Immunolocalization of H+-ATPase and IRT1 enzymes in N2-fixing common bean nodules subjected to iron deficiency

Abstract
The demand for iron in leguminous plants increases during symbiosis, as the metal is utilised for the synthesis of various Fe-containing proteins in both plant and bacteroids. However, the acquisition of this micronutrient is problematic due to its low bioavailability at physiological pH under aerobic conditions. Induction of root Fe(III)-reductase activity is necessary for Fe uptake and can be coupled to the rhizosphere acidification capacity linked to the H+-ATPase activity. Fe uptake is related to the expression of a Fe2+ transporter (IRT1). In order to verify the possible role of nodules in the acquisition of Fe directly from the soil solution, the localization of H+-ATPase and IRT1 was carried out in common bean nodules by immuno-histochemical analysis. The results showed that these proteins were particularly abundant in the central nitrogen-fixing zone of nodules, around the periphery of infected and uninfected cells as well as in the vascular bundle of control nodules. Under Fe deficiency an over-accumulation of H+-ATPase and IRT1 proteins was observed especially around the cortex cells of nodules. The results obtained in this study suggest that the increase in these proteins is differentially localized in nodules of Fe-deficient plants when compared to the Fe-sufficient condition and cast new light on the possible involvement of nodules in the direct acquisition of Fe from the nutrient solution.

Abbreviations
BMbacteroid membraneCZcortex zoneEx Cexternal cortexFC-Rferric chelate reductaseICinfected cellIn Cinternal cortexIRT1iron regulated transporter 1IZinfected zoneLHleghemoglobinNRnitrogenasePBMperibacteroid membranePBSperibacteroid spaceSsiderophoreUCuninfected cellVBvascular bundle

Keywords
Fe absorptionImmunodetectionIron transporter*Phaseolus vulgaris*Symbiosis

Introduction
Iron is an important nutrient in N2-fixing legume nodules. Iron supplied to the nodule is used by the plant for the synthesis of leghemoglobin (LH), while in the bacteroid it is used as an essential cofactor for the bacterial N2-fixing enzyme, nitrogenase (NR), and Fe-containing proteins of the electron transport chain. *In situ*, bacteroids are always surrounded by a membrane of plant origin (Verma et al., 1978). This so-called peribacteroid membrane (PBM) physically isolates the microbial symbiont from the cytoplasm of the host cell to form the symbiosome, delimiting a peribacteroid space (PBS). The supply of Fe to the bacteroids requires initial transport across the plant-derived PBM. The main metabolic exchange that occurs between plant and bacteroids is related to the reduced carbon (usually malate) supplied by the plant to the bacteroids for N2-fixation (Udvardi et al., 1988). Specific transport mechanisms responsible for this exchange have been identified (Bergersen and Turner, 1990). However, the bacteroids are dependent on the plant for all micronutrients, and transporters for these must also exist on the PBM. The mechanisms involved in bacteroid Fe acquisition within the nodule have been investigated at the biochemical level and three activities were identified (Day et al., 2001). Fe3+ is transported across the PBM complexed with organic acids such as citrate and accumulates in the PBS (Le Vier et al., 1996; Moreau et al., 1995), where it is bound to siderophore (S)-like compounds (Wittenberg et al., 1996). Fe(III)-chelate reductase (FC-R) activity has been measured on isolated PBM and Fe3+ uptake into symbiosomes was dependent on the presence of NADH (Le Vier et al., 1996). However, Fe2+ is also readily

transported across the PBM and has been found to be the favoured form of Fe taken up by bacteroids (Moreau et al., 1998). One of the proteins involved in this transport has been identified by Kaiser et al. (2003) in soybean nodules. This is a *Glycine max* divalent metal transporter 1 (GmDMT1) homologue of the NRAMP/Dmt1 family of divalent metal ion transporters. In the same work, the localization and the activity of GmDMT1 found in soybean nodules suggest that the protein is involved in Fe2+ transport and Fe homeostasis in symbiotic N2-fixing nodules. In addition, another transporter was identified and characterized as a symbiotic zinc transporter (GmZip1), which does not transport Fe2+ (Moreau et al., 2002). Some work reported that ferrous Fe was taken up by symbiosomes more efficiently than the ferric form, because in the microearobic conditions and the slightly acidic pH of nodules *in situ*, Fe2+ should be more stable (Appleby, 1984). Consequently, it is possible that ferrous Fe is the main form of iron present *in vivo* (Moreau et al., 1998). Concerning the genetic approach of Fe regulation in plants, many works focused in the regulation of the expression of genes involved in Fe acquisition, transport and homeostasis in plant, among these genes are the transcription factor *AtFIT*, the ferric reductase *AtFRO*, the iron transporter *AtIRT1* and the H+-ATPase *AtAHA* (Colangelo and Guerinot, 2004; Walker and Connolly, 2008; Buckhout et al., 2009; García et al., 2011). Nevertheless, little information is available concerning the Fe-related genes involved in Fe acquisition and transport in root-nodules of legumes. Hakoyama et al. (2009) showed that the gene of nicotianamine synthase (NAS) was expressed exclusively in the vascular bundles (VBs) of *Lotus japonicas* nodules during the late state of nodule development and was involved in the intracellular Fe translocation in nodules.

Generally, upon sensing Fe limitation, strategy I plants such as common bean, induce a coordinated set of responses that, taken together, allow the plant to maximize Fe mobilization and uptake from the soil. Under symbiotic N2-fixing condition, in which the demand for Fe increases, the preferential allocation of Fe to nodules and the Fe-use efficiency for nodules growth and functions were found to be the base of tolerance of some common bean cultivars (Krouma et al., 2008; Slatni et al., 2008, 2009). This adaptive mechanism includes the induction of three activities localized at the PM of roots cells and PBM and BM of symbiosome cells: (i) a proton pump acidifies the rhizosphere or PBS, thus driving more Fe into solution, (ii) a FC-R converts Fe3+-chelates to Fe2+ and (iii) a Fe2+ transporter moves Fe across the plasma or PBM into cells (Slatni et al., 2011).

In spite of several studies clearly elucidating the biochemical and molecular aspects involved in the regulation of the enzymes cited above in roots, little is known about their involvement in Fe uptake and mobilization in nodules and the role of these organs in enhancing Fe acquisition. Previous results focused to verify the ability of nodules to acquire Fe directly from the soil solution were obtained and showed, by detection both "*in vivo*" and "*in vitro*", the stimulation of FC-R activity in nodulated roots and detached nodules of Fe-deficient plants (Slatni et al., 2009, 2011). Two common bean cultivars were used in these studies, Coco blanc, sensitive and Flamingo, tolerant to Fe deficiency (Slatni et al., 2008). The performance of Flamingo in Fe deficiency is related to (i) its ability to maintain a better symbiotic N2 fixation linked to a better Fe allocation to nodules and a significant Fe use efficiency for nodule growth and N2 fixation, and (ii) an important ammonium assimilation linked to the maintenance of an important GS activity and particularly to a significant induction of GDH activity (Slatni et al., 2008). The same studies suggested that the nodules of tolerant cultivar contributed to the rhizosphere acidification and to the reduction of apoplastic Fe3+. It is also noteworthy that the activity of FC-R under Fe deficiency was higher in nodules than in roots (about two fold), suggesting that under this condition nodules play an important role in Fe3+ reduction and Fe uptake and consequently in Fe supply to the bacteroids (Slatni et al., 2011). Like in roots, transporters

homologues to iron regulated transporter (IRT1) should be present in nodules. The amount of this protein was increased under Fe deficiency also in nodules enabling them to acquire Fe directly from the soil nutrient solution (Slatni et al., 2011). Additionally, Slatni et al. (2011) showed that also H+-ATPase activity and protein level were increased under Fe deficiency in nodules of both cultivars, mainly in Flamingo. From the data obtained in this previous work we might now hypothesise that, when common bean plants grow with low Fe availability, nodules acquire the capacity to take up Fe not only from the plant cytosol, but also directly from the nutrient solution. The aim of the present work was: to provide more evidence about the implication of different tissues of nodules in Fe availability and uptake by immuno-histochemical analysis of H+-ATPase and IRT1 in common bean nodules.

Materials and methods

Plant materials and growth conditions

Two common bean cultivars, Coco blanc, sensitive and Flamingo, tolerant to Fe deficiency (Slatni et al., 2008), were inoculated with *Rhizobium tropici* CIAT 899 originating from International Center of Tropical Agriculture, Colombia, maintained in the “Laboratory of legumes” (CBBC, Hammam-Lif, Tunisia) and grown with the following N-free nutrient solution (Hewitt, 1966): KH_2PO_4 (1.60 mM), $MgSO_4$ (1.50 mM), K_2SO_4 (1.50 mM), $CaSO_4$ (3.50 mM), H_3BO_3 (4 μM), $MnSO_4$ (4 μM), $ZnSO_4$ (1 μM), $CuSO_4$ (1 μM), $CoCl_2$ (0.12 μM), $(Na)_6(Mo)_7O_{24}$ (0.12 μM) added with 5 μM Fe as K–Fe–EDTA and 1 mM urea as starter N needed for growth during the first two weeks. The nutrient solution was aerated with a flow of filtered air. After two weeks of pre-treatment (appearance of nodules), plants were separated into two plots; the first one did not receive Fe (Fe-deficient plants), while the second one received 45 μM Fe(III)EDTA (Fe-sufficient plants). Nodules from three-week-old plants were harvested and used for the analysis.

Fixing, embedding and sectioning

Nodules were detached from roots and fixed overnight at 4 °C either in ethanol–acetic acid (3:1 (v/v), for immunolocalization) or in 100 mM sodium phosphate buffer (pH 7.0) containing 2% paraformaldehyde (w/v) and 2.5% glutaraldehyde, then dehydrated through an ethanol–tertiary butanol series and embedded in paraffin (Paraplast plus, Sigma) as described by Villalba et al. (1991). Serial sections of 7 μm were cut with a microtome. Sections were mounted on polylysine-treated slides, deparaffinised in xylene and rehydrated through an ethanol series.

Immunolocalization

Immunological detection in tissue sections fixed as above described was performed as in Parets-Soler et al. (1990) with some modifications. Sections were incubated for 30 min at room temperature in 3% H_2O_2, then blocked for 1 h in TBS (150 mM NaCl, 25 mM Tris–HCl, pH 7.6) with 2% BSA (w/v). Polyclonal antibodies raised against a IRT1 synthetic peptide (a kind gift from Dr. Connolly; Connolly et al., 2002), and the central domain of *Arabidopsis thaliana* PM H+-ATPase (a kind gift from Dr. Serrano, Universidad Politécnica, Valenci2a, Spain) were used. Antibodies were diluted 1:1000 in TBS with 0.5% BSA and incubated overnight at 4 °C with the sections. After 3× 5 min washes in TBS, the sections were incubated 2 h at room temperature with a biotinylated secondary antibody (anti-rabbit IgG biotin conjugate developed against goat, Sigma) diluted 1:1000 in TBS with 0.5% BSA. After 3× 5 min washes in TBS the sections were incubated 30 min in the ExtrAvidin-Peroxidase (ExtrAvidin Peroxidase Staining Kit, Sigma). After 3× 5 min washes in TBS the sections were incubated 5–10 min in 0.05 M acetate buffer pH 5.0 containing 5% dimethylformamide, 0.04% 3-amino-9-ethylcarbazole (AEC) and 0.015% H_2O_2 and finally washed in distilled water.

Results

According to the microscopy observation of nodules, three major parts can be recognized at the histological level in the control section treated without primary antibodies (Fig. 1): first, the central part of the nodules or infected zone (IZ) (Fig. 1A) containing the infected cells (IC) and interconnected aggregates of smaller, uninfected cells (UCs) (Fig. 1B); second, the nodule parenchyma or internal cortex (In C) surrounding the central tissue and including the VBs (Fig. 1A and C) and third the external cortex (Ex C) (Fig. 1A and D). Fig. 1 shows the control sections which appear not stained, indicating that a specific reaction attributable to the secondary antibody and/or extravidin–peroxidase complex does not occur during the immunolocalization experiments.

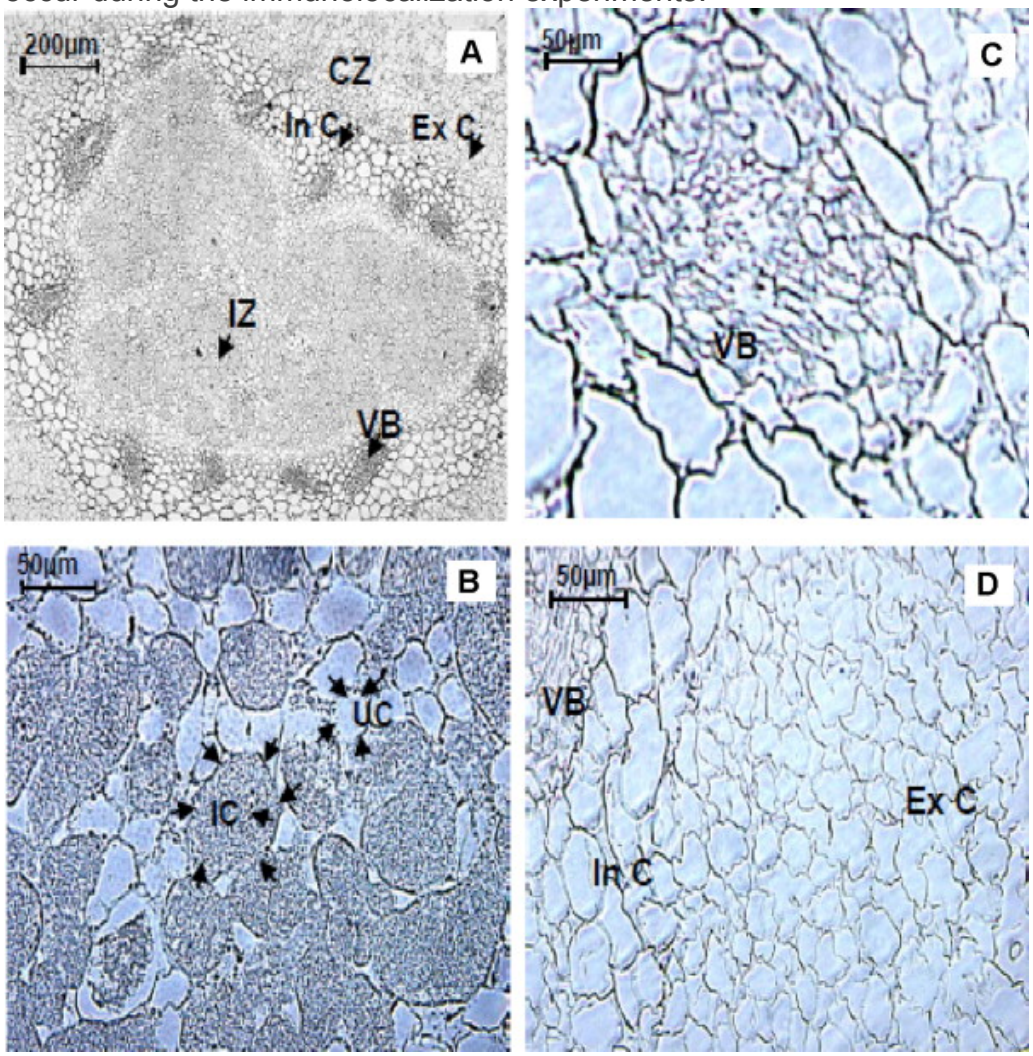

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Fig. 1. (A)–(D) Control sections of common bean nodule tissues treated without polyclonal antibodies. CZ: cortex zone; Ex C: external cortex; IC: infected cell; In C: internal cortex; IZ: infected zone; UC: uninfected cell; VB: vascular bundle.

Iron-sufficient (+Fe) and Fe-deficient (−Fe) nodule sections from both Flamingo and Coco blanc were subjected to immunoreaction with antibodies raised against H+-ATPase and IRT1 in order to localize the expression of these proteins.

In +Fe nodules of the tolerant cultivar (Flamingo) H+-ATPase protein is expressed in the VBs (Fig. 2A and B) and the IZ (Fig. 2C), but not in the Ex C (Fig. 2D). In −Fe nodules a considerable signal relative to H+-ATPase appeared in IZ (Fig. 2E and G) and also in the cortex zone (CZ), both internal and external (In C, Ex C) (Fig. 2H) but less pronounced in the VB (Fig. 2E and F) compared to +Fe nodules. In the sensitive genotype (Coco blanc),

similarly to what observed in Flamingo, H+-ATPase protein was mainly expressed in VB and IZ (Fig. 2J and K) but not in Ex C of nodules (Fig. 2L) of +Fe plants, but, conversely to Flamingo, in −Fe nodules H+-ATPase did not show any significant difference compared with the +Fe condition. Indeed, the signal intensity was similar in both the VB and Ex C between +Fe (Fig. 2J and L) and −Fe (Fig. 2N and P) conditions, while a slightly decreased signal was detected in the IZ of −Fe nodules (Fig. 2M and O).

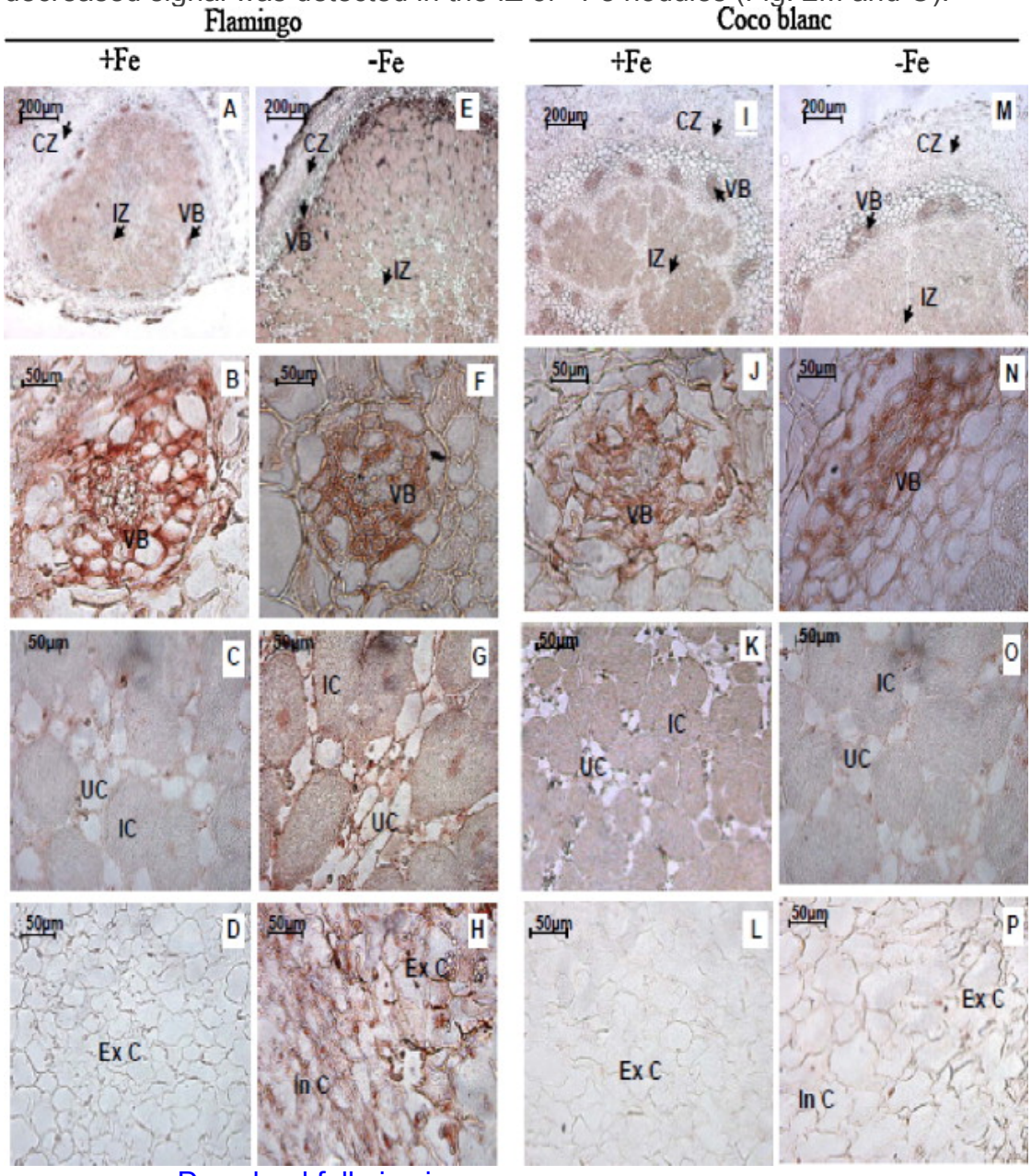

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Fig. 2. (A)–(P) Immunolocalization of H+-ATPase in common bean nodule tissues of (i) Flamingo. (A)–(D) sections of Fe sufficient nodules. (E)–(H) sections of Fe deficient nodules and of (ii) Coco blanc. (I)–(L) sections of Fe sufficient nodules. (M)–(P) sections of Fe deficient nodules treated with polyclonal antibody. Ex C: external cortex; IC: infected cell; In C: internal cortex; UC: uninfected cell; VB: vascular bundle.

Concerning IRT1, the protein is particularly expressed in VB (Fig. 3A and B) and IZ (Fig. 3A and C) of +Fe Flamingo nodules, while in −Fe ones an increased signal was detected in the IZ (Fig. 3G) and also in cortex cells (Fig. 3H) but decreased in VB (Fig. 3F) respect

to the +Fe ones. In +Fe Coco blanc nodules, IRT1 antibodies evidenced a weak signal in the VB (Fig. 3J) which slightly increased in the −Fe (Fig. 3N), while in the Ex C no appreciable difference in the signal intensity between the two conditions was observed (Fig. 3L and P). Moreover, the IZ of the −Fe nodules shows an increase in IRT1 signal (compare Fig. 3O with K).

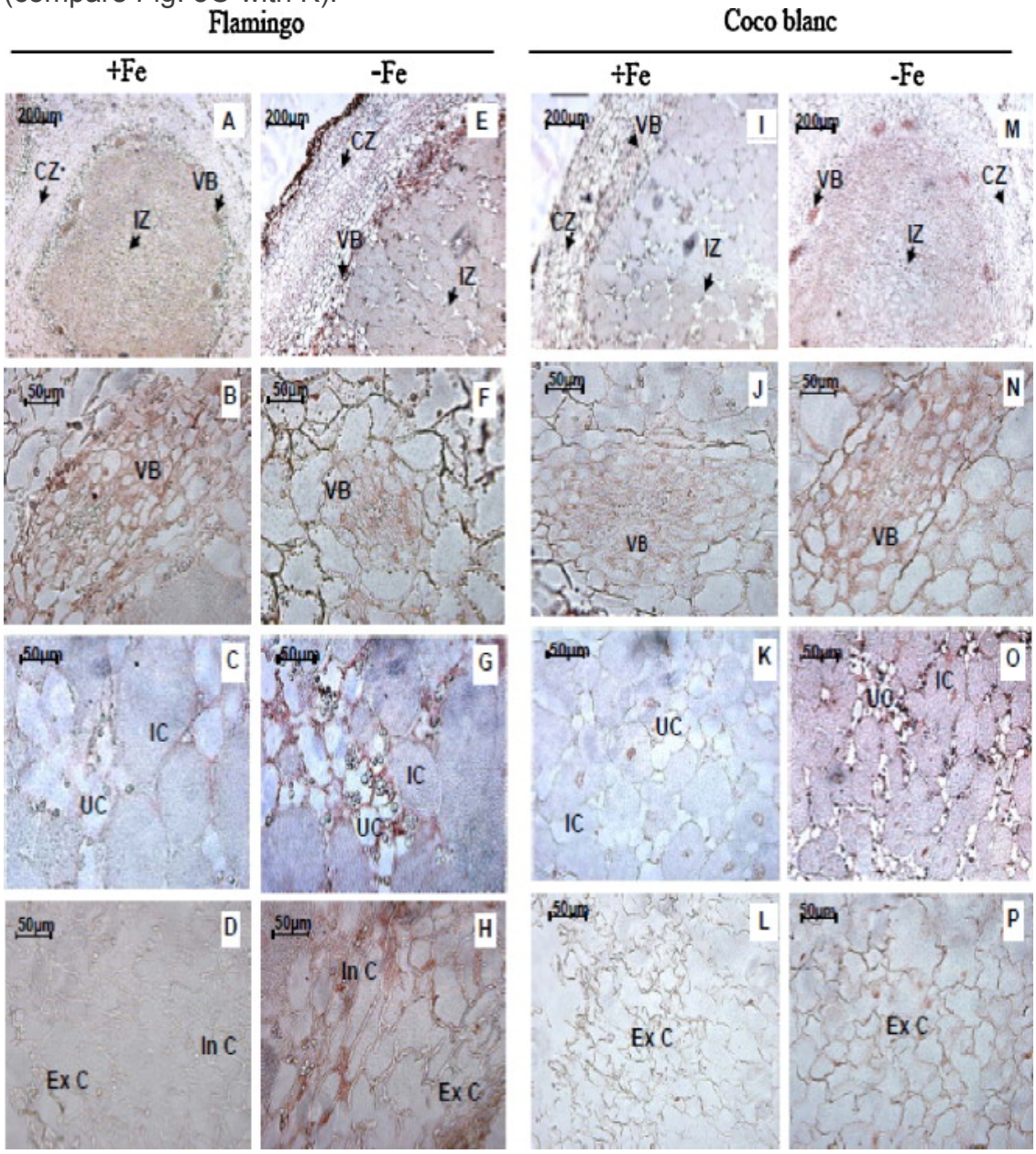

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Fig. 3. (A)–(P) Immunolocalization of IRT1 in common bean nodule tissues of (i) Flamingo. (A)–(D) sections of Fe sufficient nodules. (E)–(H) sections of Fe deficient nodules and of (ii) Coco blanc. (I)–(L) sections of Fe sufficient nodules. (M)–(P) sections of Fe deficient nodules treated with polyclonal antibody. Ex C: external cortex; IC: infected cell; In C: internal cortex; UC: uninfected cell; VB: vascular bundle.

In general two main conclusions might be drawn: (i) the signal relative to the proteins studied in this work was less intense in the sensitive cultivar than in the tolerant one, (ii) in −Fe treated plants H+-ATPase and IRT1 proteins were significantly detected in CZ of the tolerant cultivar nodules (Figs. 2H and 3H), but not in the sensitive one (Figs. 2P and 3P). In addition, enhanced signal of these proteins was also detected in the IZ of −Fe nodules

of Flamingo, while in Coco the same is true only for IRT1 (Figs. 2C, G, K, and O and 3C, G, K, and O).

Discussion

In the present study the expression and the histological distribution of H+-ATPase and IRT1 proteins in nodules of Fe-sufficient and Fe-deficient roots from two bean cultivar differently tolerant to Fe deficiency was compared. The results suggest that an increase in these proteins occurs in nodules of −Fe plants compared to +Fe ones, especially in the tolerant cultivar, Flamingo. In nodules of +Fe plants these proteins are expressed particularly in IZ and VB, while nodules of −Fe plants are characterized by high proteins accumulation in IZ and in the cortex, which is involved in the nutrient absorption and exchange with the external medium.

The accumulation of H+-ATPase and IRT1 proteins in cortex cells of Fe-deficient nodules of Flamingo suggests their participation in the acidification of the rhizosphere around the nodule and in the Fe uptake directly from the soil solution. Considerable signal of these proteins was also detected in the IZ of −Fe Flamingo nodules. This finding suggests that H+-ATPase might participate in maintaining low pH in the apoplast of IZ, contributing to the solubilisation of Fe compounds. Consequently, Fe3+ reduction via PBM is facilitated, since FC-R activity is favoured by acidic pH in the apoplast. Several studies showed that a H+-ATPase pumps protons into the PBS and generates a $\Delta\Psi$ across the PBM (Udvardi and Day, 1997; Day et al., 2001). Moreover, the bacteroid respiratory electron chain pumps protons out of the bacteroid into the PBS, resulting in additional acidification of the PBS. Thus, the pH of the PBS, determined by the relative activities of proton pumps and counter-ion movements, could be up to two pH units more acidic than the plant cytosol (Udvardi and Day, 1997). The nodule cortex is the interface between the nodule and its environment and probably, under some severe condition like Fe deficiency, it performs essential functions such as nutrient uptake. Basing on the presented data we may suggest that the driving force for the movement of nutrients across the nodule cortex is provided by the H+-ATPase located on the PM of cortex cells.

On the other hand, the determination of protein level of IRT1 in nodules by western blot analysis (Slatni et al., 2011), showed a great accumulation of this protein in both −Fe Flamingo and −Fe Coco blanc with respect to the relative control conditions. In this work we confirmed these results but we also observed that IRT1 localized in the cortex of nodules from Fe-deficient plants, suggesting that IRT1 is directly involved in Fe2+ transport from the soil solution, across the Ex C of nodules, to the bacteroid interior.

Two different behaviours can be raised in this work concerning the allocation of Fe to nodules under Fe limiting condition. The nodules of the tolerant cultivar, Flamingo, in addition to Fe supply through xylem flux from roots, depend by large part, if not exclusively, on their own ability to acquire Fe directly from nutrient solution; thus, Fe is rapidly available for nodule tissues and bacteroids to support the synthesis of Fe-depending proteins, i.e. NR and LH. This in part explains the greater efficiency of Flamingo to fix N2 under limited Fe availability (Slatni et al., 2008). On the contrary, under the same condition, the nodule of Fe-deficient plants of the sensitive cultivar, Coco blanc, depends on Fe supply through xylem flux from roots via VB and when this source is blocked or decreases the availability of Fe, the nodule is rapidly affected by Fe deficiency.

Overall, in this work the comparison between tolerant and susceptible cultivars allowed to identify the traits conferring efficiency in Fe uptake/supply to nodules. Basing on our results (data from this work; Slatni et al., 2009, 2011) and also on studies focalized on Fe uptake and transport to bacteroids (Le Vier et al., 1996; Udvardi and Day, 1997; Moreau et al., 1998), we propose a model summarising Fe acquisition and transport in nodules (Fig. 4).

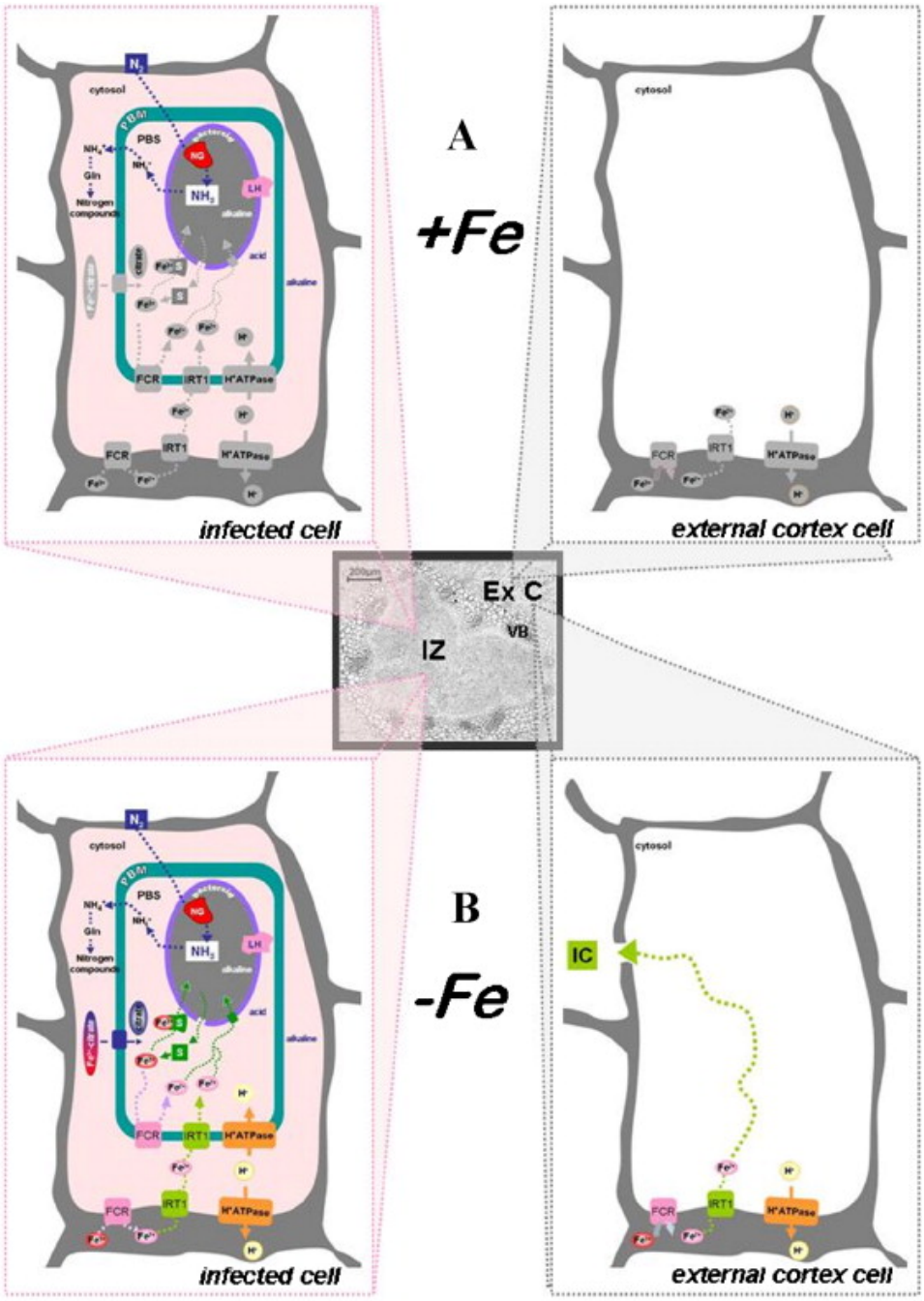

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Fig. 4. Model of Fe acquisition and transport in infected and external cortex cells of common bean nodules, of tolerant plant, under Fe sufficient (+Fe) (A) or Fe deficient (−Fe) (B) conditions. At the level of infected cells Fe deficiency induced the Fe uptake system (FC-R, IRT1, H+-ATPase) both at the PBM and the plasma membrane levels compared to +Fe condition. As well, at the level of the external cortex cells, Fe deficiency induced the Fe uptake system compared to +Fe condition, suggesting that −Fe nodules are able to acquire Fe directly from soil nutrient solution. The colored enzyme in the figure means an activated enzyme compared with the gray scale enzyme. LH: leghemoglobin; NR: nitrogenase; PBM: peribacteroid membrane; PBS: peribacteroid space; S: siderophore. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

In the presence of adequate Fe supply, plants deliver Fe to nodules via the VBs. Iron

coming from roots reaches the cytoplasm of nodule cells either as Fe2+ or Fe3+ form. Fe3+ can be transported across the PBM complexed with organic acids such as citrate and accumulates in the PBS (Le Vier et al., 1996; Moreau et al., 1995), where it is bound to S-like compounds by the bacteroids (Wittenberg et al., 1996) (Fig. 4A). On the other hand, the PBM possesses a FC-R capable to reduce Fe3+ to Fe2+. In Fe-deficient condition (Fig. 4B), we suggest that nodules are able to acquire Fe directly from soil nutrient solution. This ability implies the induction, in the Ex C of nodule, of (a) H+-ATPase which, acidifying the rhizosphere, increases the availability of Fe and creates a favourable pH condition for (b) FC-R activity which is increased in external cells of nodules (Slatni et al., 2009, 2011), and (c) IRT1 to transport Fe2+ from the rhizosphere into cytoplasm of nodule cells. Once Fe2+ has reached the cytoplasm of nodule cells, it is transported into the PBS through the same mechanism as in Fe-sufficient nodules (i.e. by IRT1 of the PBM). In addition to the direct absorption of iron by the cortex cells of nodules to support the iron requirement in the bacteroids, the xylem way from the root to the nodules of Fe uptake and transport is also present.

The main results of the present study suggest that under Fe deficiency, similarly to what occurs at the PBM, the nodule cortex shows a higher protein level of H+-ATPase and IRT1. At the whole, the data suggest that (i) these proteins play a critical role in the regulation of Fe uptake in nodules; (ii) nodules, especially from Flamingo roots, could be able to acquire Fe directly from the nutrient solution, particularly upon Fe-limitation. This would confer a considerable advantage to this cultivar, allowing the N2 fixation process to take place even in such a stress-inducing condition.

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