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THE DOMON FAMILY OF PLANT PLASMA MEMBRANE B-TYPE CYTOCHROMES: HETEROLOGOUS EXPRESSION, BIOCHEMICAL CHARACTERIZATION AND PHYSIOLOGICAL ROLES IN VIVO

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Abbreviations

AA: Ascorbic Acid

AIR12: Auxin-Induced in Root cultures 12

aNOS: apoplastic Nitric Oxide Synthase

AO: Ascorbate Oxidase

APX: Ascorbate Peroxidase

AtAIR12: *Arabidopsis thaliana* AIR12

AtPrx: *Arabidopsis thaliana* Peroxidases

CAT: Catalase

CcPs: Cytochrome c Peroxidases

cNR: Cytosolic Nitrate Reductase

CPK: Corey, Pauling, Kultin

CPs: Catalase Peroxidases

Cyt b: b-type cytochrome

DAOs: Diamine Oxidases

DHA: Dehydroascorbate

DOMON: Dopamine beta-Monooxygenase N-terminal

EPR: Electron Paramagnetic Resonance

FQR: Flavodoxin-like Quinone Reductase

gl-OXO: Germin-like Oxalate Oxidase

GR: Glutathione Reductase

GSH: Glutathione

GSSG: Glutathione disulphide

HR: Hypersensitive Response

MAPK*:* Mitogen-Activated Protein Kinase

MAPKK: MAPK Kinase

MAPKKK: MAPKK Kinase

MDAR: Monodehydroascorbate Reductase

MDH: Malate Dehydrogenase

MDHA: Monodehydroascorbate

MDHAR: Monodehydroascorbate Reductase

NADH-QR: NADH-dependent Quinone Reductase

NDR1: Non race-specific Disease Resistance 1

NI-NOR: Nitrite-NO reductase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NQR: NAD(P)H Quinone Reductase

NADPH OX: NADPH oxidase

PAOs: Polyamine Oxidases

Pas: Polyamines

PCD: Programmed Cell Death

PDB: Protein Data Bank

PM: Plasma membrane

PM-NR: Plasma membrane-bound Nitrate Reductase

pmPOX: Plasma membrane-bound Peroxidase

PR: Pathogenesis-Related

Prxs: Peroxidases

QRs: Quinone Reductases

Rboh: Respiratory burst oxidase homolog

RCBI: Resource for Studying Biological Macromolecules

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SA: Salicylic Acid

SOD: Superoxide Dismutase

Abstract

The DOMON domain is a domain widespread in nature, predicted to fold in a β-sandwich structure. In plants, AIR12 is constituted by a single DOMON domain located in the apoplastic space and is GPI-modified for anchoring to the plasma membrane. *Arabidopsis thaliana* AIR12 has been heterologously expressed as a recombinant protein (recAtAIR12) in *Pichia pastoris.* Spectrophotometrical analysis of the purified protein showed that recAtAir12 is a cytochrome *b*. RecAtAIR12 is highly glycosylated, it is reduced by ascorbate, superoxide and naftoquinones, oxidised by monodehydroascorbate and oxygen and insensitive to hydrogen peroxide. The addition of recAtAIR12 to permeabilized plasma membranes containing NADH, FeEDTA and menadione, caused a statistically significant increase in hydroxyl radicals as detected by electron paramagnetic resonance. In these conditions, recAtAIR12 has thus a pro-oxidant role. Interestingly, AIR12 is related to the cytochrome domain of cellobiose dehydrogenase which is involved in lignin degradation, possibly via reactive oxygen species (ROS) production. In *Arabidopsis* the *Air12* promoter is specifically activated at sites where cell separations occur and ROS, including ·OH, are involved in cell wall modifications. *air12* knock-out plants infected with *Botrytis cinerea* are more resistant than wild-type and *air12* complemented plants. Also during *B. cinerea* infection, cell wall modifications and ROS are involved. Our results thus suggest that AIR12 could be involved in cell wall modifying reactions by interacting with ROS and ascorbate.

CyDOMs are plasma membrane redox proteins of plants that are predicted to contain an apoplastic DOMON fused with a transmembrane cytochrome b_{561} domain. CyDOMs have never been purified nor characterised. The trans-membrane portion of a soybean CyDOM was expressed in *E. coli* but purification could not be achieved. The DOMON domain was expressed in *P. pastoris* and shown to be itself a cytochrome *b* that could be reduced by ascorbate.

A. Introduction

A-1 Plant cell wall

Plant cells are encased in rigid walls which define the cell shape and play an important role in various physiological functions such as growth, intercellular communication, defence against pathogen attack, mechanical resistance and interaction with the environment (Cosgrove et al., 1997, 1999; Fry et al., 2004). Recent discoveries have changed the idea that cell wall is simply a static structure into a new view of it as a virtual extension of the cytoplasm. This is consistent with the fact that cell walls contain surface markers that foretell patterns of development and mark positions within the plant; moreover they contain components for signalling and communication with cytoplasm.

Plant cells have two types of cell wall depositions: the primary and secondary cell walls. The primary cell wall is essentially a composite material consisting of a framework of cellulose microfibrils embedded in a matrix of other polymers which are principally polysaccharides (Fig. 1). This polysaccharide composition can be different and it defines two types of primary cell wall called type I and II. In type I cell walls the non-cellulosic biopolymers are xyloglucan and pectins. This type of primary cell walls are typical of all dicotyledons, non graminaceous monocotyledons and gymnosperms (Carpita and Gibeaut, 1993).

Type II cell walls have a low content of pectin and xyloglucan content and a high arabinoxylan content (Carpita et al.,1996).

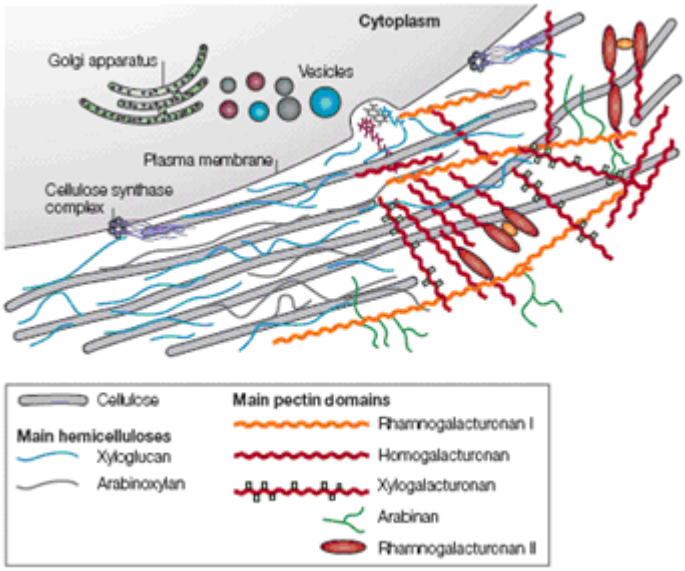


Fig. 1: **Primary cell wall growth and structure.** The polysaccharide matrix polymers, hemicelluloses and pectins, are synthesized in the Golgi apparatus and are moved to the wall surface by vesicles. Cellulose microfibriles are synthesized by large hexameric complexes present in the plasma membrane. For clarity the hemicellulose-cellulose network is shown on the left of the picture without pectins that are emphasized on the right. The main pectin polysaccharides in several plant species are rhamnogalacturonan I and homogalacturonan and the main hemicellulose is xyloglucan. It is considered that pectins are covalently bound together and are covalently and non-covalently bound to xyloglucan. Neutral pectin polisaccharides (green) can also bind cellulose surface. Figure adapted from Cosgrove et al., 2005.

Type II primary walls also contain mixed linked β-D-glucan and posses ester linked ferulic bridges in the xylan, which have never been found in type I walls.

The secondary cell wall, which is present in fully expanded and specialized cells (xylem and fibers), contain more cellulose then the primary one with a higher degree of polymerization and crystallinity (Mellerowicz et al., 2001). This secondary cell wall is characterized to be particularly thick because of the deposition of polysaccharides and accumulation of phenolic polymers, such as lignins in xylem vessels or sclerenchyma fibers. Moreover the secondary cell wall is deposited after cells stop enlargement; it is not present in all the cells, but it is typical of cells which need great mechanical strength and structural reinforcement.

Polysaccharides constitute the 90% of the plant cell wall while the remaining 10% is made up by proteins and enzymes which are involved in a lot of physiological processes such as cell enlargement, signalling, response to biotic or abiotic stresses and so on, in addition to cell wall structure and architecture.

A-1.a Cell wall components

In this paragraph I report the main polysaccharides present in the primary cell wall and the principal informations about them.

A-1.a.1 Cellulose

Cellulose primary structure is an unbranched (1,4)-linked β-D-glucan chain; dozens of these linear chains, bundled and crystallized through hydrogen bonds, form a cellulose microfibril which is mechanically strong and highly resistant to enzymatic attack. Cellulose is synthesized by membrane complexes and directly extruded into the apoplast, while the other cell wall components are synthesised in the Golgi apparatus and then moved to the plasma membrane.

Arabidopsis thaliana has 10 cellulose synthase (CESA) genes (Pear et al., 1996; Arioli et., 1998) expressed in different cell types and tissues. CESA proteins form in the plasma membrane particular hexameric structures called rosettes (Kimura et al., 1999) in which each subunit synthesizes a sugar chain. Several associated rosettes form a big complex from which is produced the microfibril. The assembly of CESA protein into hexamers is not completely clear, but is thought to require CESA dimerization, which can be mediated by two zinc fingers in the N-terminal region of the CESA proteins: a conserved domain among the CESA proteins (Kurek et al., 1999).

CESA enzymes add glucose residues to elongate the cellulose chain and the initial acceptor for this elongation seems to be a sterol glucoside, which is normally synthesized in the plasma membranes (Peng et al., 2002) and which can be used by CESA together with uridine 5'-diphosphate-glucose to form short sterol-linked glucans.

A-1.a.2 Hemicelluloses

The backbone of these polysaccharides is similar to that of cellulose, but they have branches and other modifications that avoid them to form microfibrils themselves and so they can bind only cellulose. The major hemicelluloses present in the angiosperms are xyloglucan and arabinoxylan.

Xyloglucan has xylose branches on 3 out of 4 glucose residues, while arabinoxylan is made up of a (1,4)-linked β-D-xylan backbone with arabinose branches.

A-1.a.3 Pectins

These polysaccharides are rich in galacturonic acid which forms three domains that are present in all pectin types: homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan–II (RG-II). RG-I consists of alternating residues of galacturonic acid and rhamnose, and probably has side branches that contain other pectin domains. HGA is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid residues in which some of the carboxyl groups are methyl esterified and some secondary hydroxyl groups are acetyl esterified (Ridley et al., 2001); some HGAs can be substituted to form RG-II or xylogalacturonan. This last one is modified by the addition of xylose branches, whereas RG-II is a complex pectin domain that contains 11 different sugar residues and forms dimers through borate esters. Neutral pectins, such as arabinans, have the properties to bind cellulose surfaces implying that pectins might crosslink cellulose microfibrils (Zykwinska et al., 2005) and it has been proposed that this interaction can promote wall flexibility.

Pectins are particularly abundant in the middle lamella which is the first layer formed during cell division. It makes up the outer wall of the cell and it is shared by adjacent cells.

Pectins are also primary targets of attack by invading microbes and their breakdown products function as strong elicitors of plant-defence responses (Cosgrove et al., 2005).

After the production of the different polysaccharides into the Golgi apparatus, they are secreted into the wall where they can link the newly synthesized cellulose microfibrils and the pre-existing wall polymers through spontaneous physico-chemical interactions as well as through enzymatic crosslinking.

Xyloglucan is the hemicellulose considered to crosslink cellulose microfibrils and the cellulose-xyloglucan matrix is the major load-bearing element within the cell wall, with additional contributions of Ca^{2+} cross-links between homogalacturonans (Carpita and Gibeaut, 1993). Moreover the integration of new polysaccharides into the pre-existing matrix is probably mediated by enzymes such as endotransglycosylase, which cut and ligate glycans together. The cleavage and reconnection of xyloglucan cross-links are considered the central point during chemical processes essential for wall expansion as well as for cell growth and differentiation (Nishitani and Tominaga, 1992; Purugganan et al., 1997).

A-1.b Cell wall modifications

Plant cell wall has to be considered as a dynamic structure which connect the outside of the cell, that is the apoplastic space, and the inner side. So the cell wall has a crucial role in the signalling between this two cellular compartments. There are a lot of physiological processes which induce cell wall modifications and among them the two most important are the following: cell growth and cell separation.

A-1.b.1 Cell growth

During plant life it is necessary to have some modifications in order to grow. The growth presumes cell wall expansion and at the same time the preservation of the wall mechanical integrity in order to keep the turgor pressure. This is a delicate equilibrium that must be controlled. So the cell wall must be sufficiently strong to contrast the turgor pressure but also compliant to allow modifications. There is a great difference between plant and animal cells in the mode of enlargement. Animal body enlarges essentially increasing in plasma content in an isotonic surrounding. In contrast, the plant protoplast is encased in a rigid wall infiltrated of water containing only moderate amount of solutes which are osmotically not much significant. Because of this there is a large difference in osmotic pressure between protoplast and apoplast which, in the fully turgid state, is compensated by a hydrostatic pressure (turgor) of the same magnitude. So plant cells can enlarge through water uptake favoured by a difference in water potential between protoplast and apoplast. The increase in the protoplast volume, that is the cell growth, can be accomplished by a decrease in cell wall tension which produces a decrease in turgor and so a difference in water potential (Schopfer et al., 2006).
During the so called acid growth the auxin-mediated cell wall weakening results is the cell expansion. During this process auxin increases and activates plasma membrane proton pumps which extrude protons in the apoplastic space. This acidification activates pH-dependent wall-loosening enzymes such as expansins (see below) causing cell wall relaxation, which allows cell extension under the internal turgor pressure.
Wall expansion can be localized at specific points, as in the root hair tips or in pollen tubes, or it can be very diffused as in potato tuber parenchyma. Of course there are intermediate cases in which expansion is limited to some cell walls or in which there is a gradient along cell walls. The result of these different situations is the large and different number of cell shapes

that we can recognize in a plant. During this spatial localized expansion there is the secretion of new materials in the wall even if the precise molecular mechanisms are not completely clear. Moreover the growth can be very slow, as in the meristematic zone of roots or stems, or it can show dynamic fluctuations as in response to pH or reactive oxygen species (ROS) changes. In the first case the growth happens in days and it involves the synthesis and integration of new materials into the wall, while in the faster growth, that is in the range of seconds to hours, there are changes in wall extensibility without modifications in its structure and composition (Cosgrove et al., 2000).

The walls of growing cells are under a high pressure (100-1000 atmosphere) due to polymers stretching, which allows to balance the turgor pressure and gives the energy to extend cell wall. This means that the wall stress provides the possibility to move polymers in the walls, even if the control of the process is the loosening and shifting of the linkages of cellulose microfibrils (Marga et al, 2005).

The expansive growth of plant cells implies wall loosening obtained through wall stress relaxation. This relaxation can be the result of a stress-bearing crosslink scission or of crosslink sliding along a scaffold; in any case at the end there will be a reduction in wall stress without a substantial change in wall dimensions. Actual enlargement of the wall occurs secondarily, as a consequence of cellular water uptake, in response to the turgor relaxation that inevitably accompanies wall stress relaxation. There are different hypothesis for the relaxation event and the principal candidates responsible for that event are: expansins, xyloglucan endotransglycolase/hydrolase, endo-(1,4)-β-d-glucanase, hydroxyl radicals. I will report some important informations on the first three candidates while I will discuss a little bit more on the last one because it is of major interest for this thesis.

A-1.b.2 Expansins

Expansins are wall-loosening proteins activated by acidic pH, which is typical of the so called acid growth. Expansins are activated (McQueen-Mason et al., 1992) at pH values between 4.5 and 6.0; there are different growth conditions characterized by pH modifications as the following: stem and root tropisms; hormone-induced growth; light-induced stimulation of leaf expansion and inhibition of stem elongation; responses of shoots, roots and leaves to water deficits and salt stress; initiation and early outgrowth of root hairs; and rapid growth in response to the fungal toxin fusicoccin.

Although there are a lot of papers showing that expansins are the primary wall loosing agents (Link et al, 1998; Pien et al, 2001; Cho et al, 1997, 2000) it is still unclear the mechanism of action of these enzymes. It is hypothesized that they disrupt the non-covalent bonds (the hydrogen ones) between cellulose and hemicellulose (McQueen-Mason and Cosgrove, 1994).

A-1.b.3 Xyloglucan endotransglycolase/ hydrolase

It is thought that these enzymes have different functions including wall loosening (Fry et al., 1992), wall trengthening (Antosiewicz et al., 1997), new xyloglucans integration into the wall (Thompson et al., 2001), trimming of xyloglucan strands that are not tightly stuck to the surface of cellulose (Thompson et al., 2000) even if there aren't clear evidences that they play a primary role in these events.

A-1.b.4 Endo-(1,4)-β-d- glucanase

There is a small number of papers about the possible involvement of these proteins in wall loosening and about their biochemical properties, substrate specificities and so on. Ohmiya and co-workers analyzed such enzymes from poplar and they hypothesised that these enzymes are involved in wall loosening by causing the release of xyloglucans trapped in the cellulose microfibrils (Ohmiya et al., 2000).

A-1.b.5 Hydroxyl radical

Hydroxyl radical (·OH) is one of the Reactive Oxygen Species and it is an extremely reactive molecule which is physiologically produced in the plant apoplast and which is supposed to be involved in plant cell-wall loosening (Schopfer et al., 2001). It was shown that ·OH can cleave wall polysaccharides by non-enzymatic removing of hydrogen atoms from polysaccharides (Fry et al., 1998). Moreover in plants the hormone auxin induces hydroxyl radical production, in fact it was demonstrated that the use of free radical quenchers abolished auxin-induced growth (Schopfer et al., 2002). I will discuss the hydroxyl radical role in wall loosening and also its production in plant in the following, then I will talk also about the other ROS produced in the plant apoplast.

A-1.b.6 Cell separation

Plant cell are joined together by an adhesive matrix, principally made up by pectins, called middle lamella. Even if this adhesive structure is fundamental to give strength and rigidity to the wall, it is also necessary to have cell separation during plant cell cycle, for example for roots to explore the soil or for aerial part to intercept solar irradiation (Roberts et al., 2002) and of course in events such as abscission and dehiscence. Moreover the cells that will be involved in the abscission or dehiscence events are pre-programmed compared to the adjacent ones: in fact they are morphologically different also before wall degradation. (Roberts et al., 2000).

Different physiological processes are based on cell separation events in plants:

- leaves abscission;
- fruit softening;
- seed germination;
- lateral roots emergence.

All these processes are characterized by both cell wall modifications and middle lamella degradation which is the primary site of wall breakdown. The final result is the production of a gap between cells. During this events it was shown that there is a great production of enzymes involved in degradation processes such as expansins (Cosgrove et al., 2000), β-1,4-glucanase (cellulase) and polygalacturonas. The latter ones are essential enzymes of pectin degradation because they catalyze random hydrolysis of α-1,4-glycosidic linkages in galacturonic acid, a polymer that forms the main chain of the homogalacturan region of pectin (Markovic et al., 2001; Swain et al., 2011).

Moreover cell separation events can be induced by environmental conditions as, for example during aerenchyma formation. The aerenchyma is a set of cavities which facilitates gaseous exchange in the roots; its formation is quicker when roots are in conditions of anaerobiosis and it shows cell separation and collapse (Drew et al., 2000).

It is possible that also in cell separation events ROS may be involved.

A-2 ROS and plant cell wall: production and roles

ROS are normally produced as intermediates in the reduction of O_2 to H_2O; they are usually defined as molecules containing oxygen with unpaired electrons in their pi orbital (Halliwell et al., 2006). Although ROS can be harmful for cells in high concentrations because they can

oxidise lipids and thereby compromise the plasma membrane (Halliwell and Gutteridge, 1984; Halliwell and Gutteridge, 1992), in low concentrations they are used as signaling molecules in several processes such as root hair development (Foreman et al., 2003), axillary branching of aerial tissue (Sagi et al., 2004; Sagi and Fluhr, 2006), and stomata control (Li et al., 2006). ROS include all chemically reactive molecules derived from ground state dioxygen and so they include also the singlet oxygen. In this thesis I will only talk about ROS produced by univalent reduction of O_2, that are: superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH).

Their production in the apoplast can occur both through enzymatic or non enzymatic ways (Fig. 2).

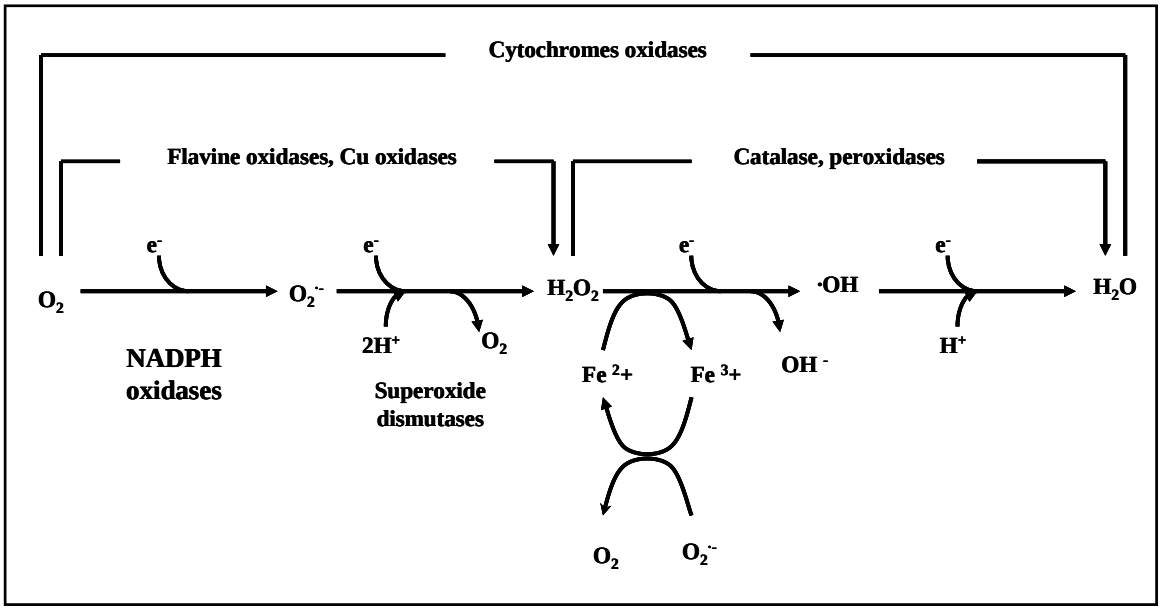


Fig. 2: **Production of ROS from univalent reduction of oxygen**. Different enzymes involved in ROS production are reported. Fe ions can catalyze the reduction of H_2O_2 to ·OH (Fenton reaction), this reaction can be associated to the oxidation of $O_2^{\cdot-}$ (Haber-Weiss reaction). Adapted from Schopfer et al., 2006.

For a long time ROS have been considered because of their involvement in plant protection from biotic and abiotic stresses, such as during the oxidative burst in response to pathogen attack. Nowadays it's clear that ROS are also important during plant development because they are involved in different processes such as hormonal signal transduction and modification of cell wall polysaccharides (Joo et al., 2005; Muller et al., 2004; Pedreira et al., 2004).

As you can see from fig. 2 there are different metabolic processes, and several different enzymes, which can give rise to ROS. One of these enzymes is the NADPH-oxidase, which is similar to that present in mammalian neutrophils. As shown in figure fig. 2 the first step of ROS production is the univalent electron transfer to oxygen that can be catalyzed by the NADPH-oxidase: an enzyme located in the plasma membrane and which produces $O_2^{\cdot-}$ in the extraprotoplasmatic space, that is the apoplast. $O_2^{\cdot-}$ can spontaneously dismutate, and this is favoured by the acidic apoplastic pH (5.5), or promoted by superoxide dismutase, forming H_2O_2. In the presence of iron or copper, kept in the reduced form by a reductant such as ascorbate which is normally present in the apoplast, H_2O_2 can be converted to ·OH through the so called Fenton reaction (Fig. 3a). The Fenton reaction can be operated by peroxydases, that are heme-containing enzymes normally present in the cell wall. $O_2^{\cdot-}$ can also be used as a reductant in the Haber-Weiss reaction (Fig. 3b). The most aggressive ROS is the ·OH, which can cause for example lipid peroxidation, DNA and protein damage, polysaccharides scission leading to cell wall loosening and so to cell expansion.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \quad (a)$$

$$O_2^{\cdot-} + H_2O_2 \longrightarrow \bullet OH + OH^- + O_2 \quad (b)$$

Fig. 3: **a: Fenton reaction; b: Haber-Weiss reaction**

Up to day there are no known ·OH scavengers and so the only way to avoid its damages is to control the reactions that lead to its generation; this means strategies to keep the concentrations of superoxide, hydrogen peroxide and transition metals tightly controlled.

As previously discussed, cell growth requires wall relaxation, i.e. loosening of bonds between or within cell wall polymers. Recently ·OH has been proposed to be involved in this bond loosening. In experiments of auxin-induced coleoptile growth, it was observed that the wall-loosening reaction was initiated 15 min after the treatment of auxin and terminated within 30 min after the removal of the hormone. This rapid auxin effect suggests that the hormone quickly activated a pre-existing wall-loosening machinery and was not compatible with de novo synthesis of wall-degrading hydrolytic enzymes.

·OH radicals have a very short half-life, in the range of nanoseconds, and can be directly produced in the cell wall by peroxidases so they can act on wall polysaccharides close to the site of their generation.

There are different papers which show the interaction between auxin and ROS. For example, incubation of maize seedlings with auxin caused an increase of $O_2^{\cdot-}$ and ·OH production in

the root elongation zone. It is important to underline that although the auxin treatment induced ROS production, also in basal conditions it was possible to record ROS production in the root elongation zone (Schopfer et al., 2002).

Using ROS scavengers during auxin-mediated growth the ROS involvement in the growth process was demonstrated. For example auxin-mediated growth was inhibited by incubation of maize coleoptile sections with Mn-TMP, a catalytic antioxidant showing both catalase and superoxide dismutase activity (Schopfer et al., 2006); whereas there was no effect of this drug on growth induced by an acidic buffer (Kutschera et al., 1994). Similar experiments were carried on using other inhibitors such as ·OH scavengers (adenine, histidine, etc.), an NADPH-oxidase inhibitor (DPI), and peroxidase inhibitors (KCN, etc.) and the result was always the same: auxin-mediated growth was inhibited while acid-mediated growth was not (Schopfer et al., 2002, 2001). These results can be explained by the effect of ·OH produced by peroxidases in destroying wall polysaccharides.

Auxin is involved in different plant cell wall modifications both during extension growth and cell separation events. The signalling induced by auxin, at least during growth, is very quick and so all the components involved in the process have to be activated and removed in a short time.

If auxin induction implied an intracellular signalling mechanism through the nucleus to synthesize degrading enzymes or protonic pumps, the effect would have required more time. So induction mediated by auxin should not be explained only by modification of gene expression. Recently it was shown that auxine-induced gene activation occurs together with the recruitment and activation of pre-existing components which are already present in the cell wall (Schenck et al.,2010). Auxin should both activate the recruitment and synthesis of degrading enzymes (as expansins, etc), but also induce NADPH-oxidases promoting in this way the ROS production which could act in short time as happens during auxin-induced cell expansion.

A-3 The apoplast compartment

The apoplast was defined for the first time by O. Munch in 1927 as opposed to symplast, and corresponding to the extracellular compartment at the outside of the plasma membrane, which includes xylem and cell wall. For a long time the apoplastic space has been considered as a passive space being simply a barrier between the cytosol and the environment. Nowadays this idea is completely changed. Several studies in fact have demonstrated that apoplast is

involved in a lot of cell processes as in plant growth, host-pathogen interactions and cell signalling pathways, thanks to its chemico-physical composition, to proteins which includes and to its position it may be defined as a bridge between the out and the inside of the cell.

The apoplast has a continuous cross-talk with the plasma membrane and in this way any environmental change is perceived by the apoplast and transmitted into the cell through plasma membrane; but on the other side, also the cytosolic changes are transmitted to the apoplast creating a continuous exchange of informations between the two compartments divided by the plasma membrane. In stress conditions or in situation of cellular damage, this cross-talk and the following responses determine the difference between survival or cell death.

As I said, the apoplast has particular chemico-physical characteristics which are very different from that of the symplast.

The apoplastic space has a more acidic pH, between 4.5 and 5.5, than the symplastic one, which is near neutrality. Moreover, the apoplast is relatively unbuffered and its pH changes after internal and external stimuli and signals. Another important difference between apoplast and symplast is that the first space contains a lower amount of solutes compared to the second one.

The apoplastic matrix includes solutes (e. g. ions, sugar, amino acides, phenolic compounds and organic acids), proteins (e. g. extensins, peroxidises and hydrolases) and, of course, cell-wall components (e. g. celluloses, pectin and glycoproteins).

A-3.a The solute composition of apoplast

After the extraction of apoplastic fluid it was possible to evaluate its solute composition. Cations and anions occur to millimolar levels and neutral metabolites, principally sugars, add up to 5-10 mM. The principal ions present in the leaf apoplast are K^+, Ca^+, Mg^{2+}, Cl^-, NO^{3-}, $H_2PO_4^-$; they are considered to be a reservoir buffering the cytoplasmic ion pools which are homeostatically controlled. Amino acids, sugars, phenolic compounds and other solutes are present in low amounts.

Apoplast contains also redox molecules and principally ascorbic acid (AA) and its oxidised form, dehydroascorbate (DHA), which together represent the major antioxidant pool of this compartment. Other antioxidants found are flavonoids and polyamines but however the redox buffering capability of the apoplast is weaker then the symplastic one (Horemans et al., 2000; Pignocchi et al., 2003).

A-3.b Ascorbic acid and its importance for apoplastic redox buffering

Plants can synthesize and store AA in several different cell compartments and it can have different functions some of which are well characterized as, for example, primary antioxidant thanks to its ability to react with hydroxyl radicals, superoxide and singlet oxygen; while other are still unclear. AA is synthesized both in green and non-green tissues, in fact its production is not dependent on photosynthesis. It is interesting that up to 10% of the AA content of the whole leaf is localised in the apoplast (Noctor et al., 1998); moreover enzymes involved in the AA metabolism are present in the apoplast or on the plasma membrane. AA is particularly important for developing plants, in fact mutants with reduced AA content are characterized by slow growth and late flowering phenotypes, while total depletion of AA results in nonviable plants.

In the apoplast AA is considered the primary or the only significant redox buffer (Foyer and Noctor, 2009; Pignocchi et al., 2003); it is involved in the regulation of cell wall synthesis and cross-linking, in the defence against pathogens and it plays a central role against ozone (Sandermann et al., 2008, Baier et al., 2005). There are experiments demonstrating that the enhancement of ascorbate oxidase activity in transformed tobacco apoplast leads to enhanced ozone sensitivity (Sanmartin et al., 2003). Ozone is extremely dangerous for cell because it can oxidize a lot of different cell components such as sulfhydryl groups, double bonds of fatty acids, amino acids etc; in water it can produce ROS and so also the dangerous hydroxyl radical. Ozone is not produced inside the cell but it can enter into the plants through open stomata; so the first structures that ozone meets in the plant cell are the apoplast and the PM, where it can be detoxified, especially by AA. In the apoplast AA reacting with ozone becomes DHA which is transported into the cytosol where it is reduced back to AA through the action of the reduced glutathione. So AA comes back again into the apoplast and is ready to detoxify ozone again (Zechmann et al., 2011).

The apoplastic AA pool is fundamental to sense the environmental perturbation and to modulate the redox gradient across the PM. The ascorbate redox system consists of reduced (AA), semi- reduced (monodehydroascorbate, MDHA), or fully oxidized (DHA) molecules. The intracellular ascorbate pool is predominantly kept in the reduced form through the action of different enzymatic recycling pathways. By contrast, the apoplastic AA pool is more oxidized with respect to the cytoplasm, although this redox status can vary between species and depends on the physiological status of the plant. In the symplast there are different

systems that can regenerate AA from MDHA, including MDHA-reductase (MDHAR). MDHA is an unstable radical and it quickly disproportionates into AA and DHA. In the symplast the presence of the acid ascorbic-glutathione (AA-GSH) cycle assures the regeneration of AA from DHA (Fig. 4); this mechanism of regeneration is not supposable in the apoplast because of the absence of the NADH or NADPH which is essential for the reaction. Several papers have demonstrated the idea that DHA has to come back to the cytosol to be reduced to AA and so this implies the action of plasma membrane AA and DHA transporters. These transporters are specific only for one of these species.

Moreover the plant plasma membrane were reported to contain a high-potential cytochrome b_{561} (cyt b_{561}) which is reduced by AA. Experiments using Phaseolus plasma-membrane vesicles loaded with AA showed that cyt b_{561} was reduced by AA and oxidized by the addition of an impermeable electron acceptor (e. g. ferricyanide) suggesting the involvement of the cytochrome in the electron transfer across the plasma membrane (Asard et al., 1992). MDHA is also reduced by this vesicle system, so it was proposed that cyt b_{561} could use cytoplasmic AA as electron donor to reduce apoplastic MDHA, like cytochrome b_{561} in animal chromaffin granules (Njus et al., 1987). However, until now the high-potential cytochrome *b* of the plasma membrane involved in MDHA reduction in the apoplast escaped molecular identification.

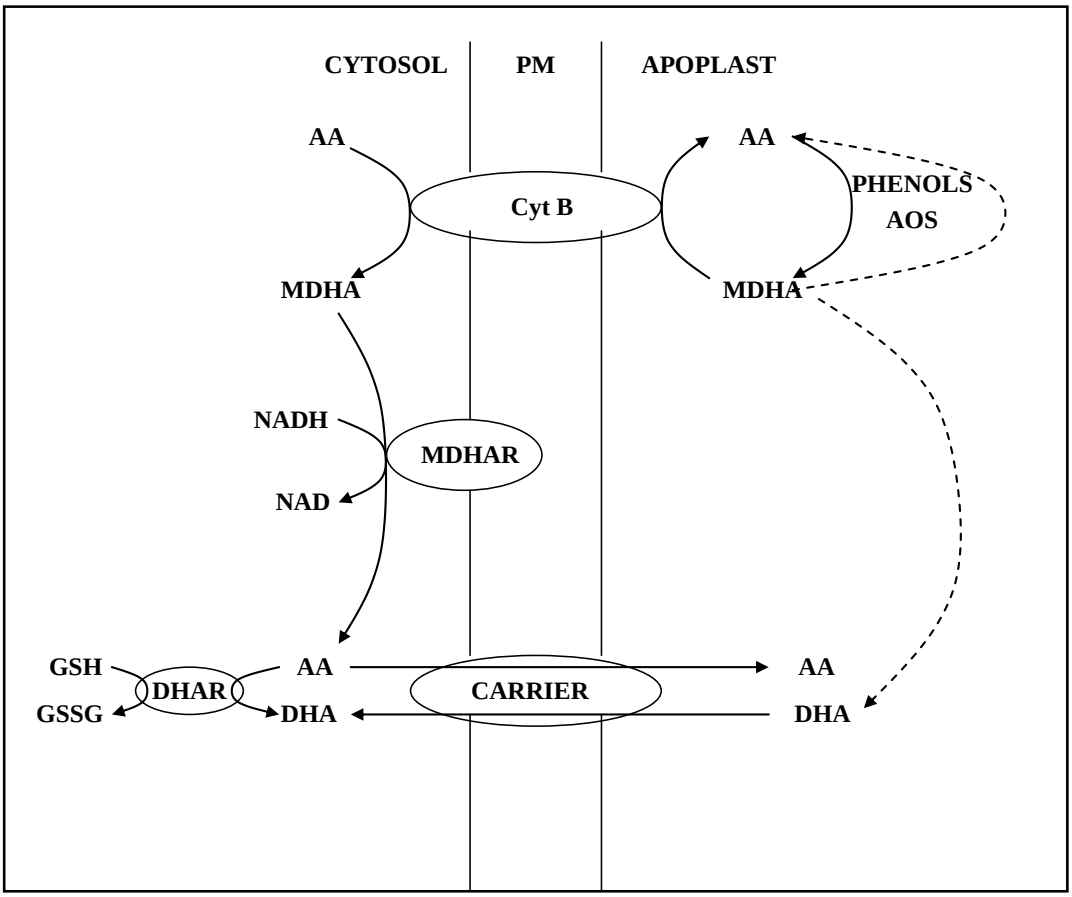


Fig. 4: S**heme of AA regeneration at PM.** After production of monodehydroascorbate (MDHA), AA is either directly re-reduced by a cytochrome *b*-mediated electron transfer (Cyt *b*) or by the action of cytoplasmic NADH–dependent MDHA oxidoreductase (MDHAR), or it disproportionates to AA and fully oxidized dehydroascorbate (DHA) (broken line). The extracellular DHA is transported to the cytosol where it is re-reduced by DHA reductase (DHAR) using glutathione (GSH) as an electron donor. Moreover, the plasmamembrane AA–DHA carrier is supposed to operate as an exchange carrier, therefore keeping extracellular AA levels constant (adapted from Horemans et., al 2000).

Ascorbate oxidase (AO) is a cell wall localised enzyme which is present in all higher plants and which catalyses the oxidation of AA to MDHA together with the four-electron reduction of oxygen to water. AA oxidation clearly favours enhanced growth, probably involving vacuolation and cell wall loosening (Hidalgo et al., 1989). Another enzyme which is present in the apoplast and which is able to oxidize AA is ascorbate peroxidase through the following reaction:

$$2AA + H_2O_2 \rightarrow 2\ MDHA + H_2O.$$

Beside these enzymatic ways to oxidize AA to MDHA in the apoplast there are also non enzymatic ways like the interaction with ROS, or with transition metals (e. g. iron, Fry et al., 1998) or other radical species (e. g. phenolic radicals).

A-3.c Redox proteins of the apoplast

The apoplast has a very weak redox buffering capability and so any change in the redox state of the apoplast may trigger reactions which involve redox interacting-enzymes (peroxidases, superoxide dismutases, oxidases) or specific substrate binding proteins (ascorbate oxidases, oxalate oxidases, diamine or polyamine oxidases). In the next pages I report the main information on these proteins.

Species	Enzyme	Acc. no.	Tissue	$MW_{nat.}$ (kDa)	MW_{SDS} (kDa)	$MW_{calc.}$ (kDa)	Cofactor, prostetic group	pI
A. cepa	NQR		Root	n.d.	27		FMN	6
	NADH-QR		Root	n.d.	31		FAD	8
	MDH		Root	n.d.	40		–	
A. thaliana	Asc-cyt b-559.8		Leaf	n.d.	27, 114		Heme	
	NQR	Q9LK88	–	92	24	21.6	FMN	$6.8^{calc.}$
B. oleracea	MDH		Inflorescences	296, 103	41		–	
C. pepo	NQR		Hypocotyl	300, 90	24		FMN	
G. max	NQR		Hypocotyl	85	22		FMN	
L. esculentum	Fe^{3+}-chelate reductase		Root	200	28			5.5
N. tabaccum	RbohD	Q8RVJ9	Leaf	n.d.	106	105.8	FMN	$8.9^{calc.}$
	nitrate reductase		Root	200	63			
	nitrate reductase		Leaf	n.d.	93			
	nitrite reductase		Root	310	n.d.			
Ph. vulgaris	Asc-cyt b-561		Hook	130, 70	$55–63^{m)}$		Heme	>8
	NAD(P)H oxidase		Hook	n.d.	n.d.		FMN	
P. sativum	Fe^{3+}-chelate reductase		Root	129	92	81		9.6
S. oleracea	MDAR		Leaf	160	45		FAD	5.3–5.6
	MDH		Leaf	103	41		–	
Z. mays	NQR		Root	n.d	27		FMN	
	NADH-QR		Root	n.d	31		FAD	
	Asc-cyt b-559.9		Root	116	$86^{m)}$		Heme	
	MDH1		Root	69	41	36	–	7.9
	MDH2		Root	69	41		–	6.5
	MDH3		Leaf	69	41		–	
	pmPOX1 (ZmPOX1)	A5H8G4	Root	70	$98^{m)}$	70	Heme	5.2
	pmPOX2a		Root	150	$118^{m)}$		Heme	
	pmPOX2b	A5H452	Root	40	$55^{m)}$	38	Heme	10.1
	pmPOX3	A5H454	Root	38	$57^{m)}$	38	Heme	9.2

Fig. 5: **Redox proteins of plant plasma membrane:** Acc. no., accession number according to UniProtKB (http://www.pir.uniprot.org/); MWnat., molecular mass detected by SEC or hrCNE; m) modified SDS-PAGE; MWcalc., molecular mass calculated; n.d., not detected; pI, isoelectric point; Calc., calculated pI based on amino acid sequence; ASC-cyt, ascorbate-reducible cytochrome; MDAR, monodehydroascorbate reductase; MDH, malate dehydrogenase. NQR, NAD(P)H quinone reductase; pmPOX, plasma membrane-bound peroxidase; NADH-QR, NADH-dependent quinone reductase.

A-3.c.1 Ascorbate oxidase

Ascorbate oxidase (AO) is a glycosylated blue multicopper oxidase which oxidises AA to MDHA which then oginates DHA and AA. Its expression is modulated by transcriptional and translational controls (Esaka et al., 1992).

AO expression is correlated to plant growth (Kato and Esaka , 2000), it is increased by growth promoters like auxin (Pignocchi et al., 2003) and jasmonates (Sanmartin et al., 2003), while it is reduced by growth suppressor like salicylic acid (Sanmartin et al., 2003).

AA and AO are involved in plant growth even if the precise mechanism is still unknown. Probably they are involved in plant growth because they can change the plant sensitivity to hormonal stimuli.

Experiments using plants that express AO gene in the sense or antisense have demonstrated that this enzyme regulates ascorbate redox state in the apoplast (Pignocchi et al., 2003) even if the precise function of this enzyme is still a matter of debate.

AO over-expressing tobacco, which have higher amount of DHA in the apoplast than the wild-type, are characterized by enhanced growth and stem elongation in respect to wild-type plants, moreover this over-expressing plants are more sensitive to ozone because of the reduced amount of AA (Pignocchi et al., 2003; Sanmartin et al., 2003). Interestingly, AO over-expressing plants don't show any change in the AA-DHA content in the apoplast, while they present an oxidation of apoplastic AA together with a shift in the redox state of AA and GSH in the symplast, where the AA is more oxidised while the GSH is more reduced. This change is consistent with the known redox coupling AA/GSH (Noctor et al., 2000) (Fig. 6).

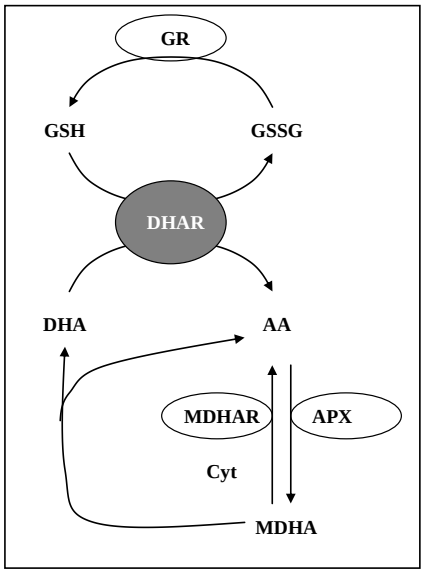


Fig. 6: **Model of Ascorbate-Glutathione redox coupling.** The scheme shows the link between ascorbate and glutathione pools in the cytosol; dehydroascorbate reductase (DHAR) catalyses the reduction of dehydroascorbate (DHA) by reduced glutathione (GSH) ensuring that the two antioxidants remain coupled. APX, ascorbate peroxidase; ASC, ascorbate; Cyt, cytochromes; GR, glutathione reductase; GSSG, glutathione disulphide; MDHA, monodehydroascorbate, AA.

The decreased AA redox state in the symplast maybe suggests that the amount of DHA coming from the apoplast is higher then the capability of the symplast to reduce it back to AA; whereas the increase in GSH reduction may be operated to balance the decrease of AA in the antioxidative metabolism.

A-3.c.2 Peroxidases

Peroxidases (Prxs) are heme-containing protein which use H_2O_2 as electron acceptor to catalyze a lot of different reactions. The general reaction catalyzed by Prxs is the following:

$$2RH + H_2O_2 = 2R\cdot + 2H_2O$$

Moreover they can produce superoxide and H_2O_2 through NAD(P)H oxidation (Vianello and Macrì, 1998). In *Arabidopsis* (Welinder et al., 2002) and in rice (Passardi et al., 2004) there are more then a hundred Prxs, in fact there are a lot of enzymatic Prxs isoforms (isoenzymes) which can catalyze several different reactions and this explain the reason why these proteins are involved in different processes during the plant life. For example they are involved in ROS and RNS metabolisms, in lignin and suberin formation, in auxin metabolism, in cell wall components crosslinkings etc. Prxs are widely distributed in nature in fact they are present in bacteria, fungi, plants and animals. On the basis of their sequence and catalytic properties, plant heme-containing Prxs are divided into three classes. Class I includes Prxs derived from prokaryotes, probably this class is at the origin of the other two classes (Passardi et al., 2007). Prxs belonging to this class are principally involved in H_2O_2 detoxification (Skulachev et al., 1998; Shigeoka et al., 2002), they are not glycosylated, they don't have signal peptide, calcium ions or disulfide bridges, and include: ascorbate peroxidases (APXs), cytochrome c peroxidases (CcPs) and catalase-peroxidases (CPs). APXs have a high affinity for AA and are present in photosynthetic organisms.

Class II Prxs are exclusively present in fungi and have the capability to degrade soil debris: a typical characteristic of this Prxs class because no other Prx is able to degrade lignin (Martinez et al., 2005). This class includes: manganese Prxs (EC 1.11.1.13), lignin Prxs (EC 1.11.1.14) and versatile Prxs, a group which includes multifunctional Prxs sharing the catalytic properties of lignin and manganese Prxs (Ruiz-Dueñas et al., 2000). PRXs belonging to this class are glycosylated, contain calcium ions, disulfide bridges and signal peptide to be directed to the endoplasmic reticulum and then to be secreted.

Class III Prxs are secreted proteins which include a large number of isoenzymatic forms involved in several physiological functions like the following: stiffening of the cell wall;

lignin and suberin deposition (Quiroga et al., 2000; Pomar et al., 2002); defence against biotic and abiotic stresses (Bolwell et al., 2001; Sottomayor and Ros Barcelò, 2003). These Prxs were probably fundamental during land colonization by plants allowing plants to survive in a more oxygenated environment or helping them to have a more resistant structure (Passardi et al., 2004; Duroux et al., 2003).

In general class III Prxs don't use AA as electron donor but they oxidise other molecules, principally phenolic compounds, altough there are two class III Prxs, purified by tea leaves which have a high affinity for AA (Kvaratskhelia et al., 1997).

Besides the classical mechanism of action in which Prxs reduce H_2O_2 there is another mechanism in which these enzymes are involved, the so called "hydroxylic cycle". In this cycle Prxs pass through a Fe(II) state using superoxide anion to produce hydroxyl radical (Passardi et al., 2004; Liszkay et al., 2003); so through this cycle Prxs increase ROS production and are involved in processes like the oxidative burst, the hypersensitive response (HR) or cell elongation (Liszkay et al., 2004; Schopfer et al, 2002). The precise *in vivo* role of a single Prx is still unknown because of the wide range of Prxs substrates, the probable functional redundancy of these enzymes and their capacity to react to several internal and external factors.

In *Arabidopsis thaliana* there are 73 genes encoding Prxs (Welinder et al., 2002) and their expression is not organ-specific. Prxs genes expression is enhanced by some factor like the following: metals (Kumari et al., 2008), pathogens (Mohr and Cahill, 2007; Chassot et al. 2008), anoxia (Klok et al. 2002); ozone stress (Ludwikow et al., 2004), cold (Llorente et al., 2002).

A-3.c.3 Superoxide dismutase

Superoxide dismutases (SODs) are enzymes that convert superoxide radical to oxygen and hydrogen peroxide involving a metal center which can be iron, copper, zinc or nickel. Consequently SODs can be classified into four groups: FeSOD, CuZcSOD, MnSOD and NiSOD. All the four groups are present in prokaryotes, while in eukaryotes FeSOD is present in the chloroplast, MnSOD is typical of mitochondria but can be also found in peroxisomes, and CuZn SOD is the most aboundant in the chloroplast, in the cytosol and in the apoplast. Phospholipid membranes are impermeable to superoxide radicals, so it is fundamental that this radical is removed in the compartment where it is present. Extracellular forms of SODs have been identified in animals, prokaryotes and plants. The CuZnSOD was found in the

apoplast of Scots pine needles (Streller and Wingsle, 1994) and there are immunolocalization experiments showing the apoplastic localization of CuZnSOD (Ogawa et al., 1996) and the presence of a high-isoelectri-point isoform on the plasma membrane of Scots pine needles (Karpinska et al., 2001). Moreover it was demonstrated a SOD activity in isolated and purified plasma membrane maize roots (Vuletić et al., 2003). At least three CuZnSOD isoforms are present in maize roots, in the apoplast and linked to the plasma membrane, moreover there is a Mn-containing isoform with germin-like protein characteristics and SOD activity (Kukavica et al., 2005). Extracellular SOD associated to plasma membrane-bound superoxide synthase are involved in H_2O_2 production, which if fundamental for cell wall polymer metabolism. On the other side the precise role of different SOD isoforms and of germin-like protein is still unknown.

A-3.c.4 Oxalate oxidase

Germin was identified for the first time during wheat embryo germination in which germin signals the onset of early plant development (Rahman et al., 1988). Germin synthesis and translation are concomitant with the initiation of growth in germinating wheat embryos. After the discovery of germin in cereals, germin-like proteins were identified in protists (Lane et al., 1991), dicotyledonous angiosperms (Hofte et al., 1993) and gymnosperms. These germin-like proteins are both soluble or apoplastic proteins. In 1993 it has been shown that germinated related germin of cereal embryos have an oxalate oxidase activity (EC 1.2.3.4): it degrades oxalic acid into H_2O_2 and CO_2 (Dumas et al., 1993; Lane et al., 1993).

Ten years ago it has been shown that oxalate oxidase (OxO) belongs to the superfamily of cupins (Dunwell et al., 2000), which includes germin-like proteins, seed strorage globulin and sucrose-binding proteins. OxO is a manganese containing homohexameric glycoprotein (Woo et al., 2000) and germin-like protein oxalate oxidase activity is particularly intresting considering the linkage of cell wall bound germin and oxalate degradation, because it can be hypothesized a role for germin in cell wall reinforcement by producing Ca^{2+} and H_2O_2 for pectic cross-linking and peroxidase mediated cross-linking of cell wall polymers respectively (Caliskan and Cuming, 1998).

Moreover, germin-like oxalate oxidase is a pathogen inducible protein (Zhou et al.,1998; Hurkman and Tanaka, 1996). It has been demonstrated an increase in the activity of germin-like oxalate oxidase in barley response to fungus infection (Dumas et al., 1993) suggesting a role of oxalate oxidase in response to pathogen attack.

The transient expression of the germin gene in wheat epidermal cells confers disease resistance suggesting that germin might be involved in cell wall cross-linking by the pathogen induced oxidative microburst. However, H_2O_2 generated by OxO might either directly inhibit pathogen infection (Wojtaszek et al., 1997) or act as a signal leading to the synthesis of defence proteins, such as pathogenesis-related (PR) proteins (Chamnongpol et al. 1998).
OxO is also involved in senescence process and leaves aging possibly contributing to H_2O_2 accumulation. The same authors demonstrated that the OxO activity is located principally around the vascular bundles and in the lower epidermis suggesting a role in the protection against pathogens allowing the remobilization of nutrients towards the regrowing tissues (Le Deunff et al., 2004).

A-3.c.5 Diamine/polyamine oxidase

Polyamine, such as putrescine, spermidine ad spermine, are ubiquitous cationic compounds involved in several physiological processes like cell growth and differentiation. Their levels are controlled through biosynthetic and biodegradation pathways. Polyamine degradation is catalyzed by copper-containing amine oxidases (CAO, EC 1.4.3.6) and flavoprotein polyamine oxidases (PAO, EC 1.5.3.11). CAOs attack the amino groups of polyamines, whereas PAOs attack secondary amino groups. The latter class use FAD as cofactor to oxidize spermine, spermidine, or their acetylated derivatives while CAOs preferentially oxidize diamines like putresceine and cadaverin and for this reason they are usually called diamine oxidases or DAOs. The result of PAOs reaction are 4-aminobutanal (from spermidine) or *N*-(3- aminopropyl)-4-aminobutanal (from spermine), H_2O_2 and 1,3-propanediamine. The product of CAOs catalyzed reaction are aldehydes, ammonium ions and still H_2O_2.
These two classes of amine oxidases are predominantly localized at plant cell wall (Sebela et al., 2001) and H_2O_2 derived from their activity has been correlated to cell wall stiffening during plant development in different plant species (Cona et al., 2006, 2006b; Paschalidis and Roubelakis-Angelakis, 2005). COAs activity is involved in H_2O_2 production against pathogen attacks and wound healing (Cona et al., 2006); for example COAs expression is induced by jasmonic acid: a wound signal (Rea et al., 2002), and H_2O_2 should be necessary for the apoplastic peroxidases for cell wall lignosuberization (Angelini et al., 2008). H_2O_2 produced by polyamine oxidase contributes also to hypersensitive response (HR) in plants, as it was demonstrated by tobacco plants infected with *Tobacco mosaic virus*. During this infection, in fact, there is an increase in polyamine content and in its biosynthetic and catabolic enzymes

(Yoda et al., 2003) and the H_2O_2 produced has been proposed as the cause of HR cell death. Interestingly, some experiments of infection using *Sclerotinia sclerotiorum* don't show H_2O_2 accumulation but increased levels of superoxide radical. The use of amine oxidase inhibitors in these experiments, however, shows decreasing superoxide levels, suggesting also in this case the polyamine oxidases involvement.

A-4 Plant plasma membrane redox components

Biological plasma membranes contain different redox elements which are ubiquitous and which are related to several fundamental cell processes including nutrients assimilation, control of cell growth, defence against pathogens attack, detoxification of xenobiotic compounds and signal transduction.

Electron transfer reactions have been demonstrated for mitochondria, chloroplasts, bacteria and plasmic reticulum membranes. Moreover, redox activity was also found in animal, plant and fungi plasma membrane, and so this activity is considered to be essential for living organisms.

Here I report on the plant redox plasma membrane components giving particular attention to the components which will be useful for the topic of this thesis.

A-4.a MAPK cascade

Mitogen-activated protein kinase (MAPK) class play a central role in plant signalling of several abiotic and biotic stresses. In general, MAPK are highly conserved modules in all eukaryotes and in plants they are involved in the regulation of growth, development, programme cell death and also in response to external stimuli such as cold, heat, drought, UV, ROS and pathogens (Colcombet and Hirt, 2008). MAPK cascades link upstream receptors to downstream targets through phosphorilation of series of proteins. The simplest cascade is made up by MAPK, MAPKK (MAPK kinase) and MAPKKK (MAPK kinase kinase). *Arabidopsis* has at least 110 genes coding for putative MAPK proteins (Mizoguchi et al., 2000). The best characterized MAPKs are MPK3, MPK4, and MPK6. MPK4 negatively regulates biotic stress signalling and in fact *mpk4* mutant has a dwarfed phenotype, while MPK3 and MPK6 positively mediates defence responses and their fundamental role for normal plant is demonstrated by the embryo lethal phenotype of *mpk3/mpk6* double mutants

(Qiu et al., 2008; Petersen et al., 2000). These three well known MAPK have also been associated to pathogen signalling, even if other MAPKs might be involved.

Flg-22 is a 22-amino acid peptide derived from flagellin, the main structural protein of the eubacterial flagellum, and it is largely used to study the signalling events after pathogen attack. In *Arabidopsis*, Flg22 is recognized by a plasma membrane receptor-like kinase complex (FLS2 and BAK1) which induces a signalling cascade including an early oxidative burst (Gomez-Gomez et al., 1999), the activation of different kinases and the consecutive phosphorylation of many cellular proteins (Nuhse et al., 2007) and gene modulation (Zipfel et al., 2004).

Experimental evidences suggest that the prolonged activation of MAPK cause redox disequilibrium ends in ROS production contributing to HR cell death regulation (Ren et al., 2002).

As already said, ROS are important in programmed cell death (PCD) and plant cells attacked by HR-inducing pathogens display two peaks of H_2O_2. The first burst of H_2O_2 happens within minutes after pathogen contact but is not specific of HR. This burst, in fact, is caused also by elicitors which don't cause HR cell death and for this reason it is considered as a general and non specific plant response to any pathogen. The second H_2O_2 burst (phase II) always precedes the onset of HR cell death and is specific for HR-inducing pathogens. MAPKs don't seem to regulate the first H_2O_2 burst, whereas their activation and the phase II H_2O_2 production precede and correlate with the HR cell death suggesting that MAPK-induced HR-like cell death might be mediated by the H_2O_2 generation. The precise mechanism of H_2O_2 production and the following HR cell death induced by MAPKs is still unclear. Moreover there are several possible mechanisms for H_2O_2 production at the PM after pathogen attack such as NADPH-oxidase, cell wall peroxidises, apoplastic polymine-oxidases. Of course, it is also possible that different mechanisms are involved in response to pathogen interaction through ROS production.

A-4.b NADPH oxidase

NADPH oxidase is a membrane bound enzyme complex, widely studied in mammals in which it is also called "respiratory burst oxidase "(Rbo). The neutrophil enzyme consists of a flavocytochrome b_{558} ($gp91^{phox}$) and of different citosolic regulatory proteins which include phosphoprotein and a small GTP-binding protein (Lambeth et al., 2004). The flavocytochrome b_{558} is able to transfer electrons from intracellular NADPH to molecular

oxygen outside the cell or inside the lytic vacuole producing superoxide radical. Cytosolic proteins are translocated to the PM in order to realize NADPH oxidase activation. Superoxide radicals produced by NADPH oxidase have a central role against pathogens. This anti-microbial action is due not only to the ROS toxicity, but also to the signalling role of superoxide anion which lead to a K^+ flux that activates specific proteases (Reeves et al., 2002).

The first plant NADPH oxidase gene was found in rice and then different respiratory burst oxidase homolog (Rboh) were found in *Arabidopsis*, tomato, tobacco and potato (Torres and Dangl, 2005). The rice NADPH oxidase, like in mammals, is involved in ROS production and so plays a central role during the hypersensitive reaction (HR) against pathogens infections. Moreover, Arabidopsis (AtrbohA-F) and tomato (Lerboh1) Rboh have a molecular weight of about 150 kDa, with a C-terminal region that shows a great similarity to the 69 kDa apoprotein of the $gp91^{phox}$. On the other side, plant plasma membrane NADPH oxidases can produce superoxide radical in the absence of cytosolic components and are directly activated by Ca^{2+} (Sage and Fluhr, 2001). Consistently with the latter observation, it was shown that all plant Rboh have a sequence of 300 amino acids containing two calcium-binding EF hand motifs, which are absent in mammals NADPH oxidases. The presence of these motifs is interesting in situations in which the Ca^{2+} signalling precedes ROS production. As said above, plant NADPH oxidases are involved in HR and in fact Rboh genes are transcriptionally upregulated by pathogen or fungal elicitors (Torres and Dangl, 2005). Experiments using AtrbohD and AtrbohF knockout mutants clearly showed that AtrbohD is responsible of ROS production during avirulent bacterial or micotic infections whereas AtrbohD is fundamental in the regulation of HR (Torres et al., 2002). The phenotype of the double mutant atrbohD/atrbohF is more severe then the phenotype of the single mutant for one of the two genes (Kwak et al., 2003). Downregulation or removal of Rboh have different effect on pathogen growth and on HR. Nicotiana tabacum rboh silenced plants are susceptible to normally avirulent *Phytophthora infestans* and don't show HR.

It seems clear that Rboh are required for pathogen-induced ROS production and then ROS can have different signalling functions in disease resistant and HR. The result of ROS signalling can be influenced by other defence regulators. For example, exogenously applied ROS can cooperate with salicylic acid, amplifying the signal to have HR; moreover, coordinated levels of ROS and nitric oxide are required to have HR (Delledonne et al., 2001). Thus, ROS produced by Rboh can have different roles depending on the situation, and the exact ROS function during pathogen infections is still unclear.

A-4.c NAD(P)H quinone reductases

Plant NAD(P)H quinone oxidoreductase (NAD(P)H-QR) use either NADH or NADPH as electron donors and a hydrophilic quinone as electron acceptor. NAD(P)H-QR is usually present in the soluble cell fraction (Trost et al., 1995; Sparla et al., 1996) but certain NAD(P)H-dependent duroquinone reductases were also found in the mithocondra, microsomes and plasma membranes (Luethy et al., 1991; Serrano et al., 1994, Trost et al., 1997)

Plant PM show transmembrane electron transport from cytoplasmic NAD(P)H to various external acceptors, together with a depolariziation of the membrane potential and secretion of H+ into the apoplast (Bérczi and Moller, 2000; Luthje et al., 2007). Menckhoff and Luthje used right-side out PM vesicles to demonstrate the characteristics of this redox system, which are the following: the use of NADH and NADPH as electron donors; the use of artificial substrates as ferricyanide (FeCN) or quinones as electron acceptors; the insensitivity to KCN (Menckhoff and Luthje, 2004).

Partially purified quinone reductases (QRs) from higher plants showed the capacity to reduce the natural quinone, vitamin K1, and studies with microsomal fractions suggest that this proteins reduce benzoquinones by a two-electron transfer reaction similar to the animal DT-diapharose (Sparla et al, 1996, 1999): a flavoprotein involved in the detoxification of redox active compounds produced by the metabolism of carcinogenic aromatic hydrocarbons. *Arabidopsis* NAD(P)H-quinone reductase analysis showed that the protein reduces quinones by a two-electron transfer step contributing to cell protection from the oxidative action of semiquinones.

Recently, Schopfer and co-workers purified a NAD(P)H oxidoreductase from soybean hypocotyl plasma membrane preparations (Schopfer et al., 2008). They showed that the protein is a tetramer, which produces $O_2^{\cdot -}$ after naphtoquinone stimulation.

A-4.d Nitrite:NO reductase

Nitric oxide (NO) as a signalling molecule in plants has received a lot of attention during recent years. It has been showed the involvement of this molecule in processes such as root growth and leaf expansion, photomorphogenesis and senescence (Beligni and Lamattina, 2001), stomatal closure and cytokinin signalling pathway (Tun et al., 2001). NO is also well

known to be involved in plant pathogenic response (Delledonne et al., 2001; Klessig et al., 2000).

NO is produced in plants via the oxidation of arginine and citrulline and there's no clear evidence for the existence of plant NO synthase (NOS) similar to those found in bacteria and mammals (Butt et al., 2003). Nitrite is a physiological plant substrate used by cytosolic nitrate reductase (cNR) and by the root-specific plasma membrane bound nitrite:NO reductase (NI-NOR). NI-NOR activity differs from that of cNR, as it was demonstrated in *Nicotiana tabacum* (Stohr et al., 2001): it doesn't use nicotine adenine nucleotides and reduced cytochrome c functions as electron donor *in vitro*; even if cytochrome c involvement at the plasma membrane is unlikely and the real electron donor is still unknown. Solubilisation studies demonstrated a tight association between NI-NOR and plasma membrane bound nitrate reductase (PM-NR) which reduces nitrate to nitrite in the root apoplast using succinate as electron donor (Stohr and Ullrich, 2002). Moreover, in the apoplast the non-enzymatic production of NO by the reaction of NO_2^- with ascorbate (Klepper, 1990) or by the light-mediated conversion of nitrogen dioxide with carotenoids (Cooney et al., 1994) and reducing agents such as AA can accelerate the rate of NO formation from nitrite (Bethke and Jones, 2004). Experiments using root plasma membrane vesicles demonstrated the increasing in NO production together with the increased external nitrate concentration and the highest NI-NOR activity was detected at 35 mM nitrate supply: a condition in which plant growth was markedly reduced. Thus, the toxic nitrate concentration may be the result of toxic NO levels at the apoplast. Moreover NI-NOR activity is reversibly inhibited by the presence of oxygen indicating a possible NO function as a signal for oxygen deficiency. It seems clear that there is a relation among the presence of PM-NR and NI-NOR at the plasma membrane and apoplastic NO production, even if the exact reactions have to be clarified especially for the interesting effects that NO could have.

A-4.e NADH-Fe^{3+} -chelate reductase

Iron is an important element for plants, it is able to accept and donate electrons and so it is a fundamental cofactor of several metalloproteins. Earth's crust has a high amount of iron, but it is not easy for plants to use this element because it tends to form insoluble ferric hydroxide complexes in aerobic environments either at neutral or basic pH (Guerinot and Yi, 1994). Moreover, iron, thanks to its catalytical properties, can generate hydroxyl radicals that are very dangerous for the cell, so it is necessary for cell survival to limit iron accumulation

(Halliwell and Gutteridge, 1992). Plants have evolved two different strategies for the acquisition of iron (Fig. 7). Strategy I occurs in all plant species except grasses and it consists on the iron solubilisation and transport into the roots. It includes the induction of three plasma membrane proteins: a proton pump which acidifies the rhizosphere taking more iron in solution; a ferric chelate reductase which converts Fe(III)-chelates to Fe(II); and a Fe(II) transporter which moves iron across the PM into cells. In *Arabidopsis* were identified the genes that encode the iron-regulated ferric chelate reductase (FRO2) and the ferrous iron transporter (IRT1) that function in the iron uptake as part of strategy I response (Robinson et al., 1999; Vert et al., 2002). FRO2 belongs to the flavocytochromes *b* family; it is predicted to have FAD- and NADH- binding sites and 8 transmembrane helices. It conserves the main features of flavocytochromes *b* suggesting that also the mechanism of action has been conserved. So, FRO2 and NADPH oxidase are related proteins (Schagerlof et al., 2006) and then it can be hypothesized also the transmembrane electron flow: NADPH is oxidised on the cytoplasmic side then electrons are transferred through b-haems on the external side of PM where they promote oxygen or FeIII reduction.

On the contrast there are evidences that FeIII-chelates reductases of different plant are similar to NADH-cytochrome *b* reductases (Karplus et al., 1999).

There are no doubts concerning FRO2 over-expression in roots growing under iron deficiency conditions; while fro2 deletion results into reduced growth under low iron availability (Robinson et al., 1999).

Strategy II consists on the production by plants of phytosiderophores (PSs) into the rizosphere were they chelate Fe(III), forming a complex (Fe(III)-PS) which is taken up into root cells through the Yellow Stripe1 proteins (Curie et al., 2001; Murata et al., 2006). This strategy is based on an uptake system characterized by a high iron affinity.

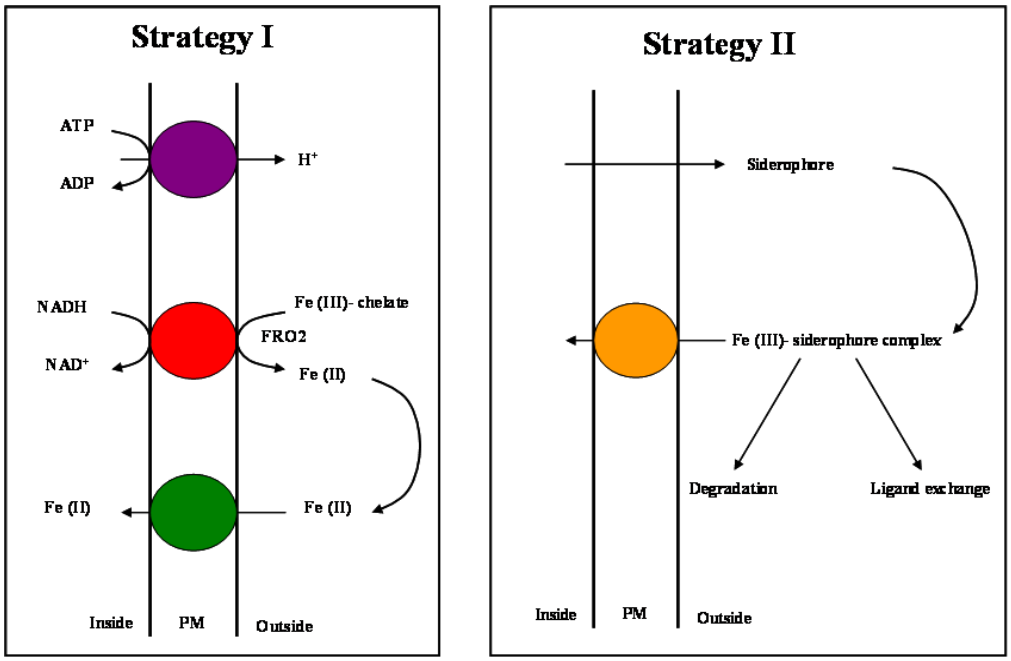


Fig. 7: **Iron uptake strategies of higher plants.** Plants can acquire iron from soils low iron availability using one of two strategies. For example pea, tomato and Arabidopsis use strategy I while barley, maize and rice use strategy II. Nature Biotechnology 19, 417-418 (2001). The two strategies are described in the text.

A-5 CYTOCHROMES b_{561}

Cytochromes (cyts) b_{561} are transmembrane proteins of about 25 kDa made up of six membrane- spanning alpha- helices which bind two hemes b; one heme with Em around +70 mV is predicted to be close to an ascorbate binding site facing the cytocol, while the second heme, with Em around +170 mV, faces the opposite side of the membrane and it can be oxidised by either MDHA and ferrichelates (Tsubaki et al., 1997; Bérczi et al., 2005; Kamensky et al., 2007; Ludwiczek et al., 2008). Analysis of PM preparations from different plant species showed the presence of *b*-type cytochromes; moreover there was always the presence of a major *b*-type cyt with a redox potential between +110 mV and +165 mV and with a typical α-band around 561nm and so called cyt b_{561} (Asard H, 2001). This cyt b_{561} can be also discriminated from the PM cyt pool by using AA as reducing agent. AA has a high redox potential (E_0 +58 mV, Washko PW, 1992) so this means that AA can donate electrons to cyt with high E_0 but not to low potential cyt *b* components.

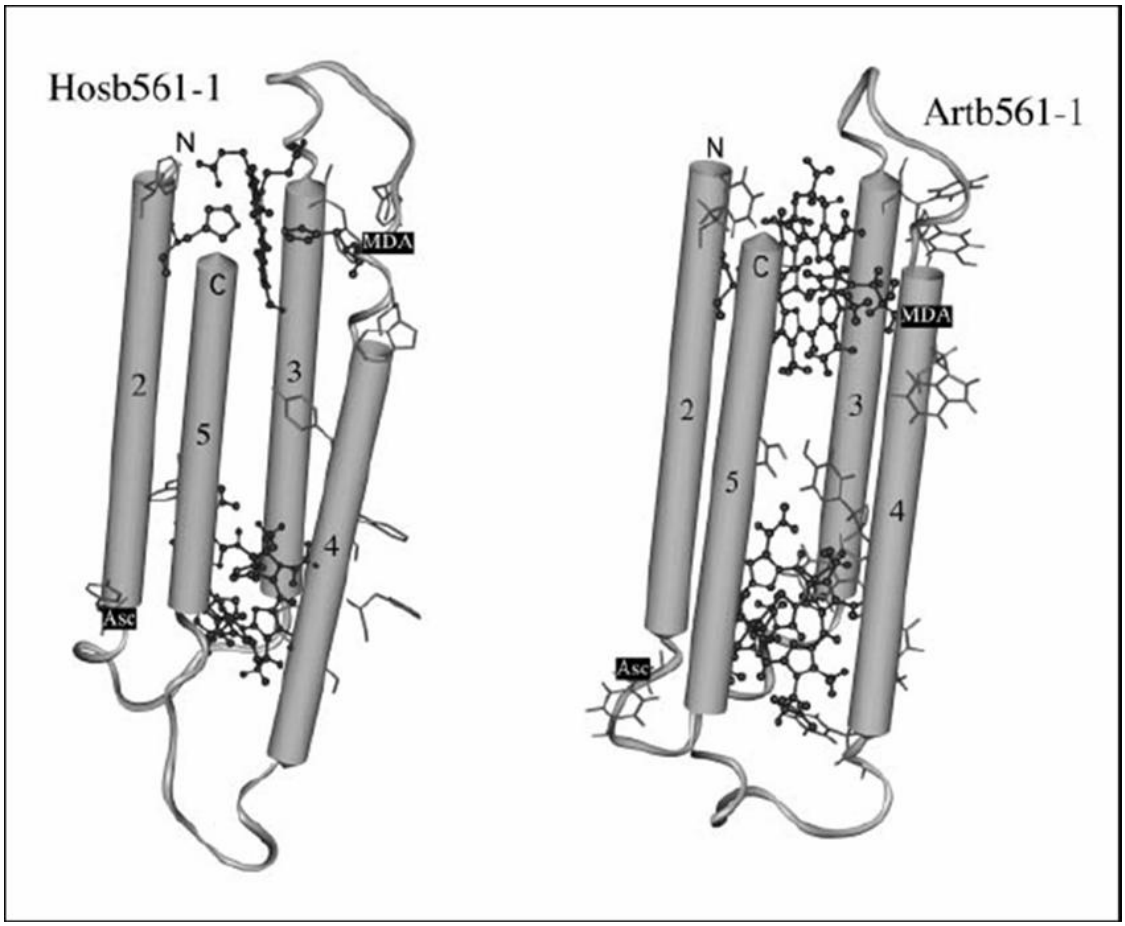


Fig. 8: **Predicted 3-D structures of *Homo sapiens* and *Arabidopsis thaliana* cyt *b*561 sequences**
Hosb561-1 and Artb561-1stand for human and Arabidopsis *b*561 structures respectively. The most conserved helices TMH2 to -5 are shown with interconnecting loops; highly conserved aromatic amino acid residues are indicated in light grey thin bar presentation. The two pairs of conserved His residues and the haems bounded by them are shown in dark grey ball-and-stick presentation. The N- and C-termini of helices 2 and 5 are indicated with N and C, respectively. The TM helix numbers are indicated on the cylinders. Putative MDHA and AA binding sites are indicated with corresponding text boxes. The helix axes are perpendicular to the membrane plane and the cytoplasmic side is below the structures.

Plants contain several hortologous genes to animal cyts *b561* (Asard et al., 2000; Tsubaki et al., 2005). *Arabidopsis thaliana* contains four genes belonging to this family and one of these (At4g25570, CYBASC1) showed similar biochemical properties to animal cyt b_{561} when expressed in recombinant form (Bérczi et al., 2007). The *in vivo* localization of AtCYBASC1 is, however, controversial, because it was found associated to tonoplast membranes (Griesen et al., 2004) but from proteomic studies results it seems to be either a tonoplast protein (Carter

et al., 2004; Shimaoka et al., 2004) or a chloroplast protein (Zybailov et al., 2008). Tonoplast localization was also demonstrated for bean CYBASC1 in etiolated hypocotyls (Preger et al., 2005), but in contrast, GFP construct of CYBLASC1 from watermelon seems to localize into the plasma membrane of transformed onion epidermal cells (Nanasato et al.,2005). No data are available for any other isoform of plant cyt b_{561}.

A plasma membrane, ascorbate reducible cyt *b* was purified from etiolated bean hypocotyls as a glycosilated protein of 55-63 kDa (Trost et al., 2000; Preger et al., 2005). A similar cyt, however, has been purified from *Arabidopsis* plasma membranes and it resulted as a 120 kDa protein which dissociates into 27 and 23 kDa polypeptides upon heat treatment (Bérczi et al, 2003).

Two years ago Preger and co-workers purified the major ascorbate reducible cyt *b* associated to the plasma membrane of soybean etiolated hypocotyls, together with the cloning and biochemical characterization of the recombinant form expressed in *Pichia pastoris.* This cyt, called AIR12, is codified by one gene (At3g07390) that is early induced during Arabidopsis lateral root formation by auxin treatment (Laskowski et al., 2006).

Sequence analysis showed that AIR12 belongs to the DOMON domain superfamily (Ponting et al., 2001). It's interesting to note that AIR12 sequence was found only in flowering plants, while DOMON-containing protein is widespread in nature (Preger et al., 2009).

A-6 DOMON domain

The "DOMON" acronym stands for DOpamine β-MONoxygenase from dopamine β-monooxygenase, the enzyme in which it was identified for the first time and which is involved in the conversion of dopamine to noradrenaline through the reduction of AA in animal chromaffin granules.

This domain is widespread in nature, in fact it is present in animal, plants, fungi, various protists, bacteria and archea and it was found both in secreted and cell surface proteins.

It consists of 110-125 residues and it can be associated to a lot of other different domains such as cytochrome b_{561}, adhesion modules such as EGF, reelin and SEA or it can have different DOMON domains associated (Lakshminarayan et al., 2007). Only four DOMON-containing proteins have been crystallized, with three of them containing a bound soluble ligand (Fig. 9).

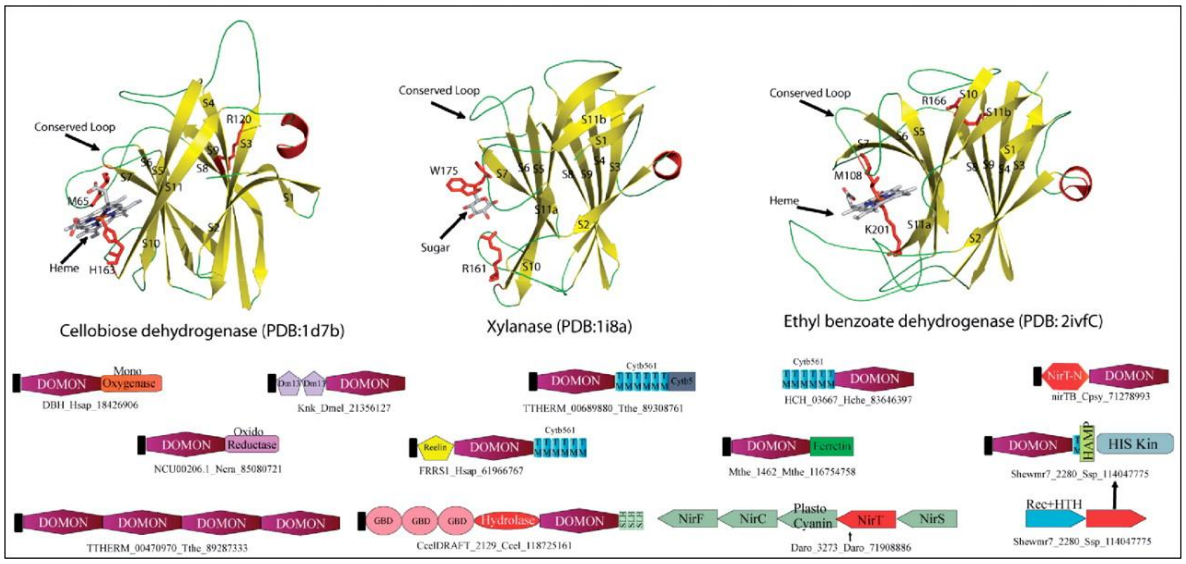


Fig. 9: **DOMON domains structurally defined and conserved gene neighborhoods.** In the upper part of the figure there are the three resolved structure of DOMON domains with the conserved loop, ligand-binding residues and conserved arginine as ribbon diagrams. In the bottom of the figure DOMON domain is reported associated to other domains (from Iyer et al., 2007)

Starting from these structures the DOMON domain is predicted to form a β-sandwich structure with 7-8 strands and likely to be involved in protein-protein interactions, even if there are still very few data on its biochemical role. It was found both in heme and sugar binding proteins.

The heme-binding versions of the DOMON domain show some conserved features which include:

- multiple hydrophobic residues, fundamental for the hydrophobic core of the β-sandwich,
- a conserved methionine in the loop between strands 5 and 6 ,
- a histidine or lysine residue in the terminal strand that are involved in the heme linking (Hallberg et al., 2000; Kloer et al.,2006).

The N-terminal cytochrome domain of cellobiose dehydrogenases (CDH) was found to be a DOMON domain and some recent studies have demonstrated that the heme bound by this DOMON domain transfers electrons to the flavin ligand of the oxidoreductase domain during oxidation of cellobiose or cello-oligosaccharides (Stoica et al.,2006). Moreover stromal cell derived receptor 2 (SDR2, a DOMON domain homolog)-like proteins, fused to a cytochrome *b561*, functions as ferric reductase (Vargas et al., 2003). So it was speculated that the heme-binding versions of the DOMON domains are cytochromes involved in redox reactions.

A-7 Plant responses to biotic stresses

Any biotic or abiotic stress is perceived by plant cells and induces in them different responses such as modified gene expression, cellular metabolism change, ROS production. In this paragraph I will focus on plant response to biotic stress.

Pathogens can be divided into three great group on the basis of their attack strategy:

- necrotrophs which kill plant cells such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*;
- biotrophs which keep plant cells alive;
- hemibiotroph which initially keep plant cells alive but kill them at later stage of infection such as *Phytophthora infestans*.

Plant cells defend themselves from pathogen attack producing ROS in the so called oxidative burst. This oxidative burst represents a ubiquitous early part of plant resistance and it involves the apoplastic space and the PM: the first structure perceiving pathogen presence. PM oxidative burst plays a central role in plant immune response (Baker et al., 1995; Wojtaszek et al., 1997) and it is principaly catalyzed by NADPH oxidases and activating calcium-dependent protein kinases (Kobayashi et al., 2007). All the responses occurring within 24 h after pathogen contact, contribute to the establishment of the hypersensitive reaction (HR). During HR plant recognizes genetically incompatible pathogen, creates unfavourable conditions for pathogen proliferation, hampers toxins and harmful enzymes and leads to localized cell death in order to stop pathogen diffusion.

Following biotic stress, in fact, one of the principle enzyme involved in ROS production at the PM is the NADPH oxidase through the production of superoxide radical, like the defence system led by neutrophils. Superoxide radical dismutation to H_2O_2 can be spontaneous or mediated by superoxide dismutase. However, PM can produce H_2O_2 thanks to different enzymes located in it such as peroxidases, amine oxidases and oxalate oxidases. H_2O_2 is a toxic compound for pathogens, and it may be also involved in plant cell wall reinforcement either by cross-linking proline and hydroxiproline-rich proteins to the polysaccharide matrix or by increasing the rate of lignin polymer formation by peroxidase activity. Plant cell reinforcement results in a strength barrier against pathogens penetration. Moreover H_2O_2 can participate to the Fenton reaction producing ·OH which is itself toxic for pathogens and can induce for protein involved in cell protection, such as glutathione-S-transferase (Orozco-Cardenas et al., 2001; de Torres et al., 2006).

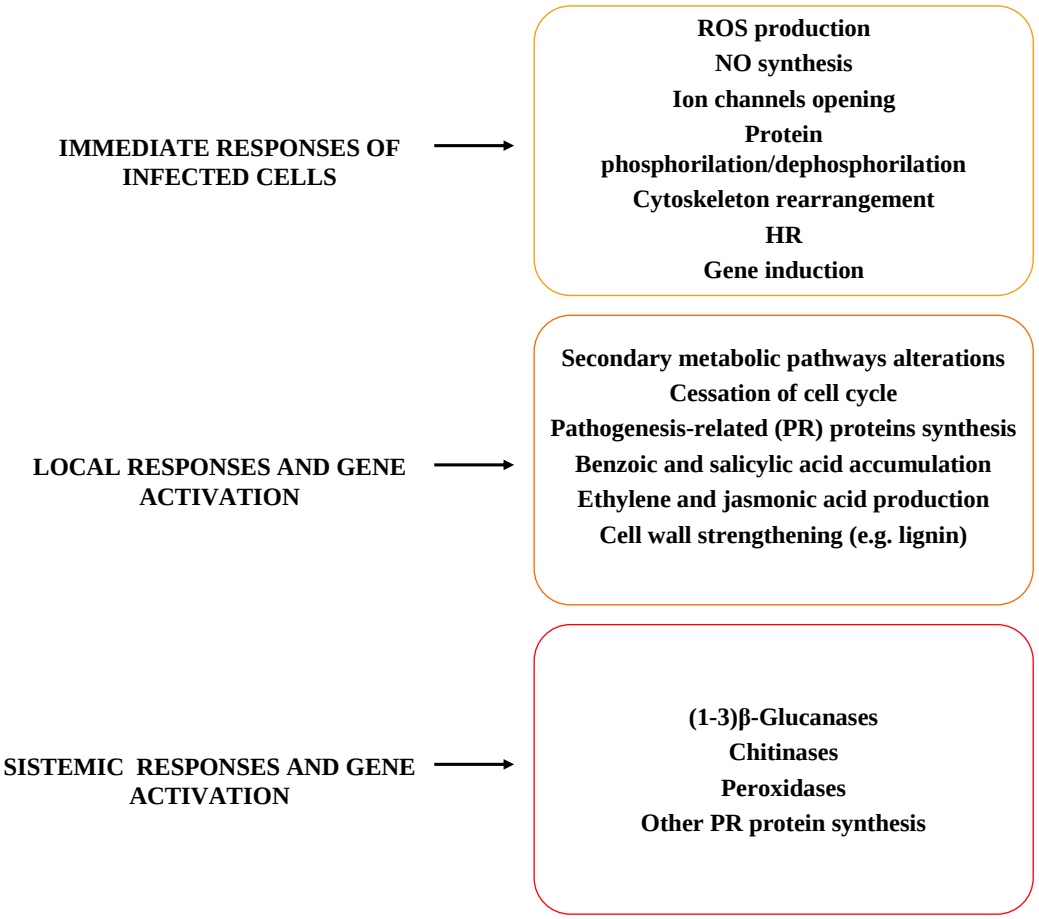
Another early characteristic of plant response to pathogen attack is the extracellular alkalinisation, which occurs as a result of the Ca^{2+} and proton influxes and the K^{+} efflux. A lot of heme containing proteins can produce H_2O_2 at alkaline pH as it was demonstrated for bean peroxidase involved in the oxidative burst (Blee et al., 2001).

ROS over-production against pathogen is also linked to programmed cell death (PCD) (Torres and Dangl, 2005; Dat et al., 2003) because ROS are involved in signalling processes that induce defence gene expression in cells surrounding infected cells which show PCD.

Together with ROS production there is another fundamental compound involved in plant defence against pathogen attack: nitric oxide (NO). NO de novo synthesis, in fact, accompanies the recognition of avirulent pathogen bacteria and potentiates the induction of PCD began by ROS. Moreover NO can bind heme of peroxidises or ascorbate oxidases (Clark et al., 2000) which detoxify H_2O_2 taking this species active. It was shown that PCD requires both H_2O_2 and NO in planta and in cultured cells (Delledonne et al., 1998; De Pinto et al., 2002). The use of NO production inhibitors, there is a reduction of HR, symptoms become more severe and bacterial growth increases. NO and ROS play a synergistic role in the activation of defence responses after pathogen attack and in the PCD.

The relationship between ROS accumulation at the plasmalemma and that potentially occurring inside the cell during pathogen responses remains unclear.

A lot of other molecules and molecular pathways are involved in plant response to pathogen attack creating a very complex network in which a lot of aspects remain unknown. The following picture summarizes the principal steps of pathogen response.



A-8 *Botrytis cinerea*: an overview

Botrytis cinerea is an ascomycete responsible for gray mould on several dicot plants. It is a necrotrophic fungus, this means that its final target is to decompose plant biomass and convert it into fungal mass. It preferentially infect plant with a high pectin content in the cell wall, probably, also because *B. cinerea* has different pectinolytic enzymes (ten Have et al., 2002) . In order to obtain cell death, *B. cinerea* produces toxic metabolites and oxalic acid and induces oxidative burst. As said above *B. cinerea* has a large host range, unlike other necrotrophic pathogen, as *Cochliobolus* and *Alternaria* spp., which produces host-selective toxins and so have a limited host range. *B. cinerea* produces different toxic compounds of low molecular weight and the most studied one is botrydial (Colmenares et al., 2002).

Oxidative burst induction is the common result after plant-pathogen interaction and during the HR: a mechanism to prevent pathogen spread. *B. cinerea*, and necrotrophic pathogens in general, act to kill host cells and so they induce a very strong oxidative burst and produce themselves ROS; moreover these pathogens induce the production of free radical in infected and non tissues (Muckenschnabel et al., 2001) resulting in lipid peroxidation and antioxidant

decreasing (Muckenschnabel et al., 2002). All these processes create redox perturbation that favours disease and finally cell death.

Oxalate acid produced by pathogen acidifies the environment creating the conditions to have the highest activity of pathogen enzymes such as laccases, proteinases and pectinases (Manteau et al., 2003); in addition this acid can bind Calcium ions, that are normally linked to pectic fibrils producing accessible sites for pectin degradation. Finally, oxalate acid decreases plant defence responses (Prins et al., 2000) and induces programmed cell death (Cessna et al., 2000).

During *B. cinerea* infection, and in general during pathogen attack, there is a change in the amount of free radical produced; rotted tissues infected by *B. cinerea* show an increase in free radical compared to normal tissues (Muckenschnabel et al., 2001, 2002). It is not easy to define if these radicals are produced by the fungus or by the host. Interestingly, uninfected tissues of infected leaves show the presence of the same radicals of infected parts (Muckenschnabel et al., 2002). It is fair to say that even if it is clear that *B. cinerea* alters the normal levels of plant free radical and so the scavenging mechanisms, it would be possible that free radical detected are the result of experimental steps, such as excision of sections of tissues, because plant physical damages are known to produce free radical changes (Walters et al., 2007).

The following picture summerizes the principal steps of *B. cynerea* infection. Briefly**,** after conidium attachment and germination on plant tissue, there is the production of an apressorium, a structure which secretes enzymes to breach the plant surface. The penetrating peg grows into the walls of the epidermal cells, then *B. cinerea* starts to produce different toxic metabolites which favours plant tissue decomposition.

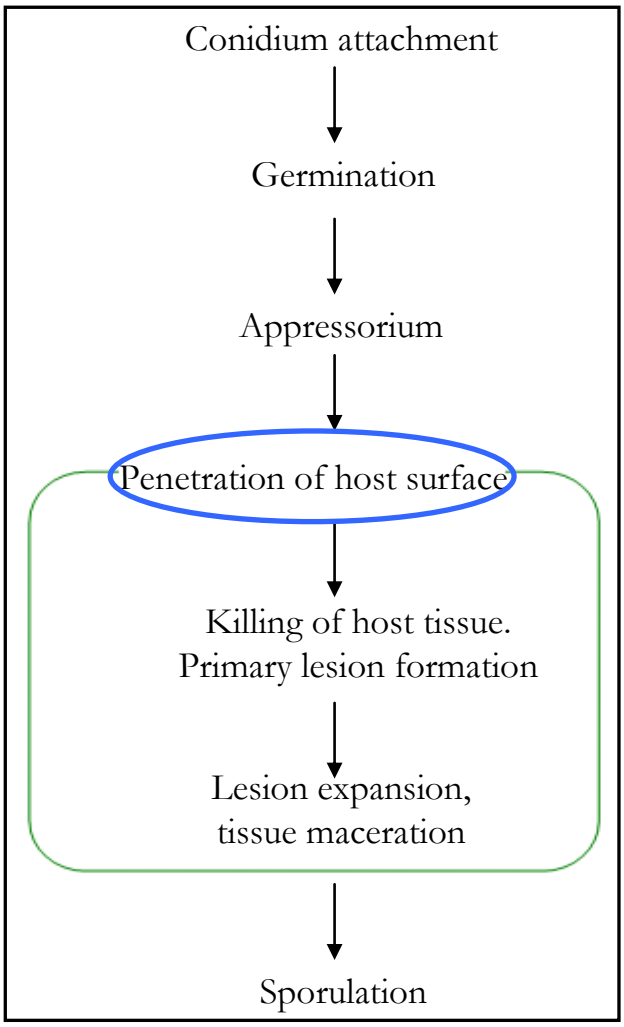


Fig. 10: **Developmental and physiological stages of *B. cinerea* infection.**

The exact role of ROS in *B. cinerea* pathogenesis is still a matter of debate. A very recent paper by L'Haridon and co-workers, in fact, shows that plant mutants with a permeable cuticle are characterized by a higher ROS production and a parallel higher resistance to *B. cinerea* then wild-type plants (L'Haridon et al., 2011) contrasting the diffuse idea that B. *cinerea* infection is favoured by ROS.

B. Chapter I: AIR12, a b-type cytochrome of the plasma membrane of *Arabidopsis thaliana* is a negative regulator of resistance against *Botrytis cinerea*

Aim of project

The aim of my PhD project was to study DOMON containing cytochromes *b* of plant plasma membrane. I focused on a protein of *Arabidopsis thaliana* called AIR12, from Auxin Induced in Root cultures during first 12h of treatment with auxin, that has never been described before. Arabidopsis has only one gene coding for AIR12 and one of the aims of my PhD has been to express AtAIR12 in an heterologous system in order to study its biochemical characteristics. Supposing that the protein was a cytochrome, from literature data, we were particularly interested in knowing the redox reactions in which AIR12 could be involved to speculate an *in vivo* role.

Together with this *in vitro* AIR12 characterisation we also looked for a phenotype in air12-knockout plants. The use of complemented plants, that are *air12*-knockout plants in which it was inserted again air12 gene, was fundamental to confirm AIR12 involvement in any observed knockout phenotype.

Introduction

This chapter and the results reported in it are the material for a manuscript that is going to be submitted to Molecular Plant

AIR12, a b-type cytochrome of the plasma membrane of *Arabidopsis thaliana* is a negative regulator of resistance against *Botrytis cinerea*

Maria Raffaella Barbaro, Valeria Preger, Alex Costa, Anja Krieger-Liszkay, Francesca Sicilia, Paolo Trost.

The extracellular matrix of plants, known as the apoplast, is constituted by the tridimensional scaffold of cell walls, including intercellular spaces and the aqueous phase permeating cell walls and filling the lumen of mature xylem vessels (McNeil et al., 1984). The aqueous phase of the apoplast is a highly dynamic compartment where several chemical reactions take place and many of these are oxido-reductions (Pignocchi and Foyer, 2003). All major ROS can be generated in the apoplast, both in pathological and physiological conditions (Torres and Dangl, 2005; Sandermann et al., 2008; Schopfer and Liszkay, 2006; Fry, 1998; Angelini et al., 2008). Nonetheless, the apoplast contains also antioxidants like AA (Foyer et al., 2008, Padu et al., 2005, Foyer and Noctor, 2011), which is probably the most abundant antioxidant of this compartment with concentrations that are often between 0.1 and 1 mM, (Pignocchi and Foyer, 2003; Padu et al., 2005). The redox state of the ascorbate system in the wall may result from the concomitant activity of ascorbate oxidase (Pignocchi et. al, 2006), the non enzymatic oxidation of ascorbic acid by ROS and degradation of dehydroascorbate by H_2O_2 (Kärkönen and Fry, 2006), the different transport systems capable of exporting AA to the cell wall and those importing extracellular DHA for internal reduction (Horemans et al., 2000), and possibly by the systems of apoplastic AA regeneration based on cytochrome b_{561} (Nanasato et al., 2005) and DOMON proteins such as Air12 (Preger et al., 2009). In any case, the ascorbate system in the apoplast is commonly found more oxidized then in the cytosol or in chloroplasts (Pignocchi and Foyer, 2003). Nevertheless, leaf apoplastic ascorbate is believed to provide a first antioxidant barrier against ozone (Sandermann et al., 2008), and it has been proposed that variations to the redox state of the apoplast may be translated into intracellular

signals that orchestrate adaptive responses (Pignocchi et al., 2006; Fotopoulos et al., 2006; Ding et al., 2009).

ROS can be produced by several pathways in the apoplast. Superoxide can be released in the cell wall by NADPH-oxidases, and different genes of the rboh family have been implicated in several different processes such as apical growth of root hairs (Foreman et al., 2003), stomata closure (Jannat et al., 2011), hypersensitive response (Yoshioka et al., 2009; Levine et al., 1994). Other redox proteins of the cell wall as amino oxidases (Angelini et al., 2010), oxalate oxidase (Lane et al., 1994) and peroxidases (Heyno et al., 2011) do also contribute to ROS production by directly generating H_2O_2 at the expenses of different electron donors, and are generally believed to be often implicated in cell wall stiffening (dos Santos et al., 2004) during differentiation or pathogen attack (Johrde et al., 2008).

Though the low pH of the apoplast and the presence of extracellular SOD isoforms do accelerate the spontaneous dismutation of superoxide to hydrogen peroxide, superoxide may also reduce transition metals (free Fe^{3+} or Cu^{2+} -chelates, or even heme-iron of a peroxidase) and give rise to a Fenton reaction that generates hydroxyl radicals from H_2O_2 (Schopfer et al., 2006). Hydroxyl radicals may attack cell wall polymers and have been implicated in auxine-induced cell wall elongation (Schopfer et al., 2002), fruit softening (Dumville et al., 2003) and separation of the micropilar endosperm cells during germination of garden cress (Müller et al., 2009).

On the other hand, hydrogen peroxide can permeate through the plasma membrane and play itself a signalling role, for instance by activating a MAPK cascade through Oxidative Signal-Inducible1 (OXI1) (Rentel et al., 2004).

H_2O_2 may also play a major role in the polymerization of lignin, a prominent redox reaction occurring in cell walls, particularly (but not exclusively) in secondary cell walls, which reinforces the cell wall. The formation of the lignin polymer is a non enzymatic process that is triggered by the dehydrogenation of monolignols into radicals. Type II peroxidases can perform this reaction by using H_2O_2 as an oxidant. Alternatively, the extraction of one electron from monolignols is catalyzed by laccases (with O_2) or metals like manganese that would be reoxidized by peroxidases (Vanholme et al., 2010) or other redox systems on the plasma membrane. Puzzlingly, the role of H_2O_2 in lignification appears just the opposite of the proposed roles of hydroxyl radicals (which derive from H_2O_2) in cell wall weakening.

AIR12 is a redox protein associated with the plasma membrane that was first described as the product of an auxin early induced gene of *Arabidopsis* root cultures (Laskowski et al., 2006). In whole plants AIR12 gene expression is associated to auxin-induced lateral root formation

(Laskowski et al., 2006). Only recently AIR12 was shown to be an ascorbate–reducible cytochrome *b*, likely associated to the external face of the plasma membrane of plants by means of a GPI-anchor (Preger et al. 2009).

Bioinformatic studies have shown that AIR12 belongs to the DOMON (Dopamine monoxigenase) domain superfamily (Iyer et al., 2007; Ponting et al., 2001; Aravind et al., 2001). DOMON domains are all beta-structures organized in a beta-sandwich as shown by the few DOMON structures solved to date (Hallberg et al., 2000; Notenboom et al., 2001; Kloer et al., 2006). Although DOMON domains are normally part of multidomain proteins that are ubiquitous in living organisms, AIR12 is constituted by single DOMON domain and AIR12 genes are exclusively found in angiosperms, often in single copy (Preger et al., 2009). Close relatives of AIR12 are constituted by a cytochrome b_{561} domain linked to one or more DOMON domains that are widely distributed in living organisms (Verelst et al., 2003; Tsubaki et al., 2005). DOMON domains are not always cytochomes, but one of the best characterized DOMON domain is the cytochrome *b* component of cellulose dehydrogenase of lignilolytic fungi (Hallberg et al., 2000).

The redox activity of AIR12 suggests a link with ascorbate or other redox compound of the cell wall (Lefebvre et al., 2007), but the physiological role of this protein *in vivo* is unknown.

Here we show that AIR12 is indeed a b-type cytochrome localized on the plasma membrane of plant cells, particularly in specific cell types of *Arabidopsis* plants where cell separations occur (micropilar endosperm, hydatodes, floral organs abscission zones, sites of lateral root initiation, lateral root cap) and in the vascular tissue of mature leaves. As expected, the expression of the AIR12 gene *in vivo* is strongly induced by auxin. As a cytochrome, AIR12 interacts with both ascorbate/monodehydroascorbate and oxygen/superoxide couples, and was shown to promote hydroxyl radical production by a plasma membrane reconstituted system. *air12* knock out mutants have no clear phenotype but surprisingly are less susceptible to *Botrytis cinerea* infection, indicating a role of AIR12 as a negative regulator of resistance against this necroptrophic fungus. Possible functions of an apoplastic redox protein as AIR12 in the establishment of *B. cinerea* infection in Arabidopsis are discussed.

Results

B-1 Heterologous expression of AtAIR12 in *Pichia pastoris*: SDS-PAGE and spectrophotometrical analysis.

In *Arabidopsis thaliana*, AIR12 is coded by a single gene (At3g07390). The coding sequence of 273 residues includes a predicted N-terminal signal peptide of 52 residues and a C-terminal sequence of 23 residues downstream the predicted GPI insertion site. The coding sequence of mature AIR12 (aa 48-227), cloned downstream an alpha-factor secretion signal sequence, was expressed in *Pichia pastoris*. Recombinant AIR12 (recAtAIR12) was purified from culture medium by means of three chromatographic steps, including a Phenyl Sepharose hydrophobic interaction column, followed by an anion exchange column and a gel filtration chromatography. The cytochrome eluted from the final purification step, a Superdex 200 gel filtration column, as a single symmetric peak of 89 ± 25 kDa (n=6). Two typical chromatograms of anion exchange and gel filtration chromatographies are reported in fig. 10.

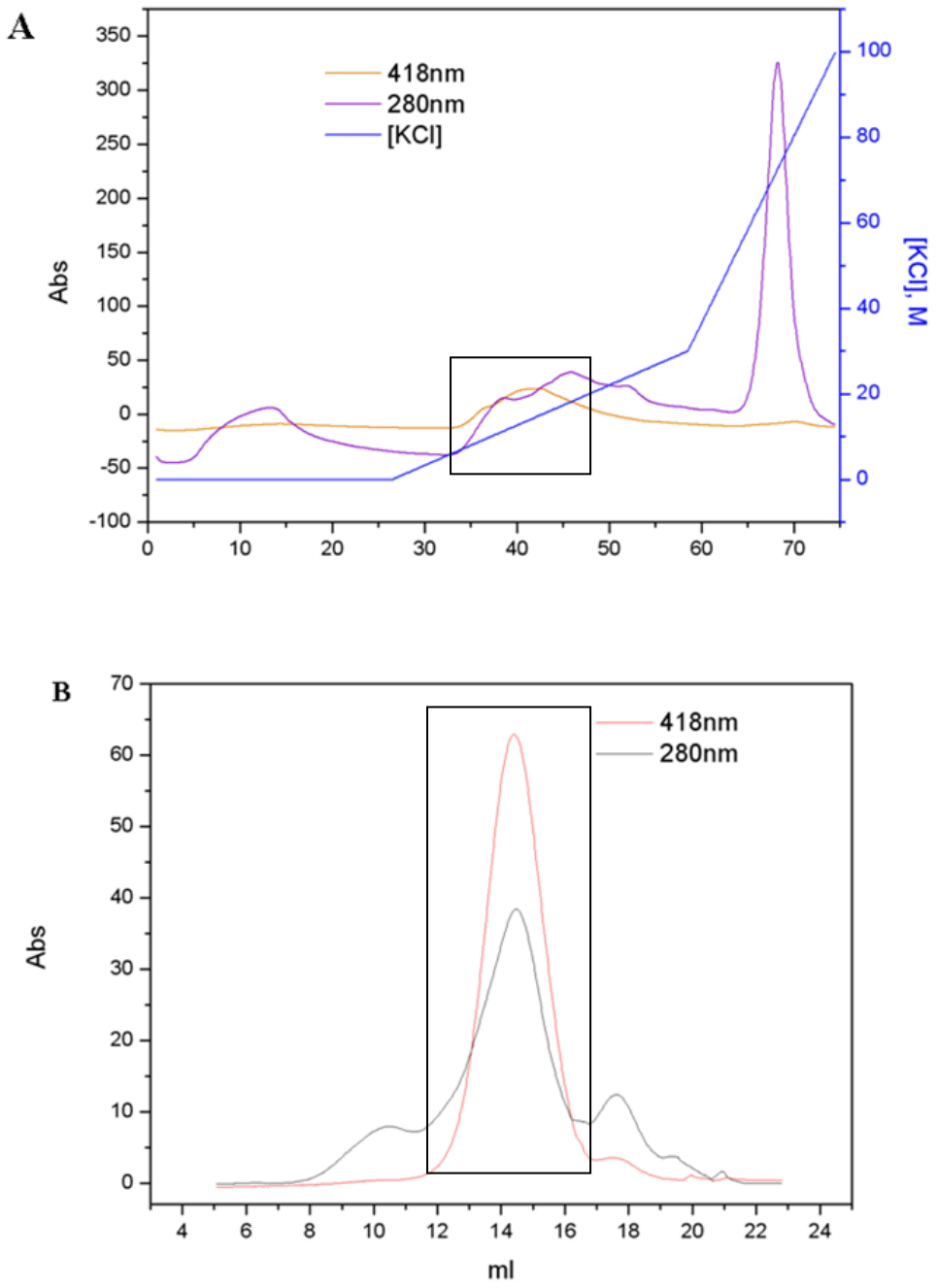


Fig. 10: **Anion exchange and gel filtration chromatographies of recAtAIR12 purification.** A**:** Chromatogram obtained from SourceQ chromatography at pH 7. 0. RecAtAIR12 (in the black square) eluted was concentrated to 0.2 ml prior to loading on the Superdex 200 column. B: The Superdex 200 HR 10/30 column was equilibrated with 50 mM Tris-HCl, pH 7.0, 150 mM KCl. The purified recAtAIR12 eluting from the gel filtration chromatography (in the black square) was then used for biochemical characterization experiments. Abs stands for absorbance.

The calculated molecular mass of recAtAIR12 (after cleavage of the alpha-factor signal, but including cMyc- and His-tags at the C-terminus) was 21.8 kDa. Analysis by SDS-PAGE of purified recAtAIR12 preparations revealed a broad, diffused band migrating from less than 45 kDa to over 100 kDa (Coomassie-staining), more heavily stained in the 45-55 kDa region (Fig. 11). Uneven migration was apparently due to glycosylation (Fig. 11), since treatment with endoglycosydase-H revealed a new sharp band of 22 kDa, nicely corresponding to the calculated molecular weight of recAtAR12.

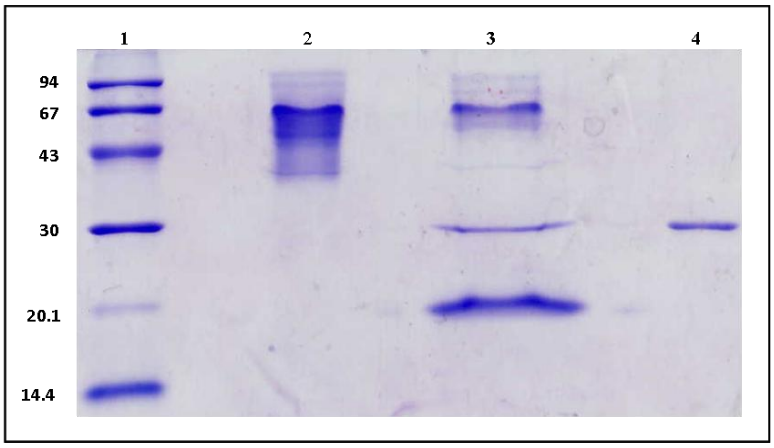


Fig. 11: **SDS-PAGE analysis of purified recombinant AtAIR12 expressed in *P. pastoris*.** Lane 1, molecular weight standards; lane 2, purified recAtAIR12; lane 3, recAtAIR12 treated with endoglycosydase-H; lane 4, endoglycosydase-H. 9 µg of purified recAtAIR12 were loaded in each lane.

Carbohydrate staining, obtained using Periodic Acid Schiff (PAS) staining, confirmed that recAtAIR12 was glycosylated (Fig.12). After endoglycosydase-H (endo-H) treatment there was a partial reduction of carbohydrate content. Coomassie staining of polyacrylamide gel after PAS staining clearly showed the appearance of a band around 22 kDa, likely AIR12, after endo-H treatment (Fig. 12). The appearance of this band confirmed the glycosylation of AIR12 even if complete deglycosylation by endo-H was apparently not achieved.

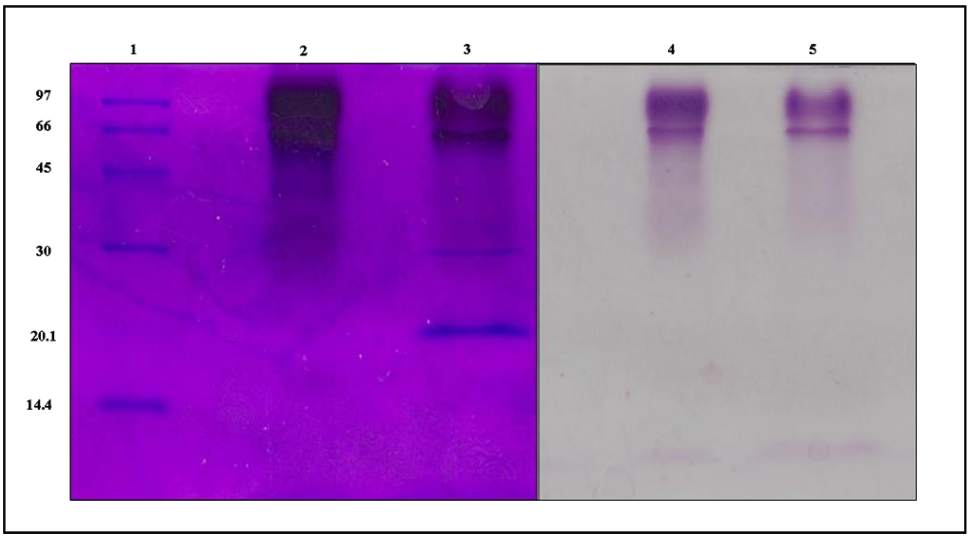


Fig. 12: **PAS staining and coomassie staining of purified recombinant AtAIR12 expressed in *P. pastoris*.** The gel on the right (lanes 4 and 5) was obtained using PAS staining, this gel was then stained with coomassie in order to verify the appearance of recAtAIR12 band. Lane 1, molecular weight standards; lane 2 and 4, purified recAtAIR12; lane 3 and 5, recAtAIR12 treated with endoglycosydase-H. 9 μg of purified recAtAIR12 were loaded in each lane.

Purified recAtAIR12 has typical spectral properties of a b-type cytochrome (Fig. 13) with an alpha band at 559 nm in the fully (dithionite-) reduced form. The reduced-minus-oxidized extinction coefficient of recAIR12 heme at 560 nm was determined by the hemochrome method as 20 mM^{-1}. The cytochrome was also almost completely reducible by ascorbate (Fig.13).

Ascorbate reduction of recAtAIR12 reached 90% of the total reduction that was obtained with dithionite.

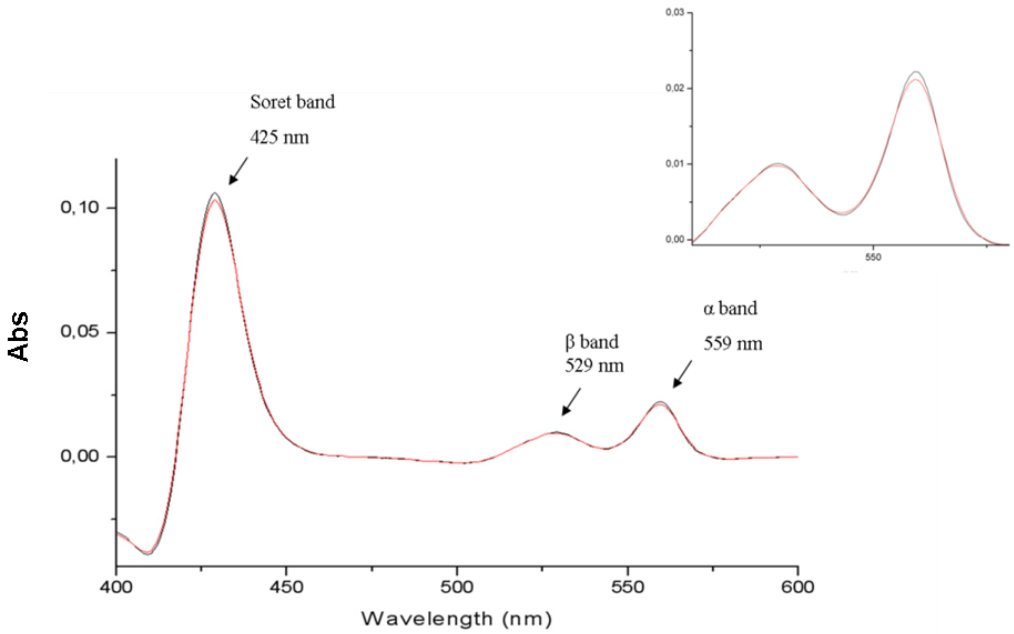


Fig. 13: **Differential absorption spectra of purified recAtAIR12 (1 µM).** The red line represents the ascorbate-reduced minus oxidized spectrum. The black line represents the dithionite-reduced minus oxidized spectrum. Both ascorbate and dithionite were added in grains. The three typical bands of a cytochrome *b* are indicated. The alpha and beta regions are enlarged in the inset.

B-1.a recAtAIR12 interactions with the ascorbate pool

Maximal rate of reduction by saturating ascorbate was 0.25 µM recAtAIR12/min (with 1.9 µM recAtAIR12, at pH5.5), but oxidation by monodehydroascorbate produced by the addition of ascorbate oxidase was faster (0.5 µM/min; Fig. 14). At equilibrium, a solution of 1.9 µM recAtAIR12 was half reduced by ascorbate around 80 µM and fully reduced by millimolar concentrations of the reductant (Fig. 15A). The alpha-band was symmetric at any stage of reduction (Fig. 15B) and the ascorbate-titration curve included a single inflection point (Fig. 15A), these results both suggest that recAtAIR12 could bind one single heme, as previously shown by other methods for soybean AIR12 (Preger et al., 2009).

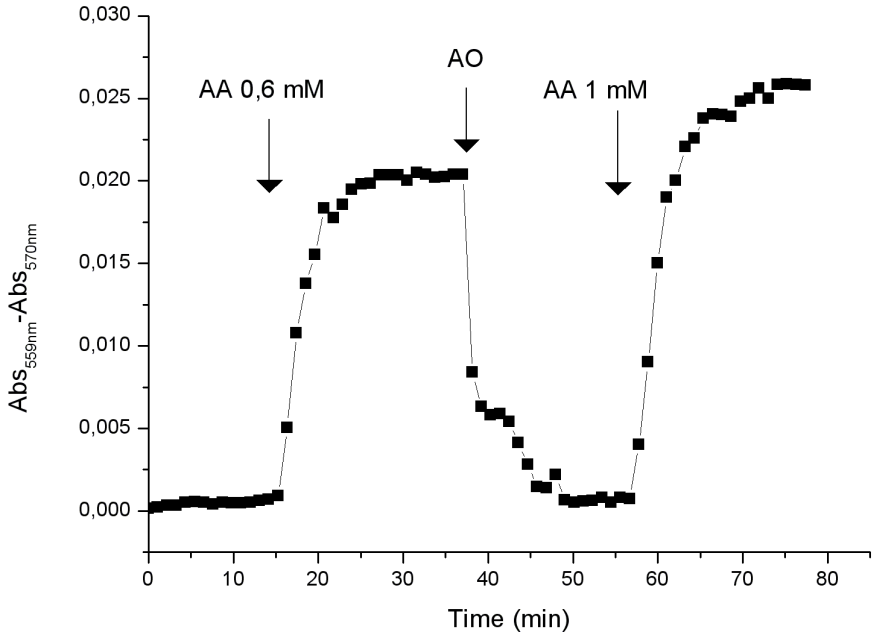
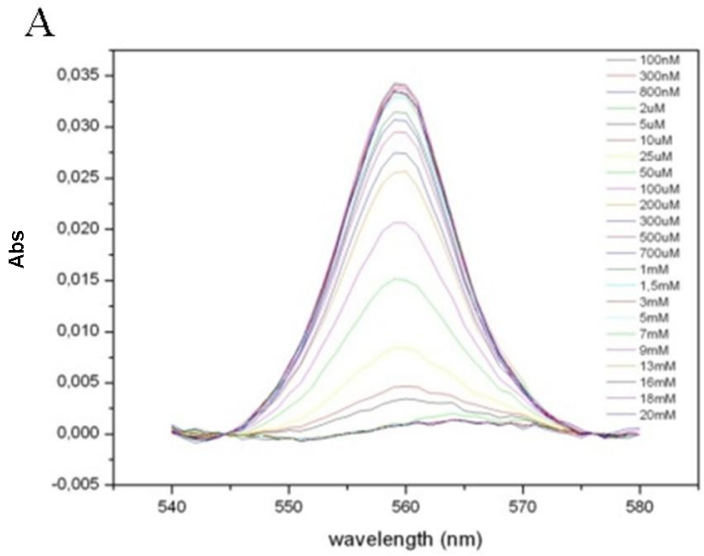


Fig. 14: **RecAtAIR12 oxidation by monodehydroascorbate.** Time course of the absorbance change at 559 nm of recAIR12 upon reduction with ascorbate (AA; 0.6 mM) followed by oxidation through the addition of ascorbate oxidase (AO; 1U) and re-reduction by ascorbate (1 mM). RecAtAIR12 (1.3 μM) was in 50 mM MES-KOH pH 5.5.



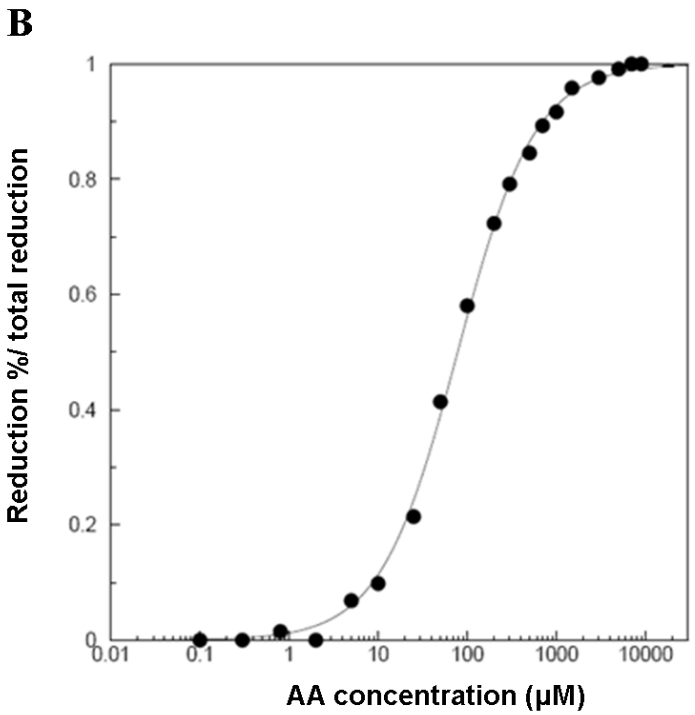


Fig. 15: **RecAtAIR12 reduction by ascorbate.** A: optical absorbance of alpha band intensity changes during ascorbate titration of recAtAIR12; B: percentage of reduced cytochrome in respect to total reduction are plotted as a function of ascorbate concentration through a non linear regression

B-1.b recAtAIR12 interactions with oxygen and ROS

In the search for alternative reductants for recAtAIR12, the xanthine/xanthine oxidase system was used as a source of superoxide, and complete reduction of recAtAIR12 was indeed observed in a matter of minutes (Fig. 16).

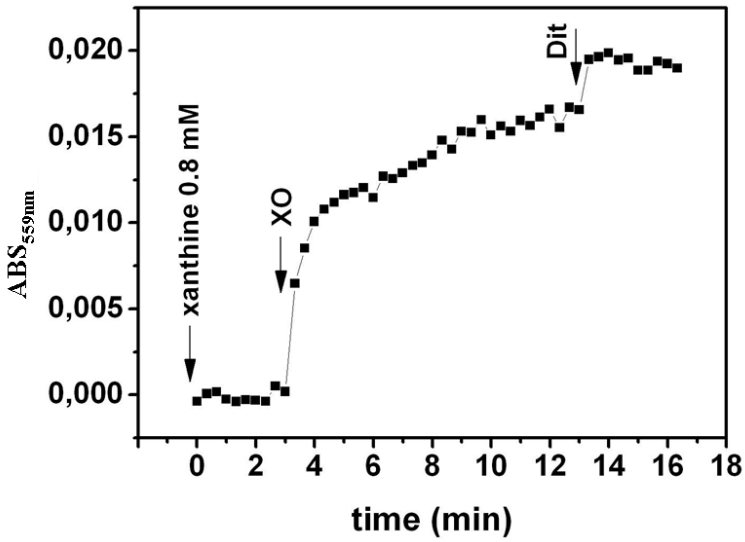


Fig. 16: **RecAtAIR12 reduction by superoxide.** Firstly xanthine was added to be sure that there was no interaction with recAtAIR12; at the end of the experiment dithionite (Dit) was added to have the total reduction of AIR12. XO: xanthine oxidase.

Reduction of recAtAIR12 (1 μM) by superoxide was initially rapid (1 μM/min, Fig. 16), 4-fold faster than the reduction by saturating ascorbate (calculated in Fig. 14), and almost complete. For comparison, we performed the same experiment using cytochrome c (from horse heart) and also in this case the reduction rate of the protein by superoxide was approximately in the same range (Fig. 17).

Cytochrome c was selected for comparison because of the standard redox potential similar to AIR12 and because both cytochromes have a hexacoordinated heme.

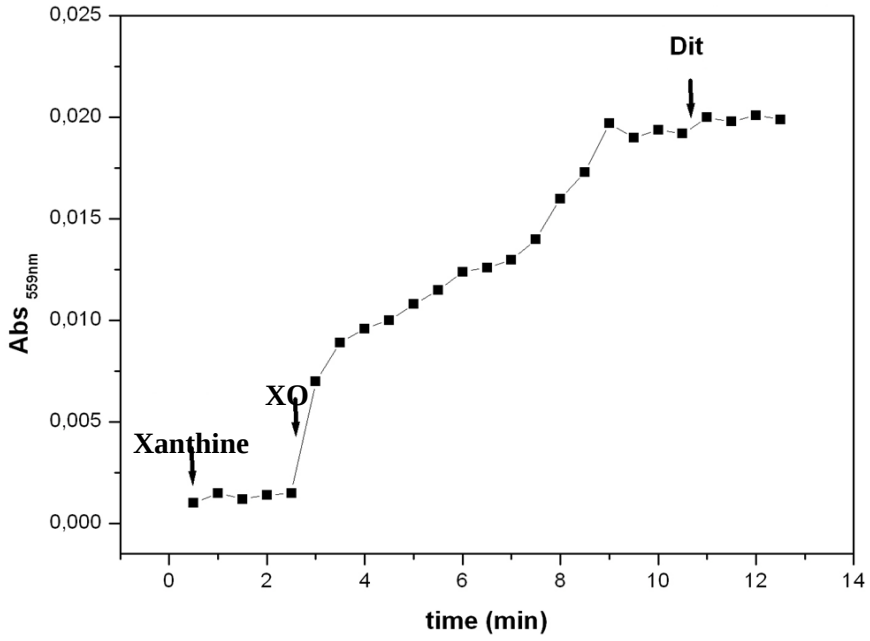


Fig. 17: **Cytochrome c reduction by superoxide.** 1 μM of cytochrome c from horse heart was used. XO: xanthine oxidase.

At difference with cytochrome c, reduced recAtAIR12 was unstable in open air when reductans were removed from the solution, and autoxidation could be slowed down by lowering the oxygen tension (not shown). At similar conditions used for superoxide tests, the maximal rate of recAtAIR12 autoxidation was 0.08 μM/min, i.e. 12-fold slower than the reduction by superoxide (Fig. 16), but 5-fold faster than cytochrome c autoxidation rate (Fig. 18).

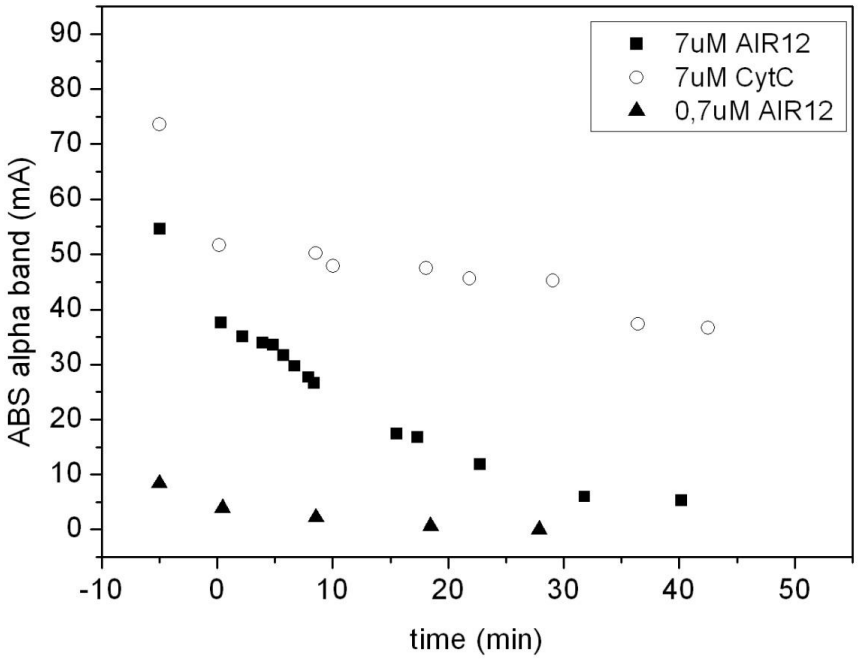


Fig. 18: **RecAtAIR12 autooxidation**. After the reduction with dithionite, recAtAIR12 and cytochrome c were passed through a Nap5 column in order to remove dithionite. The first point of the curve represents the fully reduced cytochrome before loading onto the coloumn; absorbance at time zero was obtained from the first measured spectrum after the desalting step.

Different from oxygen, hydrogen peroxide was instead unable to oxidize either recAtAIR12 or cytochrome c.

B-1.c AIR12 can work as pro-oxidant by promoting hydroxyl radical production by PM redox systems.

Plasma membrane preparations from different plants species and tissues have been previously shown to contain redox components that constitute a system able to produce hydroxyl radicals in the presence of suitable reductants (e.g. NADH) and transition metals (e.g. FeEDTA). A reasonable explanation to this activity is that plant PM preparations usually display (low) capacity to reduce oxygen with NADH, thereby producing superoxide that rapidly dismutates to hydrogen peroxide, either spontaneously or in a SOD-accelerated manner. NADH-

FeEDTA reductase activities are also a common property of PM preparations, and Fe^{2+} ions spontaneously react with H_2O_2 to produce hydroxyl radicals by the Fenton reaction. Provided that AIR12 is a PM protein that can react with both superoxide and oxygen, we wanted to test how it could interact with the hydroxyl-radical production system of the PM. In principle, AIR12 could either depress hydroxyl-radical production by removing superoxide and thereby displaying an antioxidant role, or stimulate hydroxyl-radical production by adding more superoxide to the system (AIR12 can reduce oxygen) and work as a pro-oxidant. To this aim we have purified PM vesicles from soybean etiolated hypocotyls. Hydroxyl radicals were detected by EPR spectroscopy using ethanol/a-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN) as spin-trapping system (Schopfer et al., 2002; Liszkay et al., 2004).

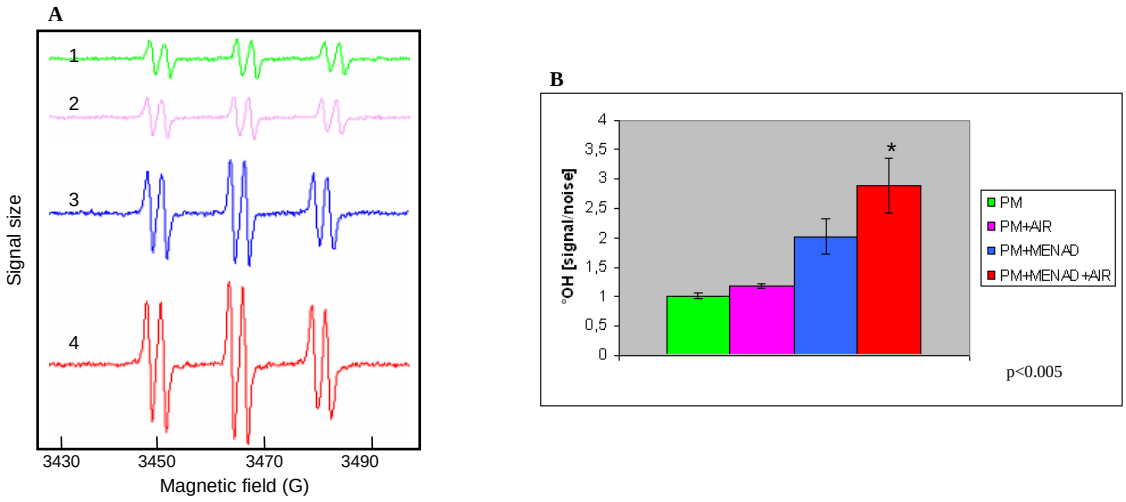


Fig. 19: **recAtAIR12 role in hydroxyl radical production by isolated PM.** A: EPR signals in different experimental conditions; 1: signal obtained by PM; 2: signal obtained by PM plus 1 µM of recAtAIR12; 3: signal obtained by PM plus 100 µM of menadione; 4: signal obtained by PM plus 100 µM of menadione and 1 µM of recAtAIR12. B: the same conditions as in panel A are reported as hydroxyl radical concentrations and the same colours are maintained. Values are means ± SE of 5 different experiments. Difference between PM plus menadione with or out of AIR12 is statistically significant ($p<0,005$). Amount of recAtAIR12 higher then 1 µM didn't give differences from results reported here.

In the presence of NADH (200 µM) and FeEDTA (50 µM), permeabilized PM vesicles (80 µg protein ml^{-1}) produced a basal amount of hydroxyl radical (Fig. 19A,B). The signal was low and unaffected by exogenous recAtAIR12 added at 1 µM. Hydroxyl radical production could be increased (2-fold) by the addition of a naftoquinone (100 µM menadione), suggesting that menadione semiquinone (MQ-) and/or hydroquinone (MQH_2) produced by NADH-dependent quinone reductases, that are also associated with the PM (Schopfer et al., 2008), may also

contribute to hydroxyl radical production. Interestingly, addition of 1 μM recAtAIR12 further stimulated this activity (3-fold; Fig. 19A,B) suggesting that AIR12, that is efficiently reduced by MQH2 (Fig. 20) but not by NADH, can work as an additional source of superoxide in this system. At least in these conditions, AIR12 behaves as a pro-oxidant, and alternative antioxidant roles (e.g. superoxide detoxification) seem less likely.

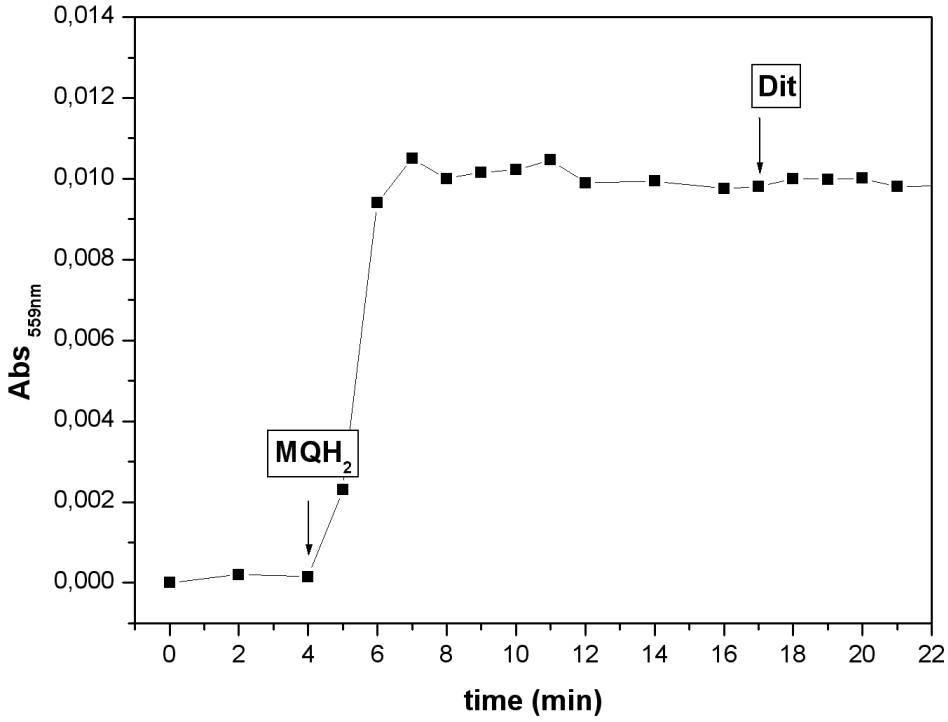


Fig. 20: **recAtAIR12 reduction by menadione.** MQH_2 : menadione; Dit: dithionite. Menadione (100 μM) efficiently reduces recAtAIR12 (0,5 μM). Further addition of dithionite didn't increase recAtAIR12 reduction indicating that menadione gave the total reduction of the protein. This is a representative experiment of three repetitions.

B-1.d *In vivo* localization of AtAIR12

These experiments were performed in collaboration with Dr. Alex Costa, department of Biology, Padova University.

AIR12 coding sequences typically contain a predicted N-terminal signal peptide for PM localization and a GPI-modification site in the C-terminal part of the protein (Preger et al., 2009). Both the N-terminal signal peptide, and the C-terminal peptide downstream the GPI modification site are expected to be cleaved off in the course of AIR12 maturation *in vivo*. Based on these predictions, AIR12 proteins are believed to be associated to plasma membranes.

In order to verify this prediction, a construct was made in which the coding sequence of the YFP was inserted between the N-terminal signal peptide (aa 1-34) and the rest of the coding sequence of soybean AIR12 (Glyma03g22260). The construct was placed under the 35S promoter and used for either transient transformation of tobacco leaves, or stable transformation of Arabidopsis plants (*Landsberg erecta* ecotype). The rationale was to obtain a chimeric protein that *in vivo* could be targeted to the correct location while keeping fluorescence after maturation. Fig. 21A,B shows very clearly that the 35S::AIR12-YFP-AIR12 construct drove the expression of the fluorescent chimeric protein in epidermal cells of both systems, and that YFP fluorescence was localized in the PM in these cells.

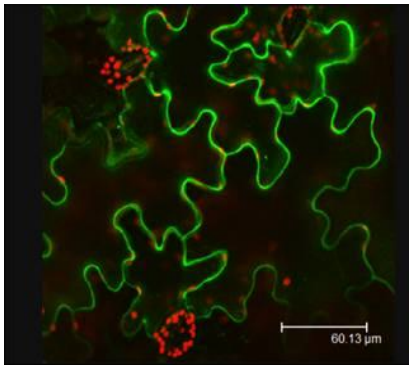


Fig. 21A. **AIR12 localization in tobacco leaves.** Tobacco leaves were infected with *Agrobacterium tumefaciens* carrying 35S::AIR12-YFP-AIR12construct. PM of epidermal cells is clearly colored in green, while stomata cells are in red.

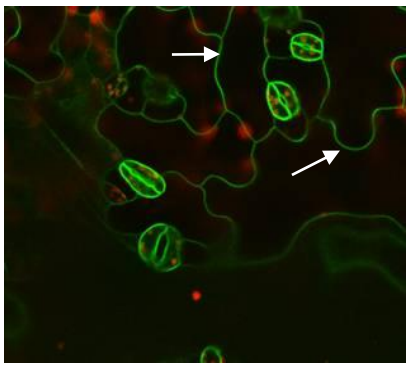


Fig. 21B. **AIR12 localization in Arabidopsis leaves.** Arabidopsis plants transformed with *Agrobacterium tumefaciens* carrying 35S::AIR12-YFP-AIR12 construct. PM of epidermal cells are green colored and indicated by white arrows.

The promoter region of Arabidopsis AIR12 (1554 bp) was cloned into suitable vectors to drive the expression of reporter genes (GUS and YFP) into stably transformed Arabidopsis plants. Analysis of promAIR12::GUS and promAIR12::YFP plants clearly showed that the expression of AIR12 tends to be very specific, both in space and time (Fig. 22). No promAIR12 activity was detected in early germinating seeds, but promAIR12::GUS seeds displayed a strong signal in the micropilar endosperm that is ruptured upon protrusion of the radicle (Fig. 22A). In seedlings, AIR12::GUS activity was first detected in stipules, and in tips and developing veins of cotyledons (Fig. 22B,C). Expression in roots was mostly associated to the root tip and sites of lateral root initiation (Fig. 22B,D). Detailed analysis of proAIR12::YFP seedlings roots indicated that AIR12 was indeed mostly confined to the lateral root cap (Fig. 22E) and in few epidermal cells covering lateral root primordia, or in epidermal cells surrounding the base of emerging, or even developed lateral roots (Fig. 22F,G,H).

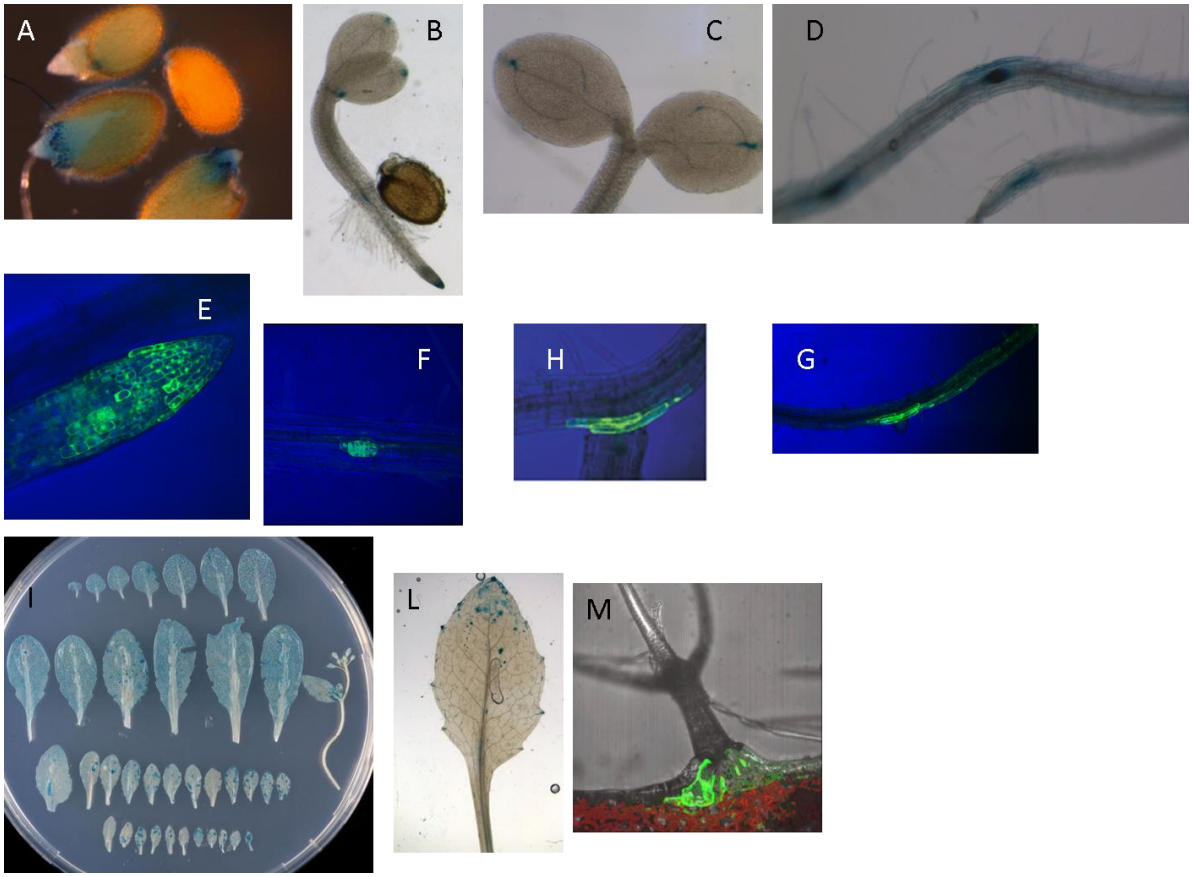


Fig. 22: **GUS and YFP expression driven by *Air12* promoter**. AIR12 expression was reported for different parts of the plant. A: AIR12::GUS activity in the endosperm of seed ruptured by radicle emerging; B, C: cotyledons showed AIR12::GUS activity in points associated to vein development and characterized by auxin accumulation; D, F: AIR12::GUS and AIR12::GFP activity was strongly

associated to sites of lateral root initiation; E: AIR12::GUS activity in cells of the root cap; H, G: AIR12::GUS activity was present in cells surrounding the site of emerged lateral root; I: AIR12::GUS activity in young leaves; L: AIR12::GUS activity in leaf was associated to hydatodes that are sites where water is released in order to transport the nutrients, contained in the water, from the roots to the leaves; M: the base of trichomes was characterized by a strong *Air12* promoter activity.

In adult plants, the expression of AIR12 was unevenly spotted in young leaf blades (Fig. 22I), but it was also associated to hydatodes (Fig. 22L) and to the base of trichomes (Fig. 22M) (Aloni et al., 2003). Finally, AIR12 was expressed in flowers, especially in pollen, in anther filaments, in the stigma and, very neatly, in the abscission zones of floral organs after shedding. Moreover it was expressed in seeds during silique maturation, possibly in seeds abscission zones (Fig. 23A, B, C).

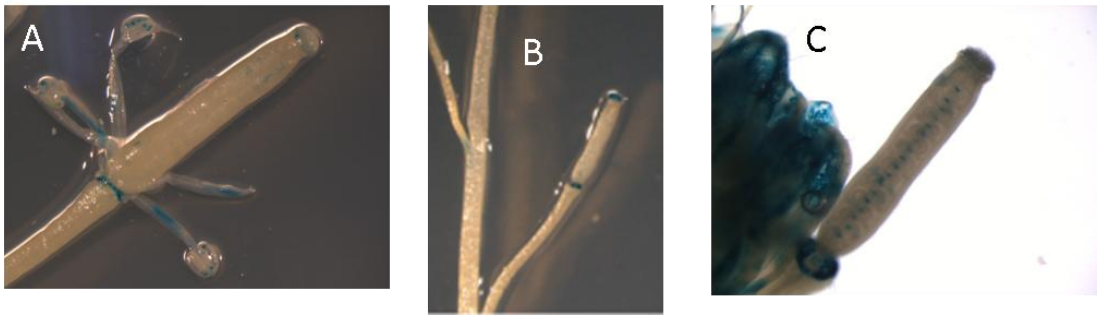


Fig. 23: **AIR12 expression in different abscission zones.**

B-1.e AIR12 expression in roots is induced by auxin

As expected, AIR12 expression was strongly induced by auxins. Two different synthetic auxins, 2,4-Dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (NAA), were tested on seedlings and AIR12::GUS activity was evaluated and compared to that of untreated seedlings. Control seedlings showed AIR12::GUS activity especially in root curves, in root tip and in some points of cotyledons,. All these sites are known to be characterized by auxin accumulation. Interestingly, the treatment with 2,4-D and NAA induced a generalised AIR12::GUS activity (Fig. 24) along seedlings indicating a strong induction of AIR12 expression.

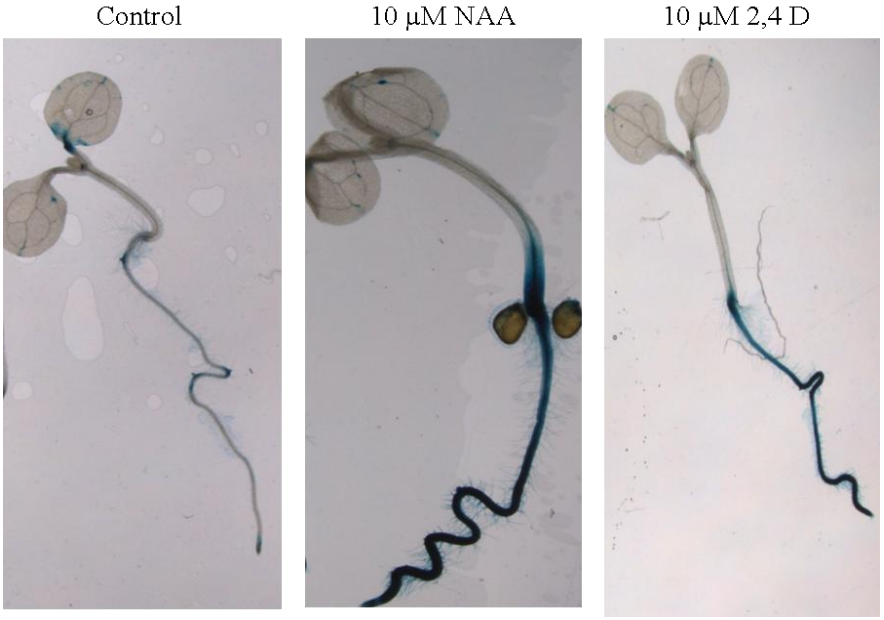


Fig. 24: **Auxins induced AIR12 expression.** Seedlings were grown in the presence or not of NAA or 2,4-D in plates on conventional medium (MS1/2).

B-1.f *Botrytis cinerea* infection

These experiments were performed in collaboration with Dr. Francesca Sicilia, Department of Biology and Biotecnology, University "La Sapienza", Roma.

The possible role of AIR12 in resistance to fungal infection was also tested. To this aim, adult control plants (wild type,wt), *air12* knock out mutants (*air12*-ko)and complemented plants were inoculated with *Botrytis cinerea*. Two days after inoculation, control plants displayed rapidly expanding water-soaked lesions of comparable diameter, while the lesions of *air12* – ko plants were significantly smaller indicating 60% increased resistance to *B. cinerea* infection compared to wt (Fig. 25). The acquired resistance in *air12* mutants was not lost in complemented plants that showed comparable susceptibility to wild type plants. Taken together, these results indicate that AIR12 is a negative regulator of resistance against the necrotrophic fungus *B. cinerea*.

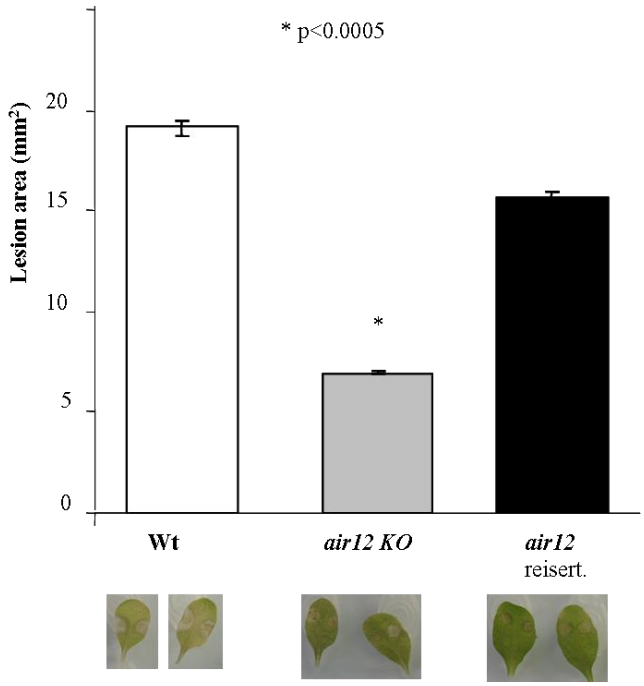


Fig. 25: ***B. cinerea* infection.** Lesion development 48 h after treatment with *B. cinerea* (3.9 x 103 spores per drop). Lesion area of air12-KO plants is smaller then that of wt and this difference is statistically significant (p‹0,0005). Values are means ± SE of 16 lesions. Asterisks indicate statistically significant differences against wt, according to Student's *t* test..

DISCUSSION

AIR12 is a b-type cytochrome made up by a DOMON domain and a GPI-anchor through which it is linked to the PM and exposed in the apoplast. There is only one gene (At3g07390) for AIR12 in *A. thaliana* genome. Looking for a function of AIR12 we started to characterize it *in vitro* with the aim of understanding its biochemical properties. Its redox activity shows a relatively wide specificity: AIR12 can accept electrons from ascorbate, quinones, superoxide, and donate them to monodehydroascorbate and oxygen and most likely the list of possible electron donors and acceptors is much longer than this. AIR12 capability to perceive AA redox state may be important in the apoplastic compartment where AA is considered the main antioxidant pool. But in this hypothesis, how AIR12 might transmit a signal derived from the apoplastic redox state of the ascorbate system is unknown.

In a membrane reconstituted system AIR12 increased the capability of the system to produce hydroxyl radicals in the presence of NADH, FeEDTA and a naftoquinone.

A possible mechanism of action for recAtAIR12 production of hydroxyl radicals *in vitro* is proposed in the following.

In our experimental conditions MQ would be reduced to MQH_2 by NAD(P)H:quinone reductase (NQR, Trost et al., 1995). MQH_2 is more stable at pH 5.5 than at alkaline pH (Liszkay et al., 2004) and it would react with AIR12 rather than with oxygen. Reduced AIR12 would instead react with oxygen thereby generating superoxide anion ($O_2^{\cdot -}$) which can dismutate into hydrogen peroxyde (H_2O_2) and oxygen. This reaction of dismutation is favoured by acidic pH and could easily happen in the apoplast. Superoxide can also reduce Fe^{3+} into Fe^{2+} (provided as Fe-EDTA in our *in vitro* assay) and H_2O_2 would then generate hydroxyl radical (·OH) by the Fenton reaction through the oxidation of Fe^{2+}. The following scheme resumes this hypothetical mechanism of action (Fig. 26).

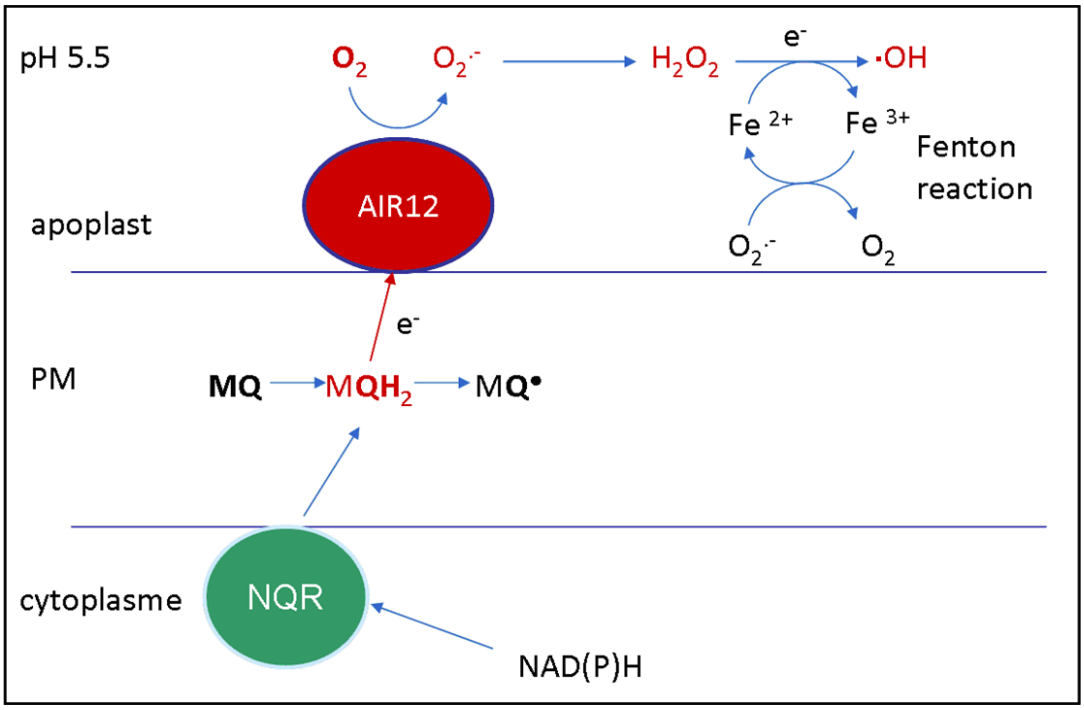


Fig. 26: **Hypothetical mechanism of action for AIR12.**

In spite of the presence of only one gene coding for AIR12 in *A. thaliana air12*-knock out (*air12*-ko) plants showed no phenotypic alterations. Different growth conditions were tried by changing temperature, photoperiod, light intensity as well as hormone treatments, but no differences were detected. The absence of a clear phenotype may depend on AIR12 belonging to a great protein family including several members also in *Arabidopsis* in which a DOMON domain similar to AIR12 is fused with a cytochrome b_{561} domain. In several conditions the absence of AIR12 was possibly balanced by an up-regulation of other members of these family of DOMON containg cytochromes.

Surprisingly we found out a particular condition in which a clear difference was observed between wild type plants and *air12*-ko plants: the latter ones showed an increased resistance against a necrotrophic fungus, *Botrytis cinerea*. Complemented plants, that is *air12*-ko plants transformed with the *air12* gene under the control of its own promoter, completely recovered the wild type phenotype, indicating that AIR12 was a negative regulator of *Arabidopsis* resistance against *B. cinerea*. The molecular reactions involved in *B. cinerea* infection cycle are still unclear. It seems that *B. cinerea*, like other necrotrophic fungi, uses oxalic acid as a virulent factor. Oxalic acid is secreted in the apolastic space of the host where it likely inhibits

the oxidative burst of the plant. Oxalic acid would induce an hyper-reduced state in infected cells and this condition would block the signalling pathway that involves calcium influx and leads to activation of NADPH-oxidases. In the absence of superoxide production, *B. cinerea* can establish the infection. However subsequent proliferation of the pathogen requires, in a second phase, a ROS production by the host to induce programmed cell death (PCD), thereby generating the substrate (dead cells) for its growth. So, necrotrophic fungi would suppress ROS production by the host in the first step of infection, but favour ROS production in a second step. We don't know how AIR12 may be involved in this infection cycle, but it is apparent that its redox activity and capacity to interact with ROS may be involved. Although any speculation on this point might be premature, we like to remind that AIR12 is structurally related to the cytochrome *b* domain of cellobiose dehydrogenase of lignolitic fungi, and this domain has been suggested to be involved in ROS production for lignocellulose degradation (Mason et al, 2002). Moreover, GUS and YFP expression driven by *air12* promoter was specifically localized at sites where cell separation processes occur such as micropilar endosperm, lateral root cap, sites of lateral root initiation, idatodes, floral organs and silique abscission zones. Cell wall modifications play a major role in cell separation and the involvement of ROS in these modifications was proposed by different groups (Schopfer et al., 2001, 2002, 2006; Fry et al., 1998).

The first site of contact between plant and pathogen is, of course, the cell wall and modifications of this structure are necessary in order to establish the infection. The apoplast is involved both in sensing this danger and alert cell to start defence responses, and in directly countering the offensive. The oxidative burst, for example, is an early response against pathogen attack and takes place in the apoplast. The molecular pathways through which environmental changes are transmitted by the apoplast to the inner cell are still largely unknown. As far as as redox changes are implied (e.g. ROS production and alteration of the redox state of ascorbate), the possibility of the existence of an electron flux through the PM is likely and transmembrane cytochromes may well be involved in such signalling pathway. AIR12 is not a transmembrane protein but it may be either a sensor of the redox state in the cell wall or may be involved in the modification of cell wall redox state. As matter of fact AIR12 interacts with the ascorbate pool which is key regulator of the apoplastic redox state. Also during *B. cinerea* infection, it is possible that AIR12 may be involved in cell wall modifications by interacting with ROS and ascorbate or it may be involved in a still hypothetical signalling process. Whichever the function of AIR12 *in vivo*, clearly its role is somehow exploited by *B. cinerea* to favour its own pathogenesis. Future studies aimed in

unravelling the physiological function of this enigmatic protein will certainly start from this fundamental observation.

MATERIALS AND METHODS

B-2 Chemicals

All chemicals were from Sigma-Aldrich (Milan, Italy) or Merck (Milan, Italy) unless otherwise stated.

B-2.a Expression of recombinant AIR12 of Arabidopsis thaliana in *Pichia pastoris*

The full length cDNA of AIR12 of *A. thaliana* (*Columbia*) was cloned directly from amplified genomic DNA since the gene contains no introns. Arabidopsis genome was amplified by means of sense primer 5'-AAAGGATCCATGTCCCTGTGTCTTAAAATACC-3' containing the BamHI restriction site (underlined) right before the ATG transcription starting codon, and antisense primer 5'AATGAATTCGAGAGACTAGAGAGAGATCC-3' containing the EcoRI restriction site (underlined). DNA was then inserted into the plasmid p35S-2 (http://www.pgreen.ac.uk) after digestion of both plasmid and PCR products by means of the two restriction enzymes. This cloned cDNA was then used as a template for the cloning of a truncated form of it into the NotI and EcoRI sites of the expression vector pPICZαB (Invitrogen) in frame with the c-myc epitope and a polyhistidine tag. Sense and antisense primers were 5'-TAGCGGCCGCAGCAGACCCCGGCGAAG-3'and 5'-TGAATTCAGGCTTGCAAATCACAGAACTTC-3' respectively (NotI and EcoRI sites are underlined). The oligonucleotides amplified a region of the gene right after the N-terminal leader peptide encoding sequence. In order to recover the recombinant protein in the culture medium, avoiding GPI-anchoring to the yeast membranes, the sequence coding for the C-terminal GPI signal peptide was also omitted from the amplified cDNA. The PCR amplified cDNA was cloned into the pPICZαB vector and used to transform P. pastoris X-33 cells (Invitrogen) by electroporation. Transformants were selected on YPDS medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar] containing 1.5 mg/ml Zeocin™. Positive clones were tested for integration by direct PCR screening. For protein expression a single colony of P.pastoris X-33/ pPICZαB-AIR12 was used to inoculate 100 ml

of BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) Yeast Nitrogen Base, 4 × 10-5% (w/v) biotin, 1% (v/v) glycerol] containing 100 μg/ml Zeocin™. Cells were grown overnight (28°C, 220 rpm in buffled flasks) until OD_{600} reached a value of 4, then pelleted (1,500 g for 10 min) and resuspended in 400 ml of BMMY inducing medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) Yeast Nitrogen Base, 4 × 10-5% (w/v) biotin, 1% (v/v) methanol] containing 0.1 mM δ-aminolevulinic acid and 40 μM hemin. Cultures were grown (28°C, 220 rpm) for 48 h in inducing medium with the addition of 2% (v/v) methanol every 24 h.

B-2.b Purification of recombinant AIR12

Chromatographic media and systems used for purification were from GE Healthcare. Two days after induction cultures of P.pastoris X-33/ pPICZαB-AIR12 were spun down (10 min at 1500 g). The supernatant (220 ml) was concentrated to 10 ml (Amicon YM-10) and equilibrated to pH 5.5 with 1 M MES. The sample was then centrifuged at 35,000 g for 1 h at 4 °C, to remove excess hemin (which precipitates at pH 5.5), then $(NH4)_2SO_4$ was added to a final concentration of 1.7 M prior to loading on a Octyl Sepharose 4 Fast Flow column (12 ml) equilibrated with 50 mM potassium phosphate pH 7, $(NH4)_2SO_4$ 1.7 M. RecAtAIR12 containing fractions, eluting in the column flow through, were pooled, diluted twice with water to lower salt content and concentrated to 7.5 ml (Amicon YM-10). Proteins were then equilibrated with 50 mM Tris-HCl pH 7.0 by means of PD-10 columns and loaded on an anion-exchange SourceQ column (7 ml) equilibrated with the same buffer. A linear KCl gradient (0-0.5 M in 3 column volume) was applied at a 0.5 ml min-1 flow rate, and 0.4-ml fractions were collected. RecAtAIR12 containing fractions were pooled, concentrated to 0.2 ml and loaded on a Superdex 200 (HR 10/30) gel filtration chromatography column equilibrated with 50 mM Tris-HCl pH 7.0, 150 mM KCl. Elution was carried out at 0.5 ml min-1 and fractions of 0.2 ml were collected.

B-2.c Heme content analysis

Heme content of recAtAIR12 samples was analyzed using the pyridine hemocrome method as described in Metzger et al. (1997). Heme b content was calculated from dithionite-reduced minus ferricyanide-oxidized spectra of pyridine hemochrome using a differential extinction coefficient (557-541 nm) of 20.7 mM^{-1} (Porra and Jones, 1963). Using this assay the reduced-minus-oxidized extinction coefficient for recAtAIR12 heme at 560 nm relative to the isosbestic wavelength of 571 nm was calculated to be 20 mM^{-1}.

B-2.d Assay of protein and AIR12 content

Total protein was assayed by the method of Bradford. Recombinant AIR12 content was calculated from dithionite-reduced-minus-oxidized spectra (ε560-571 20 mM^{-1}), considering a stoichiometry of one heme per AIR12 molecule.

B-2.e Spectrophotometric analyses

All spectrophotometric analyses were performed by means of a double beam Uvikon 941 Plus spectrophotometer (Kontron, Milano). A quartz cuvette with an internal volume of 0.11 ml and a path length of 10 mm was used.

B-2.f Protein deglycosylation

For protein deglycosylation Endo H (New England BioLabs) was used according to the manufacturer's instructions. Incubation of denatured samples with Endo H was carried out at 37 °C for 1 h.

B-2.g Electrophoretic analysis

SDS-PAGE was performed on 12.5% acrylamide gels. Samples were boiled for 10 min in sample buffer prior to loading. Gels were stained with Coomassie Brilliant Blue R-250. The staining of glycoproteins was done according to Leach et al. (1980).

B-2.h Ascorbate titration and MDHA reaction

For ascorbate titrations, 1.9 μM of recAtAIR12 in 50 mM MES (pH 5.5) was placed in a 1-cm pathlength quartz cuvette and titrated with 23 increments of ascorbate between 0.1 μM and 20 mM. All ascorbate stock solutions were freshly made, and kept on ice and protected from light. The increases in Abs_{559nm} were fitted by nonlinear regression.
1.3 μM of recAtAIR12 in 50 mM MES (pH 5.5) was reduced by 0.6 mM acorbate and spetra were recorded until reduction was stable. 1 unit of ascorbate oxidase was added and when recAtAIR12 was stably oxidized 1 mM of ascorbate was added.

B-2.i Oxygen reaction

7 μM of recAtAIR12 in 50 mM MES pH 5.5 was placed in a 1-cm pathlength quartz cuvette and reduced by 500 μM of dithionite. Reduced recAtAIR12 was loaded on a Nap5 column equilibrated with 5 volumes of 50 mM MES (pH 5.5); to elute protein, 100 μl of 50 mM MES pH 5.5 were loaded and eluted material was discarded, then 600 μl of 50 mM MES pH 5.5 were loaded and eluted sample, that is free of dithionite, was collected and spectrophotometrically analysed. Subsequent spectra were recorded in order to follow recAtAIR12 oxidation by oxygen. Experiment was repeated using 0.7 μM of recAtAIR12 and 7 μM of cytochrome c from horse heart for comparison.

B-2.l Plasma membranes preparation for EPR analysis

Plasma membranes used in EPR experiments were prepared from soybean roots grown on wet vermiculite in darkness for 4.5 days at 25 °C. The procedure described by Thein and Michalke (1988) was adopted with some modifications. Batches (100 g) of soybean hypocotyls were homogenized with a blender in 400 mL of cold medium containing 10 mM Tris-HCl (pH 8.0), 20 mM Na2EDTA, 300 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 g L^{-1} bovine serum albumin. After filtering the slurry through nylon cloth, a membrane fraction was isolated by differential centrifugation (30 min at 1,500g, 45 min at 91,000g, 4°C) and resuspended in 180 mL of medium (without PMSF and albumin). Intracellular membranes were precipitated by adding 0.043 g mL^{-1} polyethylene glycol 6000 and stirring for 30 min on ice. After removing the precipitate by centrifugation (30 min at 1,500g, 4°C), the plasma membranes contained in the supernatant were pelleted (45 min at 91,000g, 4°C), resuspended in 20 mm Hepes (pH 7.5) and stored at −70°C. Plasma membrane preparations were permeabilized by adding an equal volume of 0.05% (w/v) Triton X-100 in 20 mM Hepes, pH 7.5, and incubated for 30 min at 4° C before starting the assays.

B-2.m ·OH detection by EPR spectroscopy

Plasma membranes (80 μg protein ml-1) were incubated in tubes for 10 min in Britton-Robinson buffer, pH 5.5, 50 mM POBN, 4% ethanol, 200 μM NADH and 50 μM FeEDTA with or without recAtAIR12 (1μM) and menadione (100 μM) in a total volume of 120 μl at 25°C (Janzen et al. 1978). Aliquots of 100 μl were then transferred to a flat cell and EPR spectra were recorded at room temperature with a Bruker 300 X-band spectrometer (Bruker, Rheinstetten, Germany) at 9.69 GHz microwave frequency, 63 mW microwave power and 100 kHz modulation frequency, 1 G modulation amplitude.

A stock solution of menadione was prepared fresh in dimethyl sulfoxide before each experiment.

B-2.n Generation of *AIR12* promoter YFP and GUS lines

The YFP (Yellow Fluorescent Protein)-coding sequence and the GUS (β-glucuronidase)-coding sequence were both fused to the *AIR12* promoter (base positions -1554 to -1). The

1554 bp promoter fragment was amplified by PCR using genomic DNA extracted from Arabidopsis leaves as template. The pair of primers, both carrying an EcoRI restriction site, were as follows: forward primer 5'-CATGGAATTCTGACCGAGAGATATTCAGTTCA-3' and reverse primer 5'- CATGGAATTCGTGATGTTTATATAGAAGGGCAATG-3'. After digestion, the promoter was cloned upstream of GUS or YFP coding regions into a modified pGreen0029 binary vector (Hellens et al., 2000, Valerio et al., 2011), where the GUS or YFP coding sequence, fused with the *nos* terminator, was previously inserted in the polylinker between KpnI-SacI restriction sites. The pGreenAIR12promoter::GUS or pGreen AIR12promoter::YFP construct was transferred into GV3101-pSoup Agrobacterium strain (Hellens et al., 2000) and Arabidopsis plants (Col-0) were transformed by floral dip method (Clough and Bent, 1998) and screened on half-strength Murashige and Skoog basal salt mixture (MS) agar medium containing 50 mg L-1 kanamycin. The presence of the insertions was confirmed by PCR on genomic DNA with the following specific primers: forward primer inside AIR12 promoter sequence 5'-CTCACATTGCCCACCTCTATAAAT-3'; reverse primer inside the GUS coding sequence 5'-GCGAACTGATCGTTAAAACTGC-3'; reverse primer inside the YFP coding sequence 5'-CGGTGGTGCAGATGAACTT-3'. Seven and three independent AIR12promoter::GUS and AIR12promoter::YFP T1 plants, respectively, were screened and selected for comparable localization of reporter genes. Subsequent work was conducted on T2 plants. Three independent GUS and YFP selected lines were used for the experiments. The data showed are the most representative ones.

B-2.o RT-PCR analysis

In order to perform the RT-PCR analysis total RNA extracted from 10 days old Ler wt and *air12-ko* seedlings was extracted and the cDNA was synthesized as previously described (Costa et al., 2010). The PCR was performed using primers designed at the 5'- and 3'-END of *AIR12* coding sequence. The forward and reverse primers were the follows: 5'-AAACAAGCTTCATGTCCCTGTGTCTTAAAATACC-3' and 5'-AATGAATTCGAGAGACTAGAGAGAGATCC-3'. As internal control of cDNA quality we used the *GAPC-1* (At3g04120) with the following forward and reverse primers: 5'-GCAAAGACGCTCCAATGTTTGTTG-3' and 5'-GAAGCACCTTTCCGACAGCCTTG-3'. The PCR reaction was performed by using the GoTaq polymerase (Promega) with the

following parameters: 30 sec at 94°C, 30 sec at 58°C and 60 sec at 72°C. The PCR reaction was stopped at 35 cycles for *AIR12* and 25 cycles for *GAPC-1*.

B-2.p Complementation of *air12-KO* mutant

The complemented lines were obtained by the transformation of *air12*-ko mutant with a 2802 bp genomic fragment of *AIR12*. The selected genomic region include 1554 bps of promoter region, 822 bps corresponding to the *AIR12* coding sequence and 426 bps downstream the stop codon. The genomic sequence was amplified by PCR using the same forward primer, used for the promoter amplification, in combination with the following reverse primer: 5'-CATGCTCGAGGCTAGAGAGTCTAATGTTGCCCTAATCA-3' where an XhoI restriction site was introduced. The amplicon was digested with EcoRI and XhoI restriction enzymes and ligated into the pGreen0229 binary vector (Hellens et al., 2000). The obtained clone named pGreen0229-AIR12full-genomic was introduced into the *Agrobacterium tumefaciens* GV3101 strain and the *air12-KO* line were transformed by floral dip method (Clough and Bent, 1998) and screened on half-strength MS agar medium containing 250 mM of BASTA.

B-2.q Histochemical GUS assay and detection of YFP fluorescence

Histochemical GUS assays, and YFP detection was performed as previously described (Valerio et al., 2011)

B-2.r Generation of N-YFP-AIR12 transgenic line

The coding sequence of soybean *AIR12* was spitted in two parts an N-terminal and C-terminal part. The first 34 aa of the N-terminal end were fused upstream the YFP coding sequence in the pAVA554 vector (von Arnim et al., 1997) and then the C-terminal end was placed downstream the YFP. The construct was under control of the double 35S promoter and *nos* terminator. The entire cassette 2X35S-N-YFP-soybeanAIR12-Ter was then subcloned in the pGreen0029 binary vector (Hellens et al., 2000) and introduced in *Agrobacterium tumefaciens* GV3101 strain. Wild type Arabidopsis (Ler ecotype) were afterwords transformed by floral

dip method (Clough and Bent, 1998) and screened on half-strength MS agar medium containing 50 mg/L of Kanamycin.

B-2.s Seedlings treatment with synthetic auxins

GUS line seedlings were grown on half-strength MS agar medium supplied or not with two synthetic auxins: 2,4-dichlorophenoxyacetic acid (2,4-D) or naphtalene acetic acid (NAA). 2,4-D stock solution was prepared in water, while NAA stock solution was prepares in ethanol.

B-2.t Infection of leaves with *Botrytis cinerea*

B. cinerea growth assays were performed on detached leaves as previously described (Ferrari 2007). Rosette leaves from 4-week-old soil-grown Arabidopsis plants were placed in petri dishes containing 0.8% agar, with the petiole embedded in the medium. Inoculation was performed by placing 5µl of a suspension of 5 x 105 conidiospores ml-1 in 24 g l-1 potato dextrose broth (PBD) on each side of the middle vein. The plates were incubated at 22 °C with a 12h photoperiod. High humidity was maintained by covering the plates with a clear plastic lid. Lesion size was determined by measuring the diameter or in case of oval sessions, the major axis of the necrotic area.

C. Chapter II: CyDOM: a multidomain cytochrome b looking for a function

Aim of project

The second protein I studied during my PhD is called CyDOM standing for cytochrome plus DOMON because it is predicted to be a DOMON domain linked to a transmembrane cyt b_{561} (Tsubaki et al., 2005). This protein has never been characterized and so I tried to express it heterologously in order to demonstrate its cytochromic nature and then to define its biochemical properties. Firstly I tried to express the whole protein in *P. pastoris*, but unfortunately there were any sign of expression under any conditions. Then I cloned the two parts of the protein, the DOMON domain and the transmembrane cyt b_{561}, separately in order to express the DOMON domain in *P. pastoris* and the transmembrane portion in *E. coli* following a recent protocol.

Introduction

It has been demonstrated that plasma membranes of different plant species and tissues contain ascorbate-reducible cytochromes *b* (Asard et al., 1992; Trost et al., 2000; Preger et al., 2001), moreover it has been shown that ascorbate-loaded, right-side out plasma membrane vesicles from bean hypocotyls can reduce externally added MDHA and show transient oxidation of - cyt b_{561} (Asard et al., 2001). It was then concluded that plant plasma membranes contain cyt b_{561} with similar activity to animal cyt b_{561} of chromaffin granules. Animal chromaffin glands, in fact, contain a well characterized cyt b_{561} which mediates intravesicular AA regeneration at the expense of cytosolic AA during the biosynthesis of noradrenaline by dopamine β-hydroxylase within the vesicle (Njus et al., 1983; Harnadek et al., 1985; Wakefield et al., 1986; Menniti et al., 1986; Kent et al., 1987; Seike et al., 2003). Other two cyts b_{561} have been identified in mammals in the duodenal plasma membrane (McKie et al.,2001; Vargas et al., 2003) and in macrophage lysosomes (Zhang et al., 2006). Duodenal cyt b_{561} and chromaffin granule cyt b_{561} can catalyze the reduction of ferric-iron chelates when expressed in *Xenopus* oocytes (Vargas et al., 2003), moreover these two proteins together with lysosomal cyt b_{561} showed AA-dependent transmembrane ferrireductase activity when expressed in yeast cells (Su and Asard, 2006). Ferri-reductase activity of these proteins suggests their possible involvement in iron metabolism: a new speculative hypothesis on their possible physiological functions in mammals.

Drosophila SDR-2, an ortologue of plant CyDOM, has been suggested to catalyze a trans-plasma membrane electron transfer when heterologously expressed in *Xenopus oocytes* (Vargas et al., 2003). Plants contain cyts b_{561} (Asard et al., 2001) with aminoacid sequences and *in silico* properties similar to animal counterparts and although the clear evidence of cyt b_{561} presence on plant plasma membrane, this protein has never been isolated. The only PM ascorbate-reducible cyt *b* identified is AIR12 that, however, is linked to the PM by means of a GPI anchor and so can not be directly involved in trans-plasma membrane electron transfer.

Genome *in silico* analysis showed that cyt b_{561} domain can be associated to other domains in different redox proteins in animals, fungi and plants. In plants, multi-domain proteins containing cyt b_{561} almost always contain also a DOMON domain and *A. thaliana* has eleven genes coding for proteins made up by a cyt b_{561} linked to a DOMON domain. These multi-domain proteins are easily called CyDOM and except one (At4g18260), all the others present the DOMON domain at the N-terminal respect to the cyt b_{561}, which usually has only five transmembrane helices (helices 2-5) instead of six. As said above the DOMON domain can

be itself a cyt *b* and so CyDOM proteins may represent the association of two redox domains and having a trans membrane portion they can be directly responsible for trans membrane electron transfer. Proteomic studies identified only three CyDOMs in *A. thaliana* purified plasma membrane preparations (Elortza et al., 2003; Alexandersson et al., 2004) but none of them has been characterized.

Results

C-1.a In silico CyDOM of *Glicine max* sequence analysis

CyDOM of *Glicine max* (GmCyDOM, GenBank acc. BI973773) sequence has been used to perform different prediction analysis. The first has been done using PSORT, a tool to predict protein localization in cells and which revealed the presence of an N-terminal signal peptide sequence for plasma membrane localization. (Fig. 27A).

TMHMM server analysis showed the prediction of six hypothetical transmembrane (TM) motifs in GmCyDOM sequence (Fig. 27B). Of the six, five TM motifs are located in the *b-561* domain and one corresponds to the N-terminal signal peptide.

A

MVGKLVVVRLVLAISVLSSLLLTTSAQTACKGQAFTENKVFTTCRDLPHLSSYLHWTFDQATGKLDIAFRHTGISGT

DKWVAWAINPSNNLNSAMTGAQALVAIIPSSGAPNAYTSSIANPGTTLAEAISYNHSGLTATHQSTEVTIYATLTLPS

GTTTLVHLWNDGPVSSGTPAMHSMTSSNTQSKESLDLLSGSSQAGSGNSLRRRRNVHGVLNALSWGILMPVGAIIA

TM1

RYLKVFKSADPAWFYLHVTCQTSYIVGVAGWGTGLKLGSDSVGIKYNTHRALGITLFCLGTLQVFALLLRPNKDHK

TM2 TM3

IRIYWNIYHYAVGYSTIIISIINVFKGFDALETSVGDRYNDWKHAYIGIIAALGGIAVLLEAYTWIVVLKRRNSNKTAH

TM4 TM5

GVNGTNGYGFRGQQV

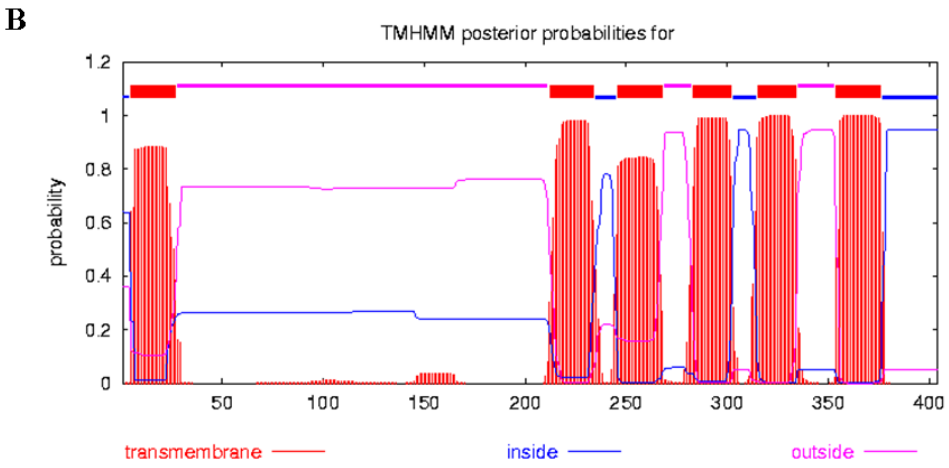


Fig. 27: **Gm CyDOM sequence analysis.** A: Signal peptide predicted sequence is red coloured, DOMON domain is yellow coloured, *b-561* core is underlined, predicted TM helices are in green and potential heme (Met and His) binding residues are in blue. Results by PSORT prediction analysis: http://psort.ims.u-tokyo.ac.jp; B: TM motifs prediction by TMHMM Server v. 2.0 analysis, CBS, Denmark.

C-1.b Gm CyDOM expression in *Pichia pastoris*

Soybean CyDOM cDNA, devoid of the N-terminal signal peptide sequence, has been cloned in the expression vector pPICZαB downstream of the alcohol oxidase 1 (*AOX1*) promoter and then used to transform *Pichia pastoris* X-33 strain by electroporation. A control culture, obtained transforming X-33 cells with pPICZαB (empty vector), has been used. *P. pastoris* transformation with exogenous DNA can give multi-integration events which results in high growth rate on increased zeocin concentrations. Transformants with multi-copies of CyDOM cDNA were screened by testing their hyper-resistance to Zeocin (Fig. 28). Positive multi-copies clones (X-33/pPicZαB/CyDOM) were cultured for 72 h and the induction of CyDOM expression was obtained by methanol, the *AOX 1* inducer. At the end of the induction solubilised membranes were prepared and spectrophotometrically tested by looking for a *b-type* cytochrome signal (Fig. 29). Unfortunately, also using different growth conditions, such as methanol concentration or induction time, no clear signal of expression was obtained.

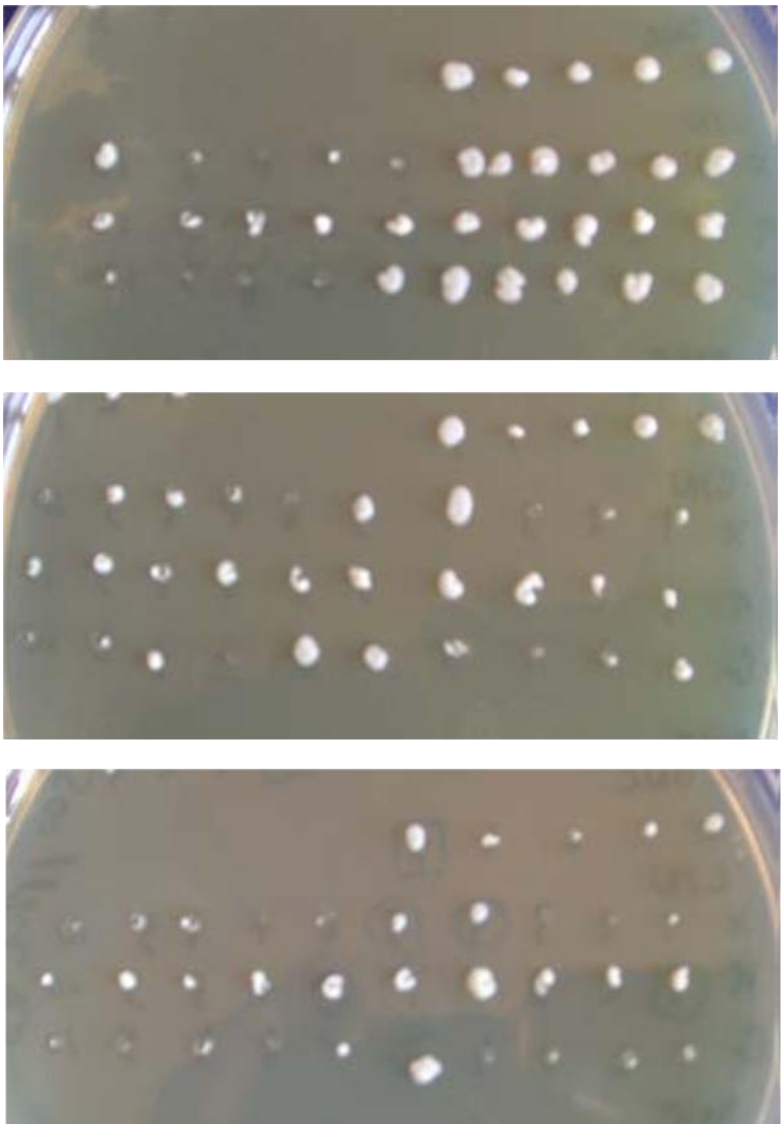


Fig. 28: **Hyper resistant transformants.** Each colony has been picked in the same position on the three plates. Multi copy recombinant grew well also on high Zeocin concentration

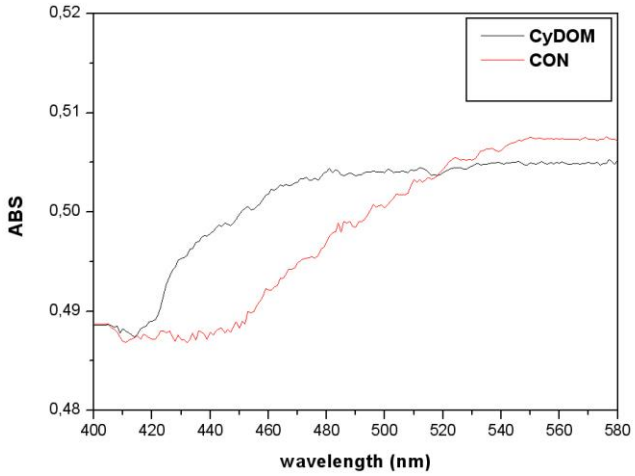


Fig. 29: **Solubilised membranes spectrophotometrical analysis**. Differential absorption spectra of solubilized microsomal membranes from *Pichia* cells expressing CyDOM (black line) compared with control culture (red line). The two spectra are ascorbate-reduced minus oxidized; ascorbate has been added in grains.CON: control culture.

C-1.c Alternative expression systems to express the two portion of CyDOM

Because of the difficulties to express the whole CyDOM in *P. pastoris* we decided to express the two portions of CyDOM in two different system. Precisely, we chose to express the DOMON domain in *P. pastoris* considering the good result obtained expressing AIR12 which is similar to CyDOM DOMON domain, and *E. coli* to express the transmembrane portion, that I will call Cy(DOM). This last domain is predicted to be a cytochrome b_{561} and we followed two similar and very recent protocols of adrenal and duodenal human cyt b_{561} expression in *E. coli* (Liu et al 2007, 2011) to express this portion.

C-1.c.1 CyDOM transmembrane prortion expression in *Escherichia coli*

E. coli BL21Star(DE3) cedlls were firstly transformed with pT-groE and then with pET29-Cy(DOM), where Cy(DOM) corresponded to transmembrane portion cDNA plus an *E. coli* signal peptide to have the correct localization of the protein in the plasma membrane . The recombinant Cy(DOM) including hexa-histidine tag is expected to have a molecular weight of 26 kDa.

BL21Star(DE3) strain carries a mutated *rne* gene that encodes a truncated RNase E protein lacking the capacity to degrade mRNA and leading to higher mRNA stability and enhanced protein expression. Together with the choice of this strain, recombinant protein expression was carried at low temperature (20°C) with the addition of heme and δ-aminolevulinic acid to help *E. coli* to synthesize the heme. A control culture consisting of BL21Star(DE3)-pTgroE transformed with pET29 was used.

Cells were collected before and after induction by IPTG (1 h, 2 h, 3 h, 24 h, 48 h after adding IPTG); they were than harvested, resuspended in SB1X and loaded on SDS-PAGE (Fig. 30).

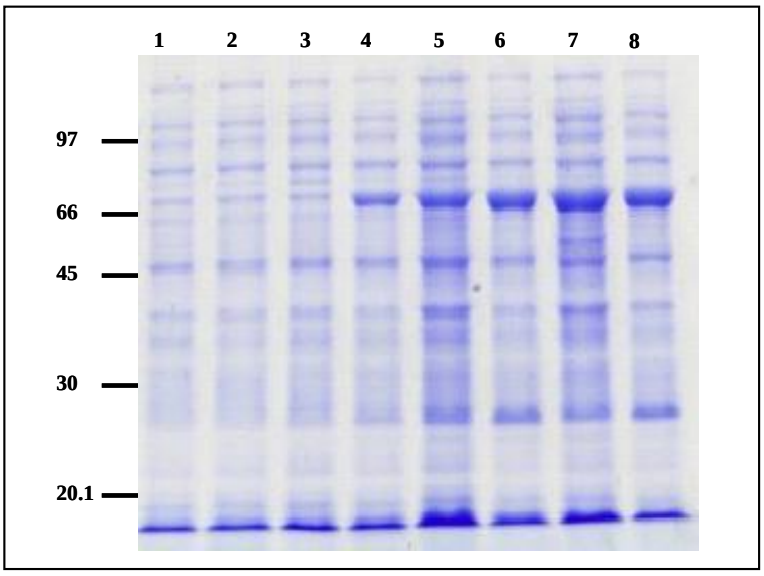


Fig. 30: **SDS-PAGE of pre- and post-induction cells.** CON= control culture cells; Cy(DOM)= pET29-Cy(DOM) transformed cells; Lanes 1 and 2= respectively CON and Cy(DOM) cells before induction; Lanes 3 and 4= respectively CON and Cy(DOM) cells 3 h post induction; Lanes 5 and 6= respectively CON and Cy(DOM) cells 24 h post induction; Lanes 7 and 8= respectively CON and Cy(DOM) cells 48 h post induction. The same amount of cells was loaded for each sample on SDS-PAGE on the basis of OD values measured for each collected sample. Molecular weight standards were reported on the right.

In the gel above the band profile of the two cultures was very similar and it was impossible to recognize a band indicating the expression of the recombinant protein. The presence in the

control culture of bands near the molecular weight expected for our recombinant protein (that is 26kDa) and their intensity increase during cell growth indicated that only a strong recombinant expression should be revealed.

C-1.c.2 Plasma membrane solubilization and recombinant protein extraction

Cells from 48 h induction were used to extract plasma membrane proteins. After high speed centrifugation, plasma membranes were separated from other cell compartments, that are present in the supernatant (SNMT), and then treated with a mild detergent, n-Dodecyl-β-D-maltoside. At the end of solubilization, solubilized proteins were collected in the supernatant, but also the precipitated material (PSOL) was resuspended and used for a Western Blot including also plasma membranes before solubilization and supernatant derived from plasma membrane (SNMT) collection (Fig. 31). All this samples were also used for spectroscopic analysis but no differences were observed, probably because of the presence of endogenous cytochrome signals from *E.coli* which hid the recombinant protein signal. The use of antibodies against the histidine tag at the C-terminus of the recombinant protein is a more sensitive method than spectrophotometric analysis to reveal recombinant Cy(DOM) (Fig. 31).

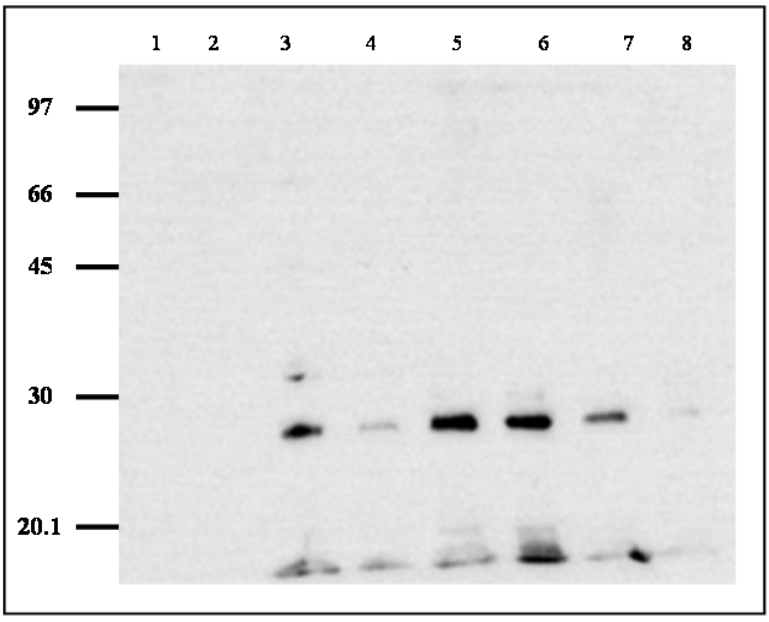


Fig. 31: **Western-blot on samples derived from different steps of solubilization**. CON= control culture cells; Cy(DOM)= pET29-Cy(DOM) transformed cells; SNMT=supernatant after total plasma membranes precipitation; MT=total plasma membranes; SOL=solubilised plasma membrane;

PSOL=precipitated material after solubilisation**;** Lanes 1 and 2= respectively Cy(DOM) and CON SNMT; Lanes 3 and 4= respectively Cy(DOM) and CON MT; Lanes 5and 6= respectively Cy(DOM) and CON PSOL; Lanes 7 and 8= respectively Cy(DOM) and CON SOL. Molecular weight standards were reported on the right.

Western Blot analysis showed principally an aspecific signal in the control lanes, possibly produced by endogenous proteins containing close histidine residues recognized by antibody. No signal was detected in other material than plasma membranes. Lane 3 corresponding to cells transformed with pET-Cy(DOM) showed a similar signal, but stronger, then the one present in the control culture (lane 4).

C-1.c.3 Purification of Cy(DOM) recombinant

Efforts to purify the recombinant protein were done using affinity chromatography on cobalt ion affinity resin, but they were unsuccessful (not shown). So I loaded solubilized samples on a MonoQ column. Two chromatographies were performed: one for each cell culture, loading the same amount of total protein (2,8 mg, estimated by Bradford method) and the same volume. The resulting chromatograms are reported below (Fig. 32).
The column (Mono Q 5/50 GL) was equilibrated with 50 mM potassium phosphate buffer (pH 7.0) and 0,08% (w/v) DM. Elution was performed by means of a continuous KCl gradient (0-1 M KCl in 25 ml, see methods) and monitored by simultaneous detection at 414 and 428 nm. Fractions of 0.5 ml were collected.

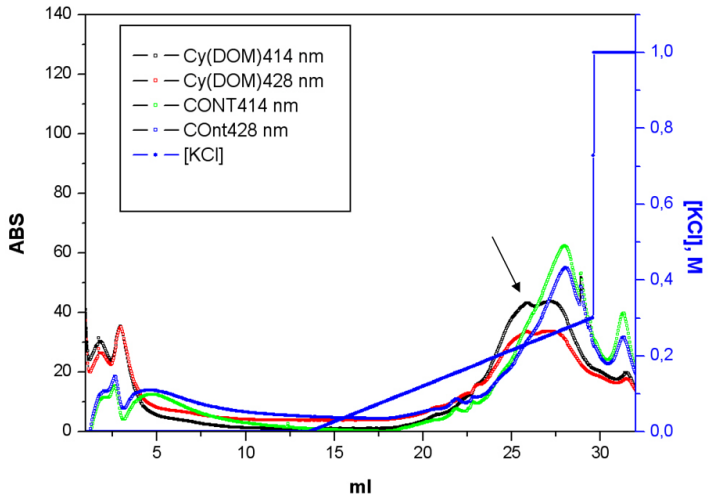


Fig. 32: **Anion exchange separation of solubilised samples.** The column (Mono Q 5/50 GL) was equilibrated with 50 mM potassium phosphate buffer (pH 7.0) and 0,08% (w/v) DM. Elution was performed by means of a continuous KCl gradient (0-1 M KCl in 25 ml, see methods) and monitored by simultaneous detection at 414 and 428 nm. Fractions of 0.5 ml were collected. Arrow indicates a component not present in control chromatogram.

The two chromatograms were very different in spite of very similar 280 nm elution profiles (not shown) confirming that the same total amount of protein was loaded. Cy(DOM) sample chromatogram showed a component that seemed to be absent in the control culture. This pick eluted at low salt concentration (17% of KCl containing buffer). Fractions eluted from Mono Q column were loaded on SDS-PAGE and interesting differences were found analysing nine fractions from 25 ml to 29 ml (Fig. 33). The first fraction (25 - 25.5 ml) in this interval was fraction number 31.

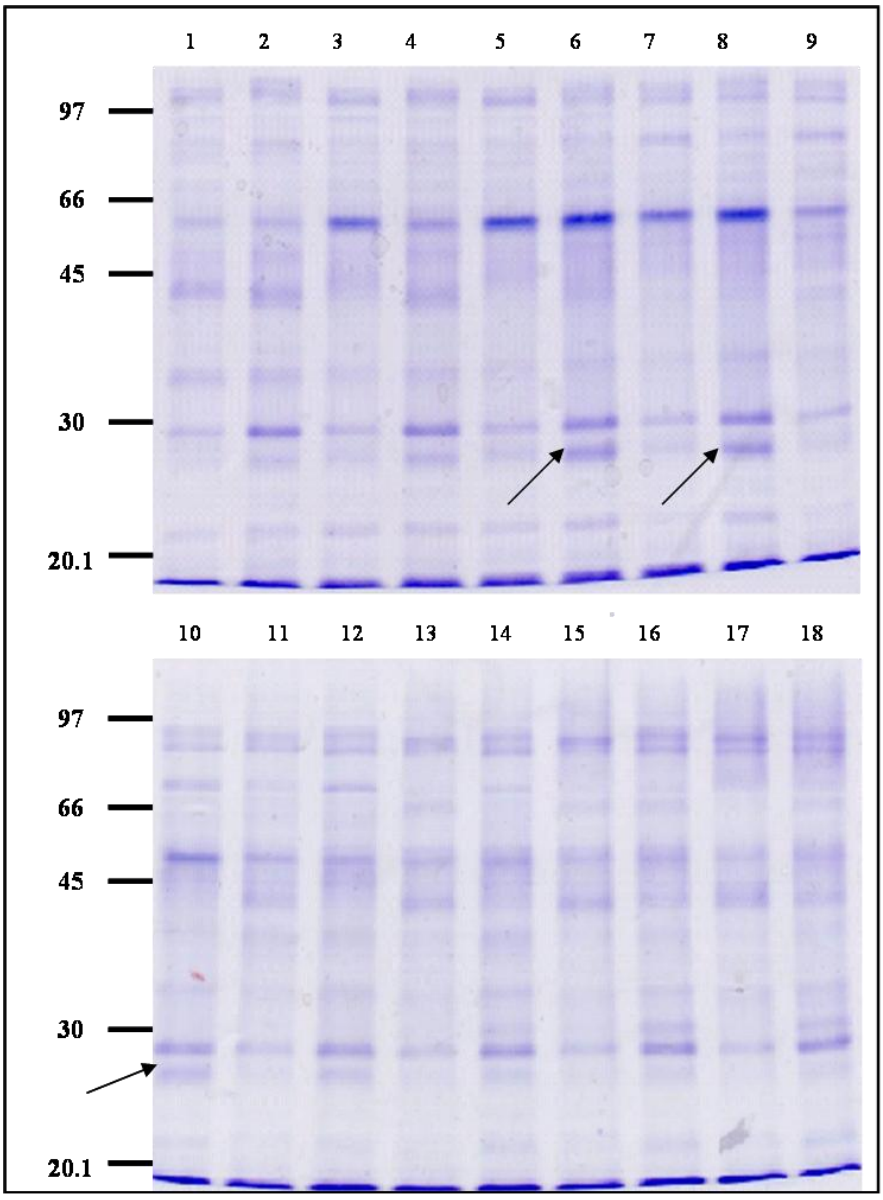


Fig. 33: **SDC-PAGE of Mono Q picks.** Lanes 1 and 2= fraction 31 of CON and Cy(DOM) respectively ; Lanes 3 and 4= fraction 32 of CON and Cy(DOM) respectively; Lanes 5 and 6= fraction 33 of CON and Cy(DOM) respectively; Lanes 7 and 8= fraction 34 of CON and Cy(DOM) respectively; Lane 9= fraction 35 of CON; Lane 10= fraction 35 of Cy(DOM); Lanes 11 and 12= fraction 36 of CON and Cy(DOM) respectively; Lanes 13 and 14= fraction 37 of CON and Cy(DOM) respectively; Lanes 15 and 16= fraction 38 of CON and Cy(DOM) respectively; Lanes 17 and 18= fraction 39 of CON and Cy(DOM) respectively; 12 µl of each fraction were added to 3 µl of SB 5X and loaded on the gel. Arrows indicate bands different from corresponding control ones.

The patterns of bands seemed to be very similar between the two cultures, but there were three fractions (lanes 6, 8 and 10) which had a band near 26 kDa stronger and a little bit higher then the corresponding control fractions (lanes 5, 7 and 9 respectively). It is possible that this band was the result of two bands corresponding to an endogenous contaminant and to the recombinant protein. The same fractions loaded on gel were also spectrophotometrically analysed but no significant difference was revealed (not shown). Samples corresponding to fractions 33, 34 and 35 were collected and concentrated to 0.2 ml using a centricon with 10 kDa cutoff and loaded on a Superdex 200 gel filtration column (Fig. 34)

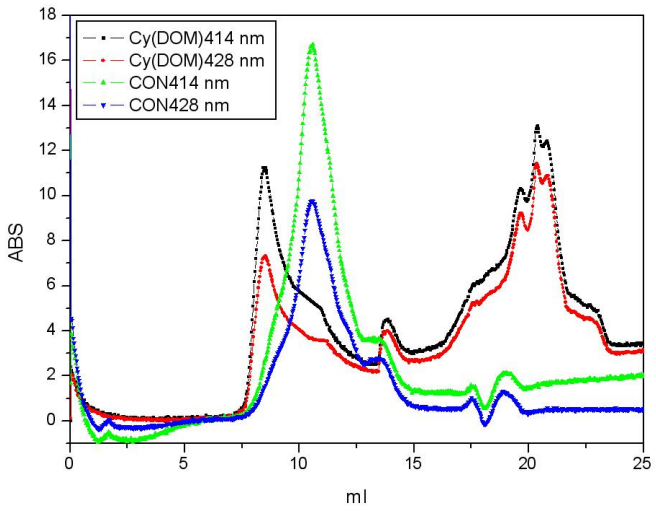


Fig. 34: **Gel filtration chromatography of fractions from control and Cy(DOM) MonoQ.** The Superdex 200 HR 10/30 column was equilibrated with 50 mM Tris-HCl, pH 7.0, 150 mM KCl and 0.08% (w/v) DM. Fractions were concentrated to 0.2 ml and then loaded on the column.

The two chromatograms were deeply different and this was not easy to explain considering that the two gels didn't show a strong difference.

A difference between control culture and Cy(DOM) containing culture is present both in SDS-PAGE and in Mono Q chromatogram; this difference might be due to recombinant protein expression because the only difference between the two cultures is the transformation with or without Cy(DOM) cDNA and so it's difficult to hypothesize different explanations.

C-1.c.4 GmCyDOM DOMON domain expression in *Pichia pastoris*

A modified version of soybean DOMON domain cDNA of GmCyDOM, that I will breafly indicate as (Cy)DOM, was introduced in the eukaryotic system *P. pastoris* for heterologous expression. Soybean (Cy)DOM cDNA was cloned in the expression vector pPicZαB downstream to alcohol oxidase 1 (*AOX1*) promoter and to an α-factor secretion signal sequence and used to transform *P. pastoris* by electroporation. Transformants with multi-copies of (Cy)DOM cDNA were screened by testing their hyper-resistance to Zeocin™ (Fig. 35).

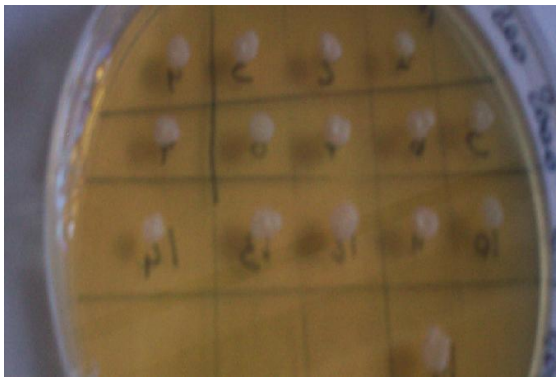


Fig. 35: **Screening of *P. Pastoris* multi-copy recombinants**. The figure shows P. pastoris colonies grown on Zeocin 2 μg/ml. These colonies were previously grown on lower Zeocin amount (0,5 and 1 μg/ml).

Positive clones were cultured and induced for production of recombinant (Cy)DOM. The induction was performed for 72 h adding 2% (v/v) methanol every 24 h. Induction media was screened for (Cy)DOM presence by spectrophotometric analysis. Ascorbate-reduced-minus-oxidized spectra revealed the presence of a cytochrome b starting from 24 h after induction. Maximum yields were obtained 48 h (Fig. 36).

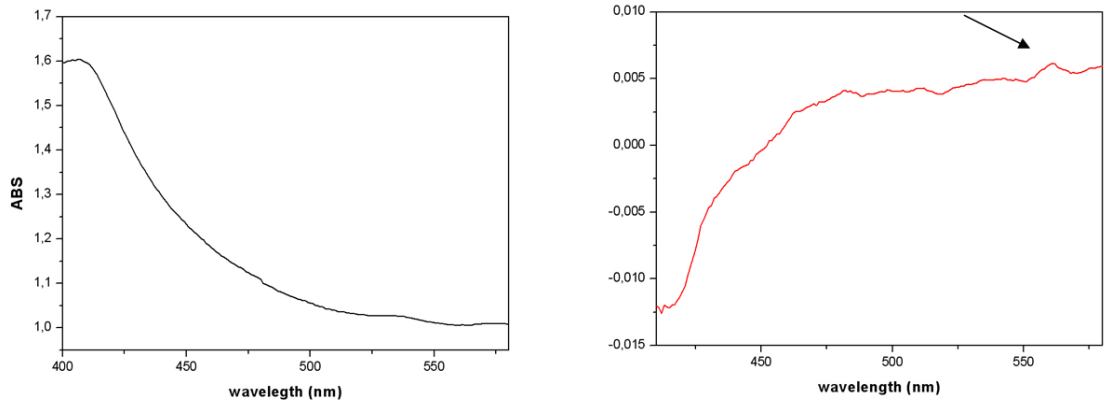


Fig. 36: **Spectrophotometric analysis of (Cy)DOM growth medium of an expressing colony.** After 48 h of induction, culture was centrifuged and supernatant analysed; no spectrum was present in the cellular component, indicating that the recombinant protein was secreted. Left panel was the oxidised spectrum, while the right one was the ascorbate-reduced-minus-oxidised; grains of ascorbate were used. Arrow indicates the appearance of a band after ascorbate treatment.

Ascorbate-reduced-minus-oxidised spectrum clearly showed the presence of a α-band at 560 nm tipycal of a cytochrome b_{561} as expected for this domain. Other experiments will be necessary to purify the recombinant protein and better characterize it.

C-1.d In vivo CyDOM localization

These experiments were performed by Dr. Alex Costa, department of Biology, Padova University.

In regenerated tocacco leaves, CyDOM is localised in the PM both in the root hairs and in the epidermal cells of root elongation zone as it is clearly seen from confocal analysis reported in Fig. 37.

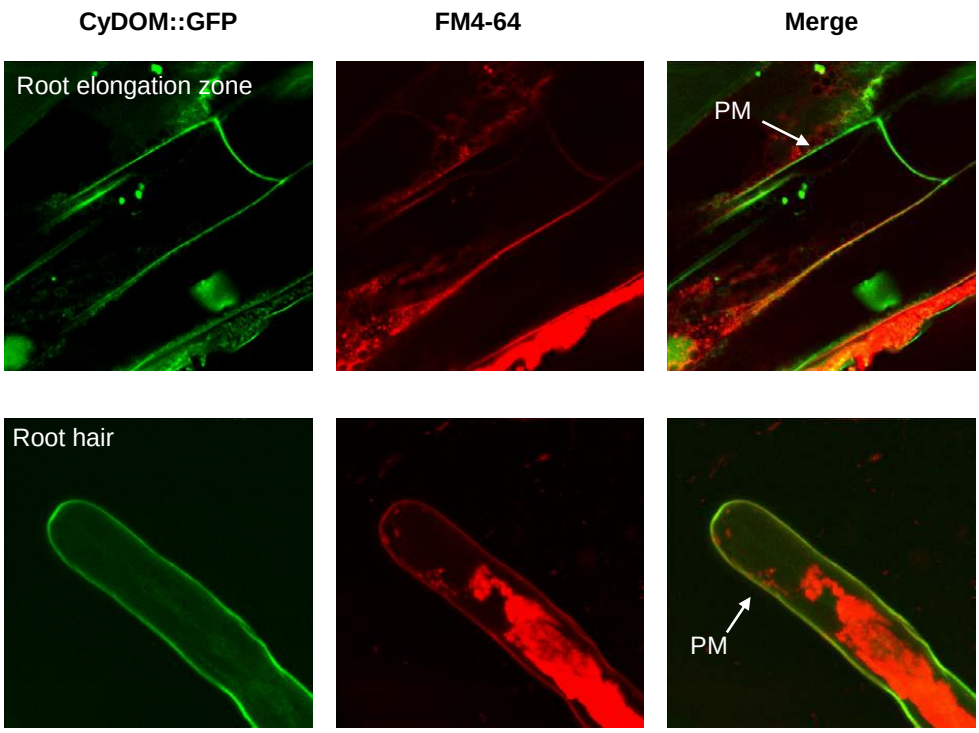


Fig. 37: **Confocal analysis of CyDOM::GFP construct localization *in vivo.*** FM4-64 is a marker for plasma membrane localization and the inner signal is the result of FM4-64 mislocalization probably due to endocytotic process.

Discussion

Recent experiments on Medicago truncatula lipid rafts from roots identified PM redox proteins (Lefebvre et al., 2007) including AIR12 and CyDOM. Lipid rafts were also isolated as sterol and sphingolipid-enriched, but glycerolipid-depleted, Detergent-Resistant Membranes (DRMs) from tobacco leaves (Peskan et al., 2000) and suspension cells (Mongrand et al., 2004) as well as Arabidopsis root-derived callus (Borner et al., 2005).

The presence of AIR12 and CyDOM in lipid rafts might indicate their involvement in maintenance or simply sensing of apoplastic redox state. As already said apoplast represents an important interface between plant and environment and its redox state can change in respect to external stimuli. The precise sensors of apoplastic redox state are still unknown, but it is possible that they are more then one considering the complexity of physiological responses in which it is involved. A protein as CyDOM, which is predicted to be made up by a DOMON domain and a transmembrane cytochrome b_{561}, that is two redox domains, might be involved in electron transfer through the PM. CyDOM plasma membrane localization was also demonstrated by CyDOM::GFP localization experiments in tobacco regenerated roots reported here. The heterologous expression of this protein in *P. pastoris* was impossible to obtain, while initial experiments of DOMON portion expression in *P. pastoris* gave positive results indicating that it might be an ascorbate reducible cytochrome *b*. Purification of this domain is fundamental to confirm this data and to characterize it.

The expression of the transmembrane portion, on the other hand, was very difficult to obtain. The presence of endogenous *E. coli* cytochromes and probably the low amount of recombinant protein produced by this system were major constraints that hindered optimization of the protocol. Liu and co-workers expressed in *E.coli* the adrenal cytochrome b_{561} and the human duodenal one: two protein apparently similar which were expressed under slightly different conditions. It is possible that we did not find the right conditions to get high amounts of our recombinant protein.

A protein as CyDOM for its redox characteristics and for its PM localization is a very good candidate for electron transport reactions. These are fundamental in several physiological responses, such as iron uptake, hormonal growth control and pathogen defence. PM redox systems, involved in these reactions, may influence the redox state of the apoplast and becomes a redox bridge between apoplast and symplast.

MATERIALS AND METHODS

C-2 Chemicals

Hemin, sodium ascorbate, δ-aminolevulinic acid, isopropyl-1-thio-β-D-galactopyranoside, guanidine, kanamycin, chloramphenicol,DNase, RNase, phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF) and egg lysozyme were from Sigma-Aldrich (Milan, Italy). *n*-Dodecyl-β-D-maltoside (DM) was from Anatrace (Maumee, OH). Restriction enzymes and other DNA modifying enzymes were purchased from M-Medical (Cornaredo, Italy). pET-29a (+) was from Novagen (Milano, Italy). Pichia pastoris X-33 cells, plasmid pPICZαB, Zeocin and *E. coli* strain BL21(DE3) were from Invitrogen (Milano, Italy). E. coli strain HB101 was from Promega (Milano, Italy).

Oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany). Reagents for DNA extraction and purification were from Qiagen (Milano, Italia). Immunoblotting reagents were from Bio-Rad (Milano, Italia). anti-His(C-term) monoclonal antibody were from GE healthcare (Milano, Italia)

C-2.a Recombinant Gm CY-DOM expression in Pichia pastoris

A truncated form of CY-DOM cDNA has already been cloned into the NotI site of the expression vectror pPICZαB in frame with the c-myc epitope and a polyhistiidine tag and has already been used to transform *P. pastoris* X-33 cells by electroporation. Multi- copies transformants were used for protein expression. A single colony of P. pastoris X-33/ pPICZαB-CYDOM was used to inoculate 100 ml of BMGY medium [1% (w/v) yeast [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) Yeast Nitrogen Base, 4 × 10-5% (w/v) biotin, 1% (v/v) glycerol] containing 100 μg/ml Zeocin™. Cells were grown overnight (28°C, 220 rpm in buffled flasks) until OD_{600} reached a value of 4, then pelleted (1,500 *g* for 10 min) and resuspended in 400 ml of BMMY inducing medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) Yeast Nitrogen Base, 4 × 10-5% (w/v) biotin, 1% (v/v) methanol] containing 0.1 mM δ-aminolevulinic acid and 40 μM hemin. Cultures were grown (28°C, 220 rpm) for 72/96 h in inducing medium with the addition of 1% (v/v) methanol every 24 h.

C-2.a.1 *P. pastoris* membranes solubilisation

After 72/96 h of induction, *P. pastoris* cells (X-33/pPicZαB/CyDOM and X-33/pPicZαB) were harvested and centrifuged at 2000g for 10 min at 4°C. The culture media was discarded and the harvested cells were washed in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol) until clarification of the pellet from hemin. The cells were resuspended to an OD_{600} of 50-100 in breaking buffer and 1 mM PMSF was added to the mixture to inhibit proteolytic activity. An equal volume of acid washed glass beads was added and the cells were broken by vortexing 8-times for 30 s, with 30 s intervals on ice. The cell debris, large organelles and glass beads were removed by centrifugation at 4°C for 20 min at 9,000 x g. The supernatant was collected and centrifuged at 4°C for 1 h at 35,000 x g. After centrifugation the membrane preparation contained in the pellet was resuspended in breaking buffer and the protein concentration was determined by the method of Bradford. The preparation was diluted to a total protein concentration of 10 μg/μL. To solubilize membrane preparation, 2% n-octyl-β-D-glucopyranoside was added and the mixture stirred on ice for 1 h. Insoluble material was removed by centrifugation at 35,000 x g for 50 min. Spectrophotometric analysis was performed on supernatants by means of a double beam Uvikon 941 Plus spectrophotometer (Kontron, Milano). A quartz cuvette with an internal volume of 0.11 ml and a path length of 10 mm was used.

C-2.b Recombinant transmembrane portion of CyDOM cloning and expression in *Escherichia coli*

C-2.b.1 Transmembrane cyt b_{561}/pET29.1a cloning

The plasmid pPICZαB containing GmCyDOM cDNA was used for two PCRs in order to add a signal peptide coding sequenceto the N-terminal of the transmembrane portion of the protein. The signal peptide coding sequence typical of E. coli to put the recombinant protein into the membrane was: MAQRIFTLILLLCSTSVFA (UniProtKB/Swiss-Prot Accession Number: DSBD_ECOLI= P36655).

Sense and antisense primers used for the first PCR to insert the signal peptide were:

5’-ATCCTGCTACTTTGCAGCACTTCCGTTTTTGCCTCCAGTCAAGCAGGAAGTGG-3’ and

5’-AAACTCGAGCACCTGCTGTCCTCTAAAACC-3’(XhoI restriction site is underlined).

The amplified PCR product was used for the second PCR to add the N-terminal restriction site and the remaining signal peptide sequence, the sense primer weas:
5'-AAAAAGCTTGCTCAACGCATCTTTACGCTGATCCTGCTACTTTGCAGCAC-3'
(HindIII restriction site is underlined) and the antisense one was the same of the first PCR. The gray sequence is the signal peptide coding sequence.
The PCR product was digested with HindIIII and XhoI, purified by agarose gel electrophoresis, and inserted into the pET-29a(+) vector, digested by HindIII and XhoI, before and in frame with vector hexa-histidine sequence. The resulting plasmid was transformed into *E. coli* HB101 using calcium chloride competent cells; transformants were screened for proper insertion of the cyt b_{561} gene. The integrity of the resulting cyt b_{561}/pET29.1a construct, designated pET29.1a-b_{561}C6H, was confirmed by restriction digestion and DNA sequencing.

C-2.b.2 *E. coli* competent cells preparation and transformation

E. coli competent cells were obtained using $CaCl_2$ method. Bacterial cells were grown overnight in 5 ml of LB medium [Luria Bertani medium, 1% (w/v) tryptone, 0,5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0] at 37°C (100 rpm). 0.1 ml of the overnight culture was used to inoculate 13 ml of fresh LB medium and grown at 37°C (100 rpm) until OD_{600} reached 0.15-0.17. Cells were divided in aliquots of 1.5 ml and centrifuged at 4°C for 30 s at 8000 rpm. Supernatant was discarded and 0.45 ml of TFBI [300 mM CH_3COOK, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% (v/v) glycerol, pH 5.8] was added to each aliquot. Cells were resuspended and centrifuged at 4°C for 10 min at 3000 rpm. Supernatant was discarded and 0.12 ml of TFBII [10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% (v/v) glycerol, pH 6.5] was added to each aliquot and cells were gently resuspended and stored at -80°C or directly used for transformation.
For competent cells transformation: DNA was added to thawed competent cells and taken 30 min on ice. Heat shock was performed putting cells at 42°C for 45 s. Cells were then put on ice for 2 min before the addition of 0.25 ml of LB medium to each cell aliquot. Cells were grown at 37°C for 1 h and then plated on LB plates containing appropriate antibiotics for transformed cell screening.

C-2.b.3 Transmembrane cyt b_{561}/pET29.1a expression in *E. coli* system

The BL21Star(DE3)/pT-groE *E. coli* strain was transformed with ransmembrane cyt b_{561}/pET29.1a and grown overnight in Terrific Broth medium (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) containing chloramphenicol (34 μg/mL) and kanamycin (50 μg/mL) at 37 °C. Five milliliters of the overnight culture was used to inoculate 1 L of Terrific Broth containing chloramphenicol and kanamycin and incubated with shaking (180–240 rpm) at 37 °C until OD_{610} reached 0.6–0.8. After chilling to 20 °C, heme (5 μM final), δ-aminolevulinic acid (0.3 mM), and isopropyl-1-thio-β-D-galactopyranoside (1 mM) were added, and culture was continued for 24 h at 20 °C with shaking at 200 rpm. Cells were harvested by centrifugation and stored at −80 °C.

C-2.b.4 Purification of transmembrane cyt b_{561}

Cells from 2 L of culture were resuspended in 960 mL of 0.1 M potassium phosphate containing 5% (v/v) glycerol, pH 7.2. Egg lysozyme (0.6 g in 12 mL of buffer) or rLysozyme Solution (0.15 mL) were added and the suspension stirred on ice for 1 h and then sonicated (6 min total; 10 s intervals, 50% duty cycle). Then $MgCl_2$ (5 mM) and DNase (20μg/mL) and RNase (20μg/mL) were added and the suspension stirred gently on ice for another 1 h before centrifugation at 100,000*g* at 4 °C for 1 h. The pellet (membrane fraction containing recombinant cyt b_{561}) was resuspended with 360 mL of 0.1 M KPi (pH 7.2) containing 5% (v/v) glycerol and 2% (w/v) DM (1.5 g DM/g total protein) and stirred on ice for 1 h. Then additional DNase (20μg/mL) and RNase (20μg/mL), $MgCl_2$ (5 mM) and 1 mM of PMSF were added, and stirring continued at 4 °C overnight. Unextracted material was pelleted by centrifugation at 100,000*g* at 4 °C for 1 h and the pH of the supernatant (the crude detergent extract) was adjusted to 7.3–7.5.

Spectrophotometric analysis was performed on supernatants by means of a double beam Uvikon 941 Plus spectrophotometer (Kontron, Milano). A quartz cuvette with an internal volume of 0.11 ml and a path length of 10 mm was used.

C-2.b.5 Electrophoresis and Western blotting

SDS-PAGE was performed on 12.5% acrylamide gels. Samples were boiled for 10 min in sample buffer prior to loading. Gels were stained with Coomassie Brilliant Blue R-250.

Electrophoresis was conducted at a constant 90 V, 4 °C for 3 h and staining was performed as described by Thomas et al. (1976).

For Western blotting, proteins were separated by SDS-PAGE and electroblotted (Semi-dry cell; Schleicher-Schuell) onto nitrocellulose membranes. Membranes were stained with Red Ponceau before incubation with rabbit antiserum raised against hexa-histidin sequence, and peroxidase-conjugated secondary antibodies. Primary and secondary antibodies were diluted 1:2,000 and 1:1,000, respectively. Blots were developed by chemiluminescence according to standard procedures.

C-2.b.6 Purification of recombinant transmembrane cyt b_{561}

Chromatographic media and systems used for purification were from GE Healthcare. All buffers used contain 0,08% DM.

Solubilized material was loaded on an ion-exchange MonoQ column (1 ml) equilibrated with 50 mM potassium phosphate pH 7.0 and a linear KCl gradient (0-0.5 M) was applied at a 1 ml min-1 flow rate, and 0.5-ml fractions were collected. Rectransmembrane cyt b_{561} containing fractions were pooled, concentrated to 0.2 ml and loaded on a Superdex 200 (HR 10/30) gel filtration chromatography column equilibrated with 50 mM Tris-HCl pH 7.0, 150 mM KCl. Elution was carried out at 0.5 ml min-1 and fractions of 0.2 ml were collected.

C-2.c CyDOM DOMON domain cloning and expression in *P. pastoris*

The plasmid pPICZαB containing GmCyDOM cDNA was used for PCR to clone the DOMON domain out of the N-terminal signal peptide. The antisense primer takes a codon stop sequence to not include the transmembrane portion.

The sense and antisense primers were: 5'-AACTGCAGAATGCAAGGGCCAGGCCTTC-3' (PstI site is underlined) and 5'-ATAAGCGGCCGCCCTAAGTGAATTCCCACTTCC-3' (NotI site is underlined). The oligonucleotides amplified a region of the gene right after the putative N-terminal leader peptide encoding sequence (predicted by SignalP 3.0, Bendtsen et al. 2004).The PCR amplified cDNA was cloned into the pPicZαB vector (digested with PstI and NotI) downstreamand in frame with the α-factor coding sequence in order to have the recombinant protein secreted in the medium. pPicZαB-DOMON constructed was used to transform *P. pastoris* X-33 cells by electroporation. Next steps are the same of that described for CyDOM expression in *P. pastoris* (see section C-2)

Spectrophotometric analysis was performed on supernatants by means of a double beam Uvikon 941 Plus spectrophotometer (Kontron, Milano). A quartz cuvette with an internal volume of 0.11 ml and a path length of 10 mm was used.

C-2.d CyDOM::GFP *in vivo* localization

C-2.d.1 CyDOM::GFP cloning and Agrobacterium transformation

The CyDOM cDNA sequence was subcloned in a pBI121 modified vector (Bregante et al., 2008) upstream the GFP coding sequence under the control of the double CaMV35S promoter. The vector was then introduced into a wildtype *Agrobacterium rhizogenes* strain by a freeze-thaw method. An *Agrobacterium* strain containing an appropriate helper Ri plasmid was grown in 5 ml of YEP medium (10 g/L peptone; 10 g/L; yeast extract, 5 g/L NaCl) overnight at 28 °C. Then 2 ml of the overnight culture was added to 50 ml YEP medium in a 250-ml flask and shaked vigorously (250 rpm) at 28°C until the culture reached to an OD600 of 0.5 to 1.0. Subsequently the culture was chilled on ice and centrifuged at 3000 g for 5 min at 4°C, the supernatant was discarded and the cell pellet was resuspended in 1 ml of 20 mM CaCl2 solution (ice-cold). About 1 μg of plasmid DNA was added to the cells which were then frozen in liquid nitrogen. The frozen cells were thawed by incubation in 37°C water bath for 5 min and 1 ml of YEP medium was added to the tube and incubated at 28°C for 2 h with gentle shaking. Then the cells were centrifuged for 30 s an the supernatant discarded. The cells were resuspended in 0.1 ml YEP medium and spread on YEP agar plate (YEP media plus 2% Agar Technical Oxoid n° 3, Sigma) containing the appropriate antibiotic selection (50 mg/L kanamycin). The plate was incubated at 28°C and transformed colonies appeared in 2 days

C-2.d.2 Plant transformation

In vitro sterile shoot cultures of *Nicotiana tabacum* cv. SR1 were maintained on MS½ (Murashige and Skoog, 1962) agar medium containing 15 g/L sucrose. The pH was adjusted to 5.5 before autoclaving. The tobacco culture was grown at 25°C, with a light/dark cycle of 16/8 h. For the induction of tobacco transgenic roots formation from leaf discs, the same protocol used for the stable transformation of *Nicotiana tabacum* was adopted (Horsch et al.,

1985). Briefly, tobacco leaf discs were incubated for 10 min with a suspension of an overnight culture of *A. rhizogenes*, harbouring the pBI-*CY-DOM-GFP* plasmid, resuspended in MS liquid medium. The leaf discs were then washed with sterile milliQ water, blotted on sterile filter paper and then placed on full MS agar plates supplemented with 3% sucrose and left in the dark at 25°C for two days. After the two days co-cultivation the leaf discs where rinsed with sterile milliQ water, blotted and transferred to MS agar plates supplemented with 3% sucrose in presence of 50 mg/L kanamycin and 200 mg/L cefotaxime. The plates were then transferred to the growth chamber at 25°C with a 16/8 light/dark cycle. After 2-3 weeks the regenerated roots were assayed for GFP fluorescence.

C-2.d.3 Confocal analysis

Confocal microscope analysis were performed using a Leica PS2 laser scanning confocal imaging system. For GFP detection, excitation was at 488 nm and detection between 515 and 530 nm. For the FM4-64 detection, excitation was at 488 nm and detection over 570 nm. The images acquired from the confocal microscope were processed using Corel Photo-Paint software (Corel Corporation, Dallas, TX, USA). For FM4-64 staining, the dye was added to milliQ water at a final concentration of 17 μM (Bolte et al., 2004) and roots were directly submerged. The microscope analysis was carried out after five min of incubation

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