTR to prevent translation, or in the 5'-UTR to prevent degradation. No such a mechanism is documented in plants, although aconitase-mediated regulation cannot be excluded [*157*].

Besides the Fe signal, oxidative stress can also promote the *Arabidopsis* ferritin gene (*AtFer1*) transcription, by means of a separate and additive pathway. A NO burst (probably NOS-like-dependent) takes place in the Fe pathway, leading to the de-repression of the iron-dependent regulatory sequence (IDRS), in the *AtFer1* promoter. IDRS-containing promoters, under standard conditions, are probably inactivated by an unknown repressor, which undergoes ubiquitinylation and proteasome-dependent degradation in presence of iron [*5*; *164*].

2 AIM OF THE THESIS

The present work aims to clarify the role of reactive nitrogen species and antioxidant systems in plant iron metabolism, and to suggest the possible implications in field practice and agricultural sciences. Two main reasons led to the choice of peach rootstocks as the experimental model. Firstly, peach is an important crop in northern Italy. On the world scale, Italy ranked second among the top peach producer countries in 2007 [*71*], and about 30% of the production in Italy (50% considering only nectarines) takes place in Emilia-Romagna region, where the production is protected by geographical indication. Secondly, peach is considered a species easily undergoing chlorosis when grown in calcareous soils, which are a large share of global agricultural soils, and frequently occur in northern Italy as well [*216*]. Moreover, this species can be assumed as a model for other stone fruit trees, because of its relatively simple and well-studied genetic and biochemic features [*69*].

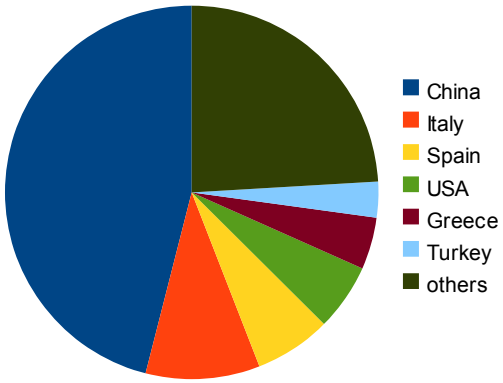

Figure 2.1: World peach production in 2007 [71].

Three commercial hybrid genotypes, normally used as peach rootstocks and characterized by different degrees of tolerance to chlorosis [*216*], and a peach cultivar, were chosen in this work to test their responses to bicarbonate. GF677 (*P. persica* × *P. amygdalus*) is the most widespread peach rootstock in calcareous soils, being considered highly resistant to bicarbonate. MrS 2/5 (*P. cerasifera* × *P. spinosa?*) is a hybrid selection, which has proven fairly tolerant to lime in fertile soils. Ishtara ((*P. cerasifera* × *P. salicina*) × (*P.cerasifera* × *P. persica*)) is generally considered a susceptible genotype. The cultivar Big Top (*P. persica* var. *nectarina*) was also included to test the performance of a non-hybrid peach genotype, although no information about its tolerance to lime was available [*210*; *216*].

The plants were micropropagated and subcultured *in vitro*; this allowed a constant supply of sample plants and a reliable control of growth conditions. The stress was imposed with 6 mM potassium bicarbonate and pH 7.6 in the medium, while control plants grew on optimal medium at pH 5.6; in order to recreate more realistic conditions, we found a better choice to include bicarbonate and alkaline pH rather than subtract iron from the medium.

Previously the *in vitro* selection for resistant plants has been successfully carried out in different species and for several abiotic stresses, including iron limitation [*62*; *147*; *176*]. Thus, a correlation between *in vitro* and *in vivo* performances could be presumed with a reasonable confidence. Moreover, despite the reports of a promoted root uptake under iron shortage, the frequent observation [*84*] of an unvarying total iron content in healthy and stressed plants, even in controlled growth conditions, suggested to consider root uptake as a possible important response, but not as a stand-alone requisite for the tolerance to bicarbonate. Thus, other responses, occurring in the shoots, were investigated.

In particular, the antioxidant capacity is considered a main component of stress tolerance. In addition, recent works pointed to the role of RNS in preventing stress, or mediating the adaptive responses to it.

Our working hypothesis assumed that the combined effect of bicarbonate and pH in the medium would induce changes in ROS and RNS contents, and those factors would interplay leading to stress damages or stress tolerance. Thus, the redox status of the plant, the H_2O_2 content, and several antioxidant enzymes were tested; the content of RNS was also estimated, and their likely source identified. These data were referred to some stress markers, as well as to the known behaviour of each genotype in presence of bicarbonate. Moreover, analogies in the response strategies of the different genotypes were analyzed.

Data obtained from this work could hopefully lead to the development of efficient and cheap tools for the treatment of iron-deficient orchards, and to the identification of suitable markers for an early selection of the resistance trait.

3 MATERIALS AND METHODS

3.1 Plant material

Three rootstock genotypes for peach and one peach cultivar, cultivated *in vitro*, were purchased from a commercial nursery (Vitroplant, Cesena, Italy). The genotypes were GF 677 (*P. persica* × *P. amygdalus*), MrS 2/5 (*P. cerasifera* × *P. spinosa?*) and Ishtara ((*P. cerasifera* × *P. salicina*) × (*P. cerasifera* × *P. persica*)), and the cultivar was the nectarine Big Top (*P. persica*).

All the genotypes were multiplicated in MS medium containing: sucrose (30 g/l), myo-inositol (100 mg/l), thiamine-HCl (1 mg/l), nicotinic acid (0.5 mg/l), pyridoxine (0.5 mg/l), glycine (2 mg/l), N^6-benzylaminopurine (1 mg/l), indolebutyric acid (0.05 mg/l), GA_3 (0.1 mg/l), and agar (6.5 g/l). The pH was adjusted to 5.6 by adding KOH before autoclaving. Standard growth conditions were: 22 ± 2 °C and a 16-h photoperiod with 30 µmol m^{-2} s^{-1} photosynthetic active radiation.

Plants grown on multiplication medium were used as control. Bicarbonate stress was imposed by growing plants on an identical medium, added with 6 mM $KHCO_3$ (0.601 g/l) and adjusted to pH 7.6. To discriminate among the source of NO, to the same medium containing $KHCO_3$, either 5 mM sodium molybdate, sodium tungstate, L-arginine or L-nitroarginine were added.

Both control (C) and stressed (K) plants were grown about 25 days before being used for the assays.

3.2 Standard extraction

The plant samples, consisting of whole regenerated plantlets devoid of callus and senescent tissues, were weighted and ground in liquid nitrogen, and the frozen powder was transferred to tubes containing cold extraction buffer. After a 20-min incubation in ice bath, the samples were centrifuged at 13,000×*g*, 4 °C, and the supernatant was collected. Sephadex G-25 gel column (NAP-25, Amersham Biosciences) were used, when specified, to desalt raw extracts and resuspend the soluble proteins in the assay buffer subsequently used.

Some methods allowed the sample to be frozen and stored at -80 °C until use for a few days.

3.3 Metabolite analysis

3.3.1 H_2O_2

The H_2O_2 content was determined as described by Wolff [*243*] with minor modifications, measuring the colorimetric reaction of xylenol orange with Fe(III), generated after the oxidation of Fe(II) by H_2O_2 and other peroxides. The Fe-xylenol orange reagent (FOX) contained 1 mM xylenol orange, 500 µM $(NH_4)_2Fe(SO_4)_2$, 100 mM sorbitol, and 50 mM H_2SO_4.

The plant material was extracted in 4 ml 0.2 M HCl + 1% PVPP. All the following operations were carried out with plastic labware. Since ascorbate interferes with the colorimetric reaction, 50 µl of the extract were incubated 5 min with 0.2 U of ascorbate oxidase in 950 µl 200 mM potassium phosphate buffer, pH 5.8. Subsequently, 200 µl of FOX reagent were added and the reaction proceeded at least 30 min.

The absorbance of the samples was read spectrophotometrically at λ=560 nm, and the concentration of hydroperoxides was obtained from a standard curve.

3.3.2 Ascorbate

Reduced and total ascorbate contents were measured according to Queval and Noctor [*185*], by following the spectrophotometrical disappearence of ascorbate in presence of ascorbate oxidase. Ascorbate oxidase was dissolved in 200 mM potassium phosphate buffer, pH 5.8, to a concentration of about 100 U/ml; the solution was subdivided into aliquotes.

The plant material was extracted in 4 ml 0.2 M HCl + 1% PVPP, and the extract was conserved a few days at -80° C. The frozen extracts were thawed on ice, and an aliquot was incubated (1:1) with 4 mM dithiotreitol; the mixture was kept at 0-4° C at least 30 min.

100 µl of raw (for reduced ascorbate) or dithiotreitol-treated (for total ascorbate) extract were suspended in 900 µl 200 mM potassium phosphate buffer, pH 5.8, and the absorbance

was read at λ=265 nm. The sample was then added with about 2 U ascorbate oxidase, and the decrease in absorbance was followed until a stable value was reached.

The difference between the initial and the final absorbance was referred to an ascorbate standard, and the concentration was calculated accounting for dilution factors. The ratio between reduced and total ascorbate was assumed as the redox status index.

3.3.3 Total thiols

The thiol concentration was assayed as described by Queval and Noctor [*185*], by means of the colorimetric reaction of sulfhydryls with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent).

The plant material was extracted in 4 ml 150 mM potassium phosphate buffer, pH 7.5 containing 6 mM EDTA and 1% PVPP.

200 µl of extract were added to 700 µl of extraction buffer and 100 µl of 10 mM DTNB, and the absorbance was read at λ=412 nm after 5 min. The concentrations were calculated by means of a standard curve.

3.3.4 MDA

The concentration of lipidic peroxides was measured as malondialdehyde (MDA) equivalents, following the method by Heat and Packer [*96*] with minor modifications. The reagent solution was 0.5% 2-thiobarbituric acid in 100 mM potassium phosphate buffer, pH 2.0.

Plant samples were extracted in 100 mM sodium-potassium phosphate, pH 7.0, + 1% PVPP, frozen and stored at -80° C.

150 µl of raw extract were added to 850 µl of reagent solution. The reaction proceeded 30 min at 100 °C, then the samples were cooled down and centrifuged (5 min, max rpm).

For each sample, the absorbance was taken at λ=532 and λ=600 nm. The concentration of MDA equivalents was calculated from the difference between the two wavelengths, basing on a standard curve.

3.3.5 NO

The NO content was estimated by the fluorescence of the probe 4,5-diaminofluorescein (DAF-2).

Fresh plantlets (about 0.2 g) were extracted in 750 µl 200 mM sodium phosphate buffer, pH 8, + 1% PVPP.

100 µl supernatant were transferred to a tube containing 900 µl 50 mM HEPES buffer, pH 7.5, and 2 µl DAF-2 solution (5 mM in DMSO) were added to each tube. A blank was included, containing 1 ml HEPES buffer and 2 µl DAF-2 solution. The samples were kept 2 hours in dark at 37 °C.

The samples were excited with a λ=495 nm and the emission at λ=512 nm was taken. Each measure was calculated as the difference between the sample and the blank, referred to the fresh weight of the sample, and expressed in arbitrary units (AU).

3.4 Enzymatic assays

3.4.1 Catalase

Catalase was assayed as described by Havir and McHale [*94*].

Plant tissue was ground and transferred in cold extraction buffer (100 mM sodium-potassium phosphate, pH 7.0, + 1% PVPP); the extract was desalted and resuspended in 50 mM sodium-potassium phosphate buffer, pH 7.0.

10 µl of the sample were added to 0.95 ml 10 mM H_2O_2 in 50 mM sodium-potassium phosphate buffer, pH 7.0, following the kinetics at λ=240 nm during 2 min. The slope was recorded, and each measure was the mean of 3 reactions.

One catalase unit is defined as the activity catalyzing the degradation of 1 µmol of H_2O_2 per minute, as calculated assuming ε_{240}=0.036 $mM^{-1}\,cm^{-1}$.

3.4.2 Superoxide dismutase

Superoxide dismutase was assayed as described by Masia [*149*], by measuring the competition for superoxide radicals produced by the illumination of riboflavin, between nitro

blue tetrazolium (NBT, producing formazan blue, which absorbs at λ=560 nm) and SOD (evolving H_2O_2).

For each sample, extraction was carried out in 100 mM sodium-potassium phosphate, pH 7.0, + 1% PVPP, and resuspension in 50 mM sodium-potassium phosphate buffer, pH 7.0.

A set of 7 glass tubes was prepared, containing 0, 10, 20, 40, 60, 80 and 500 µl of desalted extract in 3 ml reaction mixture (2 µM riboflavin 10 mM methionine, 50 µM nitroblue tetrazolium, 20 µM KCN, 6.6 mM Na_2EDTA, 65 mM sodium phosphate buffer, pH 7.8). The reaction was started by illumination under 4 fluorescent lamps in an aluminium-coated box, to provide uniform lighting. After 30 min illumination, absorbance was measured at λ=560 nm.

One superoxide dismutase unit is defined as the activity causing a 50% inhibition of the reaction producing formazan blue.

3.4.3 Ascorbate peroxidase

Ascorbate peroxidase activity was determined following the method by Chen and Asada [*37*], based on the spectrophotometrical measurement of ascorbate decrease at λ=290 nm.

Sample were extracted in 100 mM sodium-potassium phosphate, pH 7.0, + 1% PVPP, and resuspended in 50 mM sodium-potassium phosphate buffer.

The reaction mixture (1 ml) contained 100 µl desalted extract, 1 mM ascorbic acid, 0.5 mM hydrogen peroxide in 50 mM sodium phosphate buffer, pH 7.0.

One unit of ascorbate peroxidase is defined as the activity catalyzing the oxidation of 1 µmol of ascorbic acid per minute under the present conditions. An absorbance coefficient of 2.8 mM^{-1} cm^{-1} was assumed for ascorbic acid at the given wavelength.

3.4.4 Guaiacol peroxidase

Guaiacol peroxidase was assayed as described by Ushimaru *et al.* [*228*], using pyrogallol as the electron donor for the reaction.

The plant material was extracted in cold buffer (200 mM sodium phosphate, 5 mM sodium EDTA, 1% PVPP, pH 7.0) and incubated on ice for 30 min. After centrifugation, the supernatant was used for the assay.

The reaction mixture (2.5 ml) included 50 mM phosphate buffer, pH 7.0, 0.1 mM H_2O_2, 50 mM pyrogallol, and 100 µl extract; H_2O_2 and pyrogallol were prepared fresh just before use. Absorbance (λ=430 nm) was taken after 5 min incubation at room temperature, and referred to a blank with no extract added.

One unit of guaiacol peroxidase is defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of pyrogallol min^{-1} under the described conditions [*36*]. An absorbance coefficient of 2.47 mM^{-1} cm^{-1} was assumed for calculations.

3.4.5 Nitrosoglutathione reductase

Nitrosoglutathione reductase was assayed according to Corpas *et al.* [*45*] and Barroso *et al.* [*9*], by following the oxidation of NADH at λ=340 nm. Fresh solutions of GSNO and NADH were prepared before use and kept refrigerated and protected from light.

100 mM sodium-potassium phosphate, pH 7.0, + 1% PVPP was used for extraction and 50 mM sodium-potassium phosphate buffer for desalting.

The reaction mixture (0.5 ml) contained 150 µl desalted extract, 0.4 mM GSNO and 0.2 mM NADH in 50 mM sodium phosphate buffer, pH 7.8. The reaction is started by adding GSNO.

The activity is expressed as nmol NADH consumed per min, with ε_{340}=6.22 mM^{-1} cm^{-1}.

3.4.6 Nitrate reductase

Nitrate reductase was determined with the *in vivo* procedure described by Hageman and Reed [*91*] and Toselli *et al.* [*224*].

Fresh plant samples (approximately 150 mg) were weighted and transferred into test tubes containing 6 ml of infiltration buffer (50 mM potassium nitrate, 1 mM EDTA, 1% n-propanol in 100 mM potassium phosphate buffer, pH 7.5). Samples were kept under the buffer surface by a plastic screen. The tube was incubated at 32 °C and was made anoxic either by bubbling gaseous N_2 in it, or creating void with a pump; it was then wrapped with dark plastic in order to prevent any photosynthetic activity in the sample. After about 10 min, a 1-ml aliquot (t_0) was transferred to a glass tube. 30 min later, another aliquot (t_{30}) was taken.

In both t_0 and t_{30} aliquots, nitrite concentration was revealed by adding 0.5 ml sulfanilamide (1% in 3 M H_3PO_4) and 0.5 ml N-(1-naphthyl)ethylenediamine dihydrochloride (NEDD, 0.02% in distilled water). After 30 min for full color development, absorbance was read at λ=540 nm, and referred to a nitrite standard curve. The difference between t_0 and t_{30} was taken as the amount of nitrite produced during the assay time.

Nitrate reductase activity was espressed in µmol NO_2^- produced g^{-1} fresh weight min^{-1}.

3.4.7 Glutathione reductase

The glutathione reductase assay was modified from Foyer *et al.* [*74*].

The plant material was extracted at 0-4° C in 4 ml 150 mM potassium phosphate buffer, pH 7.5 containing 6 mM EDTA and 1% PVPP, and incubated on ice for 30 min. After centrifugation, the soluble proteins were desalted and resuspended in assay buffer (50 mM potassium phosphate, pH 7.5; 5 mM EDTA).

The assay consisted in following the decrease of NADPH at λ=340 nm, in a reaction mixture containing 500 µl assay buffer, 300 µl desalted extract, 100 µl 1mM NADPH and 100 µl 1mM oxidized glutathione (GSSG). The blank contained buffer instead of the extract.

One unit of glutathione reductase oxidizes 1 µmol min^{-1} of NADPH at room temperature, with ε_{340}=6.22 mM^{-1} cm^{-1}.

3.5 Soluble proteins content

The soluble proteins concentration was measured with the Pierce® BCA Protein Assay kit (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions.

3.6 Native polyacrylamide gel electrophoresis and SOD in-gel assay

Native 10% polyacrylamide gels were prepared by dissolving acrylamide in 250 mM TRIS buffer, pH 8.9, and adding 300 mM ammonium persulfate (1:20), and tetramethylethylenediamine (TEMED) (1:250).

Samples were prepared as for SOD activity assay and stored at -20° C. For each sample, the same amount of protein was treated with 2 µl 50% glycerol and 1 µl 0.25% bromophenol blue before loading.

To reveal SOD activity, the gel was incubated 20 min in NBT solution (2.45 mM in 50 mM potassium phosphate buffer, pH 7.8), then 15 min in a 28 mM riboflavin + 28 mM TEMED mixture. The bands are revealed after exposure to fluorescent light.

To distinguish among SOD isoforms, gels were pretreated 30 min with 5 mM KCN or 5 mM H_2O_2 in potassium phosphate buffer (50 mM, pH 7.8) before the incubation with NBT. KCN is able to inhibit the Cu-Zn isoform, while H_2O_2 inhibits both Cu-Zn and Fe containing isoforms.

3.7 SDS-PAGE and Western blotting

For immunoblot analyses, SDS-PAGE was carried out in 12% acrylamide slab gels, containing: 250 mM TRIS-HCl, pH 8.9, 0.1% (w/v) SDS, 5% (v/v) glycerol, 0.02% (w/v) ammonium persulfate, and TEMED (1:2000).

The samples were prepared by suspending the raw extract in 2% (w/v) SDS, 10% (v/v) glycerol and 10 mM DTT, + 1 µl 0.25% bromophenol blue in 62.5 mM TRIS-HCl, pH 6.8 (final concentrations), and heating the mixture at 95 °C for 5 min. For each sample, 40 µg of total proteins were loaded.

Polypeptides were separated using a Bio-Rad Mini-Protean II slab cell, and were transferred on to PVDF membranes (Immobilon P, Millipore Corp., Bedford, MA) using a semi-dry transfer apparatus (Novablot electrophoretic transfer unit, LKB) with 10 mM 3-(cyclohexylamino)-1-propanesulphonic (CAPS) buffer, 10% methanol, pH 11.0, at 1.5 mA cm^{-2} for 2 h.

The membranes were washed in methanol and distilled water, incubated 1 h in blocking buffer (20 mM Tris-HCl, pH 7.8; 0.18 M NaCl) containing 1.5% powdered skim milk, washed again with water, dried and incubated overnight in the anti-nitrotyrosine polyclonal antibody mix diluted 1:3000. After a new wash in blocking buffer, the membrane was incubated 1 h with the secondary antibody, and revealed with the ECL-PLUS Amersham Western Blotting Detection kit by chemiluminescence.

3.8 Confocal laser-scanning microscopy

NO was detected in leaf sections of approximately 25 mm^2, including the central vein. Tissue samples were incubated (darkness, room temperature, 1 hour) with 10 mM Tris/HCl, pH 7.4, containing 10 μM DAF-FM (Calbiochem-Novabiochem). Control samples were preincubated with 5 mM L-NAME (a NOS inhibitor) and 10 mM cPTIO (a NO scavenger) to determine the signal specificity. All the following steps were carried out in shaded light.

Subsequently, the samples were rinsed twice for 15 min in Tris buffer, and incubated (darkness, 4 °C, overnight) in 1 ml infiltration solution consisting in phosphate buffer saline (PBS, 10 mM potassium phosphate, pH 7.4, 15 mM NaCl, 3 mM KCl) added with 15% acrylamide-bisacrylamide and 0.3% (v/v) TEMED.

The solidification of the polyacrylamide bed was achieved by adding 300 mM ammonium persulfate (1:20). Leaf sections (about 0.1 mm) were obtained with a vibratome under PBS, and soaked in glycerol:PBS (1:1) on a microscopy glass.

For the specimen examination, a confocal laser scanning microscope system (Leica TCS SL; Leica Microsystems, Wetzlar, Germany) was used, with standard filters and collection modalities for DAF-2 green fluorescence (excitation 495 nm; emission 515 nm) and chlorophyll autofluorescence (chlorophyll *a* and *b*, excitation 429 and 450 nm, respectively; emission 650 nm and 670 nm, respectively) as orange [*42*].

Peroxynitrite was detected following the same procedure, with 10 μM 3'-(*p*-aminophenyl) fluorescein (APF, Invitrogen) as the fluorophore. Control samples were preincubated with 10 μM Ebselen. Laser scanning microscope detection used standard filters and collection modalities for APF green fluorescence (excitation 495 nm; emission 515 nm) [*33*].

3.9 Molecular analysis

3.9.1 GSNOR and actin primer design

Basing on known actin- and GSNOR-coding sequences, including arabidopsis (GenBank accession NM123761, GSNOR), pepper (GQ339766, actin, and AY572427, GSNOR) and pea (BV164876, actin, and DQ084382, GSNOR) sequences, a query was

submitted in the ESTree database for peach [*69*]. Among the top scores, sequences were spotted with high homology to known GSNOR genes (cluster_1_contig_479) and actin genes (cluster_16_contig_5). The following primers were designed with the software Primer3 [http://frodo.wi.mit.edu/primer3/]:

5'-TTGCATTCTTGGTCACGAG (FPrunus-GSNOR)

5'-TCTACTTTTGCCGTGTTCCA (RPrunus-GSNOR)

5'-CGTCTTCGATTGTCTTCGTC (FPrunus-Act)

5'-TGACCCATACCAACCATAACAC (RPrunus-Act)

The primers were purchased from Sigma Genosys. The hypothetical length of the amplified fragments was 389 bp (for GSNOR) and 189 bp (for actin).

3.9.2 RNA extraction

Total RNA extraction was carried out starting from at least 0.1 g fresh weight tissue, and using the commercial reactive TRIzol® (GIBCO BRL, Life Technologies). All the apparatus was sterilized in an oven at 140 °C for 3 hours. The sample was ground in liquid nitrogen, and 1 ml TRIzol reagent was added. After vortexing, the sample was incubated for 5 min on ice. Subsequently, 0.2 ml chloroform were added; the sample was gently mixed, incubated on ice 3 more minutes, and centrifuged (13,000×*g*, 4 °C, 15 min). The upper phase was transferred to a new 1.5 ml Eppendorf tube containing 0.5 ml isopropanol, and let stand 10 minutes at room temperature. The sample was centrifuged (13,000×*g*, 4 °C, 15 min), and the supernatant was discarded. The RNA pellet was washed by resuspension in 1 ml ethanol 75% and precipitated by centrifugation (7,500×*g*, 4 °C, 5 min), then ethanol was completely removed and the RNA was suspended in 20 µl DEPC water (containing 0.1% diethylpyrocarbamate). Concentration and stability of total RNA were estimated on electrophoretic agarose gel and by means of readings at λ=230, 260 and 280 nm.

3.9.3 Synthesis of cDNA

The synthesis of cDNA required a first step, where 3.7 µl RNA were treated with 2 U DNase I in 1 µl buffer, for 30 min at 37 °C. Then, EDTA (final concentration 5 mM), oligo-dT (final concentration 1 mM) and DEPC water (final volume 14 µl) were added, and the

sample was incubated for 10 min at 75 °C. Finally, dNTPs (final concentration 0.75 µM), RNase inhibitors, AMV retrotranscriptase and retrotranscriptase buffer were added to a final volume of 20 µl. After 40 min at 42 °C and 15 min at 70 °C, the cDNA was displayed by gel electrophoresis.

3.9.4 Sequencing of cDNA

The cDNA was amplified by PCR. The reaction included: 2 µl DNA polymerase buffer (10×), 1 µl 20 µM FPrunus-GSNOR primer, 1 µl 20 µM RPrunus-GSNOR primer, 0.4 µl 10 mM dNTP mix, 1 µl cDNA template, 0.2 µl Taq polymerase, 14.4 µl water. The amplification programme consisted in (1) 94 °C 3 min; (2) 35 cycles of 94 °C 40 sec, 57 °C 40 sec, 65 °C 40 sec; (3) 65 °C 10 min. The PCR product was displayed by electrophoresis; subsequently, the amplified band was excised with a scalpel, purified with the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences), according to the manifacturer's instructions, and eluted in 30 µl water.

The pGEM®-T Easy Vector System I kit (Promega) was used for the clonation of the amplified fragment. The reaction mix included: 7.5 µl T4 DNA ligase buffer (2×), 1.5 µl pGEM-T vector, 5 µl purified cDNA, 1 µl T4 DNA ligase; the mix was incubated at 4 °C overnight.

Competent *Escherichia coli* DH5α cells, stored at -80 °C, were thawed on ice. 0.5 ml cell suspension were preincubated with the ligation product for 30 min on ice, then heat-shocked at 42 °C for 1 min. 1 ml LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) was added; the cells were let grow 1 hour at 37 °C before plating on S-Gal™ LB Agar Blend (Sigma) added with ampicyllin, and incubated overnight at 37 °C. Some colonies with a white phenotype were isolated, plated on S-Gal™ LB medium with ampicyllin, and grown overnight at 37 °C. To verify the inclusion of the ligation product in the cells, single colonies were used in a colony-PCR reaction as the template.

Single positive colonies were grown overnight in liquid LB medium (at 37 °C, under shaking); the bacterial suspension was centrifuged and the supernatant was completely discarded. The pellet was used for plasmid extraction, by means of the QIAprep® Spin Miniprep Kit (Qiagen), according to the manifacturer's instructions. The isolation of plasmids

was checked by electrophoresis. Purity and concentration were extimated by spectrophotometrical reading.

6.4 pmol purified plasmid DNA, together with the primers FPrunus-GSNOR and RPrunus-GSNOR, were sent to the Instituto de Parasitologia y Biomedicina “López-Neyra” (Armilla, Granada, Spain) for sequencing.

3.9.5 Semiquantitative PCR

A preliminar PCR showed that the amplification of the cDNA template reached the saturation after 30 cycles. Therefore, the expression analysis of GSNOR genes in the different genotypes was carried out by comparing the amplification of GSNOR partial sequence to a housekeeping gene, namely actin.

To make the PCR conditions as uniform as possible, a master mix was prepared including polymerase buffer, dNTPs, water and Taq polymerase; it was subsequently splitted, and one aliquot was added with FPrunus-GSNOR and RPrunus-GSNOR primers, whereas the other aliquot was added with FPrunus-Act and RPrunus-Act primers; final concentrations were the same described in 3.9.4. Finally, the two mixes were splitted into single reaction tubes, and the sample cDNA was amplified in presence of both GSNOR and Act primers. The PCR programme was constituted by the following steps (1) 94 °C 3 min; (2) 30 cycles of 94 °C 40 sec, 57 °C 40 sec, 65 °C 40 sec.

The amplified products were run on electrophoretic agarose gel, which was rendered in an electronic format and analized with the Quantity One® 1-D Analysis software (BioRad), in order to evaluate the relative intensity of ~400 bp (GSNOR fragment) band compared to ~ 200 bp (actin fragment) band.

3.10 Statistical analysis

All measurements were taken with at least three independent replicates. When the replicates were five or more, the top and the lowest values were trimmed. Data are presented as mean ± standard error.

Significance analysis, between control and stressed samples of each genotype, was performed with the Student's unpaired *t* test. Analysis of covariance was used to assess the

significance of differences in the reduced-to-total ascorbate ratio. P scores lower than 0.1, 0.05 and 0.01 are highlighted (*, ** and ***, respectively).

4 RESULTS

4.1 Visual symptoms

About 25 days after transplantation, all the tested genotypes showed reduced growth in the medium containing $KHCO_3$ (K) compared to control (C), and Ishtara was most severely stunted. Chlorosis mainly affected the genotypes MrS 2/5 and Ishtara. The nectarine cultivar Big Top appeared nearly as healthy as in the control (fig. 4.1).

Figure 4.1: Phenotype of control (left) and bicarbonate-treated (right) plants: GF 677 (A-B), MrS 2/5 (C-D), Ishtara (E-F), Big Top (G-H).

4.2 Stress markers

In order to quantify the extent of the stress, malondialdehyde (MDA) contents and nitrated proteins were analyzed, reflecting respectively the oxidative stress on lipidic membranes, and the nitrosative stress on proteins.

In all the genotypes, the bicarbonate-enriched medium was associated to an increase in MDA contents, confirming the actual occurrence of oxidative stress; in MrS 2/5 and Ishtara significant rises could be detected.

	MDA content (nmol g^{-1} FWT)	
GF 677 - C	41.6 ± 3.9	
GF 677 - K	48.7 ± 5.7	
MrS 2/5 - C	27.1 ± 2.8	***
MrS 2/5 - K	41.0 ± 2.3	
Ishtara - C	33.8 ± 1.0	**
Ishtara - K	45.7 ± 3.5	
Big Top - C	35.3 ± 3.9	
Big Top - K	43.6 ± 4.0	

Tyrosine nitration has been proposed as a marker of nitrosative stress, since it requires the contemporary production of NO and superoxide. The Western blot profile of nitrated proteins (fig. 4.2) did not evidence any visual increase in the nitration signal, suggesting no enhanced peroxynitrite production in the considered stress conditions. Nevertheless, a qualitative difference is found in Big Top, where a ~34 kDa band appears in presence of bicarbonate.

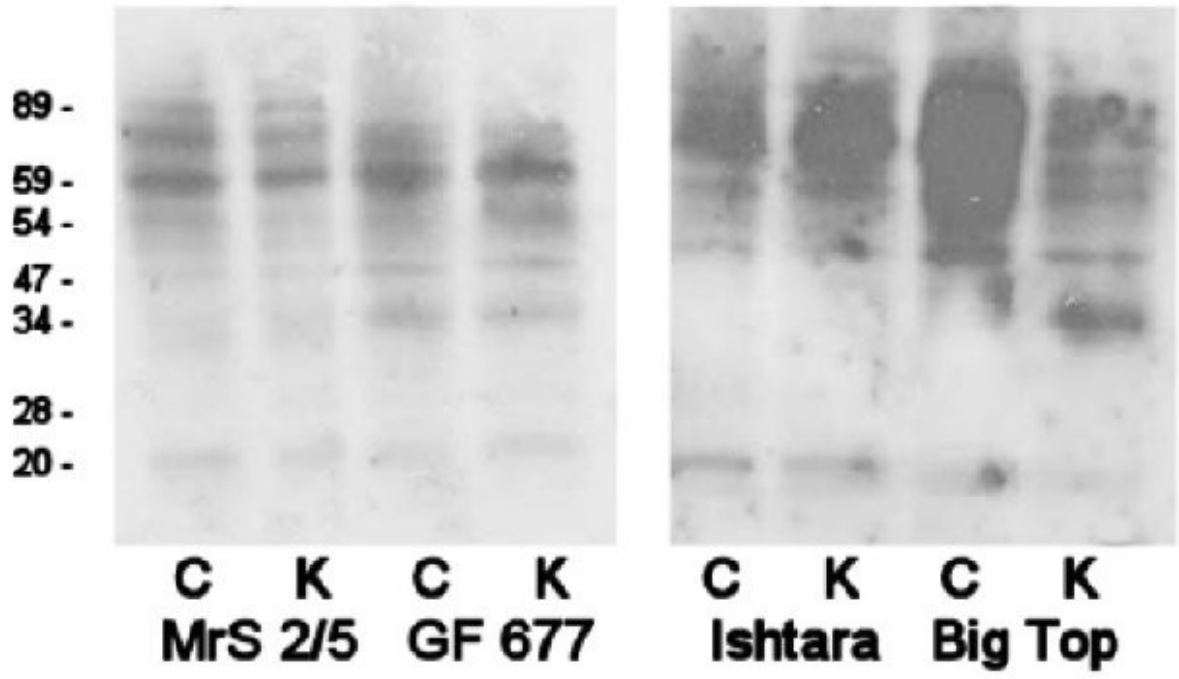

Figure 4.2: Anti-nTyr Western blot.

4.3 Hydrogen peroxide content

Assuming the oxidative stress to be due to the impaired removal of reactive oxygen species, H_2O_2 was measured.

No significant changes were found, despite a trend toward an increase in hydrgen peroxide may be proposed.

	H_2O_2 content (µmol g^{-1} FWT)
GF 677 - C	2.19 ± 0.23
GF 677 - K	2.26 ± 0.26
MrS 2/5 - C	2.00 ± 0.26
MrS 2/5 - K	2.06 ± 0.28
Ishtara - C	2.16 ± 0.26
Ishtara - K	2.68 ± 0.31
Big Top - C	2.13 ± 0.24
Big Top - K	2.45 ± 0.19

4.4 Antioxidant enzymes

Differences in oxidative stress severity and reactive oxygen species concentration could be due to differential activities of antioxidant enzymes. Thus, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) were assayed.

SOD appeared not affected by the stressing medium. CAT, APX and GPX displayed a general trend towards a reduced activity in presence of bicarbonate, but the only significant variations were for catalase in MrS 2/5, and for guaiacol peroxidase in Ishtara.

	SOD (U g^{-1} FWT)	**CAT** (U g^{-1} FWT)		**APX** (U g^{-1} FWT)	**GPX** (U g^{-1} FWT)	
GF 677 - C	169 ± 23	208 ± 83		3.4 ± 0.9	2.4 ± 0.1	
GF 677 - K	219 ± 33	225 ± 80		2.4 ± 0.2	2.5 ± 0.4	
MrS 2/5 - C	161 ± 23	336 ± 40	**	7.1 ± 0.8	3.7 ± 0.2	
MrS 2/5 - K	161 ± 17	210 ± 39		5.3 ± 0.5	3.6 ± 0.2	
Ishtara - C	205 ± 35	79 ± 25		4.2 ± 0.5	2.9 ± 0.3	**
Ishtara - K	199 ± 12	56 ± 17		3.5 ± 0.5	1.9 ± 0.1	
Big Top - C	188 ± 34	324 ± 68		3.4 ± 0.8	2.0 ± 0.4	
Big Top - K	178 ± 19	310 ± 54		2.3 ± 0.7	1.7 ± 0.1	

Since different isoforms of SOD may be induced in stress conditions, an in-gel assay was performed. No differential expression of SOD isoforms was detected, besides confirming the unvaried activity (fig. 4.3).

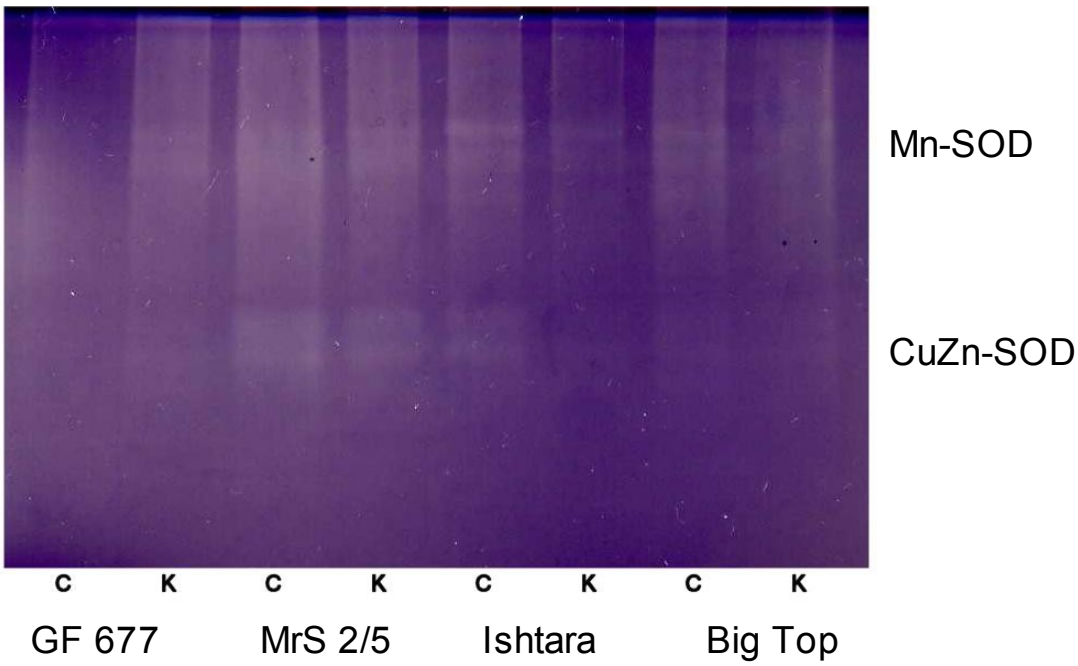

Figure 4.3: In-gel SOD assay.

4.5 RNS content

Nitric oxide (NO) and other RNS have been described either as prooxidants, or as protective factors. Thus, NO concentration has been estimated by means of DAF-2 fluorescence.

A high NO content was associated to GF 677 and Big Top. NO production was possibly promoted under stress, since a significant increase is found in GF 677, and the same trend is also found in Big Top.

	NO content (AU g^{-1} FWT)	
GF 677 - C	45.6 ± 21.8	*
GF 677 - K	100 ± 0.0	
MrS 2/5 - C	25.6 ± 4.6	
MrS 2/5 - K	26.8 ± 9.2	
Ishtara - C	11.1 ± 6.4	
Ishtara - K	13.7 ± 7.7	
Big Top - C	100.5 ± 18.4	
Big Top - K	124.0 ± 17.8	

An attempt was made to discriminate the sites of NO production in leaf tissues, by means of confocal laser-scanning microscopy (fig 4.4, A-H). Unambiguous conclusions could not be drawn, although a slight increase in fluorescence signal might be proposed in the vascular tissues of GF 677 and Big Top grown with bicarbonate in the medium.

With regard to peroxynitrite, confocal laser-scanning microscopy was carried out in GF 677 and MrS 2/5 plants (fig. 4.4, I-L). No difference emerged between stressed and control sections.

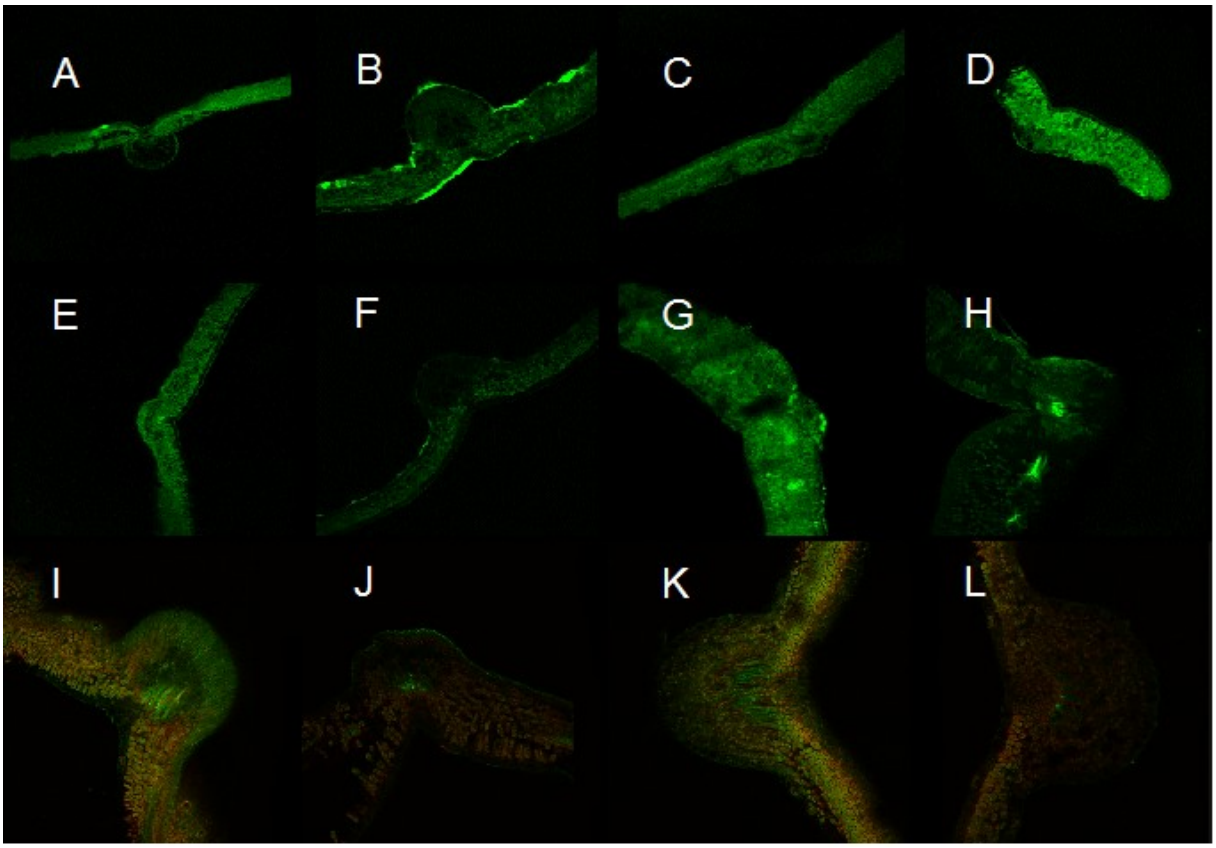

Figure 4.4: (A-H) CLSM detection of NO in leaves, by means of DAF 2-DA fluorescence. Top – control samples; bottom – bicarbonate-treated samples: GF 677 (A, E); MrS 2/5 (B, F); Ishtara (C, G); Big Top (D, H). (I-L) CLSM detection of peroxynitrite in leaves, by means of APF fluorescence. Left – control samples; right – bicarbonate-treated samples: GF 677 (I, J); MrS 2/5 (K, L).

4.6 Sources of RNS

Given the different NO production among genotypes, and its possible induction under stress, the question raised about its source. Several biochemical pathways have been established, but the enzymatic NO synthesis, catalyzed by nitrate reductase (NR) or nitric oxide synthase (NOS)-like activities, is best studied and considered probably most relevant.

Nitrate reductase activity was assayed, and no respondence emerged in relation to bicarbonate.

	NR (NO_2^- g^{-1} FWT min^{-1})
GF 677 - C	5.7 ± 1.4
GF 677 - K	6.6 ± 2.4
MrS 2/5 - C	11.3 ± 1.0
MrS 2/5 - K	11.2 ± 0.7
Ishtara - C	18.6 ± 2.5
Ishtara - K	16.5 ± 4.1
Big Top - C	14.1 ± 2.0
Big Top - K	19.1 ± 3.2

Since the genotype GF 677 displayed a significant increase in NO content when bicarbonate was included in the medium, NO fluorescence was also checked in presence of sodium tungstate (a NR inhibitor) and L-nitroarginine (a NOS inhibitor); sodium molybdate and L-arginine were used, respectively, for the controls. Although variations are not statistically significant, the tungstate salt led to an increased NO fluorescence. On the contrary, L-nitroarginine slightly reduced the NO signal, consistently with an antagonistic action towards L-arginine.

	NO content (AU g^{-1} FWT)
Na-molybdate	85.9 ± 11.0
Na-tungstate	137.7 ± 22.1
L-arginine	90.8 ± 5.3
L-nitroarginine	72.2 ± 11.2

Nitrosoglutathione has been connected to stress resistance in several experiments. Therefore, nitrosoglutathione reductase (GSNOR) could affect the performance of bicarbonate-treated plants by removing it. Indeed, GSNOR activity was halved in the most sensitive genotypes (MrS 2/5 and Ishtara) under stress, whereas no changes occurred in GF 677 and Big Top.

	GSNOR (U g^{-1} FWT)	
GF 677 - C	95.8 ± 19.4	
GF 677 - K	100.1 ± 17.7	
MrS 2/5 - C	301.4 ± 59.8	**
MrS 2/5 - K	157.4 ± 15.8	
Ishtara - C	191.4 ± 11.5	***
Ishtara - K	87.3 ± 10.6	
Big Top - C	184.7 ± 24.0	
Big Top - K	251.3 ± 26.6	

4.7 Redox status

An estimate of the redox status of the plants was based on the reduced ascorbate/total ascorbate ratio. Total ascorbate, total reduced thiols and glutathione reductase activity were also considered as parameters for redox buffering power.

In spite of a trend towards ascorbate oxidation in all genotypes under stressing conditions, changes could not be statistically validated. On the other hand, total ascorbate content was strongly reduced in Ishtara, whereas an increase was found in Big Top.

	Asc_{rd}/Asc_{tot}	Asc_{tot} (µmol g^{-1} FWT)	
GF 677 - C	0.45 ± 0.05	1.05 ± 0.40	
GF 677 - K	0.43 ± 0.00	1.10 ± 0.04	
MrS 2/5 - C	0.65 ± 0.11	1.59 ± 0.11	
MrS 2/5 - K	0.56 ± 0.08	1.64 ± 0.16	
Ishtara - C	0.65 ± 0.02	1.53 ± 0.09	***
Ishtara - K	0.51 ± 0.01	0.84 ± 0.09	
Big Top - C	0.59 ± 0.01	1.34 ± 0.15	*
Big Top - K	0.59 ± 0.04	1.92 ± 0.19	

Concerning soluble thiols, some increase was found in stressed MrS 2/5, despite the absolute content is lower than in the other genotypes. Glutathione reductase (GR) activity rose significantly in Big Top.

	Soluble thiols (µmol g^{-1} FWT)		GR (U g^{-1} FWT)	
GF 677 - C	3.65 ± 0.33		518 ± 33	
GF 677 - K	3.95 ± 0.50		570 ± 82	
MrS 2/5 - C	1.55 ± 0.08	**	426 ± 28	
MrS 2/5 - K	1.87 ± 0.07		416 ± 21	
Ishtara - C	2.89 ± 0.65		460 ± 12	
Ishtara - K	2.58 ± 0.21		547 ± 54	
Big Top - C	5.09 ± 0.44		510 ± 18	**
Big Top - K	5.73 ± 0.38		701 ± 42	

4.8 GSNOR gene fragment expression and cloning

Good quality, total mRNA could be extracted only from control plants. PCR yielded the expected ~400 bp and ~200 bp bands in GF 677, Ishtara and Big Top, but not MrS 2/5, possibly because of primers incompatibility (since MrS 2/5, unlike the other genotypes, is neither *P. persica*, nor a peach hybrid).

The ~400 bp fragment from Ishtara plants was successfully cloned. Its sequence was determined and published in the GenBank database (accession number FJ360859).

5 DISCUSSION

5.1 Choice of stress indexes

To measure the severity of stress, several parameters were taken in account. The ideal parameter in our choice would be a stable product, slowly recycled by other metabolic processes, and not involved in any protective response, since ambiguity would emerge on the stress severity and the plant's capability to cope with it.

The decrease in chlorophyll content, determining the chlorotic symptoms in leaves, was not considered suitable, as it represents not only a nutritional disorder, but also an adaptive response, aimed to limit the electron flow in plastids, and consequently electron leakage to oxygen and ROS formation. This view is substantiated by previous work [*150*] where the accessory chlorophyll *b* was shown to be more affected than chlorophyll *a*, and the chlorophyll variation not to correlate with other stress indexes. By analogy, the same considerations stand for any protective metabolites, such as osmolites, ROS scavengers or stress proteins. A further reason for caution is that the functional role of many of these compounds is inferred from the conditions of their appearance, but they are seldom sufficient to ameliorate the plant's health in such conditions. Besides, a number of biochemical pathways, such as proline biosynthesis or Krebs cycle, have been associated to stress response, often suggesting a by-product (for instance, $NAD(P)^+$ regeneration) to be the actual need for the stressed plant. Since the same by-products can be obtained through many routes, proline and other metabolites could have a secondary function as ROS scavengers or osmolites, resulting after their increased synthesis for different purposes; in any case, their reliability as stress markers would be questionable.

ROS content would be, in principle, a significant indicator of electron leakage; on the other side, H_2O_2 is a well-characterized stress response mediator, promoting, among other factors, its own scavenging by means of antioxidant enzymes. As a further variable, most antioxidant enzymes, requiring iron centres for their function, are directly affected by the stressor in our model. Thus, neither H_2O_2 concentration, nor antioxidant enzyme activities would be suitable parameters if independently taken, whereas their combination could be.

Thiobarbituric acid-reacting substances (TBARS), and notably malondialdehyde (MDA), have been chosen as markers for oxidative stress. Their formation is due to the

deregulated interaction of oxygen radicals with unsaturated fatty acids, which triggers a chain reaction with MDA as one of the end products. Thus, TBARS content is an accumulated function of oxygen radical formation during the cell development. Plant tissues recover poorly from membrane damage after the suspension of a stressing treatment [*30*]; it can be therefore supposed that the formation of new tissues, rather than the restoration of membrane functionality in existing ones, is the main recovering strategy in plants. These considerations, in our opinion, make TBARS content a reliable stress index in discrete organs mainly constituted by living tissue, such as leaves.

Protein nitration has been proposed as a marker of stress. In fact, peroxynitrite formation requires superoxide and NO to be produced at comparable rates, and its reaction with tyrosine as a preferential target could impair post-translational modification of proteins (such as phosphorilation) and lead to their degradation. Some work has been carried out, showing increased tyrosine nitration, along with NO and nitrosothiols concentrations, in salt-treated olive plants [*229*], which led to the definition of nitrosative stress, in analogy with deregulated ROS formation resulting in oxidative stress.

5.2 Bicarbonate stress occurrence

Plants yellowing and stunted growth, although not quantified, confirmed that the experimental conditions reproduced, at least partly, those leading to iron chlorosis in field.

TBARS contents were different among the genotypes, even in unstressed plants, probably reflecting their different growth modalities and basal levels. In fact, GF 677 and Big Top typically develop long leaves, with the largest near the base of the shoot, while MrS 2/5 has smaller, round leaves and greater internodal distension, and Ishtara has very small leaves, approximately constant in their size. In addition, *in vitro*-cultured Big Top plants usually become senescent earlier than the other genotypes. Thus, the amount of TBARS in control plants may depend on genetic features, on the age of the tissues, and possibly on their photosynthetic activity. Nevertheless, in all bicarbonate-treated plants, an increase in TBARS is found, and its relative extent is roughly consistent with the severity of symptoms. Taken together, these data confirm the occurrence of oxidative stress, and TBARS as a good stress index.

No obvious intensification of signal could be found by peroxynitrite fluorescence, nor by anti-nitrotyrosine immunoblotting. Therefore, nitrosative stress occurrence is not supported in the present experimental model. On the other side, one new band (~34kDa) appears in the stressed Big Top sample. Such a specificity in protein nitration would rather suggest a functional role for the affected polypeptide. Little information is available about the identity of proteins undergoing nitration; nevertheless, their number has been found to be limited in sunflower hypocotyls (about 20), including glutathione reductase, *S*-adenosyl homocysteine hydrolase (SAHH), and other proteins involved in regulation, redox exchange and carbohydrate metabolism [*33*]. SAHH was demonstrated to lose activity after nitration, which would shunt precursors from aminoacid metabolism towards ethylene or polyamine biosynthesis.

5.3 ROS and ROS scavenging

To test whether the onset of oxidative stress was due to an increased ROS production or a reduced antioxidant activity, H_2O_2 contents and enzymatic scavengers were assayed.

Hydrogen peroxide was considered an important parameter for several reasons. Despite its relatively poor reactivity, it can turn into a dangerous oxidizing factor in presence of transition metals; thus, its influence on oxidative stress can be reasonably supposed. Secondly, H_2O_2 is the most stable among ROS, is the product of superoxide dismutation, and is massively freed in stress conditions through various biochemical routes; so it can be conveniently adopted to estimate the electron leakage to oxygen or the oxidative status in plant cells. Finally, it acts as a signal to induce stress responses.

Neither H_2O_2 levels, nor SOD activity appeared to increase significantly under stress, suggesting that an increased ROS formation, although possibly present, is not a major cause of the oxidative damage. On the other hand, H_2O_2-removing enzymes, mainly catalase (in MrS 2/5) and guaiacol peroxidase (in Ishtara), suffered from significant reduction in activity. Since all of these enzymes require an heme cofactor, these data can be easily interpreted with a reduced iron availability in the cells.

Given the high sensitivity of Ishtara to bicarbonate in the present system, two considerations arise, the first regarding its low catalase activity, even in control plants, compared to the other genotypes. In our model, the CAT activity could act as a constitutive

shield against oxidative stress, rather than an induced response to stressors, since it is lowest or decreased in the sensitive genotypes, and no trend towards an increase can be detected in the resistant ones.

The second consideration concerns the relative importance of peroxidases. In fact, the mostly aspecific isoforms of this class, assayed as guaiacol peroxidase activity, are often believed to act primarily in mechanical resistance and hypersensitive response, rather than in H_2O_2 detoxification [*7*]. As no lignification was noticed in *in vitro* grown plantlets, it is possible that GPX is preferentially downregulated compared to other iron-containing antioxidant enzymes, when iron is not available. Overall, protein-bound iron could act as a buffer source of biologically active iron, and variations in protein expression under iron deprivation could be regarded in this direction.

APX, which is never significantly inactivated, is probably the major H_2O_2-scavenging activity in the tested conditions.

5.4 RNS and iron deficiency

High levels of NO fluorescence, and possibly induction under stress, were found in both the bicarbonate-tolerant genotypes (GF 677 and Big Top), whereas lower contents in MrS 2/5, and above all in Ishtara, correlated with lower tolerance. Moreover, the bicarbonate-sensitive plants, but not the resistant ones, displayed a fall in GSNOR activity.

Three possible explanations can be postulated. Both NO and GSNO act as iron chelators, keeping iron soluble and available for metabolic needs, and prevent the activation of the Fenton chemistry. Thus, plants could react to bicarbonate-induced iron precipitation by either synthesizing NO, or preventing the removal of GSNO. Secondly, NO is an effective oxygen- and lipid radicals scavenger, and can quench the lipid peroxidation chain reaction. This would justify a lower increase in TBARS in the strongest NO producers. Finally, iron uptake in the symplast could be promoted by NO action (in particular, transnitrosylation) on the proteic uptake machinery.

In this work, nitrate reductase could be excluded as a major source of NO, since no significant changes were found in NR activity between stressed and control plants; moreover, the NR inhibitor sodium tungstate did not reduce, but rather slightly increased NO fluorescence in stressed GF 677 plants. On the contrary, L-nitroarginine reduced the

fluorescence signal, although not significantly, suggesting L-arginine to be one, if not the only, source of NO in the considered experimental model.

Since a NOS-like activity has been associated to peroxisomes [*8*; *52*; *54*], it is of special interest to verify a connection between NO synthesis and ROS metabolism under stress. Peroxisomes host several oxidative metabolic pathways; superoxide and H_2O_2 originate as by-products, and are efficiently scavenged by an antioxidant apparatus including SOD, CAT and ascorbate-glutathione cycle enzymes. NO can react directly with superoxide, but not with H_2O_2; it may otherwise help peroxisomal metabolism, for instance by providing iron for catalase, as suggested by the maintenance of CAT activity in the high NO-producing genotypes. Stomatal closure, or excess O_2 production compared to carbon fixation efficiency, could lead to increased photorespiration, hence the need for catalase activity. Actually, H_2O_2 increases could not be statistically validated, so the whole hypothesis requires more evidences.

Both NO and polyamines are known to counteract ethylene effects in senescence and fruit ripening [*131*; *177*; *259*]. Moreover, polyamines have been proposed [*4*; *225*] as NO donors. Polyamines are found in virtually all cellular compartments, including mitochondria, plastids and peroxisomes, and are induced by several stressing conditions; they interplay with

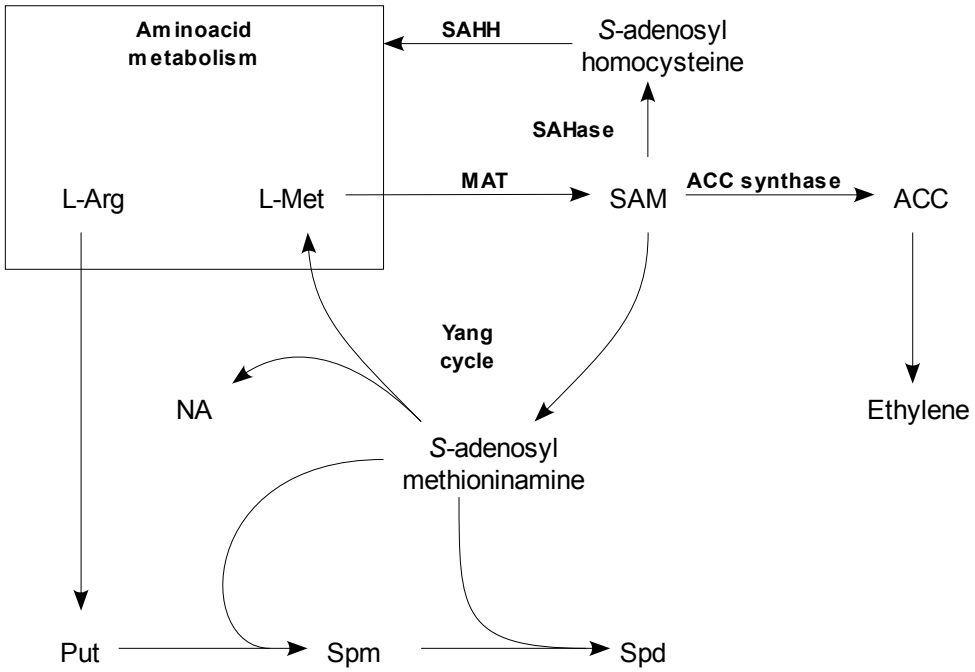

Figure 5.1: Pathways depending on S-*adenosyl methionine (SAM). L-Met – L-methionine; ACC – 1-aminocyclopropane-1-carboxylic acid; L-Arg – L-arginine; Put – putrescine; Spm – spermine; Spd – spermidine; NA – nicotianamine; MAT – methionine adenosyltransferase; SAHase –* S-*adenosyl homocysteinase; SAHH –* S-*adenosyl homocysteine hydrolase.*

ethylene metabolism (fig. 5.1) by competing for the same precursor (SAM), and downregulating ethylene synthesis and perception [*177*; *259*]; in addition, their oxidation is a H_2O_2-evolving pathway. It may be speculated that polyamines carry out their protective action by freeing NO. Besides direct competition for biochemical precursors, polyamines and ethylene also interplay by means of RNS, which have been shown to regulate several key enzymes in the pathway, such as *S*-adenosyl homocysteine hydrolase (SAHH) [*33*], methionine adenosyltransferase, *S*-adenosyl homocysteinase [*133*] and ACC synthase [*67*; *154*].

Although no conclusive evidence could be drawn from laser-scanning confocal microscopy, an increased signal was proposed in the vascular tissue of leaves from resistant plants. This would be in agreement with other experiments [*42*; *43*; *78*], and would substantiate the involvement of NO in systemic iron mobilization.

5.5 Redox balance

Assuming ascorbate pool to be the main electron buffer for antioxidant responses, a decrease in the reduced-to-total ascorbate ratio was expected in oxidizing conditions. This was not confirmed by the present data. Yet, a significantly lower amount of total ascorbate was found in the highly sensitive Ishtara under stress, whereas its concentration rose in Big Top. Similar observations were reported [*59*; *145*] for the induction of PCD, proposing that not only the redox status, but also (or mainly) the total concentrations of ascorbate or glutathione would carry a stress signal, and that a lower ascorbate content would

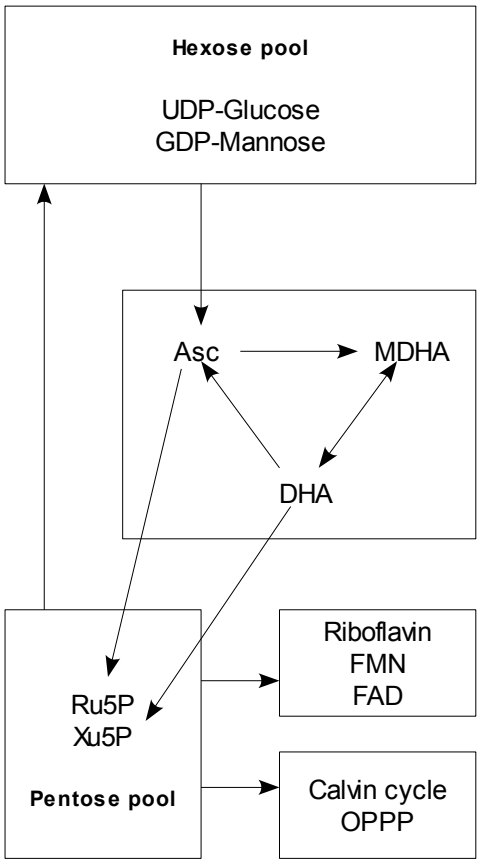

Figure 5.2: Ascorbate metabolism. Asc, ascorbate; MDHA, mono-dehydroascorbate; DHA, dehydroascorbate; Ru5P, ribulose-5-phosphate; Xu5P, xilulose-5-phosphate; OPPP, oxidative pentose-phosphate pathway.

result in a limited buffering power, thus lowering the threshold for the onset of oxidative damage. Since ascorbate peroxidase activity is not significantly decreased in bicarbonate-treated Ishtara, a possible explanation for the observed data could be the prevalence of ascorbate degradation, with respect to neosynthesis (fig. 5.2). Ribulose-5-phosphate and other pentoses, the products of this process, participate in pentose-phosphate pathways, suggesting the need to tune up the $NADP^+$/NADPH ratio. In fact, chlorotic leaves, where photosynthesis and carbon fixation are not efficient, could reduce the pentose phosphate pool through both the Calvin cycle and photorespiration, with NADPH consumption. On the other hand, an increase in ascorbate concentration, as found in Big Top, could compensate a diminished ascorbate peroxidase expression or stability when iron cofactors are not available.

Glutathione is another important redox player in plants, since it can act (1) by reducing ascorbate, (2) by directly reducing ROS, or (3) by modulating protein activity, through a post-translational modification (glutathionylation of sulfhydryls) [*75*]. Thus, glutathione reductase was supposed to react to oxidative stress. Only Big Top had a significant increase in GR activity, although the trend is present in GF 677 and Ishtara, but not in MrS 2/5.

5.6 Response strategies to bicarbonate stress

Among the four tested genotypes, two (GF 677 and Big Top) appeared to be resistant to $KHCO_3$, whereas MrS 2/5 and Ishtara were affected by chlorosis, although at different severity. Common aspects could be spotted among the different varieties, according to the performance in bicarbonate-enriched medium.

In the resistant genotypes, little lipid peroxidation increases were detected, and poor or no variations in antioxidant enzymes occurred. In addition, ascorbate content, redox status, and thiol metabolism did not vary in GF 677, while Big Top reacted to bicarbonate increasing ascorbate concentration and glutathione reduction. Overall, a severe iron shortage seemed not to take place. Both GF 677 and Big Top had high levels, and possibly promoted synthesis of NO under stress. Thus, the main role of NO, or some of its derivatives, may be keeping iron available, or even solubilizing internal iron precipitates, so that iron nutritional sufficiency was maintained in spite of the presence of bicarbonate in the growth medium. RNS may also stimulate the intake apparatus, or counteract its HCO_3^--mediated inhibition: the appearance of a nitrated protein in Big Top could reflect this phenomenon. Besides, a scavenging function of

NO against oxygen or lipid radicals cannot be excluded; coping with the onset of oxidative stress, anyway, did not require the activation of any of the tested detoxification mechanisms, with the possible exception of a generalized reduction of the redox buffers.

In the sensitive genotypes, even though H_2O_2 did not increase sensibly, antioxidant enzymes lost part of their activity, probably as a consequence of iron deprivation. Thus, unscavenged H_2O_2, and possibly deregulated iron trafficking, may be sufficient causes for the observed, significant lipid peroxidation. Alternatively, a main source of ROS not implying superoxide as an intermediate should be supposed, given the absence of response in SOD activity. Singlet oxygen formation at PSII might represent a likely candidate, also considering that carotenoids are reported to be spared from photosynthetic pigment loss possibly because of their $^1O_2^*$ quenching function.

Whatever the ROS source, their detoxification must be faced by MrS 2/5 and Ishtara genotypes, together with the restoration of iron availability. GSNO may accomplish the latter issue, forming stable iron-dinitrosyl complexes and holding iron in a bioavailable form. In addition, GSNO may undertake a role in spreading the NO signal, by trans-nitrosylating cysteine residues on specific target proteins, thus regulating their activity. Several stress-related proteins have been found to undergo Cys-nitrosylation and Tyr-nitration; among them, a CuZn-SOD [*132*], a peroxiredoxin IIE [*196*; *197*], a glutathione reductase and an alternative oxidase [*33*]. With regard to the protection from oxidative stress, given the impairment of the antioxidant enzymes, the main line of defence would be represented by the redox buffering system. Bicarbonate tolerance is found, to some extent, in MrS 2/5 compared to Ishtara (where chlorotic symptoms and stunted growth are more severe), maybe because of the different ascorbate and glutathione contents. In fact, Ishtara shows a fall in total ascorbate content, whereas MrS 2/5 has a little increase in thiols in treated samples.

The identification of the subcellular compartments where ROS are evolved could help addressing to the mechanisms of NO- and redox-mediated protection on one side, and of stress damage on the other side. To confirm NO involvement in peroxisomal activity would possibly mean increased photorespiration, or lead to the oxidation of secondary metabolites in the response to bicarbonate, whereas singlet oxygen detoxification would take place in chloroplasts by means of membrane-diffusible factors such as tocopherols and carotenoids.

5.7 Future perspectives

This work led to the individuation of different responses involving the RNS metabolism, in presence of bicarbonate, within the *Prunus* genus. Such biochemical features might be the starting point for future developments in the study and treatment of iron deficiency stress tolerance, including applicative ones.

Since an increased NO concentration was shown to correlate with higher bicarbonate tolerance, field subministration of NO might be adopted in order to prevent or cure chlorosis instances. A deeper investigation of the RNS source and action could address to efficient formulations, also accounting for cross-interactions with other components of the plant metabolism. In this sense, the comparison among polyamine and non-physiological NO donors (such as sodium nitroprusside or *S*-nitroso-*N*-acetylpenicillamine) could be of interest. Although GSNO is very expensive and labile in standard conditions, its synthesis can be achieved from nitrite and glutathione; on the other hand, nitrite alone, as a substrate of nitrate reductase, could be excluded in this work as a significant NO source. It should be noted that different donors may result in diverging responses, according to the redox status of NO, as shown for ferritin induction [*73*; *165*].

Besides supplying NO, downstream mechanisms could also be characterized. This work, where unrooted plantlets were used as the experimental model, did not consider the specific action of roots. As the resistance to chlorosis emerged even in absence of specialized uptake responses, curative treatments may be designed, not relying on external iron supply, which is an expensive and environmentally impacting practice.

If the effectiveness of GSNO were to be confirmed, the question would arise about its action. In fact, beyond its iron chelating power, GSNO is also a carrier of nitrosyl groups, and transnitrosylation of specific protein targets could take place. Thus, the screening of the nitrosylated proteome could evidence protein isoforms contributing to iron efficiency, providing biochemical markers for an early selection of bicarbonate-tolerant genotypes.

Tyrosine nitrosation did not succeed as a stress marker. Conversely, its apparent specificity suggests a form of protein regulation. The identification of the target(s) of nitration could therefore help tagging some of the metabolic routes implied in bicarbonate stress tolerance.

6 REFERENCES

1. Abat JK, Deswal R. Differential modulation of *S*-nitrosoproteome of *Brassica juncea* by low temperature: change in *S*-nitrosylation of Rubisco is responsible for the inactivation of its carboxylase activity. Proteomics. 2009 Sep;9(18):4368-80.

2. Alscher RG, Erturk N, Heath LS. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Biol. 2002;53:1331–1341.

3. Amirsadeghi S, Robson CA, McDonald AE, Vanlerberghe GC. Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signaling molecules. Plant Cell Physiol. 2006 Nov;47(11):1509-19.

4. Arasimowicz-Jelonek M, Floryszak-Wieczorek J, Kubis J. Interaction between polyamine and nitric oxide signaling in adaptive responses to drought in cucumber. J Plant Growth Regul. (2009) 28:177–186.

5. Arnaud N, Murgia I, Boucherez J, Briat JF, Cellier F, Gaymard F. An iron-induced nitric oxide burst precedes ubiquitin-dependent protein degradation for *Arabidopsis AtFer1* ferritin gene expression. J Biol Chem. 2006 Aug 18;281(33):23579-88.

6. Arnaud N, Ravet K, Borlotti A, Touraine B, Boucherez J, Fizames C, Briat JF, Cellier F, Gaymard F. The iron-responsive element (IRE)/iron-regulatory protein 1 (IRP1)-cytosolic aconitase iron-regulatory switch does not operate in plants. Biochem J. 2007 Aug 1;405(3):523-31.

7. Asada K. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol. 1999 Jun;50:601-639.

8. Barroso JB, Corpas FJ, Carreras A, Sandalio LM, Valderrama R, Palma JM, Lupiáñez JA, del Río LA. Localization of nitric-oxide synthase in plant peroxisomes. J Biol Chem. 1999 Dec 17;274(51):36729-33.

9. Barroso JB, Corpas FJ, Carreras A, Rodríguez-Serrano M, Esteban FJ, Fernández-Ocaña A, Chaki M, Romero-Puertas MC, Valderrama R, Sandalio LM, del Río LA. Localization of *S*-nitrosoglutathione and expression of *S*-nitrosoglutathione reductase in pea plants under cadmium stress. J Exp Bot. 2006;57(8):1785-93.

10. Becana M, Moran JF, Iturbe-Ormaetxe I. Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection. Plant and Soil. 1998;201: 137–147.

11. Becker R, Fritz E, Manteuffel R. Subcellular localization and characterization of excessive iron in the nicotianamine-less tomato mutant *chloronerva*. Plant Physiol. 1995 May;108(1):269-275.

12. Becker R, Manteuffel R, Neumann D, Scholz G. Excessive iron accumulation in the pea mutants *dgl* and *brz*: subcellular localization of iron and ferritin. Planta. 1998;207: 217-223.

13. Belenghi B, Romero-Puertas MC, Vercammen D, Brackenier A, Inzé D, Delledonne M, Van Breusegem F. Metacaspase activity of *Arabidopsis thaliana* is regulated by *S*-nitrosylation of a critical cysteine residue. J Biol Chem. 2007 Jan 12;282(2):1352-8.

14. Beligni MV, Lamattina L. Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta. 2000 Jan;210(2):215-21.

15. Bereczky Z, Wang HY, Schubert V, Ganal M, Bauer P. Differential regulation of *nramp* and *irt* metal transporter genes in wild type and iron uptake mutants of tomato. J Biol Chem. 2003 Jul 4;278(27):24697-704.

16. Bethke PC, Badger MR, Jones RL. Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell. 2004 Feb;16(2):332-41.

17. Bethke PC, Libourel IG, Jones RL. Nitric oxide reduces seed dormancy in *Arabidopsis*. J Exp Bot. 2006;57(3):517-26.

18. Borisjuk L, Macherel D, Benamar A, Wobus U, Rolletschek H. Low oxygen sensing and balancing in plant seeds: a role for nitric oxide. New Phytol. 2007;176(4):813-23.

19. Boucher JL, Moali C, Tenu JP. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. Cell Mol Life Sci. 1999 Jul;55(8-9):1015-28.

20. Briat JF, Fobis-Loisy I, Grignon N, Lobreaux S, Pascal N, Savino G, Thoiron S, von Wiren N, Van Wuytswinkel O. Cellular and molecular aspects of iron metabolism in plants. Biol Cell. 1995;84:69–81.

21. Briat JF, Lobréaux S, Grignon N, Vansuyt G. Regulation of plant ferritin synthesis: how and why. Cell Mol Life Sci. 1999 Oct 1;56(1-2):155-66.

22. Briat JF, Curie C, Gaymard F. Iron utilization and metabolism in plants. Curr Opin Plant Biol. 2007 Jun;10(3):276-82.

23. Briat JF, Ravet K, Arnaud N, Duc C, Boucherez J, Touraine B, Cellier F, Gaymard F. New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. Ann Bot. 2009 May 29.

24. Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J. 2006 Jan;45(1):113-22.

25. Brookes PS, Bolaños JP, Heales SJ. The assumption that nitric oxide inhibits mitochondrial ATP synthesis is correct. FEBS Lett. 1999 Mar 12;446(2-3):261-3.

26. Brumbarova T, Bauer P. Iron-mediated control of the basic helix-loop-helix protein FER, a regulator of iron uptake in tomato. Plant Physiol. 2005 Mar;137(3):1018-26.

27. Busi MV, Maliandi MV, Valdez H, Clemente M, Zabaleta EJ, Araya A, Gomez-Casati DF. Deficiency of *Arabidopsis thaliana* frataxin alters activity of mitochondrial Fe-S proteins and induces oxidative stress. Plant J. 2006 Dec;48(6):873-82.

28. Butt YK, Lum JH, Lo SC. Proteomic identification of plant proteins probed by mammalian nitric oxide synthase antibodies. Planta. 2003 Mar;216(5):762-71.

29. Carimi F, Zottini M, Costa A, Cattelan I, De Michele R, Terzi M, Lo Schiavo F. NO signalling in cytokinin-induced programmed cell death. Plant Cell Environ. 2005;28:1171-1178.

30. Cavalcanti FR, Abreu Oliveira JT, Martins-Miranda AS, Viégas RA, Gomes Silveira JA. Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. New Phytol. 2004;163:563-571.

31. Cavalcanti FR, Lima JP, Ferreira-Silva SL, Viégas RA, Silveira JA. Roots and leaves display contrasting oxidative response during salt stress and recovery in cowpea. J Plant Physiol. 2007 May;164(5):591-600.

32. Cesco S, Rombolà AD, Tagliavini M, Varanini Z, Pinton R. Phytosiderophores released by graminaceous species promote ^{59}Fe-uptake in citrus. Plant Soil. 2006;287:223-233.

33. Chaki M, Valderrama R, Fernández-Ocaña AM, Carreras A, López-Jaramillo J, Luque F, Palma JM, Pedrajas JR, Begara-Morales JC, Sánchez-Calvo B, Gómez-Rodríguez MV, Corpas FJ, Barroso JB. Protein targets of tyrosine nitration in sunflower (*Helianthus annuus* L.) hypocotyls. J Exp Bot. 2009;60(15):4221-34.

34. Chandok MR, Ytterberg AJ, van Wijk KJ, Klessig DF. The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. Cell. 2003 May 16;113(4):469-82.

35. Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci. 2004 Jan;61(2):192-208.

36. Chen GX, Asada K. Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol. 1989;30:987-998.

37. Chen GX, Asada K. Hydroxyurea and *p*-aminophenol are the suicide inhibitors of ascorbate peroxidase. J Biol Chem. 1990 Feb 15;265(5):2775-81.

38. Chinnusamy V, Schumaker K, Zhu JK. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot. 2004 Jan;55(395):225-36.

39. Colangelo EP, Guerinot ML. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell. 2004 Dec;16(12):3400-12.

40. Connolly EL, Fett JP, Guerinot ML. Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell. 2002 Jun;14(6):1347-57.

41. Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML. Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiol. 2003 Nov;133(3):1102-10.

42. Corpas FJ, Barroso JB, Carreras A, Quirós M, León AM, Romero-Puertas MC, Esteban FJ, Valderrama R, Palma JM, Sandalio LM, Gómez M, del Río LA. Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants. Plant Physiol. 2004 Sep;136(1):2722-33.

43. Corpas FJ, Barroso JB, Carreras A, Valderrama R, Palma JM, León AM, Sandalio LM, del Río LA. Constitutive arginine-dependent nitric oxide synthase activity in different organs of pea seedlings during plant development. Planta. 2006 Jul;224(2):246-54.

44. Corpas FJ, Del Río LA, Barroso JB. Post-translational modifications mediated by reactive nitrogen species: nitrosative stress responses or components of signal transduction pathways? Plant Signal Behav. 2008 May;3(5):301-3.

45. Corpas FJ, Chaki M, Fernández-Ocaña A, Valderrama R, Palma JM, Carreras A, Begara-Morales JC, Airaki M, del Río LA, Barroso JB. Metabolism of reactive nitrogen species in pea plants under abiotic stress conditions. Plant Cell Physiol. 2008 Nov;49(11):1711-22.

46. Correa-Aragunde N, Graziano M, Lamattina L. Nitric oxide plays a central role in determining lateral root development in tomato. Planta. 2004 Apr;218(6):900-5.

47. Courtois C, Besson A, Dahan J, Bourque S, Dobrowolska G, Pugin A, Wendehenne D. Nitric oxide signalling in plants: interplays with Ca^{2+} and protein kinases. J Exp Bot. 2008;59(2):155-63.

48. Crawford NM, Guo FQ. New insights into nitric oxide metabolism and regulatory functions. Trends Plant Sci. 2005 Apr;10(4):195-200.

49. Crawford NM, Gally M, Tischner R, Heimer YM, Okamoto M, Mack A. Plant nitric oxide synthase: Back to square one. Trends Plant Sci. 2006;11:526-527.

50. Cueto M, Hernández-Perera O, Martín R, Bentura ML, Rodrigo J, Lamas S, Golvano MP. Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*. FEBS Lett. 1996 Dec 2;398(2-3):159-64.

51. Curie C, Briat JF. Iron transport and signaling in plants. Annu Rev Plant Biol. 2003;54:183-206.

52. del Río LA, Corpas FJ, Sandalio LM, Palma JM, Gómez M, Barroso JB. Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. J Exp Bot. 2002 May;53(372):1255-72.

53. del Río LA, Corpas FJ, Barroso JB. Nitric oxide and nitric oxide synthase activity in plants. Phytochemistry. 2004 Apr;65(7):783-92.

54. del Río LA, Sandalio LM, Corpas FJ, Palma JM, Barroso JB. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. Plant Physiol. 2006 Jun;141(2):330-5.

55. Delledonne M, Xia Y, Dixon RA, Lamb C. Nitric oxide functions as a signal in plant disease resistance. Nature. 1998 Aug 6;394(6693):585-8.

56. Delledonne M, Zeier J, Marocco A, Lamb C. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci U S A. 2001 Nov 6;98(23):13454-9.

57. Delledonne M, Murgia I, Ederle D, Sbicego PF, Biondani A, Polverari A, Lamb C. Reactive oxygen intermediates modulate nitric oxide signaling in the plant hypersensitive disease-resistance response. Plant Physiol. Biochem. 2002;40:605-610.

58. Delledonne M, Polverari A, Murgia I. The functions of nitric oxide-mediated signaling and changes in gene expression during the hypersensitive response. Antioxid Redox Signal. 2003 Feb;5(1):33-41.

59. de Pinto MC, Paradiso A, Leonetti P, De Gara L. Hydrogen peroxide, nitric oxide and cytosolic ascorbate peroxidase at the crossroad between defence and cell death. Plant J. 2006 Dec;48(5):784-95.

60. Desikan R, Cheung MK, Bright J, Henson D, Hancock JT, Neill SJ. ABA, hydrogen peroxide and nitric oxide signalling in stomatal guard cells. J Exp Bot. 2004 Jan;55(395):205-12.

61. Díaz M, Achkor H, Titarenko E, Martínez MC. The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid. FEBS Lett. 2003 May 22;543(1-3):136-9.

62. Dolcet-Sanjuan R, Mok DWS, Mok MC. Micropropagation of Pyrus and Cydonia and their responses to Fe-limiting conditions. Plant Cell Tissue Organ Cult. 1990;21:191-199.

63. Dordas C, Rivoal J, Hill RD. Plant haemoglobins, nitric oxide and hypoxic stress. Ann Bot. 2003 Jan;91 Spec No:173-8.

64. Du S, Zhang Y, Lin X, Wang Y, Tang C. Regulation of nitrate reductase by nitric oxide in Chinese cabbage pakchoi (*Brassica chinensis* L.). Plant Cell Environ. 2008 Feb;31(2):195-204.

65. Durner J, Wendehenne D, Klessig DF. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci U S A. 1998 Aug 18;95(17):10328-33.

66. Durrett TP, Gassmann W, Rogers EE. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. Plant Physiol. 2007 May;144(1):197-205.

67. Ederli L, Morettini R, Borgogni A, Wasternack C, Miersch O, Reale L, Ferranti F, Tosti N, Pasqualini S. Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants. Plant Physiol. 2006 Oct;142(2):595-608.

68. Emerit J, Beaumont C, Trivin F. Iron metabolism, free radicals, and oxidative injury. Biomed Pharmacother. 2001 Jul;55(6):333-9.

69. ESTree database, http://www.itb.cnr.it/estree/

70. Fang W, Kao CH. Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc. Plant Sci. 2000 Sep 8;158(1-2):71-76.

71. FAO, http://faostat.fao.org/site/339/default.aspx

72. Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ. A central role for *S*-nitrosothiols in plant disease resistance. Proc Natl Acad Sci U S A. 2005 May 31;102(22):8054-9.

73. Ferrarini A, De Stefano M, Baudouin E, Pucciariello C, Polverari A, Puppo A, Delledonne M. Expression of *Medicago truncatula* genes responsive to nitric oxide in pathogenic and symbiotic conditions. Mol Plant Microbe Interact. 2008 Jun;21(6):781-90.

74. Foyer C, Lelandais M, Galap C, Kunert KJ. Effects of elevated cytosolic glutathione reductase activity on the cellular glutathione pool and photosynthesis in leaves under normal and stress conditions. Plant Physiol. 1991 Nov;97(3):863-872.

75. Foyer CH, Noctor G. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell. 2005 Jul;17(7):1866-75.

76. Garcia-Mata C, Gay R, Sokolovski S, Hills A, Lamattina L, Blatt MR. Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. Proc Natl Acad Sci U S A. 2003 Sep 16;100(19):11116-21.

77. Gas E, Flores-Pérez U, Sauret-Güeto S, Rodríguez-Concepción M. Hunting for plant nitric oxide synthase provides new evidence of a central role for plastids in nitric oxide metabolism. Plant Cell. 2009 Jan;21(1):18-23.

78. Gaupels F, Furch AC, Will T, Mur LA, Kogel KH, van Bel AJ. Nitric oxide generation in *Vicia faba* phloem cells reveals them to be sensitive detectors as well as possible systemic transducers of stress signals. New Phytol. 2008;178(3):634-46.

79. Gechev TS, Hille J. Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol. 2005 Jan 3;168(1):17-20.

80. Gogorcena Y, Abadía J, Abadía A. Induction of *in vivo* root ferric chelate reductase activity in fruit tree rootstock. Journal of Plant Nutrition. 2000;23(1):9-21.

81. Gogorcena Y, Abadía J, Abadía A. A new technique for screening iron-efficient genotypes in peach rootstocks: elicitation of root ferric chelate reductase by manipulation of external iron concentrations. Journal of Plant Nutrition. 2004;27(10):1701-1715.

82. Gould KS, Lamotte O, Klinguer A, Pugin A, Wendehenne D. Nitric oxide production in tobacco leaf cells: a generalized stress response? Plant Cell Environ. 2003;26:1851-1862.

83. Graziano M, Beligni MV, Lamattina L. Nitric oxide improves internal iron availability in plants. Plant Physiol. 2002 Dec;130(4):1852-9.

84. Graziano M, Lamattina L. Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. Plant J. 2007 Dec;52(5):949-60.

85. Green LS, Rogers EE. FRD3 controls iron localization in *Arabidopsis*. Plant Physiol. 2004 Sep;136(1):2523-31.

86. Grün S, Lindermayr C, Sell S, Durner J. Nitric oxide and gene regulation in plants. J Exp Bot. 2006;57(3):507-16.

87. Grusak MA, Pezeshgi S. Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the *dgl* mutant of pea. Plant Physiol. 1996 Jan;110(1):329-334.

88. Guerinot ML, Yi Y. Iron: nutritious, noxious, and not readily available. Plant Physiol. 1994 Mar;104(3):815-820.

89. Guo FQ, Okamoto M, Crawford NM. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science. 2003 Oct 3;302(5642):100-3.

90. Gupta KJ, Stoimenova M, Kaiser WM. In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, *in vitro* and *in situ*. J Exp Bot. 2005 Oct;56(420):2601-9.

91. Hageman RH, Reed AJ. Nitrate reductase from higher plants. Meth Enzymol. 1980;69:270-280.

92. Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. SNOSID, a proteomic method for identification of cysteine *S*-nitrosylation sites in complex protein mixtures. Proc Natl Acad Sci U S A. 2006 Jan 24;103(4):1012-7.

93. Hare PD, Cress WA, Van Staden J. Dissecting the roles of osmolyte accumulation during stress. Plant Cell Environ. 1998;21:535-553.

94. Havir EA, McHale NA. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol. 1987 Jun;84(2):450-455.

95. He Y, Tang RH, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, Fiorani F, Jackson RB, Crawford NM, Pei ZM. Nitric oxide represses the *Arabidopsis* floral transition. Science. 2004 Sep 24;305(5692):1968-71.

96. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 1968 Apr;125(1):189-98.

97. Hell R, Stephan UW. Iron uptake, trafficking and homeostasis in plants. Planta. 2003 Feb;216(4):541-51.

98. Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H. A large family of class III plant peroxidases. Plant Cell Physiol. 2001 May;42(5):462-8.

99. Hong JK, Yun BW, Kang JG, Raja MU, Kwon E, Sorhagen K, Chu C, Wang Y, Loake GJ. Nitric oxide function and signalling in plant disease resistance. J Exp Bot. 2008;59(2):147-54.

100. Huang X, Stettmaier K, Michel C, Hutzler P, Mueller MJ, Durner J. Nitric oxide is induced by wounding and influences jasmonic acid signaling in *Arabidopsis thaliana*. Planta. 2004 Apr;218(6):938-46.

101. Hughes MN. Chemistry of nitric oxide and related species. Methods Enzymol. 2008;436:3-19.

102. Hung KT, Kao CH. Nitric oxide acts as an antioxidant and delays methyl jasmonate-induced senescence of rice leaves. J Plant Physiol. 2004 Jan;161(1):43-52.

103. Ishikawa T, Shigeoka S. Recent advances in ascorbate biosynthesis and the physiological significance of ascorbate peroxidase in photosynthesizing organisms. Biosci Biotechnol Biochem. 2008 May;72(5):1143-54.

104. Ishimaru Y, Suzuki M, Tsukamoto T, Suzuki K, Nakazono M, Kobayashi T, Wada Y, Watanabe S, Matsuhashi S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK. Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. Plant J. 2006 Feb;45(3):335-46.

105. Jakoby M, Wang HY, Reidt W, Weisshaar B, Bauer P. FRU (BHLH029) is required for induction of iron mobilization genes in Arabidopsis thaliana. FEBS Lett. 2004 Nov 19;577(3):528-34.

106. Jasid S, Simontacchi M, Bartoli CG, Puntarulo S. Chloroplasts as a nitric oxide cellular source. Effect of reactive nitrogen species on chloroplastic lipids and proteins. Plant Physiol. 2006 Nov;142(3):1246-55.

107. Jasid S, Simontacchi M, Puntarulo S. Exposure to nitric oxide protects against oxidative damage but increases the labile iron pool in sorghum embryonic axes. J Exp Bot. 2008;59(14):3953-62.

108. Jiménez S, Gogorcena Y, Hevin C, Rombolà AD, Ollat N. Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of *Vitis*. Plant Soil. 2007;290:343–355.

109. Kaiser WM, Weiner H, Kandlbinder A, Tsai CB, Rockel P, Sonoda M, Planchet E. Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. J Exp Bot. 2002 Apr;53(370):875-82.

110. Kampfenkel K, Van Montagu M, Inze D. Effects of Iron Excess on Nicotiana plumbaginifolia Plants (Implications to Oxidative Stress). Plant Physiol. 1995 Mar;107(3):725-735.

111. Keyer K, Imlay JA. Superoxide accelerates DNA damage by elevating free-iron levels. Proc Natl Acad Sci U S A. 1996 Nov 26;93(24):13635-40.

112. Kim SA, Guerinot ML. Mining iron: iron uptake and transport in plants. FEBS Lett. 2007 May 25;581(12):2273-80.

113. Kim SA, Punshon T, Lanzirotti A, Li L, Alonso JM, Ecker JR, Kaplan J, Guerinot ML. Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. Science. 2006 Nov 24;314(5803):1295-8.

114. Klessig DF, Ytterberg AJ, van Wijk KJ. The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. Cell. 2004 Oct 29;119(3):445.

115. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H. Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell. 2007 Mar;19(3):1065-80.

116. Kobayashi T, Nakayama Y, Itai RN, Nakanishi H, Yoshihara T, Mori S, Nishizawa NK. Identification of novel cis-acting elements, IDE1 and IDE2, of the barley *IDS2* gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. Plant J. 2003 Dec;36(6):780-93.

117. Kolbert Z, Bartha B, Erdei L. Exogenous auxin-induced NO synthesis is nitrate reductase-associated in *Arabidopsis thaliana* root primordia. J Plant Physiol. 2008 Jun 16;165(9):967-75.

118. Kolbert Z, Bartha B, Erdei L. Osmotic stress- and indole-3-butyric acid-induced NO generation are partially distinct processes in root growth and development in *Pisum sativum*. Physiol Plant. 2008 Jun;133(2):406-16.

119. Kolla VA, Raghavendra AS. Nitric oxide is a signaling intermediate during bicarbonate-induced stomatal closure in *Pisum sativum*. Physiol Plant. 2007;130:91-98.

120. Kosegarten H, Hoffmann B, Mengel K. The paramount influence of nitrate in increasing apoplastic pH of young sunflower leaf to induce Fe deficiency chlorosis, and the re-greening effect brought about acid foliar sprays. J Plant Nutr Soil Sci. 2001;164**:**155-163.

121. Kosegarten H, Hoffmann B, Rroço E, Grolig F, Glüsenkamp KH, Mengel K. Apoplastic pH and FeIII reduction in young sunflower (*Helianthus annuus*) roots. Physiol Plant. 2004;122:96-107.

122. Kruger C, Berkowitz O, Stephan UW, Hell R. A metal-binding member of the late embryogenesis abundant protein family transports iron in the phloem of *Ricinus communis* L. J Biol Chem. 2002 Jul 12;277(28):25062-9.

123. Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I, Apel K. Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in Arabidopsis thaliana. Proc Natl Acad Sci U S A. 2007 Jan 9;104(2):672-7.

124. Lamotte O, Courtois C, Dobrowolska G, Besson A, Pugin A, Wendehenne D. Mechanisms of nitric-oxide-induced increase of free cytosolic Ca^{2+} concentration in *Nicotiana plumbaginifolia* cells. Free Radic Biol Med. 2006 Apr 15;40(8):1369-76.

125. Lanquar V, Lelièvre F, Bolte S, Hamès C, Alcon C, Neumann D, Vansuyt G, Curie C, Schröder A, Krämer U, Barbier-Brygoo H, Thomine S. Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. EMBO J. 2005 Dec 7;24(23):4041-51.

126. Lanteri ML, Pagnussat GC, Lamattina L. Calcium and calcium-dependent protein kinases are involved in nitric oxide- and auxin-induced adventitious root formation in cucumber. J Exp Bot. 2006;57(6):1341-51.

127. Laspina NV, Groppa MD, Tomaro ML, Benavides MP. Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. Plant Sci. 2005 Aug;169(2):323-330.

128. Laxalt AM, Raho N, Have AT, Lamattina L. Nitric oxide is critical for inducing phosphatidic acid accumulation in xylanase-elicited tomato cells. J Biol Chem. 2007 Jul 20;282(29):21160-8.

129. Le Jean M, Schikora A, Mari S, Briat JF, Curie C. A loss-of-function mutation in *AtYSL1* reveals its role in iron and nicotianamine seed loading. Plant J. 2005 Dec;44(5):769-82.

130. Lee U, Wie C, Fernandez BO, Feelisch M, Vierling E. Modulation of nitrosative stress by *S*-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*. Plant Cell. 2008 Mar;20(3):786-802.

131. Leshem YY, Wills RBH. Harnessing senescence delaying gases nitric oxide and nitrous oxide: a novel approach to post-harvest control of fresh horticultural produce. Biol Plant. 1998;41:1-10.

132. Lindermayr C, Saalbach G, Durner J. Proteomic identification of *S*-nitrosylated proteins in Arabidopsis. Plant Physiol. 2005 Mar;137(3):921-30.

133. Lindermayr C, Saalbach G, Bahnweg G, Durner J. Differential inhibition of *Arabidopsis* methionine adenosyltransferases by protein *S*-nitrosylation. J Biol Chem. 2006 Feb 17;281(7):4285-91.

134. Lindermayr C, Durner J. *S*-Nitrosylation in plants: pattern and function. J Proteomics. 2009 Nov 2;73(1):1-9.

135. Ling HQ, Bauer P, Bereczky Z, Keller B, Ganal M. The tomato *fer* gene encoding a bHLH protein controls iron-uptake responses in roots. Proc Natl Acad Sci U S A. 2002 Oct 15;99(21):13938-43.

136. Lombardo MC, Graziano M, Polacco JC, Lamattina L. Nitric oxide functions as a positive regulator of root hair development. Plant Signal Behav. 2006 Jan;1(1):28-33.

137. López-Millán AF, Morales F, Abadía A, Abadía J. Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris*) leaves. Physiol Plant. 2001 May;112(1):31-38.

138. López-Millán AF, Morales F, Abadía A, Abadía J. Iron deficiency-associated changes in the composition of the leaf apoplastic fluid from field-grown pear (*Pyrus communis* L.) trees. J Exp Bot. 2001 Jul;52(360):1489-98.

139. López-Millán AF, Morales F, Gogorcena Y, Abadía A, Abadía J. Metabolic responses in iron deficient tomato plants. J Plant Physiol. 2009 Mar 1;166(4):375-84.

140. Lucena JJ. Effects of bicarbonate, nitrate and other environmental factors on iron deficiency chlorosis. A review. J Plant Nutr. 2000;23(11):1591-1606.

141. Lucena C, Waters BM, Romera FJ, García MJ, Morales M, Alcántara E, Pérez-Vicente R. Ethylene could influence ferric reductase, iron transporter, and H^+-ATPase gene expression by affecting *FER* (or *FER*-like) gene activity. J Exp Bot. 2006;57(15):4145-54.

142. Ma JF, Ueno H, Ueno D, Rombola AD, Iwashita T. Characterization of phytosiderophore secretion under Fe deficiency stress in *Festuca rubra*. Plant Soil. 2003;256:131-137.

143. Maeda H, DellaPenna D. Tocopherol functions in photosynthetic organisms. Curr Opin Plant Biol. 2007 Jun;10(3):260-5.

144. Mahajan S, Tuteja N. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys. 2005 Dec 15;444(2):139-58.

145. Mahalingam R, Jambunathan N, Gunjan SK, Faustin E, Weng H, Ayoubi P. Analysis of oxidative signalling induced by ozone in *Arabidopsis thaliana*. Plant Cell Environ. 2006 Jul;29(7):1357-71.

146. Margulis A, Sitaramayya A. Rate of deactivation of nitric oxide-stimulated soluble guanylate cyclase: influence of nitric oxide scavengers and calcium. Biochemistry. 2000 Feb 8;39(5):1034-9.

147. Marino G, Beghelli S, Rombolà AD, Cabrini L. In vitro performance at high culture pH and in vivo responses to Fe-deficiency of leaf-derived quince BA 29 (*Cydonia oblonga*) somaclones regenerated at variable pH. J. Hort. Sci. & Biotech. 2000;75(4):433-440.

148. Martin M, Colman MJ, Gómez-Casati DF, Lamattina L, Zabaleta EJ. Nitric oxide accumulation is required to protect against iron-mediated oxidative stress in frataxin-deficient Arabidopsis plants. FEBS Lett. 2009 Feb 4;583(3):542-8.

149. Masia A. Superoxide dismutase and catalase activities in apple fruit during ripening and post-harvest and with special reference to ethylene. Physiol. Plant. 1998;104:668-672.

150. Masia A, Mantelli M, Cellini A, Rombolà AD. Calcareous soil and iron chlorosis in peach tree; some effects on oxidative stress physiology. 13th International Symposium on Iron Nutrition and Interactions in Plants, Montpellier 2006 (poster session).

151. McInnis SM, Desikan R, Hancock JT, Hiscock SJ. Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signalling crosstalk? New Phytol. 2006;172(2):221-8.

152. Meyer C, Lea US, Provan F, Kaiser WM, Lillo C. Is nitrate reductase a major player in the plant NO (nitric oxide) game? Photosynth Res. 2005;83(2):181-9.

153. Millar AH, Day DA. Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. FEBS Lett. 1996 Dec 2;398(2-3):155-8.

154. Mishina TE, Lamb C, Zeier J. Expression of a nitric oxide degrading enzyme induces a senescence programme in *Arabidopsis*. Plant Cell Environ. 2007 Jan;30(1):39-52.

155. Mittler R. Abiotic stress, the field environment and stress combination. Trends Plant Sci. 2006 Jan;11(1):15-9.

156. Modolo LV, Augusto O, Almeida IMG, Pinto-Maglio CAF, Oliveira HC, Seligman K, Salgado I. Decreased arginine and nitrite levels in nitrate reductase-deficient *Arabidopsis thaliana* plants impair nitric oxide synthesis and the hypersensitive response to *Pseudomonas syringae*. Plant Sci. 2006 Jul;171(1): 34-40.

157. Moeder W, Del Pozo O, Navarre DA, Martin GB, Klessig DF. Aconitase plays a role in regulating resistance to oxidative stress and cell death in Arabidopsis and *Nicotiana benthamiana*. Plant Mol Biol. 2007 Jan;63(2):273-87.

158. Molassiotis A, Tanou G, Diamantidis G, Patakas A, Therios I. Effects of 4-month Fe deficiency exposure on Fe reduction mechanism, photosynthetic gas exchange, chlorophyll fluorescence and antioxidant defense in two peach rootstocks differing in Fe deficiency tolerance. J Plant Physiol. 2006 Feb;163(2):176-85.

159. Morales F, Belkhodja R, Abadía A, Abadía J. Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.). Photosynth Res. 2000;63(1):9-21.

160. Morot-Gaudry-Talarmain Y, Rockel P, Moureaux T, Quilleré I, Leydecker MT, Kaiser WM, Morot-Gaudry JF. Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. Planta. 2002 Sep;215(5):708-15.

161. Mukherjee I, Campbell NH, Ash JS, Connolly EL. Expression profiling of the Arabidopsis ferric chelate reductase (*FRO*) gene family reveals differential regulation by iron and copper. Planta. 2006 May;223(6):1178-90.

162. Murata Y, Ma JF, Yamaji N, Ueno D, Nomoto K, Iwashita T. A specific transporter for iron(III)-phytosiderophore in barley roots. Plant J. 2006 May;46(4):563-72.

163. Murgia I, Briat JF, Tarantino D, Soave C. Plant ferritin accumulates in response to photoinhibition but its ectopic overexpression does not protect against photoinhibition. Plant Physiol. Biochem. 2001;39:797-805.

164. Murgia I, Delledonne M, Soave C. Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. Plant J. 2002 Jun;30(5):521-8.

165. Murgia I, de Pinto MC, Delledonne M, Soave C, De Gara L. Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells. J Plant Physiol. 2004 Jul;161(7):777-83.

166. Murgia I, Vazzola V, Tarantino D, Cellier F, Ravet K, Briat JF, Soave C. Knock-out of ferritin *AtFer1* causes earlier onset of age-dependent leaf senescence in *Arabidopsis*. Plant Physiol Biochem. 2007 Dec;45(12):898-907.

167. Nagata M, Murakami E, Shimoda Y, Shimoda-Sasakura F, Kucho K, Suzuki A, Abe M, Higashi S, Uchiumi T. Expression of a class 1 hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in *Lotus japonicus*. Mol Plant Microbe Interact. 2008 Sep;21(9):1175-83.

168. Negishi T, Nakanishi H, Yazaki J, Kishimoto N, Fujii F, Shimbo K, Yamamoto K, Sakata K, Sasaki T, Kikuchi S, Mori S, Nishizawa NK. cDNA microarray analysis of gene expression during Fe-deficiency stress in barley suggests that polar transport of vesicles is implicated in phytosiderophore secretion in Fe-deficient barley roots. Plant J. 2002 Apr;30(1):83-94.

169. Neill S, Desikan R, Hancock J. Hydrogen peroxide signalling. Curr Opin Plant Biol. 2002 Oct;5(5):388-95.

170. Neill S, Bright J, Desikan R, Hancock J, Harrison J, Wilson I. Nitric oxide evolution and perception. J Exp Bot. 2008;59(1):25-35.

171. Nikolic M, Römheld V. Does high bicarbonate supply to roots change availability of iron in the leaf apoplast? Plant and Soil 2002;241:67-74.

172. Nikolic M, Römheld V. Nitrate does not result in iron inactivation in the apoplast of sunflower leaves. Plant Physiol. 2003 Jul;132(3):1303-14.

173. Ogo Y, Itai RN, Nakanishi H, Inoue H, Kobayashi T, Suzuki M, Takahashi M, Mori S, Nishizawa NK. Isolation and characterization of IRO2, a novel iron-regulated bHLH transcription factor in graminaceous plants. J Exp Bot. 2006;57(11):2867-78.

174. Pagnussat GC, Lanteri ML, Lamattina L. Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiol. 2003 Jul;132(3):1241-8.

175. Palmieri MC, Sell S, Huang X, Scherf M, Werner T, Durner J, Lindermayr C. Nitric oxide-responsive genes and promoters in *Arabidopsis thaliana*: a bioinformatics approach. J Exp Bot. 2008;59(2):177-86.

176. Palombi MA, Lombardo B, Caboni E. In vitro regeneration of wild pear (*Pyrus pyraster* Burgsd) clones tolerant to Fe-chlorosis and somaclonal variation analysis by RAPD markers. Plant Cell Rep. 2007 Apr;26(4):489-96.

177. Pandey S, Ranade SA, Nagar PK, Kumar N. Role of polyamines and ethylene as modulators of plant senescence. J Biosci. 2000 Sep;25(3):291-9.

178. Perazzolli M, Romero-Puertas MC, Delledonne M. Modulation of nitric oxide bioactivity by plant haemoglobins. J Exp Bot. 2006;57(3):479-88.

179. Petit JM, van Wuytswinkel O, Briat JF, Lobréaux S. Characterization of an iron-dependent regulatory sequence involved in the transcriptional control of *AtFer1* and *ZmFer1* plant ferritin genes by iron. J Biol Chem. 2001 Feb 23;276(8):5584-90.

180. Pich A, Manteuffel R, Hillmer S, Scholz G, Schmidt W. Fe homeostasis in plant cells: does nicotianamine play multiple roles in the regulation of cytoplasmic Fe concentration? Planta. 2001 Oct;213(6):967-76.

181. Pierre JL, Fontecave M. Iron and activated oxygen species in biology: the basic chemistry. Biometals. 1999 Sep;12(3):195-9.

182. Planchet E, Jagadis Gupta K, Sonoda M, Kaiser WM. Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J. 2005 Mar;41(5):732-43.

183. Planchet E, Sonoda M, Zeier J, Kaiser WM. Nitric oxide (NO) as an intermediate in the cryptogein-induced hypersensitive response - a critical re-evaluation. Plant Cell Environ. 2006 Jan;29(1):59-69.

184. Puig S, Andrés-Colás N, García-Molina A, Peñarrubia L. Copper and iron homeostasis in *Arabidopsis*: responses to metal deficiencies, interactions and biotechnological applications. Plant Cell Environ. 2007 Mar;30(3):271-90.

185. Queval G, Noctor G. A plate reader method for the measurement of NAD, NADP, glutathione, and ascorbate in tissue extracts: Application to redox profiling during *Arabidopsis* rosette development. Anal Biochem. 2007 Apr 1;363(1):58-69.

186. Ramirez L, Zabaleta EJ, Lamattina L. Nitric oxide and frataxin: two players contributing to maintain cellular iron homeostasis. Ann Bot. 2009 Jun 25.

187. Ranieri A, Castagna A, Soldatini GF. Iron deficiency induces variations in oxidative stress bioindicators in sunflower plants. Agricoltura Mediterranea. 1999;129:180-192.

188. Ranieri A, Castagna A, Baldan B, Soldatini GF. Iron deficiency differently affects peroxidase isoforms in sunflower. J Exp Bot. 2001 Jan;52(354):25-35.

189. Ravet K, Touraine B, Boucherez J, Briat JF, Gaymard F, Cellier F. Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. Plant J. 2009 Feb;57(3):400-12.

190. Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R. When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiol. 2004 Apr;134(4):1683-96.

191. Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM. Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. J Exp Bot. 2002 Jan;53(366):103-10.

192. Rodríguez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gómez M, Del Río LA, Sandalio LM. Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots. Imaging of reactive oxygen species and nitric oxide accumulation in vivo. Plant Cell Environ. 2006 Aug;29(8):1532-44.

193. Rogers EE, Wu X, Stacey G, Nguyen HT. Two MATE proteins play a role in iron efficiency in soybean. J Plant Physiol. 2009 Sep 1;166(13):1453-9.

194. Rombolà AD, Brüggemann W, López-Millán AF, Tagliavini M,, Marangoni B, Moog PR. Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). Tree Physiol. 2002 Aug;22(12):869-75.

195. Rombolà AD, Gogorcena Y, Larbi A, Morales F, Baldi E, Marangoni B, Tagliavini M, Abadía J. Iron deficiency-induced changes in carbon fixation and leaf elemental composition of sugar beet (*Beta vulgaris*) plants. Plant Soil. 2005;271:39-45.

196. Romero-Puertas MC, Laxa M, Mattè A, Zaninotto F, Finkemeier I, Jones AM, Perazzolli M, Vandelle E, Dietz KJ, Delledonne M. *S*-nitrosylation of peroxiredoxin II E promotes peroxynitrite-mediated tyrosine nitration. Plant Cell. 2007 Dec;19(12):4120-30.

197. Romero-Puertas MC, Campostrini N, Mattè A, Righetti PG, Perazzolli M, Zolla L, Roepstorff P, Delledonne M. Proteomic analysis of *S*-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. Proteomics. 2008 Apr;8(7):1459-69.

198. Römheld V. The chlorosis paradox: Fe inactivation as a secondary event in chlorotic leaves of grapevine. J Plant Nutr. 2000;23(11):1629-1643.

199. Rouhier N, Jacquot JP. The plant multigenic family of thiol peroxidases. Free Radic Biol Med. 2005 Jun 1;38(11):1413-21.

200. Rümer S, Gupta KJ, Kaiser WM. Plant cells oxidize hydroxylamines to NO. J Exp Bot. 2009;60(7):2065-72.

201. Rustérucci C, Espunya MC, Díaz M, Chabannes M, Martínez MC. *S*-nitrosoglutathione reductase affords protection against pathogens in Arabidopsis, both locally and systemically. Plant Physiol. 2007 Mar;143(3):1282-92.

202. Saito S, Yamamoto-Katou A, Yoshioka H, Doke N, Kawakita K. Peroxynitrite generation and tyrosine nitration in defense responses in tobacco BY-2 cells. Plant Cell Physiol. 2006 Jun;47(6):689-97.

203. Sakamoto A, Ueda M, Morikawa H. *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase is an *S*-nitrosoglutathione reductase. FEBS Lett. 2002 Mar 27;515(1-3):20-4.

204. Sang J, Jiang M, Lin F, Xu S, Zhang A, Tan M. Nitric oxide reduces hydrogen peroxide accumulation involved in water stress-induced subcellular anti-oxidant defense in maize plants. J Integr Plant Biol. 2008 Feb;50(2):231-43.

205. Sasakura F, Uchiumi T, Shimoda Y, Suzuki A, Takenouchi K, Higashi S, Abe M. A class 1 hemoglobin gene from *Alnus firma* functions in symbiotic and nonsymbiotic tissues to detoxify nitric oxide. Mol Plant Microbe Interact. 2006 Apr;19(4):441-50.

206. Schaaf G, Ludewig U, Erenoglu BE, Mori S, Kitahara T, von Wirén N. ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. J Biol Chem. 2004 Mar 5;279(10):9091-6.

207. Schaaf G, Schikora A, Häberle J, Vert G, Ludewig U, Briat JF, Curie C, von Wirén N. A putative function for the *Arabidopsis* Fe-Phytosiderophore transporter homolog AtYSL2 in Fe and Zn homeostasis. Plant Cell Physiol. 2005 May;46(5):762-74.

208. Schikora A, Schmidt W. Iron stress-induced changes in root epidermal cell fate are regulated independently from physiological responses to low iron availability. Plant Physiol. 2001 Apr;125(4):1679-87.

209. Schmidt W, Tittel J, Schikora A. Role of hormones in the induction of iron deficiency responses in Arabidopsis roots. Plant Physiol. 2000 Apr;122(4):1109-18.

210. Scotti C. Portinnesti del pesco e caratteristiche del Suolo. Agricoltura. 2006 jul-aug.

211. Sharma S. Adaptation of photosynthesis under iron deficiency in maize. J Plant Physiol. 2007 Oct;164(10):1261-7.

212. Stöhr C, Strube F, Marx G, Ullrich WR, Rockel P. A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. Planta. 2001 Apr;212(5-6):835-41.

213. Stöhr C, Ullrich WR. Generation and possible roles of NO in plant roots and their apoplastic space. J Exp Bot. 2002 Dec;53(379):2293-303.

214. Stoimenova M, Hänsch R, Mendel R, Gimmler H, Kaiser WM. The role of nitrate reduction in the anoxic metabolism of roots I. Characterization of root morphology and normoxic metabolism of wild type tobacco and a transformant lacking root nitrate reductase. Plant Soil. 2003;253:145-153.

215. Sun B, Jing Y, Chen K, Song L, Chen F, Zhang L. Protective effect of nitric oxide on iron deficiency-induced oxidative stress in maize (*Zea mays*). J Plant Physiol. 2007 May;164(5):536-43.

216. Tagliavini M, Rombolà AD. Iron deficiency and chlorosis in orchard and vineyard ecosystems. Eur J Agron. 2001;15:71-92.

217. Takahashi S, Yamasaki H. Reversible inhibition of photophosphorylation in chloroplasts by nitric oxide. FEBS Lett. 2002 Feb 13;512(1-3):145-8.

218. Tanou G, Job C, Rajjou L, Arc E, Belghazi M, Diamantidis G, Molassiotis A, Job D. Proteomics reveals the overlapping roles of hydrogen peroxide and nitric oxide in the acclimation of citrus plants to salinity. Plant J. 2009 Dec;60(5):795-804.

219. Tarantino D, Petit JM, Lobreaux S, Briat JF, Soave C, Murgia I. Differential involvement of the IDRS *cis*-element in the developmental and environmental regulation of the *AtFer1* ferritin gene from *Arabidopsis*. Planta. 2003 Sep;217(5):709-16.

220. Thimm O, Essigmann B, Kloska S, Altmann T, Buckhout TJ. Response of Arabidopsis to iron deficiency stress as revealed by microarray analysis. Plant Physiol. 2001 Nov;127(3):1030-43.

221. Tian QY, Sun DH, Zhao MG, Zhang WH. Inhibition of nitric oxide synthase (NOS) underlies aluminum-induced inhibition of root elongation in Hibiscus moscheutos. New Phytol. 2007;174(2):322-31.

222. Tischner R, Planchet E, Kaiser WM. Mitochondrial electron transport as a source for nitric oxide in the unicellular green alga *Chlorella sorokiniana*. FEBS Lett. 2004 Oct 8;576(1-2):151-5.

223. Torres MA, Dangl JL. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol. 2005 Aug;8(4):397-403.

224. Toselli M, Flore JA, Marangoni B, Masia A. Effects of root-zone temperature on nitrogen accumulation by non-bearing apple trees. J. Hort. Sci & Biotechn. 1999;74(1):118-124.

225. Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh EI, Scherer GF. Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant Cell Physiol. 2006 Mar;47(3):346-54.

226. Tun NN, Livaja M, Kieber JJ, Scherer GF. Zeatin-induced nitric oxide (NO) biosynthesis in *Arabidopsis thaliana* mutants of NO biosynthesis and of two-component signaling genes. New Phytol. 2008;178(3):515-31.

227. Tuteja N, Sopory SK. Chemical signaling under abiotic stress environment in plants. Plant Signal Behav. 2008 Aug;3(8):525-36.

228. Ushimaru T, Maki Y, Sano S, Koshiba K, Asada K, Tsuji H. Induction of enzymes involved in the ascorbate-dependent antioxidative system, namely, ascorbate peroxidase, monodehydroascorbate reductase and dehydroascorbate reductase, after exposure to air of rice (*Oryza sativa*) seedlings germinated under water. Plant Cell Physiol. 1997;38:541-549.

229. Valderrama R, Corpas FJ, Carreras A, Fernández-Ocaña A, Chaki M, Luque F, Gómez-Rodríguez MV, Colmenero-Varea P, Del Río LA, Barroso JB. Nitrosative stress in plants. FEBS Lett. 2007 Feb 6;581(3):453-61.

230. Vanin AF, Svistunenko DA, Mikoyan VD, Serezhenkov VA, Fryer MJ, Baker NR, Cooper CE. Endogenous superoxide production and the nitrite/nitrate ratio control the concentration of bioavailable free nitric oxide in leaves. J Biol Chem. 2004 Jun 4;279(23):24100-7.

231. Verbruggen N, Hermans C. Proline accumulation in plants: a review. Amino Acids. 2008 Nov;35(4):753-9.

232. Vert GA, Briat JF, Curie C. Dual regulation of the Arabidopsis high-affinity root iron uptake system by local and long-distance signals. Plant Physiol. 2003 Jun;132(2):796-804.

233. Vinocur B, Altman A. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol. 2005 Apr;16(2):123-32. Review.

234. von Wiren N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC. Nicotianamine chelates both FeIII and FeII. Implications for metal transport in plants. Plant Physiol. 1999 Mar;119(3):1107-14.

235. von Wirén N, Khodr H, Hider RC. Hydroxylated phytosiderophore species possess an enhanced chelate stability and affinity for iron(III). Plant Physiol. 2000 Nov;124(3):1149-58.

236. Vorwieger A, Gryczka C, Czihal A, Douchkov D, Tiedemann J, Mock HP, Jakoby M, Weisshaar B, Saalbach I, Bäumlein H. Iron assimilation and transcription factor controlled synthesis of riboflavin in plants. Planta. 2007 Jun;226(1):147-58.

237. Vranová E, Inzé D, Van Breusegem F. Signal transduction during oxidative stress. J Exp Bot. 2002 May;53(372):1227-36.

238. Wang Y, Yun BW, Kwon E, Hong JK, Yoon J, Loake GJ. *S*-nitrosylation: an emerging redox-based post-translational modification in plants. J Exp Bot. 2006;57(8):1777-84.

239. Wang Y, Chen T, Zhang C, Hao H, Liu P, Zheng M, Baluška F, Šamaj J, Lin J. Nitric oxide modulates the influx of extracellular Ca and actin filament organization during cell wall construction in *Pinus bungeana* pollen tubes. New Phytol. 2009;182(4):851-62.

240. Waters BM, Blevins DG. Ethylene production, cluster root formation, and localization of iron(III) reducing capacity in Fe deficient squash roots. Plant Soil. 2000;225:21-31.

241. Wei J, Theil EC. Identification and characterization of the iron regulatory element in the ferritin gene of a plant (soybean). J Biol Chem. 2000 Jun 9;275(23):17488-93.

242. Wendehenne D, Durner J, Klessig DF. Nitric oxide: a new player in plant signalling and defence responses. Curr Opin Plant Biol. 2004 Aug;7(4):449-55.

243. Wolff SP. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. Meth Enzymol. 1994;233:182-189.

244. Yamaguchi-Shinozaki K, Shinozaki K. Organization of *cis*-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci. 2005 Feb;10(2):88-94.

245. Yamasaki H, Sakihama Y. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett. 2000 Feb 18;468(1):89-92.

246. Yamasaki H, Shimoji H, Ohshiro Y, Sakihama Y. Inhibitory effects of nitric oxide on oxidative phosphorylation in plant mitochondria. Nitric Oxide. 2001 Jun;5(3):261-70.

247. Yamasaki H. The NO world for plants: achieving balance in an open system. Plant Cell Environ. 2005;28:78–84.

248. Yan Y, Wei CL, Zhang WR, Cheng HP, Liu J. Cross-talk between calcium and reactive oxygen species signaling. Acta Pharmacol Sin. 2006 Jul;27(7):821-6.

249. Yang XD, Dong CJ, Liu JY. A plant mitochondrial phospholipid hydroperoxide glutathione peroxidase: its precise localization and higher enzymatic activity. Plant Mol Biol. 2006 Dec;62(6):951-62.

250. Zeidler D, Zähringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J. Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc Natl Acad Sci U S A. 2004 Nov 2;101(44):15811-6.

251. Zhang F, Wang Y, Yang Y, Wu H, Wang D, Liu J. Involvement of hydrogen peroxide and nitric oxide in salt resistance in the calluses from *Populus euphratica*. Plant Cell Environ. 2007 Jul;30(7):775-85.

252. Zhang X, Takemiya A, Kinoshita T, Shimazaki K. Nitric oxide inhibits blue light-specific stomatal opening via abscisic acid signaling pathways in *Vicia* guard cells. Plant Cell Physiol. 2007 May;48(5):715-23.

253. Zhang Y, Wang L, Liu Y, Zhang Q, Wei Q, Zhang W. Nitric oxide enhances salt tolerance in maize seedlings through increasing activities of proton-pump and Na^+/H^+ antiport in the tonoplast. Planta. 2006 Aug;224(3):545-55.

254. Zhao T, Ling HQ. Effects of pH and nitrogen forms on expression profiles of genes involved in iron homeostasis in tomato. Plant Cell Environ. 2007;30:518-527.

255. Zhao DY, Tian QY, Li LH, Zhang WH. Nitric oxide is involved in nitrate-induced inhibition of root elongation in *Zea mays*. Ann Bot. 2007 Sep;100(3):497-503.

256. Zhao MG, Tian QY, Zhang WH. Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. Plant Physiol. 2007 May;144(1):206-17.

257. Zhou B, Guo Z, Xing J, Huang B. Nitric oxide is involved in abscisic acid-induced antioxidant activities in *Stylosanthes guianensis*. J Exp Bot. 2005 Dec;56(422):3223-8.

258. Zhu SH, Zhou J. Effects of Nitric Oxide on Fatty Acid Composition in Peach Fruits during Storage. J. Agric. Food Chem. 2006, 54, 9447-9452.

259. Ziosi V, Bregoli AM, Bonghi C, Fossati T, Biondi S, Costa G, Torrigiani P. Transcription of ethylene perception and biosynthesis genes is altered by putrescine, spermidine and aminoethoxyvinylglycine (AVG) during ripening in peach fruit (*Prunus persica*). New Phytol. 2006;172(2):229-38.

260. Zottini M, Costa A, De Michele R, Ruzzene M, Carimi F, Lo Schiavo F. Salicylic acid activates nitric oxide synthesis in *Arabidopsis*. J Exp Bot. 2007;58(6):1397-405.