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New targets in plant boron deficiency response: N-glycosylation and regulation of root development.

(Nuevos blancos en la respuesta vegetal a la deficiencia de boro: N-glicosilación de proteínas y la regulación del desarrollo de la raíz)

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Abstract/ Resumen

Since Warington described in 1923 for first time boron (B) essentiality in plants, many authors have tried to understand what the micronutrient is doing, how the micronutrient is acquired, and what happens when the micronutrient is absent. First studies on B nutrition focused on physiological processes and biochemical pathways which appeared altered as a consequence of B deficiency/deprivation: from growth arrest, to transport of several solutes, and phenol, sugar or nucleotide metabolism. Next, several studies identified Rhamnogalacturonan II (RGII) pectin as the most relevant B ligand, because the micronutrient would mediate RGII dimerization and hence regulate cell wall porosity and strength. Other B complexes were identified *in vivo*, for example B-mannitol and B-sorbitol, in plants which produces such polyols instead of sucrose for sugar transport. Then molecular studies approached B transport, with the description of *BOR1* as a gene required for B transport, and some years later also with the description of *NIP5;1* . Such description was surprising because during many years it was assumed that boric acid, a neutral molecule, should not require neither channels nor carriers to move through the lipid bilayer. Since then, main efforts are focused on clarifying signaling pathways governing B deficiency response, which implies both the tight regulation of B transporters as well as the changes in transcriptome, proteome and metabolism.

However, many questions about B deficiency still remain unsolved. For example, during many years RGII dimerization was assumed to occur *in muro*, however recent evidences seems to reject such assumption. Also, to date there is no a convincing link between B-mediated RGII dimerization and the pleiotropic responses observed when the micronutrient is absent. Some authors argue claim for the existence of other B ligands, which could link B function with the observed B-deficient phenotypes. In such way, some recent reports confirmed the existence of complexes between B and polar glycolipids and glycosphingolipids. In this context our group have focused on glycoproteins because they can present *cis*-diols able to react with borate/boric acid at the same time that could be exerting a structural or signaling function due to complexion with B, and because some indirect evidences suggested a high requirement of B in biological processes which

implies a high synthesis of glycoproteins like the symbiosis between rhizobia and legumes, or the development of zebrafish.

In the present work I have first analyzed the potential of N-glycoproteins as B ligands in rhizobia-legume symbiosis, finding some proteins which could potentially interact with the micronutrient. However, during the development of such experiments was found an unexpected hyper-accumulation of N-glycoproteins under B deficient conditions. This process could be explained by either a failure in glycosylation or due to defects in secretion and sorting. Due to the lack of knowledge about N-glycosylation and protein secretion in rhizobia-legume symbiosis, and the difficulties to work with a complex process of organogenesis regulated by plant and bacteria mechanisms, I decided to switch to a best characterized model plant as *Arabidopsis thaliana*. Although some previous work were done with Arabidopsis (B transporters were primarily describe here), B deficiency response in Arabidopsis was only partially characterized. Therefore, I started characterizing B deficiency symptoms, which included growth arrest and changes in root morphology. Those are product of a reduced cell elongation, and a diminution of cell division, concomitant with an early cell differentiation prolonged close to the root tip, and the disappearance of quiescent center. In parallel was observed an accumulation of callose and lignin substances in B deficient plants. Then was confirmed that B deficiency led to an accumulation of N-glycoproteins in *Arabidopsis*, similar to that described in nodules. Because B deficiency produce an accumulation of N-glycoproteins, but apparently does not alter the N-glycosylation process, the accumulation should be caused by an alteration of the endomembrane system, as supported by some drugs which inhibits vesicle trafficking.

Nowadays has been found in plants other B ligands tan RGII, and we have the tools to find more in future. In example, N-glycoproteins are potential candidates, which could be universal B ligand in all eukaryotes and explain the requirement of B in organisms lacking RGII. But at the same time, taken together my results and most recent publications on B deficiency, RGII synthesis, cell wall perception, and root development, we have never so close to explain plant B deficiency response through a decrease of dRGII-B (B-mediated dimeric RGII) which would act as signal and trigger the activation of several signaling pathways which could explain many (if not all) phenotypes observed in response to B deficiency. Those signaling pathways activated would be: a) ethylene would be

controlling cell elongation and hairy phenotype; b) Wound/Cell Wall Damage/Pathogen-like response (involving jasmonic acid, salicylic acid, reactive oxygen species and Ca^{2+}) would control the accumulation of lignin and callose, as well the induction of stress responsive genes as jasmonic acid; c) control of root meristem size and root organization due to the auxin/cytokinin balance; d) other mechanisms would control B transporters by still unknown signaling pathways. By contrast, the accumulation of N-glycoproteins is in my opinion a mechanical consequence of some problems endomembrane trafficking. Future research will test the proposed model, and will define a fine roadmap of the B deficiency response, which will land in biotechnological targets to improve crops.

Desde que Warington describiera en el año 1923 la esencialidad del boro en plantas, numerosos autores han tratado de esclarecer la función del micronutriente, los mecanismos de adquisición y las respuestas que acompañan la ausencia del micronutriente. Los primeros estudios en la nutrición del boro se centraron en las consecuencias de la deficiencia de boro sobre distintos procesos fisiológicos y rutas bioquímicas: desde la inhibición del crecimiento al transporte de agua, al metabolismo de azúcares y nucleótidos. A continuación diversos estudios llevaron a la identificación de las pectinas, y en concreto el rhamnogalacturonano II (RGII), como el ligando de boro más relevante, donde el micronutriente mediaría la dimerización del RGII, regulando la porosidad y la rigidez de la pared celular. Otros complejos se han identificado *in vivo* en plantas, por ejemplo los complejos B-manitol y B-sorbitol, en plantas que producen estos polioles como azúcar móvil (en vez de la sacarosa). A posteriori, se analizó el transporte de boro usando abordajes moleculares, que llevaron a la descripción del gen *BOR1* para la carga xilemática, y unos años más tarde la descripción de *NIP5;1* como un importador de boro. Estos artículos supusieron una descripción sorprendente, porque durante muchos años se asumió que el boro, por ser una molécula neutra, podría atravesar la membrana lipídica sin la necesidad de canales ni transportadores. Desde entonces, se ha puesto el foco en clarificar las rutas de señalización que gobiernan la respuesta de las plantas a la deficiencia de boro, que implica tanto la fina regulación de los transportadores de boro como cambios en el transcriptoma, el proteoma y el metabolismo.

Sin embargo, muchas cuestiones sobre la deficiencia de boro siguen sin respuesta. Por ejemplo, durante muchos años se ha asumido que la dimerización del RGII ocurre *in muro.* Sin embargo, varios trabajos recientemente publicados parecen refutar esa hipótesis. Hasta la fecha tampoco hay una conexión evidente entre el papel del boro en la dimerización del RGII y los síntomas pleiotrópicos que se producen en deficiencia de boro. Ante estas lagunas, varios autores han propuesto la existencia de otros ligandos de boro, que podrían llenar ese hueco entre la función del boro y los síntomas producidos en su ausencia. En esa línea, recientemente se ha confirmado la existencia de complejos entre el boro y glicolípidos y esfingolípidos, y nuestro grupo se ha centrado en las glicoproteínas como potenciales ligandos de boro. Las glicoproteínas son candidatos interesantes porque estructuralmente presenta los grupos *cis*-diol capaces de reaccionar con ácido bórico/borato, porque biológicamente realizan funciones tanto estructurales como de señalización, y porque se ha observado un mayor requerimiento de boro en procesos biológicos que acarrean un incremento en la síntesis de glicoproteínas (como la simbiosis entre rizobios y leguminosas o el desarrollo del pez zebra).

En el presente trabajo se ha analizado el potencial de las N-glicoproteínas como ligandos de boro en la simbiosis rizobio-leguminosas, hallando que algunas proteínas podrían interaccionar con el micronutriente. Sin embargo, durante el desarrollo de estos experimentos se observó una extraordinaria acumulación de N-glicoproteínas bajo condiciones deficientes de boro. Esta acumulación de N-glicoproteínas podría explicarse por fallos en la glicosilación o en la maquinaria de secreción. Dado nuestro conocimiento limitado sobre la síntesis de glicoproteínas y sobre los mecanismos de secreción de las mismas en la simbiosis, y dada la complejidad añadida de trabajar en la misma, donde los dos simbiontes regulan la organogénesis del nódulo, se decidió cambiar a una planta modelo mejor caracterizada como *Arabidopsis thaliana*. Esta planta había sido utilizada previamente para analizar el transporte de boro, que llevó al descubrimiento de los transportadores de boro *BOR1* y *NIP5;1*. Sin embargo, no se caracterizaron en profundidad ni los requerimientos de boro de esta planta ni las respuestas a la deficiencia del micronutriente. En ese sentido comencé ese apartado caracterizando los síntomas que siguen a deficiencia de boro, que incluyen la inhibición del crecimiento y cambios en la morfología de la raíz. En concreto, la inhibición del crecimiento se debe a una menor elongación celular así como a un cese en la división celular, acompañadas de una diferenciación celular en regiones más próximas al ápice de la raíz, y a la desaparición

del centro quiescente. Paralelos a esta reorganización del meristemo se produce la acumulación de callosa y lignina en plantas crecidas en deficiencia de boro, así como la acumulación de N-glicoproteínas. Pese a esa acumulación de N-glicoproteínas en condiciones deficientes en el micronutriente, la síntesis de N-glicoproteínas no se ve afectada, por lo que esta acumulación parece responder a un fallo en el sistema de endomembranas, como así lo apoyan una serie de ensayos preliminares con ciertos inhibidores del tráfico vesicular.

Recientemente se ha descrito en plantas otros ligandos además del RGII, y poseemos las herramientas para encontrar más en el futuro. Por ejemplo, como se deriva de este trabajo, las N-glicoproteínas son potenciales candidatos, que al estar conservado en eucariotas podrían explicar el requerimiento de boro en otros organismos que no poseen RGII. Al mismo tiempo, basados en nuestros resultados, y en las recientes publicaciones sobre la deficiencia de boro, la síntesis del RGII, los mecanismos de percepción de la integridad de la pared celular y el desarrollo de la raíz, estamos en disposición de poder explicar todos la respuesta de las plantas a la deficiencia de boro en base a un descenso en el dímero de RGII mediado por boro (dRGII-B) que sería la señal que activaría diversas rutas de señalización que explicarían muchos (si no todos) los síntomas observados en la deficiencia de boro. Las rutas de señalización serían: a) Etileno, que controlaría la elongación celular y el fenotipo "peludo" de la raíz; b) La percepción por herida/daño en la pared celular - que incluiría ácido jasmónico, ácido salicílico, especies reactivas de oxígeno (ROS) y calcio – y que controlaría los depósitos de lignina y callosa, así como la expresión de ciertos genes; c) El control del tamaño del meristemo y su organización en base al balance entre auxinas y citoquininas; d) Otros mecanismos regularían la abundancia y localización de los transportadores de boro u otros fenotipos desconocidos por el momento. En mi opinión la acumulación de glicoproteínas se debe a un fallo mecánico en el sistema de endomembranas. Futuros trabajos confirmaran, desmentirán o modificarán el modelo aquí propuesto y permitirán definir en detalle una línea temporal de la respuestas a la deficiencia de boro, como futuros objetivos para la mejora biotecnológica de cultivos de interés agronómico.

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Pues somos un proceso, un ser/estar ensamblado una sola vez en un juego de presencias y ausencias, de circunstancias, tengo que dar gracias a todos, los que han sido y los que no.

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Abbreviators

α-Fuc Immonoglobulin (Ig) anti-fucose

α-Xyl Immonoglobulin (Ig) anti-xylose

AAC Amberlite IRA 7-43 Affinity Chromatography

ABA Abscisic acid

ACC 1-aminocyclopropane-1-carboxylic acid

ACN Acetonitrile

AI2 Autoinductor-2

AOX *mitochondrial Alternative OXidase*

B Boron

CHCA α-Cyano-4-hydroxycinnamic acid

CK Cytokinins

ConA Concanavalin A lectin

CW Cell Wall

CWD Cell Wall Damage response

CYCB1;1 Cyclin-dependent protein kinase CYCB1;1

dpg days post-germination

dpt days post-transference

DTT Dithiothreitol

EDTA Ethylene

ER Endoplasmic Reticulum

GA Golgi Apparatus

GFP Green Fluorescent Protein

GUS β-glucuronidase gene.

HG Homogalacturonan

HRP Horseradish Peroxidase

hpt hours post-transference

JA Jasmonic Acid

KDO 3-Deoxy-D-manno-2-octulosonic acid

LEH Length-of-the-first-Epidermal-cell-with-visible-root-Hair-bulge parameter

MALDI Matrix-Assisted Laser Desorption Ionization

TOF Time Of Flight

MES 2-(*N*-morpholino)ethanesulfonic acid

MRNG(s) Mannose Rich N-Glycan(s)

MRNGP(s) Mannose Rich N-GlycoProtein(s)

MSm Murashiegue and Skoog media

MS Mass Spectrometry

O/N Overnight

OG Oligogalacturonides

PAGE PolyAcrilamide Gel Electrophoresis

PAL Phenylalanine Ammonia Lyase

PAMPs Pathogen-Associated Molecular Patterns

PEB Protein Extraction Buffer

PB Petrobactin

pbm PeriBacteroid membrane

PI Propidium Iodide

PMF Peptide Mass Fingerprint

PVPP Polyvinylpyrrolidone

QC Quiescent Center

RF Rhizoferrin

RGI Rhamnogalacturonan I

RGII Rhamnogalacturonan II

mRGII monomeric RGII

mRGIII(B) "borated" monomeric RGII

dRGII-B B-mediated dimeric RGII

NMR Nuclear Magnetic Resonance

ROS Reactive Oxygen Species

RT Room Temperature (21ºC)

SA Salicylic Acid

SAM S-Adenosyl methionine

SDS Sodium Dodecyl Sulfate

TAIR The Arabidopsis Information Resource

TFA Trifluoroacetic acid

TGN/EE *Trans* Golgi Network/Early Endosome

Tris 2-Amino-2-hydroxymethyl-propane-1,3-diol

TY Triptone Yeast media

TLC Thin Layer Chromatography

UPR Unfolded Protein Response

VF Vibrioferrin

1. Introduction

1.1. Boron (B) chemistry.

Boron (B) occupies the number 5 in the element chart. It has two isotopes, ^{10}B and ^{11}B, with a relative abundance of 20% and 80% respectively, to give an atomic weight of 10.81 (Power and Woods, 1997). Together with Silicon (Si) and Germanium (Ge), the element is considered a metalloid, because it has intermediate properties between metals and non-metals (Power and Woods, 1997; Marschner, 2012).

It is considered a light and scarce element at Cosmos (16.9 ± 2.2 atoms/10^6 Si), bypassed during normal thermonuclear reactions, which suggest that most B is formed by spallation reactions (Power and Woods, 1997, and references therein). Although B is widely distributed in lithosphere and hydrosphere (Power and Woods, 1997), their abundance is different in marine and terrestrial environments (Shorrocks, 1997; Power and Woods, 1997; Carrano et al., 2009). In marine environments B concentration is approximately 0.4 mM, distributed in a homogenous way following a depth independent (non-nutrient-like) concentration profile (Carrano et al., 2009). In contrast, in terrestrial environments B is considered a scarce element, although distribution is heterogeneous, ranging from deficient to toxic areas worldwide distributed (Shorrocks, 1997; Power and Woods, 1997). In soil, B is present mainly as boric acid (H_3BO_3 or $B(OH)_3$), in equilibrium with borate ($H_3BO_4^-$ or $B(OH)_4^-$) (pK_a 9.24). These two molecules are the bioavailable forms of B (). Within cells (pH~7.5) more than 98% of B would be as boric acid, whereas at apoplast (pH~5.5) the borate anion will count less than 0.05% (Marschner, 2012). Boron is scarce in soils with high rainfall because the element is lixiviated. Clays increase retention of borate mediated by electrostatic interaction whereas organic matter increases retention of boric acid by esterification with *cis*-diol containing residues (Goldberg, 1997).

1.2. Boron requirement, boron ligands, and responses to boron nutrition along the life tree.

Boron is essential for some organisms in trace amounts, but to date there is not confirmed neither a requirement for all living things or a unifying biological function. Boron has been never reported as cofactor of any enzyme or as component of basic building blocks (Marcschner, 2012). However, all of described responses related to B

nutrition involved molecules containing *cis*-diol groups susceptible to react with borate or boric acid. This post-synthesis association of B increases the stability of such molecules independently of their biological function. The higher stability is explained because boric acid and borate esterify with the adjacent hydroxyl groups in *cis* configuration (Loomis and Durst, 1992), forming intramolecular or intermolecular bonds.

In this section, I summarized the organisms in which the element is essential, the responses of these organisms to different levels of B (from deficiency to toxicity), as also the molecules which has been reported to be ligands of B, if so, in each group.

1.2.1. Boron in Bacteria

In 1949, Skyes and Reed described that it prevents swarming in *Proteus vulgaris*, and after observing flagella under electron microscopy, they linked such effect with the formation of viscous substances of polyhydroxylic compounds (Skyes and Reed, 1949). However, requirement of the micronutrient for bacteria was never considered until Bonilla and coworkers described its essentiality for growth of diazotrophic filamentous heterocyst-forming cyanobacteria (Mateo et al., 1986; Bonilla et al., 1990; García-González et al., 1990). In these bacteria, the micronutrient is stabilizing some components of the heterocyst envelope, which become disassembled in the absence of the micronutrient (García-González et al., 1988; García-González et al., 1991). Recently our group described that glycolipids isolated from the heterocyst potentially interact with B (Abreu et al., 2014b). Nevertheless the exact point of action of B on the heterocyst envelope remains unsolved.

There is no extensive studies on mineral nutrition in bacteria excepting the auxotrophic photosynthetic bacteria, where boric acid is always included as constituent in media, i.e. Bg11 medium (Stainer et al., 1971); and few reviews that listed mineral requirements in bacteria associated with plants (O'Hara, 2001). Although inconsistent, somehow B is considered as beneficial element, with the exception of above described studies in some cyanobacteria (Bonilla et al., 1990). However, several recent studies have been focused in the isolation of bacteria from poor and rich B environments (Ahmed et al., 2007a; Ahmed et al., 2007b; Ahmed et al., 2007c; Ahmed et al., 2007d; Miwa et al., 2008a; Miwa and Fujiwara, 200; Miwa et al., 2009a; Yoon et al., 2010; Ahmed and Fujiwara, 2010; Ahmed et al., 2014b; Abbas et al., 2014). These authors have characterized a broad

spectra of bacteria, from those which growth better in absence of boron as *Lysinibacillus parviboronicapiens* sp. (Miwa et al., 2009a), those which do not need boron but are able to tolerate boric acid concentrations higher than 400 mM as *Gracidibacillus boraciitolerans* sp. (Ahmed et al., 2007b), to those unable to growth under B deficiency as *Bacillus boronophilus* sp. (Ahmed et al., 2006). This scenario reinforce the idea that each species, and even subspecies, varieties or cultivars could have specific requirements, making difficult to generalize a B requirement for such heterogeneous group.

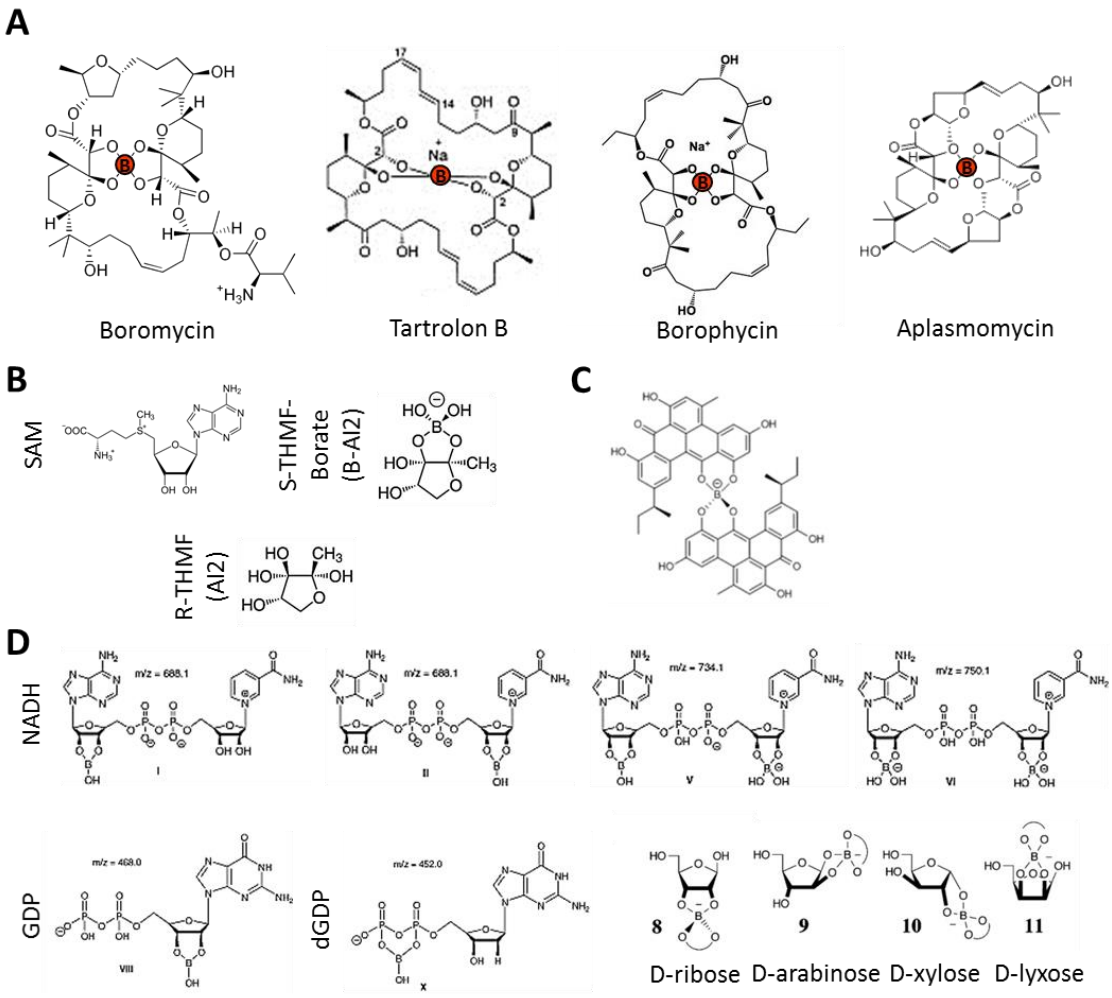


Figure 1. Boron ligands.

A) B containing bactericidal polyesther macrolides. Images taken from www.wikipedia.org

B) S-adenosylmethionine (SAM) is precursor of AI2 and an *in vitro* ligand itself. Borated (S-THMF-Borate) or non boronated AI2 (R-THMF) depend of the presence of B in the nutrient solution. Modified from Pereira et al., 2012. C) Borolithochromes from prehistoric *Solenopora jurassica*. Modified from Wolkstein et al., 2010. D) Proposed *in vitro* complexes between boron and nucleotides or monosaccharides. Modified from Kim et al., 2003; Ricardo et al., 2004, www.wikipedia.org

From the different performed studies, we cannot conclude whether the micronutrient is essential or not in all bacteria. But the methodologies are being used to test B essentiality in bacteria are also not accurate, because are mainly based in growth curves which don't consider the generational times nor the small requirements in those tiny organisms. Such methodologies only allow the detection of bacteria with high B requirements, as the filamentous heterocyst forming cyanobacteria when under auxotrophic conditions synthesize the heterocyst envelope (Mateo et al., 1986; García-González et al., 1991). In my opinion, serial experiments, where several "generations" of bacteria are constantly growing in B deficient media are more accurate, and allow a better characterization, not only of essentiality but also about the beneficial effects of B during stress, which we have proved recently in non-diazotrophic filamentous heterocyst-forming cyanobacteria and free-living rhizobia (Abreu et al., 2012, 2014b; Martín-Quijada, personal communication).

However, it is in bacteria where more and more diverse *in vivo* boron ligand molecules have been described to date, with the additional evidence that the micronutrient is increasing its stability or favoring their function. It is also interesting to notice that most of these B ligands were identified in marine prokaryotes, probably because the high availability of the micronutrient in seas (Carrano et al., 2009; Dembitsky et al., 2002, 2011).

First ever described B ligand was boromycin, isolated from *Streptomyces antibioticus* (Huetter et al., 1967; Dunitz et al., 1971). Later, B was found in other B containing antibiotics, namely aplasmomycin (Okami et al., 1976; Nakamura et al., 1977), tartrolon B (Irschick et al., 1995) and borophycin (Hemscheidt et al., 1994; Banker and Carmeli, 1998). All B containing antibiotics are polyether-macrolides, which present B in a central position stabilizing the structure of the antibiotic through intramolecular ester bonds (Fig. 1A). Their bactericidal potential is related with its lipophilic character and its function as ionophores (Kohno et al., 1996). Recently, some of these B containing antibiotics have received many attention because of its potential as anti-HIV agents (Kohno et al., 1996). Genes involved in the synthesis of tartrolon B have been recently identified in symbiotic cellulose-degrading bacteria in shipworm gills (Elshahawi et al., 2013), however B seems to be incorporated non-enzymatically as terminal step in these compounds (Chen et al., 1979; 1981; Elshahawi et al., 2013).

In 2002, Chen and coauthors reported that B was as component of Autoinductor-2 (AI2), a *quorum* sensing molecule of bacteria of the genus *Vibrio* (Bassler et al., 1993, 1994). Despite AI2 was discovered in advance, and its synthesis determined (Schauder et al., 2001), AI2 structure was not characterized untl Chen and collaborators crystallized sensor LuxP from *Vibrio harbeyi* bound to AI2. Using this unusual approach they had the chance to discover that AI2 is a furanosyl borate diester (Chen et al., 2002).

Biosynthesis of AI2 starts from S-adenosylmethionine (SAM), known to be an *in vitro* B ligand (Ralston and Hunt, 2001). SAM is transformed in dihydroxy-2,3-pentanedione (DPD) after three enzymatic steps (Schauder and Bassler, 2001; Schauder et al., 2001). DPD is unstable, and exists as an equilibrate mixture between three isomers and two hydrated versions. The formation of S-THMF-borate is not enzymatically controlled, and borated AI2 can be obtained spontaneously even from synthetic DPD. In the presence of B, cyclic DPD reacts with boric acid to form the borated-AI2 (S-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate or S-THMF-borate), which is the active quorum sensing molecule recognized by LuxP in *V. harbeyi* and other marine bacteria (Chen et al., 2002). Because genes involved in AI2 synthesis (i.e. LuxS) and AI2 perception (LuxP or LsrB) are present in numerous and very diverse groups of both Gram-positive and Gram-negative bacteria, it was proposed that AI2 would be a universal signal in bacteria (Surette et al., 1999; Winans, 2002; Coulthurst et al., 2002; Pereira et al., 2012). However, in terrestrial bacteria as *Salmonella enterica ssp. enterica serovar Typhimurium* the molecule binds to the LsrB receptor is the DPD enantiomer, non-borated *R*-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) (Miller et al., 2004). This difference is probably explained because the different abundance of B in these environments: when boron is present in nutrient solution (as sea), it reacts forming S-THMF-borate, causing a shift of the equilibrium between DPD enantiomers; in poor boron environments (as soils) formation of R-THMF is propitiated (Meijler et al., 2004; Miller et al., 2004; Semmelhack et al., 2004, 2005). Because AI2 synthesis is cytoplasmic, it is still unknown if bacteria using borated AI2 have specific mechanisms to acquire B from the nutrient solution, or the micronutrient enter passively.

Siderophores are organic low-molecular weight compounds, universally synthesized to chelate iron and other metals who are scarce in environment (Johnstone and Nolan,

2015). Amin and colleagues (207) reported that vibrioferrin (VF) isolated from *Marinobacter* sp. DG870, 893, and 979 marine bacteria associated with *Gymnodinium catenatum* bind boron. Specifically VF formed a tetraester complex with carboxylate and *α*-hydroxyl groups. It was suggested that the affinity of VF by B was high because the complex VF-B was isolated from bacteria growing in borosilicate glass flasks but in media lacking the micronutrient (Amin et al., 2007). Based on this study, the affinity to boric acid was tested in other siderophores, including the hydroxamate, catecholate and carboxylate type siderophores, as well as those that use some combination of these three functional groups. Authors found that carboxylate (citrate) siderophores VF and rhizoferrin (RF), and catecholate petrobactin (PB) present a high affinity for boron, although form 1:1 complexes. However, aerobactin, a dihydroxamate siderophore has low affinity for B, coincident with the affinity spected based on electrostatic repulsion and steric impediments (Harris et al., 2007). Moreover the formation of VF-B complexes in *Marinobacter algicola*, has been confirmed that B nutrition exert a complex control over iron transport. Under iron sufficient conditions, boron presence didn't alter transcription nor translation of iron transporter genes. However, under iron deficient conditions, B regulates expression of genes related with iron transport in two fashions: a) down-regulation of the periplasmic ferric binding protein Mb-FbpA, and b) upregulation of other iron uptake genes (Weerasinghe et al., 2013; Romano et al., 2013). Under iron deficient conditions (-Fe), several periplasmic proteins were upregulated in –B when compared with B sufficient conditions (+B, 0.4mM), including the protein FbpA, e periplasmic member of an iron (Fe^{3+}) ABC transporter. Coincident with these results, *fbpA* gene expression decrease with the addition of 0.4mM or 10mM of boric acid to the nutrient solution (Romano et al., 2013). In parallel, the same authors demonstrate a direct binding of borate to Mb-FbpA, which would be acting synergistically in iron (Fe^{3+}) sequestration (Weerasinghe et al., 2013). In the other hand, iron deficiency causes the accumulation of several proteins involved in iron uptake, which at protein level are not affected by boron (*pvuA, pvsA, fhuA, vciA* and *aldolase*) but whose transcription requires B. Moreover, an iron regulator protein called Fur, which act as a transcriptional repressor of the above mentioned genes (excepting FbpA which has no Fur box sequence) when iron is present, showed the same gene transcriptional pattern (Barker, 2013; Romano et al., 2013; Weerasinghe et al., 2013).

The above mentioned studies on prokaryotic ligands of B suggest that they perform *'non vital'* functions, and cannot explain in any case the essentiality of B by filamentous heterocyst-forming cyanobacteria (Mateo et al., 1986; García-González et al., 1988; García-González et al., 1991) and *Bacillus boroniphilus* (Ahmed et al., 2007b). However, the ability to incorporate B in those molecules could improve the competitiveness of bacteria and the ability to adapt to environmental changes (Abreu et al., 2012; Abreu et al., 2014b; Martín-Quijada, personal communication).

1.2.2. Boron in Archaea

No single reports have studied B requirement in archaea. However, studies of microbial diversity in extreme environments, as boron-rich hot springs, have revealed the presence of archaea, but not bacteria, in the places with the highest boric acid concentration (~3500mg/L or ~56 mM). Specifically, analyzed RNA fragments showed homology with the species *Acidianus infernus* and uncultured *Staphylothermus* (Stout et al., 2009).

1.2.3. Boron in algae

Different studies have covered the requirement of B for algae, leading to different conclusions depending of the group. In diatoms the element is considered essential (Lewin, 1966a, b; Lewin and Chen, 1976; Smyth and Dugger, 1980), although the author found differences between marine and freshwater diatoms (Lewin et al., 1966a). Two functions have been proposed, that the micronutrient is incorporated in the cell wall, probably forming ester bonds with polysaccharide matrix, or that the is forming part of the borosilicate capsule (Loomis and Durst, 1992; Deyhle et al., 2004). In *Chlorophyta* (green algae) several authors found that boron is not essential in species as *Chlorella vulgaris*, *Scenedesmus* sp., or *Chlamydomonas reinhardtii* (Bowen et al., 1965; Dear and Aronoff, 1968; Gerloff, 1968; McBride et al., 1971; Kropat et al., 2011), although some authors defend a beneficial role of the micronutrient (McIlrath and Skok, 1958). As in other organisms, high concentration the element results toxic (Bowen et al., 1965; Reid et al., 2004), and have been described in *Chlorella pyrenoidosa* a delay in cell division, increase in size and accumulation of proteins (Fernández et al., 1984; Sánchez-Maeso et al., 1985). Another green algae, *Chara*, has been used in several studies of B transport, because its big cells which are easy to manipulate, and become essential in the

understanding of B transport at cellular level (Stangoulis et al., 2001), because in conjunction to other studies (Dordas and Brown, 2001a, b) suggest that passive diffusion of boric acid it is only sufficient as driven force only when the gradient is high (Tanaka and Fujiwara, 2008).

In *Chlorophyta* has been described some ligands, although it is not clear its biological relevance. In seaweed *Ulva* was found a complex between ulvan (gel-forming water-soluble cell-wall sulphated polysaccharides) and boron at pH ≥ 9 (Lahaye et al., 1998). Also, the analysis of cytoplasm from *Chara* led to the identification of a bis-*N*-acetyl-serine borate complex (Stangoulis et al., 2010). Recently, it has been identified a family of compounds called Jurassic borolithochromes, presenting B in its structure, which has been identified in prehistoric algae deposits (Wolkenstein et al., 2010, 2015). Borolithochromes have been described as borated aromatic polyketides which differ from other borated polyketides, as the above mentioned bacterial antibiotics. Based on its localization, borolithochromes were associated with prehistoric coralline red algae *Solenopora jurassica* (Wolkenstein et al., 2010, 2015), however the only molecule showing a similar structure to borolithochrome is the clostrubin A, isolated recently from the anaerobic bacteria *Clostridium beijerinckii* (Pidot et al., 2014). Future studies will unravel the origin, synthesis and function of such complex but metastable compounds.

1.2.4. Boron in plants.

In 1910, Agulhon demonstrated a beneficial but not essential role of B for plants growth, and some years later Mazé (1912-1919) also provided evidences about the importance of B in plant growth. However, the report generally accepted to first describe the essentiality of B was authored by Katherine Warington in 1923. In her work, Warrington provided solid evidences about the requirement of B in broad beans (*Vicia faba*), later reinforced by other authors (Sommer and Lipman, 1926). Despite this recognition the study did not satisfied the three requirements later pronounced by Arnon & Stout for essential elements (Arnon and Stout, 1939). Early reports suggest that B can be substituted by germanium (Ge) (McIlrath and Skok, 1966), however later results confirmed that the micronutrient cannot substitute B essential functions but compete with B when Ge is present in high concentration (Brown and Jones, 1972; Ishii et al., 2002; Hayes et al., 2013). The third requirement to consider B essentiality is to know the function/s exerted by the micronutrient (Arnon and Stout, 1939). During many years were

proposed and discussed many diverse B functions (reviewed in Goldbach, 1997), until it was convincingly demonstrated that Boron mediates the dimerization of Rhamnogalacturonan II (RGII) in plant cell walls (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; Matoh et al., 1996; O'Neill et al., 1996; Kaneko et al., 1997).

Below we summarized most relevant boron nutrition investigations, which were focused on the next topics: i) B requirement in different plant species and varieties; ii) B functions and ligands; iii) Short and long distance transport of B, and finally; iv) Signaling and responses to B deficiency and toxicity.

1.2.4.1. Boron requirement in plants.

To date, B has been confirmed as an essential micronutrient in all vascular plants (Epstein & Bloom, 2005; Marschner, 2012), and apparently would be essential at reproduction stage in *Selaginella apoda* and *Dryoteris dentata* (Bowen and Gauch, 1965) but not in bryophites (Lewis et al, 1980). The requirements are very diverse in plants, ranging from 0.2 to 800 mg B kg^{-1} dry weight (Epstein and Bloom, 2005). The requirement at reproductive stage seem universal, and affect both monocots and dicots. However, the requirement in meristems is not universal, and depends of both pectin content and B remobilization mechanisms (Blevins and Lukaszewski, 1998).

First reports on B nutrition described that the symptoms of deficiency in plants were growth arrest and root swelling (Warington, 1923; Sommer and Lipman, 1926; Brenchley & Warington, 1927; Sommer and Sorokin, 1928; Johnston and Dore, 1928, 1929; McHargue & Calfee, 1932, 1933; Neales, 1959, 1964; Dear and Aronoff, 1965), accumulation of brown substances (Warington, 1923; Spurr, 1952; Albert and Wilson, 1961), which finally led to a necrotic phenotype and plant death (Sommer and Sorokin, 1928; Spurr, 1952; Whittington, 1957; Albert and Wilson, 1961). Different attempts using both histological and biochemical techniques provided evidences suggesting that growth arrest was likely caused either by a reduction in cell elongation (Odhnoff, 1957; Albert & Wilson, 1961; Cohen, 1977), an arrest of cell division (Sommer and Sorokin, 1928; Whittington, 1957, 1959; Cohen and Albert. 1974; Moore and Hirsch, 1983), or by a combination of both processes (Cohen, 1976; Cohen and Lepper, 1977). Further studies using electron microscopy dealt with ultrastructure and revealed defects in cell wall thickness and architecture (Spurr, 1957), disassembly of mitochondria and chloroplast,

accumulation of multiple secretory vesicles and hydrophilic substances (Hirsch and Torrey, 1980, da Silva et al., 2008).

Then, different authors explored the processes altered under B deficiency and tried to connect such responses with primary B functions (by alphabetical order): Alkaloids synthesis (Steinberg, 1955; Tso et al, 1962); Enzymatic functions (Lukaszewski and Blevins, 1996); Indolacetic acid metabolism (Eaton, 1940; MacVicar and Tnottingham, 1947; Galston and Dalberg, 1954; Bohnsack and Albert, 1977; Hirsch and Torrey, 1980; Hirsch et al., 1982); gibbelerelic acid (Skok, 1968); Lignin metabolism (Skok, 1957; Neales, 1960; McIlrath and Skok, 1964; Lewis, 1980); Membrane integrity (Pollard et al., 1977; Pollard et al., 1977; Cakmak et al., 1995; Marschner, 2012). Nucleic acid (RNA and DNA) metabolism (Cory et al., 1966; Cory and Finch, 1967; Chapman and Jackson, 1974; Cohen and Albert. 1974; Wainwrig et al., 1980; Lovatt et al., 1981); Phenol metabolism (Spurr, 1952; Perkins and Aronoff, 1956; Watanabe et al., 1961, 1964; Dear and Aronoff, 1965); Sucrose transport (supported by Gauch and Dugger, 1953; Mitchell et al., 1953; Sisler et al., 1956; Baker et al., 1956; Mitchell et al., 1960a, 1960b; refused by McIlrath & Palser, 1956); Sugar metabolism (Scott, 1960; Yih and Clarck, 1965). However no conclusive evidences were obtained for the functions proposed.

Then researchers focused at the cell wall, analyzing structural changes produced by B deficient symptoms and characterizing biochemically the cell wall, which led to satisfactory discovery of the rhamnogalacturonan II (RGII) as a B ligand, with the micronutrient mediating the dimerization of RGII (dRGII-B) (extensively explained below). Discovery of B transporters in *Arabidopsis* during the 00's opened another interesting scenario. Since then, homologues of BORs and NIPs have been described in many species, and become clear the existence of a fine control of these B transporters. In the last 15 years, molecular biology approaches and high-throughput methodologies have led B research to study: i) Identification of genes increasing Boron Acquisition Efficiency (BAE) and Boron Use Efficiency (BUE). ii) Characterize -Omic changes in response to b deficiency and toxicity (including transcriptomic, proteomic and metabolic changes); iii) Signaling pathways in plant B deficiency response.

1.2.4.2. Boron ligands.

As previously described, a series of evidences led to researchers to be focused on the effect of B on cell wall (Warington, 1923; Berger, 1949, Skok, 1957; Lewis, 1980; Loomis and Durst, 1991). Then it was demonstrated that more than 90% of B was firmly associated with cell wall in low B conditions (Yamauchi et al., 1986; Loomis and Durst, 1992; Matoh et al., 1992; Brown and Hu, 1994; Hu and Brown, 1994, 1996), and especially to the pectin fraction (Yamauchi et al., 1986; Matoh et al., 1993).

Pectins are plant heteropolysaccharides which embrace cellulose and hemicelluloses in the primary cell wall, where provide structural support maintaining wall structure, promote cell-cell adhesion, determinate development and cell expansion, an influence signaling (Mohnen, 2008; Atmodjo et al., 2013). Pectins consist of three domains, namely: Homogalacturonan (HG), Rhamnogalacturonan I (RGI), and Rhamnogalacturonan II (RGII). HG is the simplest domain, a linear polymer of 1,4-liked α-D-galacturonic acid; RGI is more complex, consisting of a main backbone composed of alternating (1-2)α-L-rhamnose-(1-4)-α-D-galacturonic acid, with side branches of arabinan and galactan (Mohnen, 2008; Atmodjo et al., 2013). Structurally, RGII is the most complex polysaccharide identified on Earth. It has a main chain similar to HG and four branches (named A to D), composed of thirteen different residues, some very specific as 3-Deoxy-D-manno-2-octulosonic acid (KDO) or 2-methyl-substitued L-fucose or xylose, and more than 20 different linkages (Pérez et al., 2003; O'Neill et al., 2004; Mohnen, 2008; Yapo and Beda, 2011a, 2011b; Bar-Peled et al., 2012; Atmodjo et al., 2013; Funakawa and Miwa, 2015). In parallel with RGII structural characterization (Darvill et al., 1978; Stevenson et al., 1988; Whitcombe et al., 1995), B was identified to mediate the dimerization of two RGII molecules (dRGII-B) (Stevenson et al., 1988; Ishii and Matsunaga, 1996; Kobayashi et al., 1996; Matoh et al., 1996; O'Neill et al., 1996; Kaneko et al., 1997). Specifically, borate forms four ester bonds between the apiose residues located at chain A of two RGII molecules (Ishii et al., 1999; Ishii and Ono, 1999).The B-mediated RGII dimmer (dRGII-B) is a metastable complex which is important in pore size and cell wall structure (Findeklee and Goldbach, 1996; Fleischer et al., 1999; Matoh et al., 2000; Ryden et al., 2003), being crucial for plant growth and development (O'Neill et al., 2001). The structural complexity of RGII it is proposed to be extended at genetic level, with some authors suggesting that RGII synthesis would

require more than 50 genes, although few have been identified (Funakawa and Miwa, 2015).

Several studies confirmed that RGII structure and its B-mediated dimer (dRGII-B) were highly conserved in vascular plants (Hu et al., 1996; Matoh et al., 1996; Pabst et al., 2013; Funakawa & Miwa, 2015), although there is a clear different content of B between dicotyledonous and monocotyledonous plants probably because the relative abundance of the pectin fraction in monocots (Hu et al., 1996; Matoh et al., 1996). B and dRGII-B has been also detected in trace amounts in primitive plants as lycophites, ferns, mosses and hornworts (Matsunaga et al., 2004). However, RGII is not as highly conserved as first supposed, several structural changes in RGII composition among species or developmental stages (O'Neill et al., 2004; Bar-Peled et al., 2012; Pabst et al., 2013), as well as in plant mutant lines with an altered glycome (Reuhs et al., 2004; Séveno et al., 2009; Voxeur et al., 2011).

During more than 15 years the hypothesis that dimerization of RGII occurred *in muro* was more accepted than an intracellular dimerization, although evidences were compatible with both mechanisms. *In muro* dimerization hypothesis was supported mainly on the basis that monomeric RGII (mRGII) (obtained by acid hydrolysis) spontaneously dimerizes *in vitro* in the presence of boric acid (1.2 mM, pH 3.4-5) (O'Neill et al., 1996; Ishii et al., 1999). The dimerization is favored by the presence of divalent cations as Pb^{2+}, Sr^{2+}, La^{3+}, Ba^{2+} and Ca^{2+} (O'Neill et al., 1996; Ishii et al., 1999) which would neutralize the negative charges of the mRGII. Furthermore, Fry and colleagues reported that dimerization is also favored *in vitro* by glycosphingolipids (GIPC) (Voxeur and Fry, 2014), synthetic polycations as polyhistidine and natural polycations as HydroxyProline Rich Glycoprotein (HPRG) (Chormova and Fry, 2015). *In vivo* studies confirmed a correlation between B levels and RGII dimerization, and between dRGII-B and growth or cell wall structure. In tobacco B-deficient cells the amount of RGII was marginal, however addition of B in B-deprived cell increases the amount of dRGII-B (Kobayashi et al., 1997; Matoh et al., 2000). Also, in low-B acclimated cells B addition produced a rapid decrease in cell pore (Fleischer et al., 1999). However, these results are compatible with the "intracellular dimerization" hypothesis, because none of these studies answer whether crosslinked RGII proceed from new synthesis or from mRGII previously secreted to the cell wall. Recent experiments

performed by Fry's group reinforce the "intracellular dimerization" hypothesis (Chormova et al., 2014a, b). They observed that B-deficiency acclimated cells resupplied with B form dRGII-B, but when B resupply is accompanied by treatments altering sugar metabolism they are not able to form dRGII-B. Also, exogenous labelled mRGII added in cells growing with B was not incorporated in the cell wall. In my opinion this experiments were inconclusive because they inhibited sugar metabolism instead of using drugs that specifically inhibit secretion, and because experiments of addition of labeled RGII were performed on mature cells, whose already built wall could indeed impede that RGII reached the plasma membrane-cell wall interface, where the assembly of new cell wall should take place.

From a theoretical perspective, B could enter in secretion vesicles by passive diffusion or through the action of B transporters (Miwa et al., 2013). Secretion vesicles perfectly satisfy the pH (5-5.5) but not the Ca^{2+} concentrations (>1 mM) required for *in vitro* RGII dimerization. Other evidence suggesting that *in vivo* RGII could be dimerized intracellularly came from RGII localization studies. First studies reported that RGII occurred in primary cell wall, but not in middle lamella or in secondary cell walls (Williams et al., 1996; Matoh et al., 1998; Willats et al., 2001). However, more recently other reports found RGII in nascent cell plates (Baluska et al; 2002, 2005; Dhonukshe et al., 2006). Cell plate formation could be divided in different stages: at the beginning, secretion of proteins and polysaccharides predominate, and at later stages, endocytosis contributes to recycling plasma membrane proteins (Drakakaki, 2015). So RGII could be dimerized within vesicles before secretion or recycling from mature cell wall respectively. Because in dividing cells treated with BFA, RGII was accumulated within the BFA compartments (Yu et al., 2002; Baluska et al; 2002, 2005; Dhonukshe et al., 2006), the authors assumed that pectin were endocyted from cell wall. Further progress in our understanding of the cytokinesis (Reichhardt et al., 2007; Viotti et al., 2010), and some inconsistencies found in the above mentioned literature, led us to reinterpret available data.

On one hand, authors misunderstood that anti-RGII antibodies recognize both mRGII and dRGII-B, not only dRGII-B as the authors assumed (Matoh et al., 1998). On the other hand, recent evidences suggest that BFA inhibits both Golgi secretion (Golgi and ER are somehow fused and typical Golgi markers are found in ER) and endosome recycling.

Because both secretion and endosomal pathways converge at *Trans* Golgi Network/Early Endosomal (TGN/EE) compartment, a secretory origin of the RGII synthesized in the cell plate cannot be ruled out. Indeed, other cell wall polysaccharides secreted to the cell plate as xyloglucans seem to be originated mainly from new synthesis instead of secretion (Drakakaki, 2015).

Although RGII is the universal plant B ligand, polyol sugars also form stable B complexes in phloem sap (Hu and Brown, 1997; Stangoulis et al., 2010). To date, it has been reported the presence of sorbitol-B (2:1), mannitol-B (2-1), sucrose (2:1) and N-acetyl-serine (2:1) complexes. Also, indirect evidences suggest that glycolipids (Abreu et al., 2014b; Voxeur and Fry, 2014) and glycoproteins (Wimmer et al., 2009) are forming, at least transitorily, complexes with B and RGII. Fry and collaborators isolate GIPC-B and GIPC-B-RGII complexes from plants, and proposed that those ligands could be intracellular intermediaries in the formation of dRGII-B (Voxeur and Fry, 2014). Using co-immunoprecipitation assays, our group identified a stable complex between a legume specific Arabinogalactan Protein Extensin-like (AGPE) and RGII (AGPE-B-RGII) in symbiotic rhizobia-legume root nodules, although still we have no direct evidence about the interacting sites (Reguera et al., 2010a). Overall, those data reinforce the hypothesis that RGII is not the only B ligand in plants, although it is probably the most stable. Future research is needed to clarify the function of the B-dependent complexes between glycolipids or glycoproteins with RGII, and the existence, if so, of other B complexes in plants.

1.2.4.3. Transport of boron in plants.

Boron transport has been analyzed both at cellular and at whole plant levels. At cellular level several authors have described either passive or mediated mechanisms of transport through the lipid bilayer. At plant level, it has been assumed that B is transported upstream by transpiration, and depending of the carbon compound used to translocate photosynthates B could be phloem mobile or immobile (reviewed in Brown and Shelp, 1997; Brown et al., 2002; Dannel et al., 2002; Takano et al., 2008; Tanaka and Fujiwara, 2008; Miwa et al., 2008b; Fuji et al., 2009; Fuertes et al., 2010; Miwa and Fujiwara, 2010; Miwa et al., 2010; Reid, 2014).

At cellular level was assumed that boric acid, a small (molecular radius is 2.573 Å) non-charged molecule whom theoretical lipid permeability coefficient was 8×10^{-6} cm S^{-1}, could cross passively the plasma membrane (Raven, 1980). Under sufficient boron the microelement moves merely by passive diffusion and permeability coefficient was similar to the theoretical (Dordas and Brown, 2000). However, the permeability coefficient calculated in plasma membrane of *Cucurbita pepo* (Dordas and Brown, 2001b) or *Chara corallina* (Stangoulis et al., 2001) were lower, which implies the existence of B transporters. Those results were supported with the description of an active B transport induced by low B conditions (Dannel et al., 2000). In this scenario, where an active transport mechanism was supported we still missing the actors, which would arrive during 00's. The first B transporter, *BOR1*, was described in *Arabidopsis* (Noguchi et al., 1997; Noguchi et al., 2000; Takano et al., 2001; Takano et al. 2002). BOR1 (At2g47160) is member of a family of $Na^+/HCO3^-$ antiporters (Parker and Boron, 2013), which have several paralogs in *Arabidopsis*, some already described as B transporters: *BOR2* (At3g62270) and *BOR4* (At1g15460) (Miwa et al. 2007; Miwa et al. 2013). Then NIP5;1 was described as a B channel (Takano et al., 2006). NIP5;1 (At4g10380), is an aquaporin of the *Nodulin-26 like Integral Protein* (*NIP*) family (Pommerrenig et al., 2015). In this second group have been characterized as B transporters *NIP5;1*, *NIP6;1* (At1g80760), and NIP7;1 (At3g06100) (Takano et al. 2006; Tanaka et al. 2008; Li et al., 2011). Other aquaporins have also the potential to transport boric acid, as *TIP5;1* (At3g47440; Pang et al., 2010) or *Solanaceae XIPs* (Bienert et al., 2011). The above mentioned BORs and NIPs act coordinately in *Arabidopsis* to control B homeostasis (under both deficient and toxic conditions) (Takano et al., 2008; Fuji et al., 2009; Miwa et al., 2009b; Miwa et al., 2010; Reid et al., 2014; Pommerrenig et al., 2015; Shimotohno et al., 2015). Because there is homologs of these transporters in other angiosperms [as *Zea mays* (Chatterjee et al., 2014; Durbak et al., 2014; Leonard et al., 2014), *Oryza sativa* (Nakagawa et al. 2007; Tanaka et al. 2013, 2014; Liu et al., 2015), *Vitis vinifera* (Pérez-Castro et al. 2012), *Hordeum vulgare* (Reid 2007; Sutton et al. 2007; Schnurbusch et al., 2010), *Citrus* (An et al., 2012; Cañon et al., 2013), *Medicago truncatula* (Bogacki et al., 2013), *Triticum aestivum* (Reid 2007; Leaungthitikanchana et al. 2013. 2014; Pallotta et al., 2014)], in primitive plants (Wakuta et al., 2015), yeast (Nozawa et al., 2006b; Takano et al., 2007; Jennings et al., 2007; Kaya et al., 2009; Bozdag et al., 2011) and even in humans (Park et al., 2004, 2005), we could conclude the importance of B homeostasis in living organisms, even in those where B requirement has not been proved.

In the next paragraphs we briefly expose the specific function, location, and regulation mechanisms for the *Arabidopsis* B transporters, in order to understand B homeostasis in this model plant:

- Aquaporin *NIP5;1* is a channel which acts as an importer, loading boron in the cytosol which then would move through the symplast way. *NIP5;1* defective mutants are susceptible to B deficiency, but indistinguishable of wt plant under high B concentrations (Takano et al. 2006). It is located in root outer epidermis, and this polar location requires some specific secretion machinery, because when TGN is altered there is an intracellular accumulation of NIP5;1 (Takano et al. 2006; Alassimone et al., 2010). In contrast, *NIP5;1* gene is regulated at post-transcriptional level, through the stability of NIP5;1 mRNA by a mechanism which probably implies the ribosomal machinery (Takano et al., 2010; Tanaka et al., 2011; Uehara et al., 2015).

- *BOR1* is a boron exporter, located at the inner face of the endodermis. When mutated, plants grew in low B are smaller than wt and B content is reduced in shoot but not in roots. However plants grew under optimal B are indistinguishable from the wild type (Noguchi et al., 1997; Noguchi et al., 2000; Takano et al., 2001; Takano et al., 2002). Because its location and the phenotype observed, BOR1 plays an essential function in removing B from symplastic route, once the nutrient have passed the Caspary band, and loading it in xylem (Takano et al., 2002). Then, B can be move upward by transpiration. *BOR1* is regulated at post-traductional level. Under low B level (<100 μM) the protein is located at plasma membrane, but when B increases (>100 μM) BOR is endocyted, ubiquitinated and transported to lytic vacuoles to be degraded. Several key residues have been identified in BOR1 sequence involved in the mentioned regulation: tyrosine residues Y398, Y405 and Y414 are critical for B-dependent endocytosis, lysine reside K590 is required for ubiquitination and subsequent degradation, and leucine residues L455 and L456 seem important in the polar localization and in the B-dependent internalization (Takano et al., 2005, 2007, 2010; Kasai et al., 2011; Yoshinari et al., 2012; Yamauchi et al., 2013; Kasai et al., 2014; Wakuta et al., 2015). By this tight control *BOR1* has become a model to study regulated secretion, endocytosis, and protein degradation (Alassimone et al., 2010; Viotti et al., 2010).

- *BOR2* is another BOR family member, the closest homolog of *BOR1*, expressed in lateral root cap and root epidermis. Under low B levels, *bor2-1* mutants showed a drastic reduction in cell elongation, higher than wt and *bor1-1* mutant, however plants grew

normally under normal B concentrations. Because all *bor2* mutants alleles showed no significant changes in B content but changes in RGII dimerization, it was proposed that this transporter is supplying B to cell wall or to vesicles favoring RGII dimerization (Miwa et al., 2013).

- *NIP6;1* is another B responsive aquaporin able to transport the micronutrient, which is located in nodal sites at shoots and stems, specifically in companion cells. *nip6;1-1* and *nip6;1-2* mutants showed small shoots, a decrease in the B content of young rosette leaves and stems, but no significant changes in old rosette leaves nor roots, suggesting that the protein is probably controlling the xylem-phloem transfer under low B conditions (Tanaka et al., 2008).

- *NIP7;1* is another aquaporin mainly expressed in the anther developmental (stage 9 to 11), which are coincident in *Arabidopsis* with the meiosis to form the tetrad microspores, the most sensitive stage to B deficiency in pollen development. However, this transporter showed a high specificity but low affinity for boron, which opens many questions about their function (Li et al., 2011).

- *BOR4* is another member of BORs family, which is stably expressed under B toxicity at the outer face of the epidermis. *bor4* mutants were hypersensitive to B toxicity whereas lines overexpressing *BOR4* in Arabidopsis showed an increased B toxicity tolerance, because the content of B in both root and shoots decreases. Due to its specific location, which is the opposite of BOR1 and BOR2, and the phenotypes observed with mutants and overexpressing lines, BOR4 would be pumping out excess of boric acid (Miwa and Fujiwara, 2011; Miwa et al., 2014).

At plant level, we can distinguish upward (via xylem) and downward (via phloem) B transport. All plants studied to date present isoforms of NIPs and BORs to control B homeostasis and favor both acquisition under low B conditions and excretion under high B conditions. Once B is incorporated in roots and loaded at xylem (passively or mediated by B transporters), it is assumed that B should move upward via xylem forced by the transpiration stream, in a passive process which would not involve any chelating molecule (Raven, 1980; Brown and Shelp, 1997; Tanako and Fujiwara, 2008). This results would explain why first B deficiency symptoms on shoots were observed in meristems, where the transpiration rate is minor if compared with mature leaves. Because numerous plants showed its maximum B in older organs, and B deficient symptoms at meristems, the micronutrient was considered immobile in these plants. However, there is other plants

where first B deficient symptoms are observed in mature leaves and not in meristem, suggesting the existence of a recycling/retranslocation/remobilization mechanism (Brown and Hu, 1996, 1997; Brown and Shelp, 1997; Bellaloui and Brown, 1998; Brown and Hu, 1998; Brown et al., 1999b). Several studies led to identify that these plants are characterized by the presence of polyols as photosynthate translocation molecule, and it was confirmed the presence in phloem sap of B-sorbitol and B-manitol complexes (Hu et al., 1997; Penn et al., 1997). Also was confirmed that increasing sorbitol synthesis by insertion of a limiting enzyme in tobacco turned B mobile in this plant (Bellaloui et al., 1999; Brown et al., 1999a; Bellaloui et al., 2003). Also some recent evidences suggest that the synthesis of polyols is regulated by the B nutritional state in olive (*Olea europaea)* (Perica et al., 2001a, 2001b, 2002; Liakopoulos et al., 2005, 2009). If this is the case, we should consider the existence of a systemic mechanism connecting meristems as the organ perceiving B deficiency with the source organs which would produce polyols to remobilize B to the meristem.

1.2.4.4. Boron levels perception by plants.

One of the main question in plant B nutrition is whether the nutrient itself, as free boric acid/borate, or any of the ligands (the amount of dRGII-B, or an uncharacterized B complex), is perceived by the plant, so plant is able to respond to the changing nutrient levels. Because phenylboronic derivatives lack one bond with the phenol moiety, they are able to form only one side complexes, so compete with borate anion. Some studies were performed using phenylboronic acids as competitor to test whether the ability of borate to form two side complexes is in the basis of the B deficiency response. Indeed BY2 tobacco cells treated with phenylboronic acids showed some morphological similitudes with B-starved cells (Bassil et al., 2004). A similar approach was followed in *Arabidopsis*, however, there is not conclusive results when analyzing expression of genes typically induced under B deficient conditions (Wimmer, personal comunication). Therefore there are not enough data to either confirm or discard that free boric acid/borate is directly perceived by plants. An alternative explanation would be that B levels are indirectly perceived by the amount of dRGII-B. Several mutants with an altered B transport or RGII structure (O'Neill et al., 2001; Delmas et al., 2008; Hayashi et al., 2008; Kobayashi et al., 2011; Miwa et al., 2013; Dumont et al., 2014) and treatments altering RGII synthesis (2β-Deoxy-Kdo and 2-Fluoro-L-Fucose; Smyth et al., 2013; Villalobos et al., 2015; Dumont et al., 2015) showed morphological symptoms similar to B-deficient plants (growth arrest,

root swelling, diminished cell elongation, abnormal cell differentiation, pollen sterile), and the increase of B in the nutrient solution rescued wild type phenotype. However expression of B transporter or other B-responsive genes have not been analyzed so far in these RGII mutants.

In the last years, plant B perception and response have received much attention due to the development of cheap high-throughput -omics. Hopefully, those methodologies would allow to clearly define early and late responses to B deficiency, as well as to define the connections between changes at biochemical/molecular level and early symptoms of B deficiency. Two main approaches have been used: top-down non targeted approaches and bottom-up targeted approaches.

Top down non-targeted approaches consist of a first characterization of the overall gene expression, proteins or metabolites which changed under B deficiency/toxicity. A second stage aims to validate and characterize the potential receptors and signaling cascades which could be participating in B perception. At transcriptomic level, several studies have been performed using both microarray (Kobayashi et al., 2004; Camacho-Cristóbal et al., 2008; Kasajima et al., 2010; Camacho-Cristóbal et al., 2011; Redondo-Nieto et al., 2012; Koshiba et al., 2013; Quiles-Pando et al., 2013; Zhou et al., 2015a, 2015b) and RNAseq (Yang et al, 2013a; Lu et al., 2014b, 2015; Yang et al., 2015) methodologies. At proteomic and metabolic level there is less available data, but has been described defects in cytoskeleton, energy metabolism or protein folding (Alves et al., 2011; Yang et al., 2013b; Chen et al., 2014; Lu et al., 2014a; Liu et al., 2015). Finally, at genomic level, a screening of mutants with a high requirement of B for elongation has also been performed, from which the ethylene receptor *CONSTITUTIVE TRIPLE RESPONSE1 (CTR1)* was identified (Tabata et al., 2003), suggesting an interaction between the hormone and the micronutrient (see below).The other approach, complementary to the above described, is a bottom up targeted approach. In this approach, agonist or antagonist drugs, as well as mutants in signaling pathways of interest were used with the objective to look for treatments which alleviate B deficiency symptoms or that mimic deficiency symptoms in B-sufficient plants. From both targeted and non-targeted studies have emerged a picture suggesting that B deficiency response requires the activation of several potential signaling cascades, including transcription factors (Kasajima et al., 2010; Camacho-Cristóbal et al., 2011; González-Fontes et al., 2013),

ROS (Koshiba et al., 2009; Oiwa et al., 2013; Camacho-Cristóbal et al., 2015); Ca^{2+} signaling (Koshiba et al., 2010; Redondo-Nieto et al., 2012; Quiles-Pando et al., 2013; González-Fontes et al., 2014), MAPK (Koshiba et al., 2010; Oiwa et al., 2013), ethylene (Martín-Rejano et al., 2011; Camacho-Cristóbal et al., 2015), auxin (Martín-Rejano et al., 2011; Camacho-Cristóbal et al., 2015; Li et al., 2015), cytokinin (Yang et al., 2013a) and wounding/pathogen-like responses (Koshiba et al., 2010; Oiwa et al., 2013).

A third line of evidence is being explored studying *BOR1* and *NIP5;1* boron transporters. As documented above, these transporters are tightly regulated by the content of B. Different reporter lines have allow identification of proteins controlling the important polar location of *BOR1* and *NIP5;1* (Alassimore et al., 2010; Viotti et al., 2010; Uehara et al., 2013) and the control mechanism executed by B sufficient or B deficient conditions (Takano et al., 2005; Fuji et al., 2009; Miwa et al., 2009b; Takano et al., 2010; Kasai et al., 2011; Tanaka et al., 2011; Yoshinari et al., 2012; Wakuta et al., 2015).

1.2.5. Boron in bacteria-plant symbioses:

An important amount if information about roles of B in plant developmental processes and ligands of B have been obtained from investigations of symbioses of plants with N_2-fixing bacteria. Below, we summarized those studies.

1.2.5.1. Rhizobia-legume symbioses

Interaction of legumes with rhizobia lead to the formation of a new organ called nodule, where bacteria can fix atmospheric nitrogen (N_2) into ammonia (NH_3), in a complex and tightly regulated process which require a molecular dialogue between both symbionts. Depending of the legume and the rhizobia, nodules are classified in determinate (i.e. *Phaseolus vulgaris-Rhizobium leguminosarum* B625) or indeterminate (*Pisum sativum-R. leguminosarum* 3841 or *Medicago sativa-Ensifer meliloti* 1021), characterized respectively by a determinate (spherical nodules) or apical indeterminate (cylindrical nodules) growth habit. Several stages can be highlighted during the establishment of the symbiosis: bacterial-plant recognition, infection, endocytosis of the bacteria, differentiation of the bacteria to bacteroid (which actively express nitrogenase enzyme), and finally senescence. In indeterminate nodules these stages coexist but are spatially organized, so in a mature nodule we can found a nodule meristem (I), an

infection/differentiation zone (II), a fixation zone (III), and a senescent zone (IV). In determinate nodules these stages occur sequentially, so all nodule structure is in one stage at each time (Beringer et al., 1979; Brewin et al., 1991, 2004; Oldroyd and Downie, 2008; Popp and Ott, 2011; Haag et al., 2013).

Boron was first described essential for rhizobia-legume symbiosis leading both to determinate or indeterminate nodules, and the requirement is B higher than not nodulated legumes (Brenchley y Thornton, 1925; Gárate et al., 1993; Yamagishi and Yamamoto, 1994; Bolaños et al, 1994; Bonilla et al., 1997). During three decades, our group focused the investigation on this topic, characterizing at physiological, histological and biochemical level the symptoms of deficiency in nodules. In *P. sativum-R. leguminosarum* symbiosis, our best characterized model, morphologically, plant roots and shoot showed typical symptoms of B deficiency (growth arrest, root swelling, "browning"…) (Gárate et al., 1993; Bolaños et al., 1994; Bonilla et al., 1997). B deficient plants also had a reduced number of nodules, which were small, round and pale brown instead of cylindrical and pink as control nodules. In B-deficient nodules, they are not functional nitrogenase activity is not detected, so plants suffering not only B but also N starvation, did not grow and finally died (Bolaños et al, 1994; Carpena et al., 2000).

In sections of B-deficient nodules, we observed that they were poorly invaded (Bolaños et al., 1996). Two processes are important for bacterial invasion: progression along infection threads and symbiosome formation after endocytosis. Infection thread (IT) is a transcellular channel-like structure surrounded by a cell wall with cellulose and pectins. The lumen if the IT is composed of a glycoprotein matrix which become case-harden by oxidative crosslinking (Brewin, 1991; Rae et al., 1992; Brewin, 2000, 2004). In B deficient nodules, IT were short and usually aborted (Redondo-Nieto et al., 2001). Close examination of –B IT showed that bacteria were "trapped" in the lumen and glycoprotein matrix attached to the bacterial cell surface (Reguera et al., 2010a). Once bacteria reach the forming nodule, they invade the host cell by endocytosis forming an organelle-like compartment named symbiosome, where bacteria are multiplied and then differentiate in N_2-fixing bacteroids. Bacteroids are engulfed by the peribacteroid membrane (pbm), and surrounded by the peribacteroid space, being the pbm particularly enriched in transporters of carboxylic acids which provide energy and nutrients to the bacteroid, and ammonium transporters which rescue the NH_3 produced by the bacteroid

(Whitehead and Day, 1997; Clarke et al, 2014; González-Guerrero et al., 2014). In B deficient plants, very few bacteria reach the endocytosis stage, and furthermore, symbiosome formation and bacteroid differentiation are aberrant (Bolaños et al., 1996; Bolaños et al. 2001; Redondo-Nieto et al., 2001).

At biochemical level our group demonstrated that early plant-bacterial recognition through Nod factors were affected by B starvation (Redondo-Nieto et al., 2001), but once plant and bacteria are in contact, bacteria is recognized as pathogen so we found induction of *PR* (*Pathogenesis Related*) proteins (Bolaños et al., 2004; Reguera et al., 2010b). In –B nodules, occurred cell proliferation but not proper cell differentiation, and nodule cells were smaller, especially at fixation zone (III), which was associated with a failure in endoreduplication of plant cells (Reguera et al, 2009). In B-deficient nodules we also observed a diminution of HPRG (HydroxyProline Rich Glycoproteins) (Bonilla et al., 1997), and abnormal glycosylation of PsNLEC1, a symbiosome targeted protein (Bolaños et al., 2001). Anti-RGII antiserum revealed abnormal intracellular localization in B-deficient nodules (Bonilla et al., 1997; Redondo-Nieto et al, 2007, 2008), suggesting an incorrect synthesis and/or targeting of pectins and glycoproteins. Another interesting biochemical phenotype was the description of a B-mediated complex between RGII and AGPE, the glycoprotein present in the IT. In B deficient nodules, this complex is reduced, which could explain the abortion of ITs (Reguera et al, 2010a). Last but not least, using Ambelite IRA 7-43, our group described the presence of potential B ligands, those identified to date were involved in O_2 protection of the nitrogenase (Reguera et al., 2010c).

1.2.5.2. Symbiosis *Frankia*-actinorhizal plants.

The interaction between actinorhizal plants and Frankia is also sensitive to B deficiency. Our group found that B deficiency altered nodulation and plant growth in *Discaria trinervis*-*Frankia* BCU110501 symbiosis. Also, free-living *Frankia* growth and vesicle formation were reduced under B and N deprived media, probably because a requirement of the micronutrient in the formation of the hopanoid layer which act as O_2 barrier and prevent nitrogenase inactivation. According to its structure, which present several *cis*-diol groups, so it was proposed that boron would crosslink hopanoids in a similar way that crossling glycolipids and polysaccharides in the heterocyst envelope (Bolaños et al., 2002).

1.2.6. Boron in yeast

Boron is not considered an essential element for yeast, because the micronutrient is not required for completing their life cycle. However, the element is considered beneficial because increases growth (Bennett et al., 1999). Much more attention has been paid to the toxic effect of high doses of B on fungi, which has been extensively used as treatments in urinary infections produced by *Candida* (De Seta et al., 2009) or *Saccharomyces* or in defense of *Botrytis* (Qin et al., 2010).

In *Saccharomyces cerevisiae*, boric acid concentrations higher than 10 mM reduces growth in liquid media (Abreu et al., unpublished results), although in plates there is some growth up to 80 mM (Nozawa et al., 2006b; Takano et al., 2007; Jennings et al., 2007; Kaya et al., 2009; Bozdag et al., 2011; Uluisik et al., 2011b), indicating the high tolerance of this organism to B. In relation with B toxicity, several B exporters have been described in yeast: BOR1, DUR3, FPS1 (Nozawa et al., 2006b), ATR1 (Kaya et al., 2009) and ATR-like genes (Bozdag et al., 2011), whose function is to prevent adverse effects of high boron in the cytoplasm (Nozawa et al., 2006b; Takano et al., 2007; Jennings et al., 2007; Kaya et al., 2009; Bozdag et al., 2011). Besides B transporters, several other genes have been identified conferring tolerance or sensitivity to boron treatments (Nozawa et al., 2006a). Genes conferring tolerance to high B are implicated in tRNA modifications, whereas those conferring sensitivity are involved in sugar and lipid metabolism, vesicular and transport system, and amino acid metabolism (Kaya et al., 2009; Uluisik et al., 2011b). These defects in amino acid metabolism were also confirmed after describing that B stress caused an induction of amino acid metabolism at the same time that decreased protein synthesis (Uluisik et al., 2011a). Other studies suggest that boric acid perturbs yeast cell wall and stress is perceived by the high osmolarity/glycerol (HOG) signalling pathway, the trehalose-synthesis (TPS1/TPS2) pathway, and the copper zinc superoxide dismutase SOD1 (Schmidt et al., 2012), suggesting that those are the pathways which made boric acid a good antimycotic agent. However, to date none B ligand has been identified in yeast.

1.2.7. Boron in animals

Mineral nutrition studies in animals have to face with the difficulties associated with a heterotrophic habit, which makes difficult to generate accurate mineral nutrient deficiencies. B essentiality has been described in *Xenophus*, *Oncorhynchus mykiss* (trout) and *Danio rerio* (zebrafish) (Eckhert, 1998; Rowe et al., 1998; Rowe and Eckhert, 1999; Fort et al., 1999; 2002; Nielsen, 2008). In the case of zebrafish B deficiency was accompanied by several embryo developmental defects (Fort et al., 1999, 2002; Reguera, 2009) which coincides with a period of high synthesis of membrane and glycoproteins in zebrafish embryos (Laughlin et al., 2008). In tetrapod, specifically in rodents, pig and humans the micronutrient is considered beneficial, but not essential (Hunt et al., 1996 Nielsen, 1996; Lanoue et al., 1998, 1999, 2000; Nielsen and Forrest, 2014). B function in animals is not clear, although the micronutrient has been connected with calcium metabolism (Barranco et al., 2009; Henderson et al., 2009), vitamin D metabolism (Hunt et al., 1994; Kurtoğlu et al., 2001), cell growth (Gallardo et al., 2004; Park et al., 2004, 2005), bone growth and maintenance (Hakki et al., 2010; Ying et al., 2011; Taşli et al., 2013) and inflammatory process (Hunt and Idso, 1999). Other evidences suggest that boric acid inhibits proliferation and migration of some prostate cancer cell lines (Barranco and Eckhert, 2004, 2006; Barranco et al., 2007). As in yeast, there are not B ligands described in animals, so at the moment we cannot propose a plausible scenario to explain such diverse responses to B deficiency. In humans, a B transporter was identified, *SLC4A1*, a member of the $Na^{+}/HCO3^{-}$ family that shows some homology with AtBOR1 (Park et al., 2004, 2005). It was confirmed that this protein transported B against gradient and regulate growth in cell lines (Park et al., 2005) and mutations in this gene was associated with several corneal endothelial dystrophy (OMIM 610206) (Lopez et al., 2009; Desir et al., 2007; Han et al., 2013; Jalimarada et al., 2013; Kodaganur et al., 2013; Siddiqui et al., 2014).

1.2.8. Boron ligands during *in vitro* studies: ribose and ribonucleotides in prebiotic world

Several *in vitro* assays have confirmed the ability of B to interact with *cis*-diol containing molecules including carbohydrates (Loomis and Durst 1992; Ricardo et al., 2004; Scorei and Cimpoiaşu, 2006), molecules conjugated with a glycan (glycoconjugates) (Nishikaze et al., 2013) and nucleotides (Ralston and Hunt, 2001; Kim

et al., 2003, 2004). Also this property has been exploited in the development of sensors or purification procedures for: monosaccharides, ribonucleotides or nucleic acids, glycopeptides and glycoproteins (Sparbier et al., 2005, 2006; Martin et al., 2013; Lacina et al., 2014; Li et al., 2014).

Complexes between B and monosaccharides, especially ribose, have been highlighted in relation with a potential role of B during origin of life on Earth (Ricardo et al. 2004; Scorei and Cimpoiaşu 2006; Benner 2007; Scorei, 2012; Grew and Edward, 2015). Several studies confirmed that under primitive conditions the micronutrient could stabilize ribose, and favor several reactions so increasing the amount of monosaccharides (Amaral et al., 2008; Kim et al., 2011; Furukawa et al., 2013; Gunther et al., 2013). This presumptive function is compatible with both "genetics first" and "metabolism first" models for the origin of life (Benner et al., 2010, 2011, 2012; Robertson and Joyce, 2012).

1.3. Open questions in boron nutrition: is there a universal requirement of the micronutrient? What is the *clé de voûte* in boron?

Arnon and Stout (1939) described the three requirements that an element have to satisfy to be considered essential in plants. We can extend such definition to other organisms, as animals or bacteria. Plant macronutrient requirement is conserved along the life tree, and include the following elements: Carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphate (P), sulfur (S), calcium (Ca), potassium (K) and Magnesium (Mg). Several micronutrients, are also universally required, as Iron (Fe), Manganese (Mn), zinc (Zn), copper (Cu) or chlorine (Cl). These common nutrients exert the same (i.e. Fe is a cofactor in metaloenzymes) or comparable functions (i.e. Ca, further it functions as second manager or cofactor, play a structural role stabilizing different extracellular substances). However, some elements are specifically required by particular organisms or phylogenetic groups, i.e. animals require iodide (I) and sodium (Na); or some plants and diatoms require silicon (Si). In this context, emerge some basic questions:

i) Is boron universally required for life? So far, we know that boron is essential in some bacteria, some algae, all vascular plants and some animals, and beneficial for many others.

ii) What essential function is boron performing? What building block, cofactor, or ligand require boron? To date, the boron ligands identified *in vivo* are not conserved along life tree, which would suggest an independent "evolution" of B essentiality in those organism. Moreover, the B ligands identified in bacteria are insufficient to explain their B

requirement. Because there are potential B ligands demonstrated *in vitro* which has been never probed *in vivo* (as saccharides, glycolipids and glycoproteins), and because there is other *cis*-diol containing molecules which never has been tested *in vitro* nor *in vivo* as potential B ligands (as phosphoinositides), we still have the possibility to find an universal B ligand and a universal B function.

1.3.1. How can we identify new boron ligands? Glycoproteins and glycolipids as potential boron ligands with signaling function.

Isolation and identification of *in vivo* B ligands is being an elusive task due to the low amount of B, the labile nature of the ester bonds that B forms with *cis*-diol containing molecules (susceptible to changes in pH), the absence of radioisotopes, or the relative simple isotopic signature (Goldbach, 1997). For that reasons only metastable B complexes have been detected, as RGII (O'Neill et al., 1996), AI2 (Chen et al., 2002), or B containing antibiotics. Two indirect methods have been proposed to identify potential ligands, one method is based on the ability of phenylboronic acid (PBA) to interact with *cis*-diol containing molecules (Bassil et al., 2004; Wimmer et al., 2009); the other method is based on the ability of Amberlite IRA 7-43 to sequestrate boric acid/borate, or one-side B complexes (Reguera et al., 2010c). These methodologies could be coupled online or offline with different separation, detection, and identification systems, depending of the research objective. To confirm the presence of B in such molecules, detection by Mass Spectrometry (MS) or Nuclear Magnetic Resonance (NMR) have proved to be valuable (Hu et al., 1997; Chen et al., 2002). Furthermore, once identified potential targets we should try to identify two side complexes which would require laborious work using yeast two hybrid system (only if both ligands are proteins), co-immunoprecipitation (which would require the development of specific antibodies), or other approaches used to study interactions between biomolecules (surface plasmon resonance spectroscopy, electrospray ionization mass spectrometry…; Piehler, 2005; Berggård et al, 2007; Kitova et al., 2012). Both PBA and Amberlite Chromatographic methods have been already used in plants, and from that studies emerge a scenario where glycolipids and glycoproteins could be B ligands (Wimmer et al., 2009; Reguera et al., 2010c; Abreu et al., 2012).

Other evidences supporting the hypothesis that glycoproteins are potential B ligands are the identification of B complexes with N-glycans *in vitro* (Nishikaze et al., 2013),

changes in Ps-NLEC1 glycoprotein pattern in legume nodules (Bolaños et al., 2001), and purification of glycoproteins and glycopeptides with phenylboronic acid resins (Wimmer et al., 2009). In this work, we will focus on N-glycoproteins because the available tools to analyze it, and because theoretically typical plant N-glycans posse at least three free *cis*-diol groups (Fig. 4A) so the number of N-glycosylation sites present in each N-glycoprotein would determine the available sites to interact with B (Fig. 5). Also, N-glycoprotein synthesis and secretion of both proteins and glycoproteins are better characterized in *Arabidopsis* (Etzler and Mohnen, 2009; Stanley et al., 2009; Ruiz-May et al., 2012; Strasser, 2014). Extracellular, plasma membrane, or endomembrane-localized proteins and glycoproteins are synthesized in ribosomes associated to ER, internalized co-translationally, and folded within ER lumen. After being checked by several folding quality tests, proteins progress along the endomembrane system to their final destination (Etzler and Mohnen, 2009; Ruiz-May et al., 2012; Strasser, 2014, Lannoo and Van Damme, 2015). In the case of N-glycoproteins, the N-glycan previously synthesized over a dolichol precursor by the action of several enzymes including *ALG3* (Henquet et al., 2008; Kajiura et al., 2010), *ALG10* (Farid et al., 2011), *ALG11* (Zhang et al., 2009), and *ALG12* (Hong et al., 2009), whose final structure is $Glu_2Man_9GlcNAc_2$. This N-glycan is transferred to the nascent protein in the ER by the action of the OST complex (Olygosaccharide Transferase) (Koiwa et al., 2003; Lerouxel et al., 2005; Frank et al., 2008; Hüttner et al., 2012; Su et al., 2012; Farid et al., 2013). Then, N-glycan is processed, including the removal of two terminal glucose residues (which act as quality control mechanisms) to obtain the Mannose Rich N-Glycan (MRNG), whose structure is $Man_9GlcNAc_2$ (Ruiz-May et al., 2012; Strasser, 2014; Lannoo and Van Damme, 2015). The synthesis and structure of the N-glycan in the ER is highly conserved among eukaryotes, whereas the structure of the N-glycan processed during Golgi diverge (Varki et al., 2009). In plants, those proteins which are not located at ER can suffer a co-secretion cleavage and decoration of the N-glycan, including removal of some mannose at ER by *MNS3, an* alfa-mannosidase type I, which in combination with Golgi Apparatus (GA)-located *MAN1* and *MAN2* remove four mannose residues (Liebminger et al., 2009; Kajiura et al., 2010) to form the $Man_5GlcNAc_2$ N-glycan. After that stage, maturation proceed in three interchangeable pathways, whose final products are: i) Complex N-glycans, whose general structure is $GlcNAc_2XylFucMan_3GlcNAc_2$ (GnGnXF), typical of

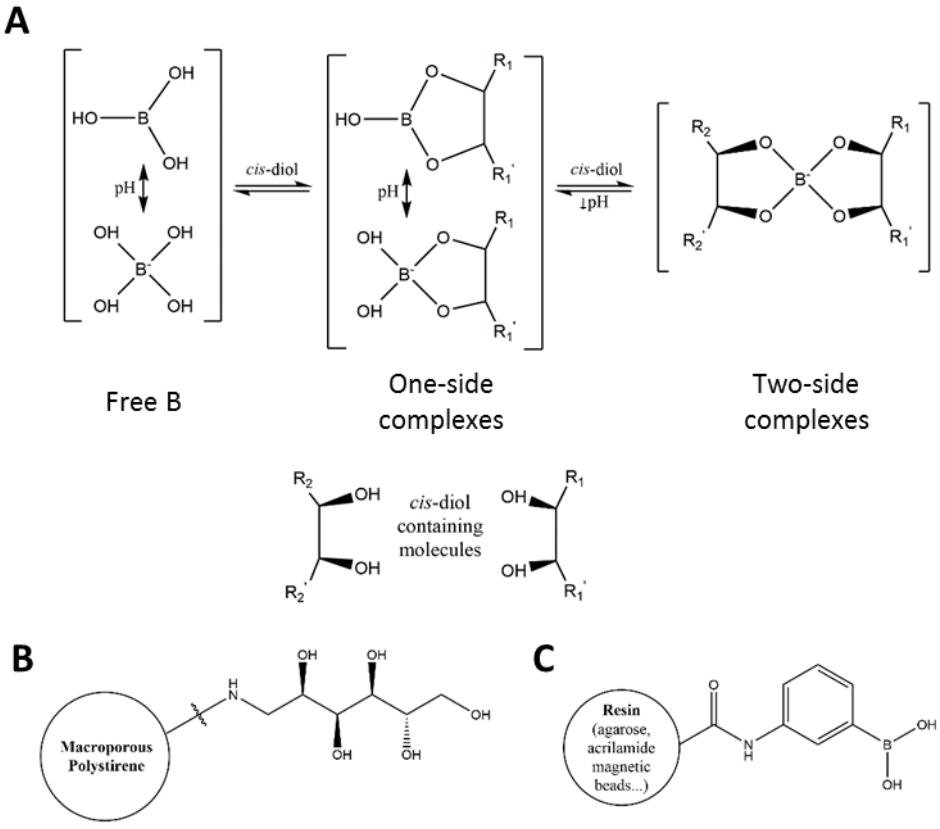


Figure 4. B mediated *cis*-diol crosslinking.

A) Boric acid/borate react with *cis*-diol containing molecules forming one side complexes. Under the presence of more *cis*-diol containing molecules two side complex could be formed. B) Structure of Amberlite IRA 7-43 and C) Phenylboronic Acid resins.

plasma membrane or secreted proteins. The synthesis of Complex N-glycan require several enzymatic activities, including GA-located N-acetylglucosamine transferase (*GnTI* and *GnTII*) (von Schaewen et al., 1993; Strasser et al., 2005; Frank et al., 2008; Kang et al., 2008), the removal of two mannose residues by GA-located alfa-mannosidase type II (*HGL1*) (Kaulfürst-Soboll et al., 2011) and the action of GA-located xylosyl and fucosyl transferases, *XYLT* and *FUT11/12* respectively (Strasser et al., 2004); ii) a second pathway led to the formation of a Lewis type-epitope whose structure is $Gal_2Fuc_2GlcNAc_2XylFucMan_3GlcNAc_2$, share the steps described for Complex-N-Glycan and is characterized by the presence of two terminal galactose and the presence of α1-4 fucose (Fitchette et al., 1999; Wilson et al., 2001; Léonard et al., 2002; Strasser et al., 2007); iii) a third pathway led to the formation of paucimannosidic N-glycan, usually proteins located at vacuoles but also in some extracellular proteins, whom structure is $XylFucMan_3GlcNAc_2$ (PNGXF), and it's the most abundant N-glycan found in plants. Paucimannosidic N-glycan requires the activity of *GNTI, HGL, XYLT* and

FUT11/12 enzymes, but also the action of β-N-acetylhexosaminidase *HEXO1* and *HEXO3* (Liebminger et al., 2011; Yoo et al., 2015).

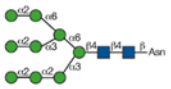
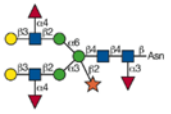
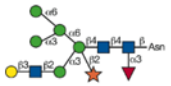
N-Glycan	Name	Synthesis	*cis*-diols
	High Mannose N-glycan	ER	3 Man
	Complex N-glycan	*medial*- & *trans*-Golgi, TGN	3 Fuc 2 Gal
	Hybrid N-glycan	*medial*- & *trans*-Golgi	1 Fuc 1 Gal 2 Man
	Paucimannose N-glycan	*medial*- & *trans*-Golgi, TGN, Vacuole	1 Fuc 2Man

Figure 5. Plant N-glycan types: structure, name, synthesis and number of free *cis*-diol moieties.

In the present work we will not include a detailed description of secretory pathways present in plants because they are beyond our objectives. However, some brief considerations are important for understanding potential effects of B deficiency. As mentioned above, glycoprotein synthesis and modification, and cell wall polysaccharides synthesis are coupled with endomembrane transport. In plants, there is a default secretion pathway used by proteins containing peptide signal, and recently it has been described the existence of a non-default pathway because proteins lacking secretion peptide could be transported to extracellular spaces (Drakakaki and Dandekar, 2013). In the default pathway, proteins are incorporated translationally into the ER, and depending of their final destination they can remain in the ER, or progress to the GA. At GA the road bifurcates, so proteins can go to the vacuole, plasma membrane, cell wall/apoplast but passing through the *Trans* Golgi Network/ Early Endosomal compartment (TGN/EE), a vesicular network structure where converged components newly secreted or endocyted from plasma membrane/cell wall. Proteins with lysosome or storing vacuole destination also go through the Pre-Vacuolar Compartment (PVC) (Driouich et al., 1993; Hanton et al., 2007; Bassham et al., 2008; Richter et al., 2009; Kim and Brandizzi, 2014). Synthesis of cell wall polysaccharides (hemicellulose and pectins) starts within GA, and their secretion also occur through the default secretion pathway, so once they are synthesized they are incorporated in vesicles which progress through the TGN/EE compartment to the

plasma membrane or the apoplast (Driouich et al., 1993; Driouich et al., 2012; Worden et al., 2012; Kim and Brandizzi, 2014; Drakakaki, 2015). As mentioned in section 1.2.4.2, some authors suggest that cell wall polysaccharides could be endocyted (Yu et al., 2002; Baluska et al; 2002, 2005; Dhonukshe et al., 2006), however there is not a general agreement about this claim (Reichhardt et al., 2007; Viotti et al., 2010). It's interesting to note here the existence of *echidna* mutant, which among other symptoms showed an accumulation of polysaccharides in the vauole, suggesting that failure in secretion produce a misslocalization not only of proteins but also of polysaccharides (Gendre et al., 2011, 2013; Boute et al., 2013; McFarlane et al., 2013).

O-glycoproteins, including Hydroxyproline Rich O-GlycoProteins (HRGPs) subtypes (extensins (EXTs), arabinogalactan proteins (AGPs) and Hyp/Pro-rich proteins (H/PRPs)), play very important roles in *Arabidopsis* development and stress responses. However O-glycosylation is worse characterized, i.e. the addition of the O-glycan to serine, threonine, and also to hydroxyproline residues occurs in Golgi, but it is not clear if the O-glycan is transferred in its final form, or it is further processed during secretion. Then, O-glycoproteins would be secreted via the default pathway indicated above (Driouich et al., 1993; Hanton et al., 2007; Bassham et al., 2008; Richter et al., 2009; Kim and Brandizzi, 2014). Also, the methodologies to analyze properly the O-glycosylation process are not standardized (Willats and Know, 1996; Nguema-Ona et al., 2007, 2012, 2013; Hijazi et al., 2014; Basu et al., 2015). So, although O-glycoproteins posse several *cis*-diol groups, in this work we will not explore its potential as B ligands.

Glycolipids are also potential B ligands because the *cis*-diol groups present in the glycan moiety, as recently explored (Abreu et al., 2014b; Voxeur and Fry, 2014). In plants can be found neutral glycolipids and glycosphingolipids, playing very diverse structural and signaling functions in plants (Hölzl and Dörmann, 2007; Sperling and Heinz, 2003; Pata et al., 2010; Markham et al., 2013). Also, several proteins are retained in plasma membrane through glycosylphosphatidylinositol-type (GPI) anchors, and are crucial in growth and elongation (Schindelman et al., 2001; Borner et al., 2002, 2003; Gillmor et al., 2005; Roudier et al., 2005; Etzler and Mohnen, 2009; Cheung et al., 2014).

Other potential B ligands are phosphoinositides (PIs), which possess two hydroxyl groups at positions 2 and 3 in *cis* configuration, and several hydroxyl groups in *trans*. A

potential complexation of B with PI would interfere the activity of PI kinases and modulate directly the formation of PI3P and PI(3,5)P_2 (Brown et al., 2002; Bolaños et al., 2004; Nielsen et al., 2014). PIs are particularly interesting because they have functions in vesicle trafficking and signaling (Thole and Nielsen, 2008; Boss and Im, 2012; Gillapsy, 2013; Krishnamoorthy et al., 2014). However, to date the hypothesis has not been properly explored and it is also beyond the scope of the present work.

1.3.2. Distribution and synthesis of boron ligands. When is the micronutrient added?

Another interesting question in B research is whether B is incorporated enzymatically or non-enzymatically to form *in vivo* B ligands. Prokaryotic ligands (AI2, B antibiotics and siderophores) only have the *cis*-diol groups where B is incorporated by a passive non-enzymatically terminal process. However, in RGII there are two apiose residues in chain A and B, but RGII dimerization occurs only through the apiose residue of chain A (Ishii et al., 1999). This specificity suggests some steric impediment and also open the possibility that the process is catalyzed by an enzyme. Fry's group failed in the identification of a "boron transferase", but maybe genes *shv3* and *slv1*, encoding glycerophosphoryl diester phosphodiesterase-like proteins (Hayashi et al., 2008), are interesting candidates now that glycolipid-B complexes has been described (Voxeur and Fry, 2014).

1.3.3. Gaps in boron deficiency response: passive defects vs active signaling.

To date, most B deficient symptoms have been considered the result of a failure in cellular processes or enzymatic activities. However, recent evidences suggest the activation of signaling processes during the B deficiency response (Martín-Rejano et al. 2011; Camacho-Cristóbal et al., 2015). During our work, additionally to the analysis of N-glycosylation during B deficiency response, we have characterize the effect of B on root organization and development, and the accumulation of different cell wall substances in response to B deficiency. In order to reevaluate B deficiency symptoms based on the complex networks which control and integrate plant development with environment perception, in the next paragraphs we summarize the organization of the *Arabidopsis* root and provide some basic concepts about the signaling pathways controlling root development and cell wall synthesis.

1.3.3.1. Root organization and development.

Arabidopsis root is organized in two axis. Longitudinally, we can distinguish a developmental gradient, from totipotent quiescent cells located in the root organization zone (ROM), to dividing cells in the division/proliferation zone, and finally to elongated cells, which also present specific morphological features, in the cell elongation/differentiation zone. Between division and elongation zone there is a transition zone, where there is a readjustment of the gene networks which would be transcribed to fulfill the different requirements in terms of that proteins and metabolites which are synthesized. Radially, *Arabidopsis* root consists of four concentric monolayers: epidermis, cortex, endodermis, pericycle; and centrally is localized the stele (with xylem and phloem). There is two exceptions to this organization, the columella and the root cap which are located at the root tip, with protecting and tracking functions respectively (Dolan et al., 1993; Scheres et al., 1995).

Once defined root organization, it is clear that root organization and development require several levels of regulation. A first level would determine the cell cycle phase, namely if a certain cell is at interphase, being divided or entering endoreduplication (Inzé and De Veylder, 2006; Takahashi and Umeda, 2014). Simultaneously, cell fate decisions are based on the position of the cell, asymmetric cell division, and the autonomous and non-autonomous signals present in those cells (Hove and Heidstra et al, 2008; Petricka et al., 2009, 2012a, 2012b; Perilli et al., 2012; Sozzani and Iyer-Pascuzzi, 2014). Once cells enter in elongation there is a massive increase of cell surface and volume, which implies other level of regulation to activate both protein and polysaccharide synthesis (Sanmartín et al., 2011; Liberman et al., 2015). In plants, elongation is a prerequisite to cell differentiation, which in this case implies the acquisition of specific biochemical and morphological features (i.e. root hairs in epidermis, Caspary bands in endodermis or lignification and cell death in protoxylem cells) (Perilli et al., 2012; Petricka et al., 2012). Cell division dominates over cell elongation/differentiation during the first days post-germination, until the meristem have a critical mass which allow to conform a mature meristem, where cell division and cell elongation/differentiation are balanced (Moubayidin et al., 2013; Sozzani and Iyer-Pascuzzi, 2014; Pacifici et al., 2015).

Cell cycle, cell fate/identity, the developmental program and the maintenance of the meristem organization are controlled by an intricate, balanced and dynamic network of signals including phytohormones (auxin, cytokinin, brassinosteroids, gibberellic acid, ethylene, ABA…), RNAs, miRNAs, proteins, small peptides, ROS and other metabolites (Hove and Heidstra et al, 2008; Petricka et al., 2009; Tsukagoshi et al., 2010; Wells et al., 2010; Garay-Arrroyo et al., 2012; Perilli et al., 2012; Petricka et al., 2012; Delay et al., 2013; Sozzani and Iyer-Pascuzzi, 2014; Takatsuka and Umeda, 2014; Tsukagoshi et al., 2014; Drisch and Stahl, 2015; Pacifici et al., 2015). Plants are able to perceive several external constrains and to develop coordinated changes in root architecture (promoting or inhibiting growth) controlled by the above mentioned mechanisms. Several examples of this control have received attention in the last years, as nutrition perception mediated by TOR (Target Of Rifampicin) signaling (Henriques et al., 2014; Xiong and Sheen, 2014; Barrada et al., 2015) or the homeostasis of metals (López-Bucio et al., 2003; Abel, 2011; Giehl et al., 2014; Giehl and von Wiren, 2014; De Smet et al., 2015).

1.3.3.2. Cell wall synthesis and perception

Cell wall components are synthesized in different compartments: glycoproteins and cell wall polysaccharides (hemicelluloses and pectins) are synthesized in endomembrane compartments; cellulose is synthesized in the plasma membrane; whereas monolignols subunits are synthesized in the cytoplasm and further secreted to the cell wall (apoplast) where they are polymerized. The synthesis and deposition of these polymers are coordinated, and usually plants compensate the lack of one polymer increasing the synthesis of others (Benatti et al., 2012; Le Gall et al., 2015).

This compensation response is now accommodated in a model where cell wall is not a passive structure, but an active and dynamic compartment similar to yeast and animal envelope. In this model, plant mechanoreceptors as well as cell wall receptors are able to perceive respectively mechanical stress and changes in composition, and activate signaling cascades which result in a compensation response (Pilling and Höfte, 2003; Humphrey et al., 2007; Seifert and Blaukopf, 2010; Steinwand and Kieber, 2010; Hamann and Denness, 2011; Benatti et al., 2012; Engelsdorf and Hamann, 2014; Hamann, 2015; Nishitani and Demura, 2015).

2. Objectives

Based in the antecedents mentioned in the introduction section, the hypothesis of this work is that B is required in all living organism for a correct assembly and reactivity of glycoconjugates (glycoproteins, glycolipids and other molecules containing a glycan moiety). Previous research demonstrated abnormal glycosylation of proteins important for a correct establishment of the legume-rhizobia symbiosis, and described some of them as ligands of B, therefore I will specifically explore in this work the potential of N-glycoproteins as boron ligands in rhizobia-legume symbioses. The aims of this thesis are:

i. To characterize N-glycoprotein patterns and N-glycoproteome in B-sufficient and B-deficient legume root and nodules.
ii. To identify potential N-glycoprotein B ligands using Amberlite IRA 7-43 affinity chromatography.

The development of the symbiotic nodule is a complex process of organogenesis regulated by two different organisms. Hence, the previously reported results and the obtained during the approach of these two objectives could be attributed to an incorrect legume-bacteria interaction, as a consequence of the long term exposure to B deficiency, rather than to responses to a starvation of the nutrient. Therefore, after describing N-glycosylation in B-deficient nodules I focused on a simplest model as *Arabidopsis thaliana* where, contrarily to legume nodules, a deep characterization of B requirements and a phenotypic description of B deficiency responses have still to be unraveled. So, using a battery of *Arabidopsis* lines I defined three tasks:

iii. To define optimal levels of boron for *Arabidopsis* growth
iv. To characterize boron deficiency responses in Arabidopsis:
    a. Morphological and developmental alterations in root meristem
    b. Accumulation of cell wall polymers
    c. N-glycoprotein pattern
v. To explore synthesis vs. secretion effects of B deficiency on N-glycosylation.

3. Material & Methods

3.1. Growth conditions

For all the experiments included in this manuscript B-free media was prepared with miliQ water (Eppendorf), in polyethylene B-free containers (VWR). Media were incubated for at least 48 hours under shaking (except when indicated) with Amberlite IRA 7-43 resin (Sigma), which strongly complexes H_3BO_3 on its N-methylglucamine functional groups with a adsorption capacity of up to 5 mg B g^{-1} (Asad et al., 1997). Boron was added as boric acid (Fluka), from sterile stocks prepared in plastic bottles with miliQ water.

All chemicals were pure grade (Sigma), except when indicated.

3.1.1. Growth of rhizobia

Rhizobia strains that nodulate legumes representative of indeterminate (*Pisum sativum*, *Medicago sativa*) or determinate (*Glycine max*) nodule development used in this study are listed in Table 1. Bacteria were cultivated in liquid tryptone-yeast (TY) medium [5 g L^{-1} tryptone, 3 g L^{-1} yeast extract, 10mM $CaCl_2$] (Beringer, 1974), in shade, at 28ºC on an orbital shaker at 200 rpm. OD was measured with a spectrophotometer (Basic Biospectrophotometer, Eppendorf) at 600 nm.

Table 1. Rhizobia strains and legume partner used in this study.

Rhizobia			Legume partner
Strain	**Description**	**Reference**	
Rhizobium leguminosarum 3841	300 *str*, biovar viciae	(Johnston and Beringer, 1975)	*Pisum sativum*
Ensifer (Sinorhizobium) meliloti 1021	*expR101::ISRm2011-1*, Sm^R 300	(Meade et al., 1982)	*Medicago sativa*
Rhizobium leguminosarum B625	3841 (pRP2JI) Sm^R 300; Spc^R	(Sindhu et al., 1990)	*Phaseolus vulgaris*

3.1.2. Growth of legumes and nodulation experiments

Experiments were performed as previously indicated (Bolaños et al., 1994) with minor modifications. Briefly, pea (*Pisum sativum* cv. Lincoln), alfalfa (*Medicago sativa* cv. Moapa), or soybean (*Glycine max* cv. Williams) seeds were surface-sterilized with 70 %

(v/v) ethanol for one minute and 10 % (v/v) sodium hypochlorite for 10 minutes, soaked for 4 hours in sterile distilled water and then germinated at darkness on wet perlite at 25 °C. After 4 days the seedlings were transferred to plastic growth pots (Riviera) and cultivated on perlite with FP medium for legumes (Table 2; Fahraeus, 1957), lacking B. When convenient, the micronutrient was added at a concentration of 10 μM of boric acid. In nodulation experiments, legume plants grew during ten days to use B reserves present in the seed. After that stage, plants were inoculated with 1 mL (about 10^8 cells) per seedling of an exponential culture of the required bacterial strain. Inoculated plants were maintained in a growth cabinet at 22 °C day/18°C night temperatures with a 16/8 h photoperiod and an irradiance of 200 μmol photons m^{-2} s^{-1}. Relative humidity was kept between 60 and 70 %.

3.1.3. Growth of *Arabidopsis thaliana*

Arabidopsis thaliana (L.) Heynh. ecotype Columbia 0 (Col 0) was used as wild type (wt) strain. Mutants and transgenic lines used in this study are summarized in Table 3 and Table 4. *Arabidopsis* lines were propagated using a soil:perlite mixture (2:1), alternatively watered with tap water or full Murashigue and Skoog media (MSm) media (Table 2; Murashigue and Skoog, 1962) twice per week. Seeds were collected and stored at room temperature (RT). Before use, seeds were surface sterilized by incubation with 96% (v/v) ethanol during 2 minutes, and then dried in a sterile hood. After disinfection, seeds were stored at 4ºC until use.

Semisolid plates were prepared using ½MSm, using 0,8% (w/v) Phytagel (Sigma) as gelling agent, because it does not contain detectable boron traces (Gruber et al., 2013). Media were prepared in polyethylene flasks by mixing homemade macro- and micro-nutrients (lacking iron and boron) stock solutions, 1 mM MES (2-(*N*-morpholino)ethanesulfonic acid; Duchefa Biochemie) and pH was adjusted at 5.7 with KOH. Before pouring the plates, we added iron, sugar and vitamins from sterile stock and dispensed 20 ml in P90 plates (Thermo Scientific) or 50 ml in P100 square plates (Thermo Scientific), and the required amount of boric acid. When necessary, drug treatments were added to the plates immediately before plating, from sterile concentrated stocks. Drugs stocks were prepared as follows: 1-aminocyclopropane-1-carboxylic acid (ACC) (Sigma, 1 mM), DL-Dithiothreitol (DTT) (Sigma, 1 M) and Silver nitrate (Fluka, 1 mM) were

prepared in miliQ water. Brefeldin A (BFA, 1 and 10 mM) (Sigma), cycloheximide (CHX) (Sigma, 100 mg mL^{-1}), Monensin (Mon) (Sigma, 0.1, 1 and 10 mM) and Tunycamicin A (TuniA) (Sigma, 5 mM) were prepared in DMSO. All drug stocks were prepared by filtration through a 0.22 μm pore filter.

Table 2: Composition of media for plant growth used in this work.

Concentrations are indicated at μM, except when indicated.

Compound	**FP** [a]	**½MSm** [b]
KNO_3	10000*	9400
NH_4NO_3		1000
$Na_2HPO_4 \cdot 2H_2O$	68	
KH_2PO_4	700	625
$MgSO_4 \cdot 7H_2O$	500	750
$CaCl_2 \cdot 2H_2O$	680	1500
FeNaEDTA	50	50
H_3BO_3	9.3	**
$MnCl_2 \cdot 4H_2O$		50
$MnSO_4 \cdot H_2O$	10.6	
KI		2.5
$ZnSO_4 \cdot 7H_2O$	0.7	15
$Na_2MoO_4 \cdot 2H_2O$	1	0.52
$CuSO_4 \cdot 5H_2O$	3.2	0.053
$Co(NO_3)_2 \cdot 6H_2O$		0.055
myo-inositol		100 mg L^{-1}
Glycine		2 mg L^{-1}
Nicotinic acid		0.5 mg L^{-1}
Pyridoxine HCl		0.5 mg L^{-1}
Thiamine HCl		0.1 mg L^{-1}
Sucrose		2% (w/v) ~ 58mM
MES		1000
Phytagel (Sigma)		0.8 % (w/v)
pH	6.5-6.7	5.6-5.8

[a] Fahreus Phosphate medium (ref).

[b] Half strength Murashiegue and Skoog medium (ref).

* Nitrate was omitted in N_2 in symbiosis experiments.

** The amount of Boron added was indicated in each experiment.

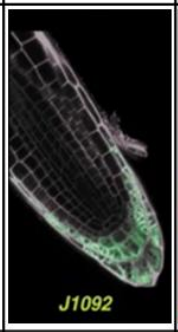
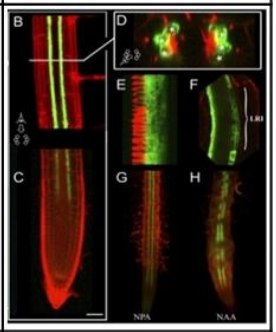
Table 3. Arabidopsis lines used in this study.

Name	Description	Reference
Col 0	A. thaliana Columbia 0 (Col 0). Wild type	-
pCYCB1;1:GUS	*A. thaliana* Col0 carrying carrying β-glucuronidase gene fused to cell cycle G2/M cyclin promoter region and D-box controlling degradation.	Colón-Carmona et al., 1999
QC46:GUS	*A. thaliana* Col 0. Transgenic line carrying unknown sequence fused to β-glucuronidase gene, which is expressed in QC	Sabatini et al., 2003
bor1-1	*A. thaliana* Col 0 Tn5 insertion line in gene *BOR1*. Sensitive to low B conditions.	Noguchi et al., 1997
alg3-2	*A. thaliana* Col 0 Tn5 insertion line in gene *ALG3*. Altered synthesis of N-glycan precursor. Sensitive to osmotic and salt stress.	Henquet et al., 2008
alg10-1	*A. thaliana* Col 0 Tn5 insertion line in gene *ALG10*. Altered synthesis of N-glycan precursor. Sensitive to osmotic and salt stress.	Farid et al., 2011
stt3a-2	*A. thaliana* Col 0 Tn5 insertion line in OST complex. Hypoglycosylation of N-glycoproteins. Sensitive to osmotic and salt stress.	Koiwa et al., 2003
mns12	*A. thaliana* Col 0. Double mutant by Tn5 insertion line in genes *MNS1* and *MNS2*. Don't synthesize Complex N-glycoproteins. Sensitive to osmotic and salt stress.	Liebminger et al., 2009
mns123	*A. thaliana* Col 0. Triple mutant by Tn5 insertion line in genes *MNS1, MNS2* and *MNS3*. Don't synthesize Complex N-glycoproteins. Growth defects, root swelling.	Liebminger et al., 2009
cgl1	*A. thaliana* Col 0 Tn5 insertion line in gene *GNTI*. Don't synthesize Complex N-glycoproteins. Sensitive to osmotic and salt stress.	von Schaewen et al., 1993
gntI	*A. thaliana* Col 0 Tn5 insertion line in gene *GNTI*. Don't synthesize Complex N-glycoproteins. Sensitive to osmotic and salt stress.	Kang et al., 2008
EBS:GUS	*A. thaliana* Col 0 background, carry a construction between the promoter region of the *EBS* gene and the β-glucuronidase gene	Stepanova et al., 2005

Mutant *bor1-1* was a kind gift of Dr. Takano. Mutants *alg3-2, alg10-1, stt3a-2, mns12, mns123, cgl1* and *gntI* were a kind gift of Dr. Strasser. Line pCYCB1;1:GUS was a kind gift of Prof. Gutierrez. Line QC46:GUS was a kind present of Dr. Sabatini. Line EBS:GUS was a kind gift of Dr. Hernández.

Table 4: *Arabidopsis thaliana* enhancer trap lines used in this study.

A. thaliana Col 0 enhancer trap lines carrying enhancer trap-driven GAL4 + *UAS:mGFP5-ER* construction (Haseloff et al., 1997). Due to enhancer-trap region GFP is expressed in different tissues, indicated below. All lines were purchased from NASC.

Name	J0192	Q0990	J2812	J2672	J0121
Picture					
Tissue	· Columella · Root Cap	· Stele · Quiescent Center	· Epidermis · Cortex · Lateral Root Cap	· Endodermis · Root Cap	· Pericycle cells

In direct growth experiments seeds were sown with a toothpick under sterile conditions, plates were sealed with Micropore tape (3MM) and then stratified at 4ºC during 24 h. *Arabidopsis* plates were maintained in a growth cabinet at 24 °C day/20°C night temperatures with a 16/8 h photoperiod and an irradiance of 120 µmol photons m^{-2} s^{-1}. Relative humidity was kept between 60 and 70 %. In transference experiments, 3 or 5 days post germination (dpg) plants were transferred with a toothpick under sterile condition from the initial plate to one with the convenient medium, ensuring that the whole seedling was straight and in contact with media.

3.2. Microscopy

3.2.1. Light and epifluorescence microscopy

For light and epifluorescence imaging, observations were made on an Olympus BX61 microscope, and images were captured with an Olympus DP70 digital camera using the analySIS software (Soft Imaging System, Olympus).

For epifluorescence, we used a mercury lamp of 100W (Osram 103W/2) with the filters indicated in Table 4.

Table 4. Filters present in the BX61 microscope

Common Name	Commercial Name	Excitation filter	Dichroic Mirror	Emission filter	Uses
DAPI	U-MNU2	BP360-370	DM400	BA420	Aniline Blue
FITC	U-MNB2	BP470-490	DM500	BA520IF	GFP

3.2.2. Confocal microscopy

For laser scanning confocal microscopy, plants were transferred from plates to slides and mounted in water or stained with propidium iodide (PI) (section 3.2.4.4). Plants were observed using an Olympus FLUOVIEW FV1000 (Germany), and images were acquired using the FluorView10 software (Olympus) with the following excitation and detection wavelengths, respectively: 488 nm and 515–545 nm for GFP; 543 nm and >650 nm for propidium iodide.

Confocal imaging was done at Institute of Cellular and Molecular Botany, Bonn University (Germany).

3.2.3. Image analysis

Images were analyzed and modified using FIJI software (Fiji Is Just ImageJ; http://fiji.sc/Fiji), version v1.49v following the instructions included in the ImageJ User Guide.

For root length measurements in Arabidopsis, plates were scanned at indicated times with a desk scan, using default parameters (color, resolution 400 ppp) and images were analyzed using FIJI software. “Set Scale” tool was used to adjust the scale manually (with the help of a ruler). To measure hypocotyl and roots, they were drawn using “Segmented lines” tool over them and using ROI (Region of Interest tools) length data was extracted. Root measurements were analyzed with IBM SPSS Statistics 22 software (see below).

Length-of-the-first-epidermal-cell-with-visible-root-hair-bulge parameter (LEH) was calculated as previously indicated (Le 2001). Briefly, using microscopic images, the distance between the first (a bulge) and the second root hairs in different cell columns at the indicated times were measured. Media and standard deviation were calculated for each time.

3.2.4. Staining methods for *Arabidopsis*

3.2.4.1. Fixing and Clearing

Arabidopsis plants were fixed in Ethanol:Acetic Acid (3:1) in a multidish plate O/N. When indicated, samples were cleared submerging them in a solution of 10& (w/v) of Chloral Hydrate in 30% glycerol until observation.

3.2.4.2. Callose staining with Aniline Blue

After clearing, plants were washed three times with 150mM of K_2HPO_4 pH 9. Then, plants were stained with a fresh made solution of Aniline Blue [0.05 % (w/v) Aniline Blue (water soluble) in 150mM of K_2HPO_4 pH 9] during at least 2 hours. Plants were mounted in 30% glycerol and observed under epifluorescence microscope (using the DAPI filter), keeping constant the exposure time between samples (Shen and Shikora, 2015).

3.2.4.3. Lignin staining with Phloroglucinol-HCl (Wiesner test)

Plants were stained directly after fixation and clearing with a fresh solution of 5 $mg \cdot mL^{-1}$ of Phloroglucinol (Sigma) in Ethanol:HCl (1:1) by submerging them during 2 minutes. Plants were immediately transferred to a dish containing 50% glycerol 1% (v/v) HCl and observed under bright field (Caño-Delgado et al., 2003).

3.2.4.4. *In vivo* localization of GFP and Propidium iodide staining

In vivo localization of GFP was performed transferring plants from plates to a home-made chamber created with a slide, and two cover slides at the edges, filled with MS liquid media. Plants were incubated 10 minutes in dark with a fresh solution of 10 $\mu g\ ml^{-1}$ propidium iodide (PI) prepared in water (100x stocks were prepared in water and stored at -20ºC) , then washed with water to remove excess of PI, and immediately observed with the confocal microscope .

3.3. Protein analysis

3.3.1. Protein extraction and quantification

Protein extraction was performed using prechilled Protein Extraction Buffer [PEB; 50 mM Tris HCl pH 7.5, 10mM DTT, 0.1% (w/v) PVPP, 1x Protease Inhibitor (Roche)]. For

each experiment, protein isolation was performed at the indicated time of development by homogenizing the material with prechilled mortar and pestle.

Protein quantification was assayed using Bradford assay (Bradford, 1976) using the BioRad Protein Assay (BioRad) and following manufacturer instructions for miniaturized assays. OD at 595 nm was measured with the microplate reader Biotek Synergy HT (Biotek), using the software Gen5 (Biotek).

3.3.2. **Amberlite IRA 7-43 Affinity Chromatography**

Amberlite IRA 7-43 Affinity Chromatography (AAC) was performed as previously described (Reguera et al., 2010c), with minor modifications. Briefly, Amberlite IRA 7-43 was buffered before starting the AAC procedure. For that purpouse we poured in an 2 mL tube approximately 300 µL of Amberlite IRA 7-43 resin, and washed it twice with miliQ water. Then we incubated the resin three times with 500 µL of AAC washing buffer [50 mM Tris HCl pH 7.5, 10mM DTT, 1x Protease Inhibitor (Roche)], 10 minutes each. Just before starting AAC all the buffer was removed.

Native protein extracts were adjusted to a final concentration of 1 mg mL^{-1} with PEB. 500 µL of native protein extracts were then incubated with the resin. For ensure maximum extract-resin contact incubation was performed during 1 hour at Room Temperature (RT) in a carrousel. Then, tubes were centrifuged during 1 min at 5000g, and the supernatant fractions were transferred to a new tube. This soluble fraction constituted the Non Retained Fraction (NRF). The pellet was washed twelve times 10 minutes each with 1 mL AAC washing buffer to remove non-specific attachment to Amberlite. Finally 250 µL of Laemmli buffer 2x (Laemmli, 1970) were added, and material was released from the resin by boiling 10 min. This final fraction was called Retained Fraction (RF). NRF and RF were separated by SDS-PAGE as indicated below.

3.3.3. Protein separation

For protein separation, native protein extracts were mixed with Laemmli buffer 5x (Laemmli, 1970) and boiled at 95ºC during 10 minutes.

SDS denaturing Polyacrilamide Gel Electrophoresis (SDS-PAGE) was performed according to standard procedures. Briefly, gels were prepared fresh using the Miniprotean system III (BioRad), with a separation and a concentration region containing 12% and 4% of acrylamide respectively. Gels ran at 100V (constant voltage) during 2 hours or until the front dye (Bromophenol blue) reach the end. Dual color Pre-stained Precision Markers were used as reference for relative molecular weight. All reactives used were electrophoresis grade, from BioRad.

3.3.4. Protein detection

Replicate gels were used for different detection methods, described below. In all cases, gels were documented with Chemidoc RC+ device (BioRad) and the ImageLab software v.3.0.1 (BioRad) using the instructions recommended by the manufacturer for each detection method.

Gel images were processed and analyzed using the ImageLab software v.3.0.1 (BioRad). In the case of semiquantification of Mannose Rich N-glycoproteins we extracted the intensities of both ConA affinoblot and Comassie stained gel for each treatment, and then we relativize them using the following formula:

$$Ratio\ MRNGPs/Prots = \frac{\frac{Signal\ MRNGP\ \text{at}\ [\text{B}]_x}{Signal\ MRNGP\ \text{at}\ [\text{B}]_{100}}}{\frac{Signal\ Coomassie\ \text{at}\ [\text{B}]_x}{Signal\ Coomassie\ \text{at}\ [\text{B}]_{100}}}$$

When comparisons involved mutants or treatments and A B gradient, we relativize data with the signal obtained in wild type plants grew in 100µM H_3BO_3 using the following formula:

$$Ratio\ MRNGPs/Prots = \frac{\frac{Signal\ MRNGP\ \text{mutant/treatment}\ [\text{B}]_x}{Signal\ MRNGP\ \text{wt}\ [\text{B}]_{100}}}{\frac{Signal\ Coomassie\ \text{mutant/treatment}\ [\text{B}]_x}{Signal\ Coomassie\ \text{wt}\ [\text{B}]_{100}}}$$

3.3.4.1. Coomassie staining

Gels were fixed in (40% (v/v) ethanol 10% (v/v) acetic acid) during at least 30 minutes and then incubated in preheated staining solution during 1 hour. Staining Solution was prepared following manufacturer instructions, 1 pill of Phastgel Blue R (Coomassie R250; GE Healthcare) was prepared in 1.6 L of 10% acetic acid. Finally, gels were distained with washing buffer (10% acetic buffer) until the background disappears.

3.3.4.2. Silver Staining

For silver staining we used a Silver Staining kit (GE Healthcare), following manufacturer instructions. Namely, after electrophoresis, gels were fixed in 40% (v/v) ethanol 10% (v/v) acetic acid buffer during at least 30 minutes. Then were sensitized during 1 hour in sensitizing solution [30% (w/v) ethanol, 0.2% (w/v) sodium thiosulfate, 6.8% (w/v) sodium acetate] and washed four times, fifteen minutes each, with miliQ water. After that we incubate the gel in Silver solution [0.25% w/v) silver nitrate] during 1 hour in dark. Then gels were washed quickly with miliQ water (<1 min) and developed until the signal appears incubating the gels in Developing solution [2.5% (w/v) sodium carbonate, 0.03% formaldehyde]. When bands were clearly observed the developing reaction was stopped incubating the gels with a 0.1M EDTA solution.

3.3.4.3. Electroblotting

For transferring separated proteins from gels to nitrocellulose membranes a standard semidry protocol was used in the TurboTranfer device (BioRad), following fabricant instructions.

Gels and 3MM paper were incubated during 10 to 30 minutes in Towbin buffer [25 mM Tris HCl pH 8.3, 192 mM glycine, 20% methanol] whereas nitrocellulose membranes (PALL) were incubated in miliQ water. Then, we assemble the sandwich system, and electrotransference was performed using standard protocol at room temperature (Maximum 2.5 V, Maximum 10 mA, 30 min). After electroblotting the membranes were processed distinctly according to the following procedures.

3.3.4.4. Affinoblot with Concanavalin A

Mannose Rich N-glycans were detected with Concanavalin A (ConA) lectin (Faye and Chrispeels, 1985) with minor modifications. Membranes were blocked O/N in Tween-TBS or TTBS buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20]. Then membrane was incubated with 25 μg ml^{-1} Concanavalin A lectin (Sigma) in TTBS buffer supplemented with 100 μM $CaCl_2$ and 100 μM $MnCl_2$ for two hours. We washed four times the membrane with TTBS, fifteen minutes each. Then membranes were incubated with 5 μg ml^{-1} horseradish peroxidase (HRP, Sigma) prepared in TTBS, for 1

hour. Membranes were washed four times with TTBS. Finally peroxidase activity signal was detected using chemiluminiscence reaction (ECL advance kit, GE Healthcare).

3.3.4.5. Immunoblots

Specific glycoproteins or N-glycans were detected using specific antibodies at indicated concentrations: Anti-Xylose (1:500; Agrisera), Anti-Fucose (1:500; Agrisera); Anti-AtPDI5 (1:1000; Gift of Dr. Strasser); Anti-BiP (1:000; Agrisera); Secondary antibody was Anti-IgG_{rabbit} –HRP conjugated (1:10000; Agrisera). Membranes were blocked in 5% (w/v) nonfat dry milk in TBS buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl] during at least 1 hour. Then membranes were incubated O/N with the proper antibody in 5% (w/v) non-fatty milk in TBS buffer. Membranes were washed with TBS four times and then incubated it with secondary antibody (HRP-conjugated) prepared in 5% (w/v) non-fatty milk in TBS buffer. Membranes were washed four times with TBS and finally the peroxidase activity signal was detected using chemiluminiscence reaction (ECL advance kit, GE Healthcare).

3.3.5. Protein identification

3.3.5.1. In-gel protein digestion and sample preparation

Protein bands of interest were excised from Coomassie-stained gels, deposited in 96-well plates, and processed automatically in a Proteineer DP (Bruker Daltonics). The digestion protocol was as follows: gel plugs were washed firstly with 50 mM ammonium bicarbonate and secondly with acetonitrile (ACN) prior to reduction with 10 mM DTT in 25 mM ammonium bicarbonate solution, and alkylation was carried out with 55 mM iodoacetamide in 50 mM ammonium bicarbonate solution. Gel pieces were then rinsed firstly with 50 mM ammonium bicarbonate and secondly with ACN, and then were dried under a stream of nitrogen. Proteomics Grade Trypsin (Sigma) at a final concentration of 16 ng/µl in 25% ACN/50 mM ammonium bicarbonate solution was added and the digestion took place at 37 ºC for 4 h. The reaction was stopped by adding 50%ACN/0.5% trifluoracetic acid (TFA) for peptide extraction. The tryptic eluted peptides were dried by speed-vacuum centrifugation and resuspended in 4 µl of MALDI solution [30% (v/v) ACN/15% isopropanol/0.5% (v/v) TFA). A 0.8 µl aliquot of each peptide mixture was deposited onto a 384-well OptiTOFTM Plate (AB SCIEX) and allowed to dry at room temperature. A 0.8 µl aliquot of matrix solution (3 mg/mL α-Cyano-4-hydroxycinnamic

acid in MALDI solution) was then deposited onto dried digest and allowed to dry at room temperature.

3.3.5.2. MALDI peptide mass fingerprinting (PMF), MS/MS analysis and database searching

For MALDI-TOF/TOF analysis, samples were automatically acquired in an ABi 4800 MALDI-TOF/TOF mass spectrometer (AB SCIEX) in positive ion reflector mode (the ion acceleration voltage was 25 kV to MS acquisition and 2 kV to MS/MS), and the obtained spectra were stored into the ABi 4000 Series Explorer Spot Set Manager. PMF and MS/MS fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in ABi 4000 Series Explorer Software v3.6. Each PMF spectrum was internally calibrated with the mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of <25 ppm. Known trypsin and keratin mass signals, as well as potential sodium and potassium adducts (+21 Da and +39 Da) were removed from the peak list. To submit the combined PMF and MS/MS data to MASCOT software v.2.5.0 (Matrix Science, London, UK), GPS Explorer v4.9 was used, searching in the Arabidopsis thaliana protein database from Uniprot/SwissProt (14095 sequences). The following search parameters were used: enzyme, trypsin; allowed missed cleavages, 1; carbamidomethyl cystein as fixed modification by the treatment with iodoacetamide; variable modifications, oxidation of methionine; mass tolerance for precursors was set to ± 50 ppm and for MS/MS fragment ions to ± 0.3 Da. The confidence interval for protein identification was set to ≥ 95% ($p < 0.05$) and only peptides with an individual ion score above the identity threshold were considered correctly identified.

3.4. Data analysis

Statistical comparison was performed with IBM SPSS Software v22.0 whereas graphical representation were done with Excel2013 and IBP SPSS Software.

4. Results

4.1. N-glycoproteins as potential boron ligands in rhizobia-legume symbiosis.

Although boron is essential for all stages of the rhizobia-legume symbiosis, interestingly, the highest requirement coincides with phases where glycoprotein synthesis are particularly relevant, as formation of infection threads and symbiosome development.

4.1.1. Boron deficiency led to accumulation of N-glycoproteins carrying both Mannose Rich N-glycan and Complex N-glycan in both indeterminate and determinate nodule forming rhizobia-legume symbiosis.

To test the hypothesis that N-glycoproteins, as *cis*-diol containing molecules, could be in vivo B ligands the N-glycoprotein pattern of B-sufficient and B-deficient root and nodules was first analyzed and compared. Mannose Rich N-glycoproteins were accumulated along the time course in B-deficient roots, indeterminate nodules of *P.*

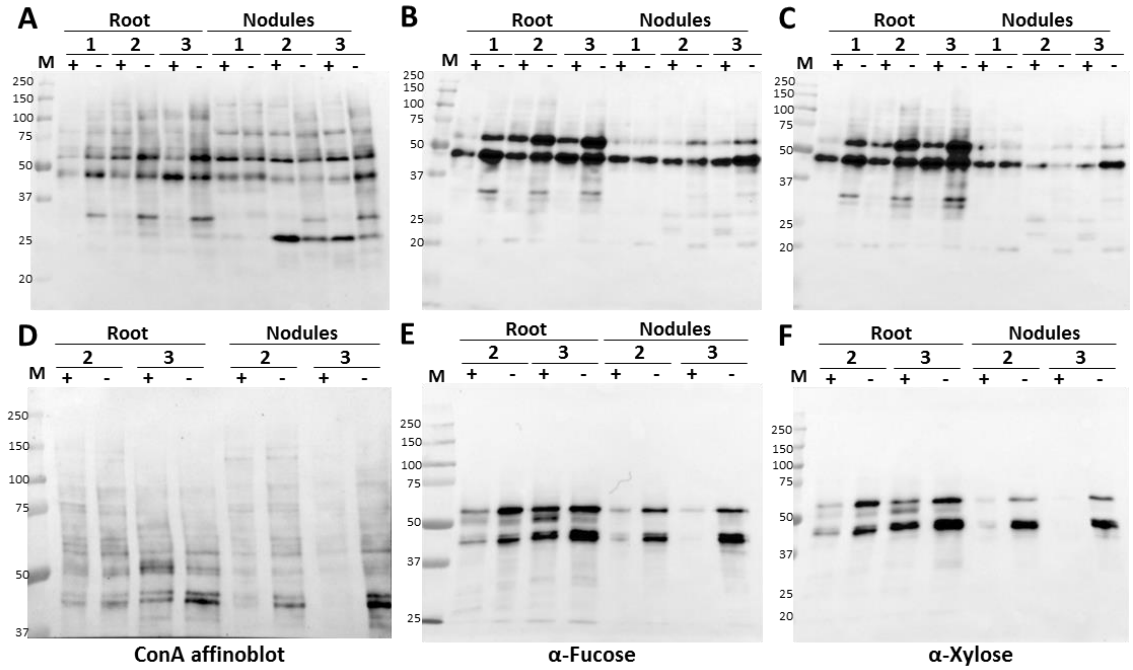


Figure 6. Boron deficiency causes a gradual accumulation of N-glycoproteins in indeterminate nodule-forming rhizobia-legume symbiosis.
Analysis of total proteins extracted from *Rhizobium leguminosarum* bv. 3841 – *Pisum sativum* (A, B, C) or *Ensifer meliloti* 1021 – *Medicago truncatula* symbiosis (D, E, F), 1, 2, or 3 weeks post inoculation. Proteins were subjected to SDS-PAGE under reducing conditions, blotted and analysed using the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (A, D); anti-fucose (B, E) or anti-xylose antibodies (C, F), which recognize complex N-glycans with

sativum – *R. leguminosarum* 3841, *M. sativa* – *E. meliloti* 1021 (Fig. 6), and in determinate nodules of *P. vulgaris* – *R. leguminosarum* B625 (Fig. 7) symbioses.

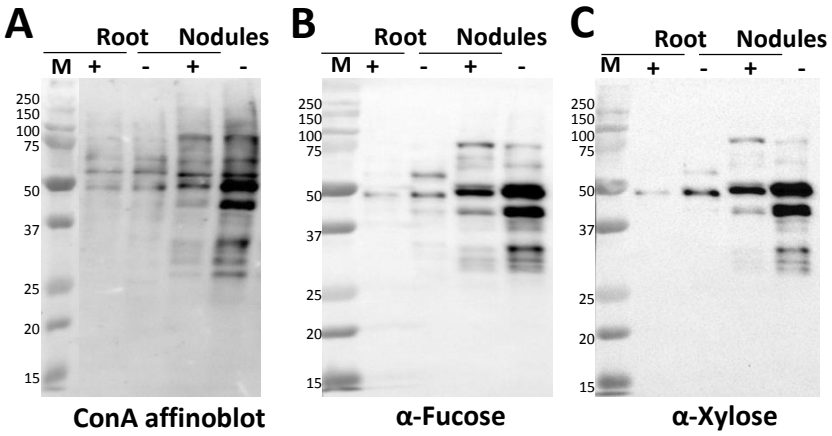


Figure 7. Boron deficiency causes an accumulation of N-glycoproteins in determinate nodule-forming rhizobia-legume symbiosis.
Analysis of total proteins extracted from *Rhizobium leguminosarum* B625-*Phaseolus vulgaris* 3 weeks post inoculation. Proteins were subjected to SDS-PAGE under reducing conditions, blotted and analysed using the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (A); anti-fucose (B) or anti-xylose antibodies (C), which recognize complex N-glycans with a core α1,3-fucose and β1,2-xylose residues respectively. (+) B sufficient samples; (-) B deficient samples.

4.1.2. Mannose Rich N-glycoproteome changes in boron deficient nodules.

N-glycoproteins carrying Mannose Rich N-glycan (MRNG) derived from B sufficient (+B) or B-deficient (-B) extracts were analyzed. For that purpose I used commercial ConA affinity chromatography, where ConA lectin is fused to agarose columns. Non-specific interactions were avoided washing with protein extraction buffer, whereas specific interactors were recovered with the sugar competitor methyl-α-D-mannopyranoside. Eluted N-glycoproteins were analyzed by LC-MS/MS and obtained peptides were identified in a *Viridiplantae*-rhizobia specific database created with sequenced organisms. Proteins identified were listed according to its presence in both +B and –B nodules, or exclusively in +B or -B samples (Table 6). Besides glycoproteins, several proteins not described as glycosylated were identified. Because the use of native extracts, we identified several proteins from *Rhizobium*, which are not described as glycoproteins, but which could interact with plant proteins and indirectly be retained in ConA columns. Also, several plant proteins retained with ConA lectin don't possess N-glycosylation sites in their sequences.

Table 5: Proteins purified with Concanvalin A Affinity Chromatography from B sufficient (+B) and B deficient (-B) nodule proteins.

+B	-B	ID	Description	Location	N-Glico sites	Size (kDa)
✓	✓	Q9XF98	Calreticulin OS=Prunus armeniaca	RE	Pot (2)	48.4
✓		Q40987	Nodule lectin OS=Pisum sativum GN=NLEC1	Apo/CW	Pot (1)	28.9
✓	✓	Q9SLY8	Calreticulin OS=Oryza sativa subsp. japonica GN=Os07g0246200	ER	Pot (1)	48.3
✓		Q9SAZ0	Leghemoglobin Lb120-34 OS=Pisum sativum	Cyt		15.9
✓		Q9FF52	60S ribosomal protein L12-3 OS=Arabidopsis thaliana GN=RPL12C	Cyt		17.8
✓		A2YVR7	Proteasome subunit alpha type-2 OS=Oryza sativa subsp. indica GN=PAB1	Cyt/ Nu		25.8
✓		O24581	Luminal-binding protein 3 OS=Zea mays GN=BIPE3	ER	Pot (1)	73.1
✓		Q03684	Luminal-binding protein 4 OS=Nicotiana tabacum GN=BIP4	ER	Pot (1)	73.5
✓		O48668	Leghemoglobin Lb5-10 OS=Pisum sativum	Cyt		15.9
✓		Q9SXU1	Proteasome subunit alpha type-7 OS=Cicer arietinum GN=PAD1	Cut/Nu		27.1
✓	✓	P93447	Elongation factor 1-delta OS=Pimpinella brachycarpa	Cyt		24.5
✓	✓	P29828	Protein disulfide-isomerase OS=Medicago sativa GN=PDI	ER	Pot (1)	57.1
	✓	P08283	Histone H1 OS=Pisum sativum	Nu		28.0
	✓	P46266	14-3-3-like protein OS=Pisum sativum	Nu		29.3
	✓	Q9S8P4	Rhicadhesin receptor OS=Pisum sativum GN=GER1	Apo/CW	Pot (2)	23.0
	✓	O81918	Elongation factor 1-delta OS=Beta vulgaris	Cyt		24.7
	✓	Q9AXQ3	Eukaryotic translation initiation factor 5A-4 OS=Solanum lycopersicum	Cyt		17.5
	✓	Q6Z6G6	Cytoplasmic tRNA 2-thiolation protein 1 OS=Oryza sativa subsp. japonica GN=NCS6	Cyt		40.9

Among relevant MRNGPs identified in +B nodules appeared PsNLEC1, a symbiosomal protein previously described by our group as not correctly N-glycosylated in B deficient nodules (Bolaños et al., 2001). Other proteins found in +B but not in -B nodules were BiP-like and calreticulin-like proteins, which are proteins involved in protein folding. (Table 5). To test whether BiP is reduced or not glycosylated under B deficiency I probed *Pisum*, *Medicago* and *Phaseolus* roots and nodules with an anti-BiP antibody. In B deficient samples was observed the appearance of several bands, corresponding to BiP isoforms, with an intensity even higher than in B sufficient samples (Fig. 8).

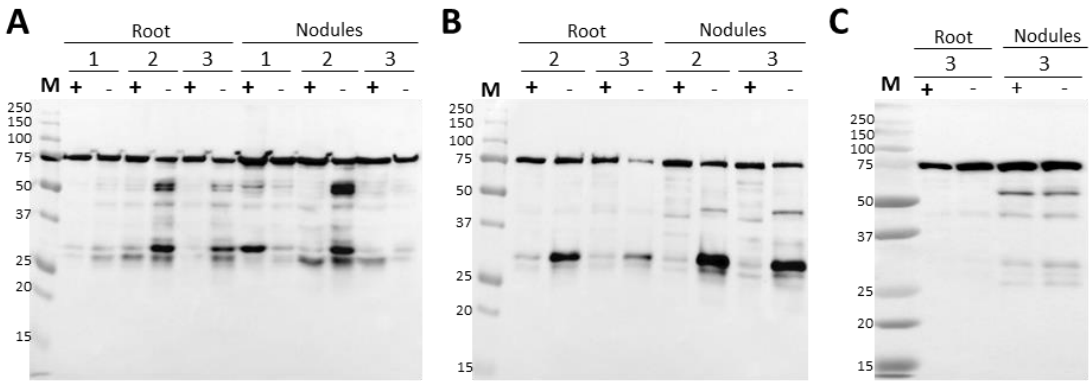


Figure 8. Boron deficiency causes changes in BiP like proteins.
Analysis of total proteins extracted from *Rhizobium leguminosarum* bv. 3841 – *Pisum sativum* (A), *Ensifer meliloti* 1021 – *Medicago truncatula* symbiosis (B), and *Rhizobium leguminosarum* B625-*Phaseolus vulgaris* (C) at different (1, 2, or 3) weeks post inoculation). Proteins were subjected to SDS-PAGE under reducing conditions, blotted and analysed using the anti-PDI antibody detection PDI-like proteins. (+) B sufficient samples; (-) B deficient samples.

4.1.3. Specific N-glycoproteins interact with Amberlite IRA 7-43.

N-glycans possesses several sugar residues with free *cis*-diol moieties which could be potential ligands of B. I tested this possibility using Amberlite IRA 7-43 affinity chromatography followed by detection in the linked material of both MRNGPs and CNGPs by ConA affinoblot or anti-fucose; anti-xylose immunodetection respectively (Fig. 9).

Most proteins were not forming one-side complexes with B *in vivo*, so they are not retained in resin Amberlite IRA 7-43. Also, the number of proteins present in the retained fraction (RF) seem higher in B sufficient samples (+B) than in the B deficient samples (-B) (Fig. 9A). I found Mannose Rich N-glycoproteins in both Non Retained Fraction

(NRF) and RF (Fig. 9B), in both +B and –B samples, although less in the –B samples. By contrast, Complex N-glycoproteins were detected only detected in the NRF (Fig. 9C, 9D).

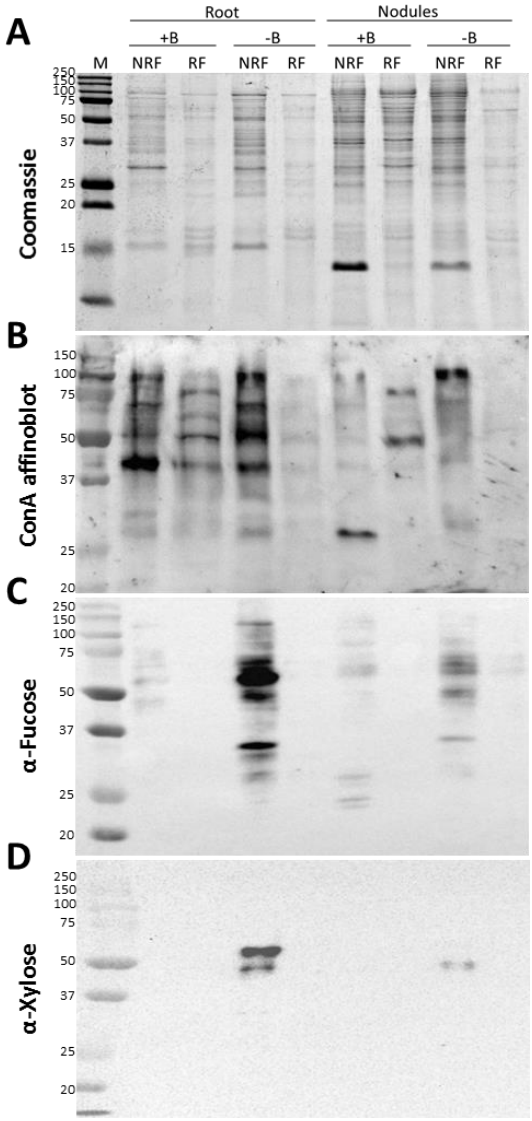


Figure 9. N-glycoproteins are potential Boron ligands.
Native proteins isolated from root and nodules of 3 weeks-old R. *leguminosarum* bv. 3841 – *P. sativum* grew under B-sufficient (+B) and B-deficient (-B) conditions were incubated with Amberlite IRA 7-43 resin. Supernatant fraction was conserved and named Non Retained Fraction (NRF). After 12 washes, proteins bound to the resin were eluted with 2x Laemmli buffer, and constituted the Retained Fraction (RF). Proteins were subjected to SDS-PAGE under reducing conditions, blotted and analysed using silver Staining (A); the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (B); anti-fucose (C) or anti-xylose antibodies (D), which recognize complex N-glycans with a core α1,3-fucose and β1,2-xylose residues respectively.

4.2. Morphological characterization of boron deficiency in Arabidopsis.

As described in the Introduction section, our group has deeply characterized the effects of B-deficiency on nodule development. Therefore, was considered crucial to perform an overall phenotyping characterization of B deficiency in *Arabidopsis* previous to explore the relationship between B nutrition and N-glycoproteins in this model plant.

4.2.1. Boron nutrition affects growth of both root and hypocotyl.

To start our study, I first analyzed growth of Arabidopsis directly germinated on plates with semisolid ½ MSm with increasing concentrations of boric acid under light conditions. (Fig 10).

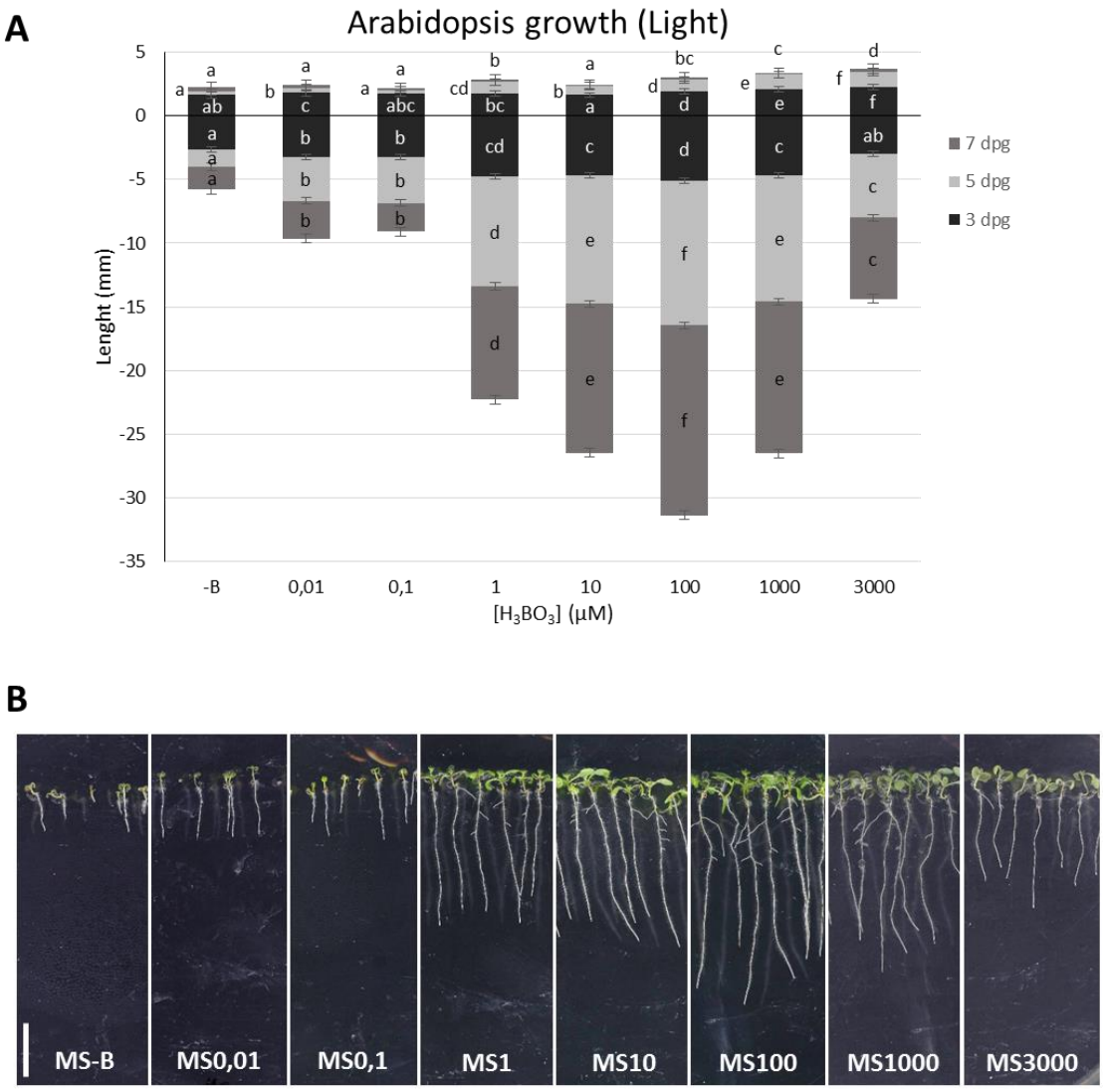


Figure 10. *Arabidopsis* growth in light conditions.
A) Root and Shoot length (mm) at 3, 5 and 7 days post germination (dpg) of *Arabidopsis* plants grown with a 16 h light/ 8 h dark photoperiod in media with the indicated concentrations of H_3BO_3 expressed in µM. B) Representative Arabidopsis seedlings 7 dpg. Scale bar in B represents 1 cm. Different letters in A represent significant differences (α=0,05).

Fig. 10A shows that significant differences of growth were observed as early as 3 days post germination (dpg). Growth was reduced under low B (from -B to 0.1 µM boric acid) and high (3 mM boric acid) B concentrations. The differences of growth increased along the time (5 and 7 dpg). Optimal root growth occurred at concentrations of boric acid ranging from 1 µM to 1 mM, although maximum growth is observed at 100 µM. By contrast, hypocotyl maximum growth was observed at 3 mM boric acid.

Dark conditions were used to study hypocotyls elongation (Fig 11). Differences of growth were observed again as early as 3dpg, and were more evident 5 and 7dpg, although growth behavior related to B seemed to change along the time in this dark conditions. Maximum growth on both root and hypocotyl at day 3 was observed with 100 µM boric acid (Fig. 11A). However, at day 7 differences of root growth were less pronounced compared with light experiments. Hypocotyl growth was reduced in low B concentrations (-B to 0.1 µM boric acid), and there were not significant differences in concentrations higher than 10 µM boric acid. It is interesting to notice that hypocotyl elongation at day 3 was reduced in 3mM boric acid treatments, but growth rate raised at day 5 and 7 (Fig. 11A, B).

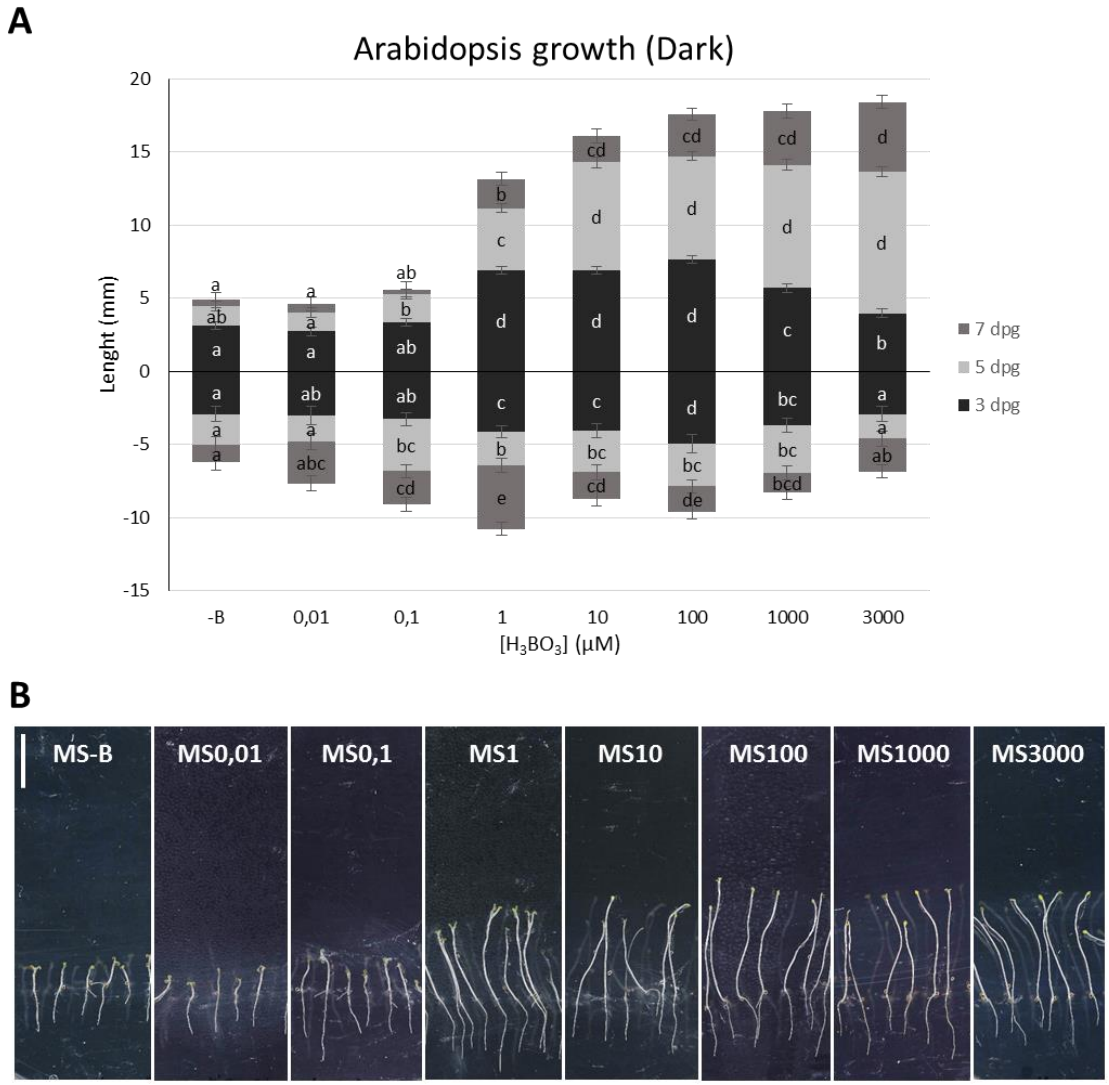


Figure 11. *Arabidopsis* growth in dark conditions.
A) Root and Shoot length (mm) at 3, 5 and 7 days post germination (dpg) of *Arabidopsis* plants grown in dark in media with the indicated concentrations of H_3BO_3 expressed in µM. B) Representative Arabidopsis seedlings 7 dpg. Scale bar in B represents 1 cm. Different letters in A represent significant differences (α=0,05).

In the next step of this study, I analyzed growth in transference experiments (Fig. 12). In those experiments, plants grown in +B (MSm100) or –B (no B added) conditions during 5 days were respectively transferred to either -B, +B, or same media. Then, growth was followed during several days. Both in light and dark experiments (Fig. 12A and 12B), I observed that root growth was inhibited 24h after transference of plants from +B to -B conditions (+B/-B), indicating that roots were particularly sensitive to B deprivation. By contrast I could not observe hypocotyls growth inhibition in light, and in dark conditions it was reduced 2 dpt (days post-transference).

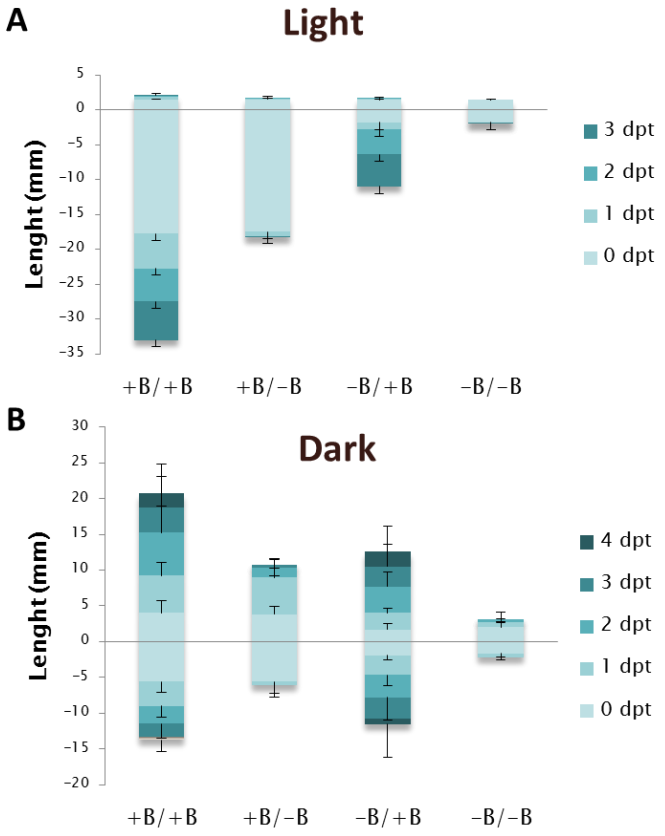


Figure 12. *Arabidopsis* growth in transference experiments.
Arabidopsis grown during 5 days in B sufficient (100 µM boric acid, +B) or B deficient media (-B)) were transferred respectively to both +B or –B media. Root and Shoot length (mm) 1 to 4 days post transference (dpt) of *Arabidopsis* plants grew in light (A) or dark (B) conditions.
+B/+B (control +); +B/-B (B deprivation); -B/+B (B resupply); -B/-B (Control -).

In resupply experiments (Fig. 12, -B/+B treatments), root growth was recovered in both dark and light, although under light conditions it took almost 48 hours. Hypocotyl growth was not significantly different in light, because hypocotyl already reached its maximum growth before transference (Fig. 12A). In dark conditions I could detect that resupply of B (-B/+B) rescued hypocotyl growth (Fig. 12B)

4.2.2. Boron deficiency affects root morphology, cell division and cell differentiation in roots of Arabidopsis.

Growth arrest was the most obvious symptom in B deficient plants (Figs. 10, 11 and 12). At morphological level it was accompanied by drastic changes in root tip, among them the appearance of swollen roots and the development of abundant long root hairs in a phenotype usually named "Hairy roots" (Fig. 13B, 17A).

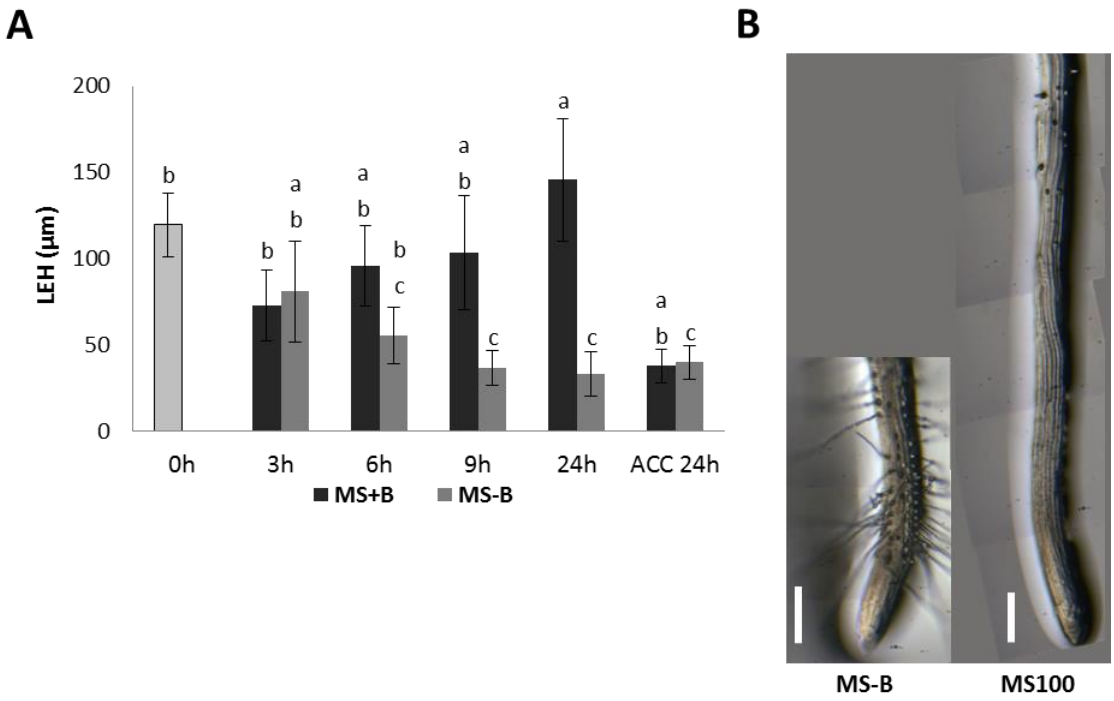


Figure 13. Cell elongation was inhibited within 24h of B deprivation in *Arabidopsis thaliana.* Plants grown during 5 days in MS100 (control) plates were transferred to B deficient or control plates and photographed at indicated times under microscope. LEH was calculates as explained in Material and methods section. A) Evolution of cell elongation along time after transference. ACC treatment was included as control known to inhibit cell elongation. B) Reconstruction of representative plants 24 after transference. Scale bar in B represents 100 µm. Different letters in A represent significant differences (α=0,05).

Next experiments were designed in order to check alterations of cellular processes (cell elongation/differentiation, cell division, quiescent center identity) that could explain reduced root growth under B deficiency.

LEH parameter was used to estimate the effect of B deficiency in elongation of recently differentiated cells. After 3 hours LEH decreased in both B deficient (-B) and B sufficient (+B) plants. After 6 hours +B plants recovered gradually (at 24hpt 145.6 ± 35.3 µm) whereas in plants transferred to B deficient media LEH dropped gradually until they

reached a minimum value at 9 hpt (32.9 ± 12.9 µm), which was maintained at 24 hpt. As control treatment I used ACC, known to inhibit cell elongation, and observed that this treatment produce a decrease in LEH in both +B and –B plants (37.6 ± 9.6 and 39.8 ± 9.6 µm respectively), to the same extend that observed in –B plants (Fig. _A). In fact, ACC-treated plants resembled B deficient plants (Figs. 13A, 38, 39).

The effect of B nutrition on cell division in direct growth (Fig. 14) and transference experiments (Fig. 15) was analyzed through GUS staining using the reporter line *pCYCB1;1:GUS*.

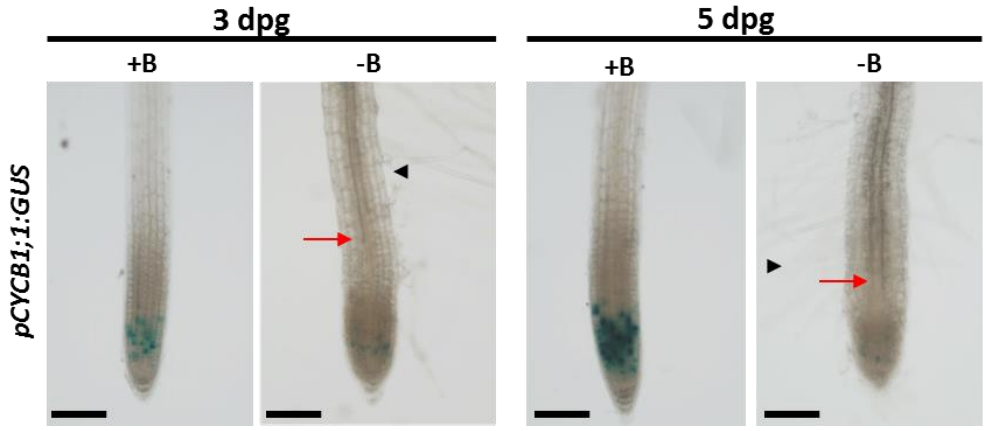


Figure 14. Cell division was diminished in *Arabidopsis* seedling germinated in B deficiency. Kinetics of *pCYCB1;1:GUS* expression in *Arabidopsis* primary root tips. Seedlings were grown on semisolid plates containing ½MS medium with boric acid (+B; 100µM) or without B (-B) and GUS stained (5 h) the indicated days post germination (dpg). Red arrow highlights protoxylem. Black arrowhead highlights root hairs. Scale bar represents 100 µm.

For direct growth experiments, seeds were germinated in boron sufficient (MSm100) and B deficient (MSm-B) plates. In B sufficient plants was observed an increase of GUS signal (cells expressing *CYCB1* gene) from 3 to 5dpg. However, in B deficient plants, staining was always low compared with +B seedling, and decreased from day 3 to 5 after germination. The reduced decrease of *CYCB1* expression in B deficient seedlings was concomitant to the appearance of root hairs (black arrowhead) and the appearance of protoxylem (red arrows) in zones typically occupied by the root meristem (Fig. 14).

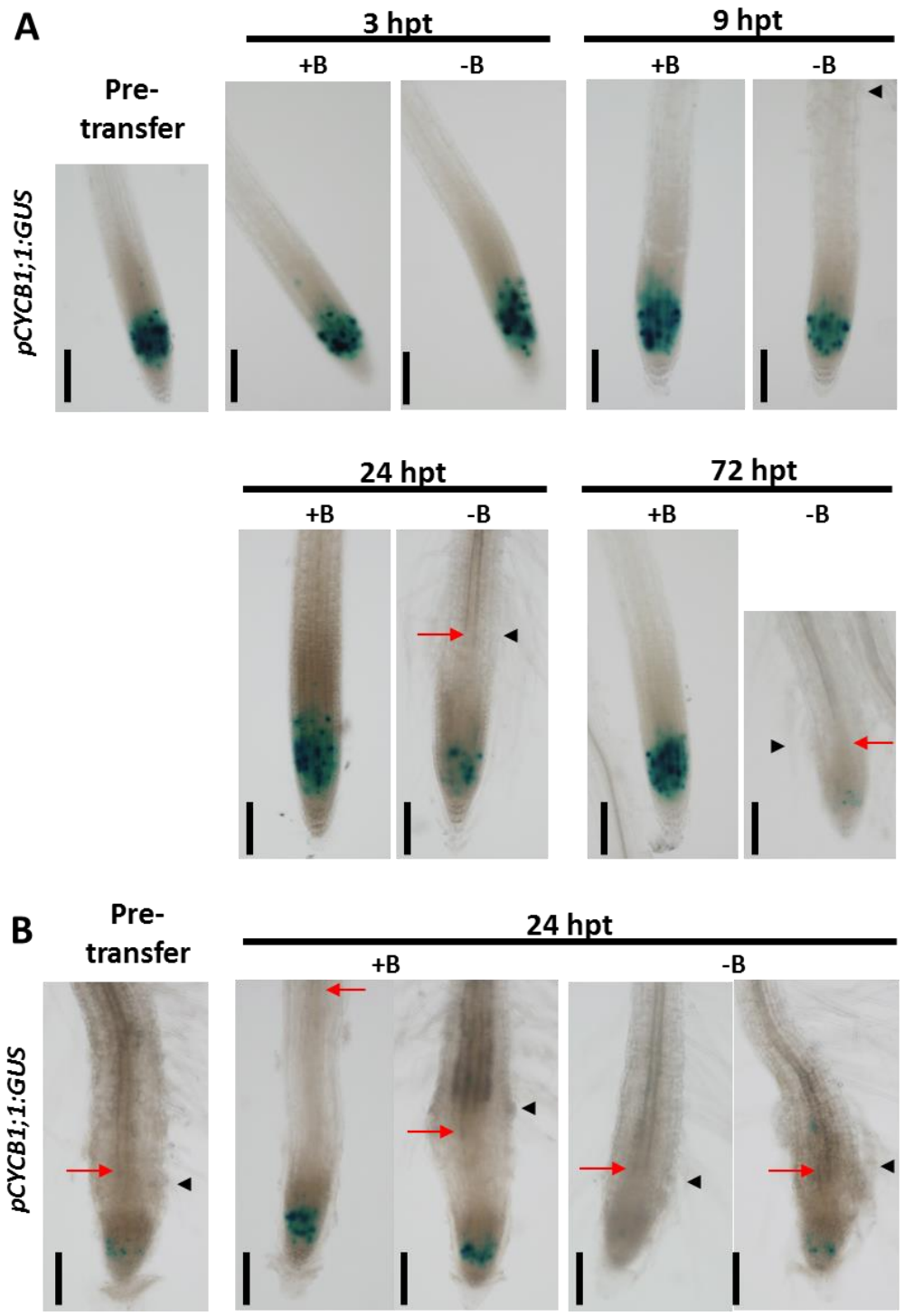


Figure 15. Cell division was inhibited 9h after B deprivation in *Arabidopsis thaliana.*
Kinetics of *pCYCB1;1:GUS* expression in *Arabidopsis* primary root tips. Seedlings were grown on semisolid plates containing ½MS medium with boron (+B) (A) or without (-B) (B), then transferred to +B or –B plates and GUS stained (5 h) at the indicated hours post transference (hpt). Red arrow highlights protoxylem. Black arrowhead highlights root hairs. Scale bar represents 100 µm.

Because changes observed in direct growth experiments are more liable to be secondary/indirect responses to B deficiency and they not allow reveal very early effects, we also analyzed *CYCB1* expression occurring after B deprivation in transference experiments (Fig. 15). In contrast to what I observed in cell elongation, transference of B sufficient plants 3 hpt to –B media did not resulted in detectable changes in GUS staining. A slight diminution of the area expressing *CYCB1* was observed 9 hpt. It was more reduced at 24 hpt, and almost disappeared 72 hpt. As in direct growth experiments, loosening of cells entering in mitosis occurred in parallel with appearance of root hairs (black arrowhead) and protoxylem (red arrows) close to the root tip. (Figs. 15A). A resupply of boron to *pCYCB1;1:GUS* plants grown in B deficient conditions during 5 days resulted in a rescued mitotic activity as reported by appearance of β-glucuronidase activity (Fig. 15B).

Finally I analyzed quiescent center identity using the reporter line *QC46:GUS* (Fig. 16). In direct growth experiments GUS staining appeared in the quiescent center region of primary roots. Meanwhile staining disappeared in primary root of B deficient plants at day 5 post-germination (Fig. 16).

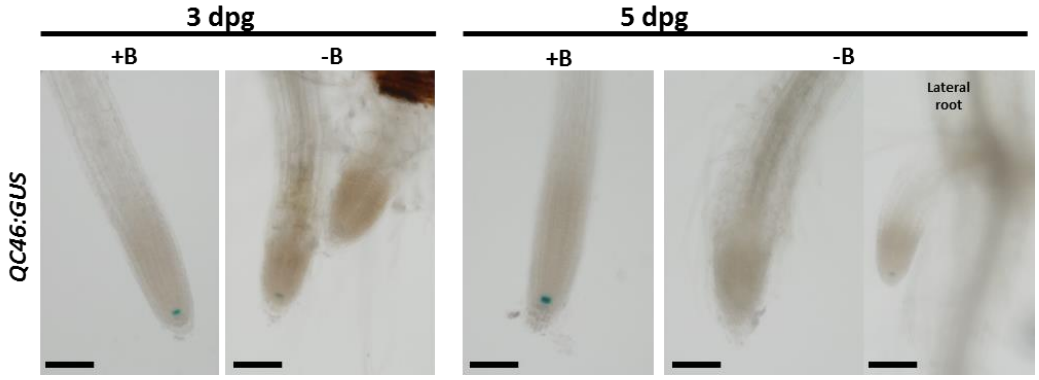


Figure16. Cell quiescence in *Arabidopsis thaliana* was diminished and disappeared under B deficiency.

Kinetics of *QC46:GUS* in *Arabidopsis* primary root tips. Seedlings were grown on semisolid plates containing ½MS medium with boron (+B) or without (-B) and GUS stained (5 h) the indicated days post germination (dpg).

Scale bar represents 100 µm.

4.2.3. Root histology is altered under boron deficiency.

Previous results suggested that B deficiency can affect the cell organization of root meristem (Figs. 13, 14, 15 and 16). To test it, I used confocal microscopy of wild type and enhancer trap lines (Tables 4 and 5), following staining of cell walls with propidium

iodide (PI). As mentioned above, B deficient plants manifested swelling of roots and a "hairy" phenotype (Fig. 17). The close examination of the meristem region revealed that in B sufficient plants PI penetrated and stained all root cells, allowing the observation of inner cell layers and the QC (Fig. 17B, +B, asterisk). In B-deficient plants staining of cell wall was regular in outer epidermis and root hairs (Fig. 17B, white arrowheads) but some irregular depositions strongly stained by PI appeared in inner epidermis and cortex (Fig. 17B, green arrowheads). Furthermore, the inner tissue layers were poorly or not stained, making difficult the observation of endodermis, vascular bundle and quiescent center.

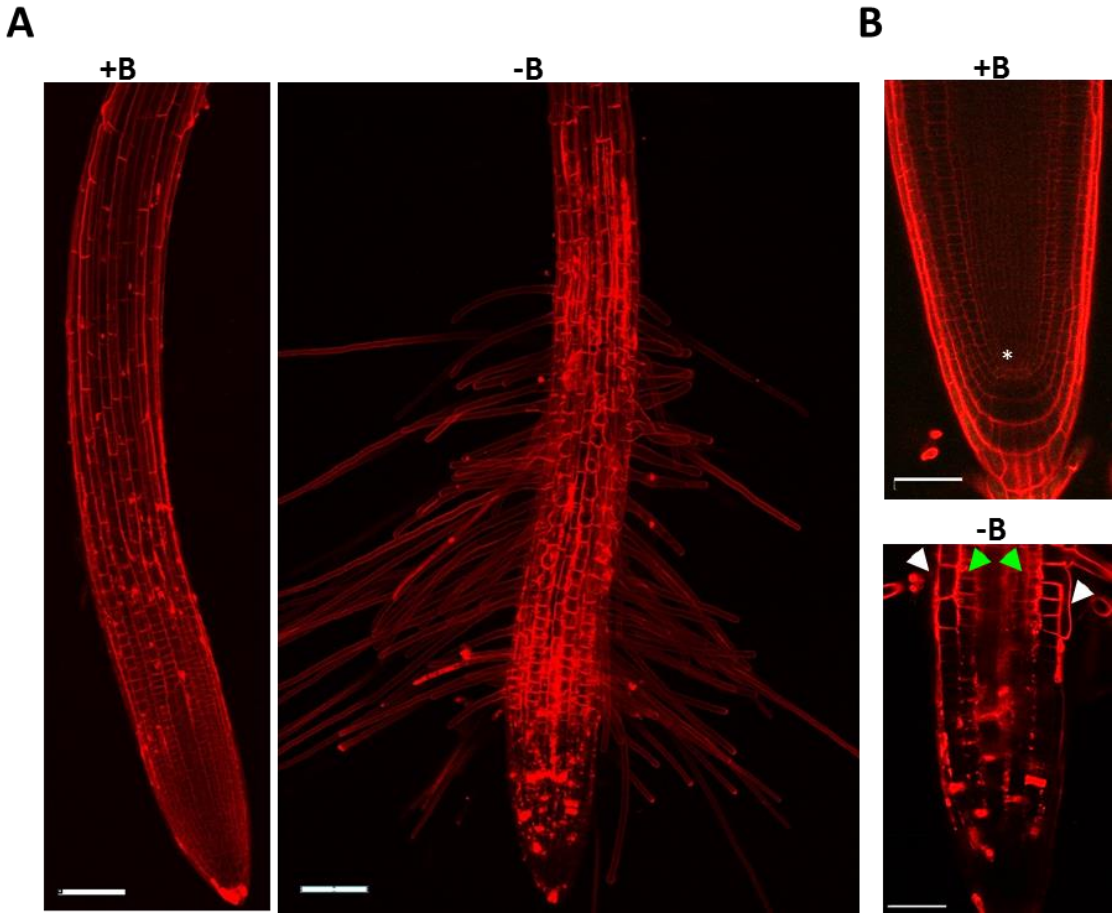


Figure 17: Propidium iodide staining of *Arabidopsis thaliana* wild type plants in transference experiments.
Arabidopsis plants grown in B sufficient conditions during 5 days were transferred to B sufficient (+B) or B deficient (-B) media and photographed at 1 days post transference (1dpt). A) Low magnification view of the *Arabidopsis* root. B) Close view of the root tip. White asterisk indicates the position of QC. White arrowheads indicate epidermis. Green arrowheads indicate cell wall deposits at cortex. Red signal: PI stained cell walls. Scale bars: 100 µm in (A) and 50 µm in (B).

To explore *in vivo* whether B deficiency alters not only cell wall deposition but also cell fate/differentiation, I used confocal microscopy on enhancer trap GAL4-GFP lines

developed by Jim Haseloff as markers for root tissues (Table 5). Plants grown during 7 days on B sufficient or B deficient media were photographied with a confocal microscope (Figs. 18, 19, 20, 21 and 22).

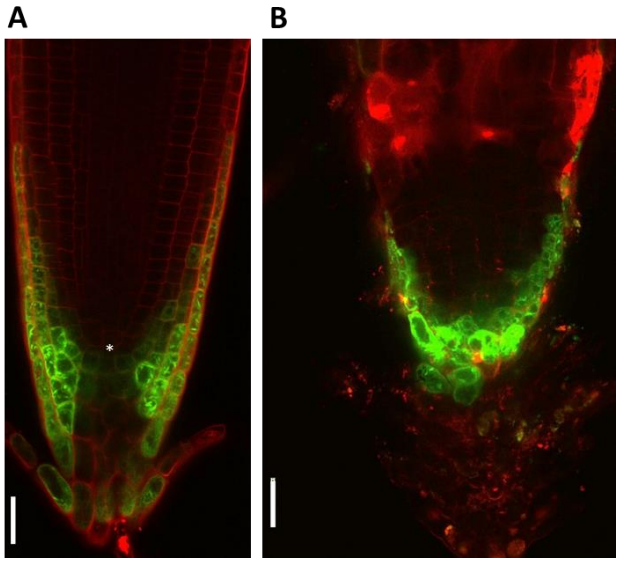


Figure 18. GFP expression in optical medial sections of *Arabidopsis thaliana* transgenic line J1092 in direct growth experiments.
Arabidopsis grown in B-sufficient (A) or in B-deficient (B) conditions during 7 days. White asterisk indicates the position of QC. Scale bars: 25 µm.

In line J0192, GFP was localized in cells forming both the columella and the root cap, according to previous descriptions. In control conditions (MSm+B) I observed a strong GFP signal in root cap and a weak signal in columella cells, which nevertheless were regularly organized in different layers (Fig. 18A). Moreover, cells were regularly shaped, and was evident the quiescent center immediately over the columella (white asterisk in Fig. 18A). In B-deficient plants (Fig. 18B) the root tip was disorganized, with columella cells not structured in different layers, and with some cell debris attached to the root tip (Fig. 18B).

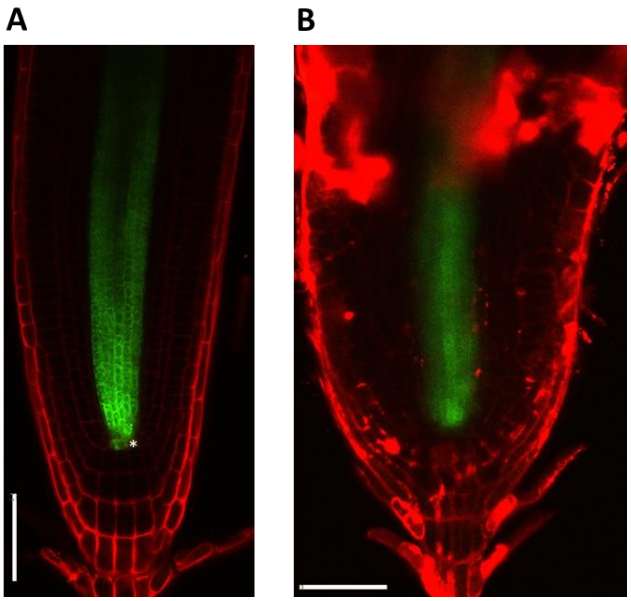


Figure 19. GFP expression in optical medial sections of *Arabidopsis thaliana* transgenic line Q0990 in direct growth experiments.
Arabidopsis grown in B-sufficient (A) or in B-deficient (B) conditions during 7 days. White asterisk indicates the position of QC. Scale bars: 50 µm.

Line Q0990 is considered a reporter of quiescent center, and vascular parenchyma at early stages (when cells fate is determined but cell differentiation has not still triggered) (Fig. 19). In this experiment, control plants (Fig. 19A) showed the expected distribution of GFP. In –B plants was observed a fuzzy appearance of the GFP signal (Fig.19B). This fuzzy appearance was not solved even increasing laser intensity nor exposition time, suggesting the presence of some CW material that makes a diffusion/optical barrier (Fig. 17B and Fig. 19).

Enhancer trap line J0121 express GFP in fully differentiated pericycle cells localized in the elongation zone. In control conditions was observed such expected expression of GFP (Fig. 20A), but in –B plants I observed that GFP signal appeared very close to the root tip (Fig. 20B), and GFP stained strands were thicker than in control plants (Fig. 20C, D).

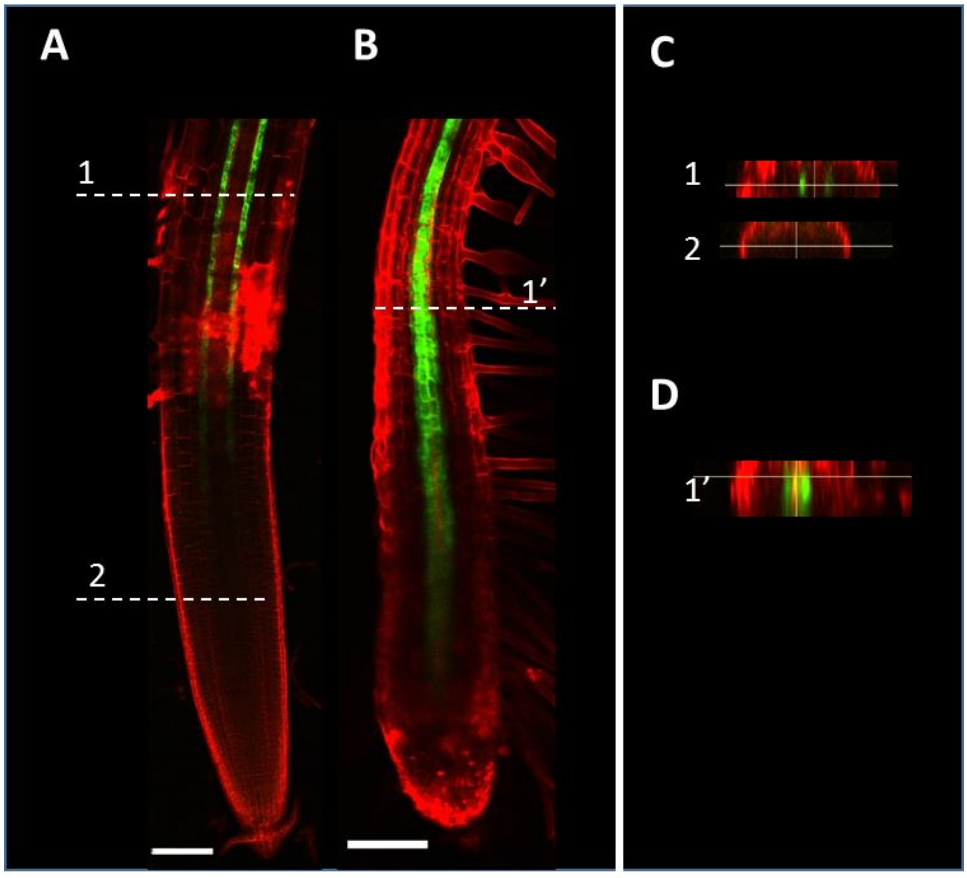


Figure 20. GFP expression in optical medial sections of *Arabidopsis thaliana* transgenic line J0121 in direct growth experiments.
Arabidopsis grown in B-sufficient (A, C) or in B-deficient (B, D) conditions during 7 days. (A, B) Root organization at medial optical section. (C, D) Orthogonal view at positions indicated in (A, B). Scale bars: 50 µm. Scale bars: 100 µm.

GFP is expressed in line J2672 in the endodermis and the root cap. In B sufficient plants, GFP signal in root cap cells covered from the root tip until the differentiation zone where root hairs emergence starts. Also, GFP was expressed in the endodermis region starting in the elongation/differentiation zone (Figs. 21A, C). In B deficient plants, root cap extension was reduced, endodermal GFP was expressed very close to the root tip and the expression region was wider than in control plants (Fig. 21B, D).

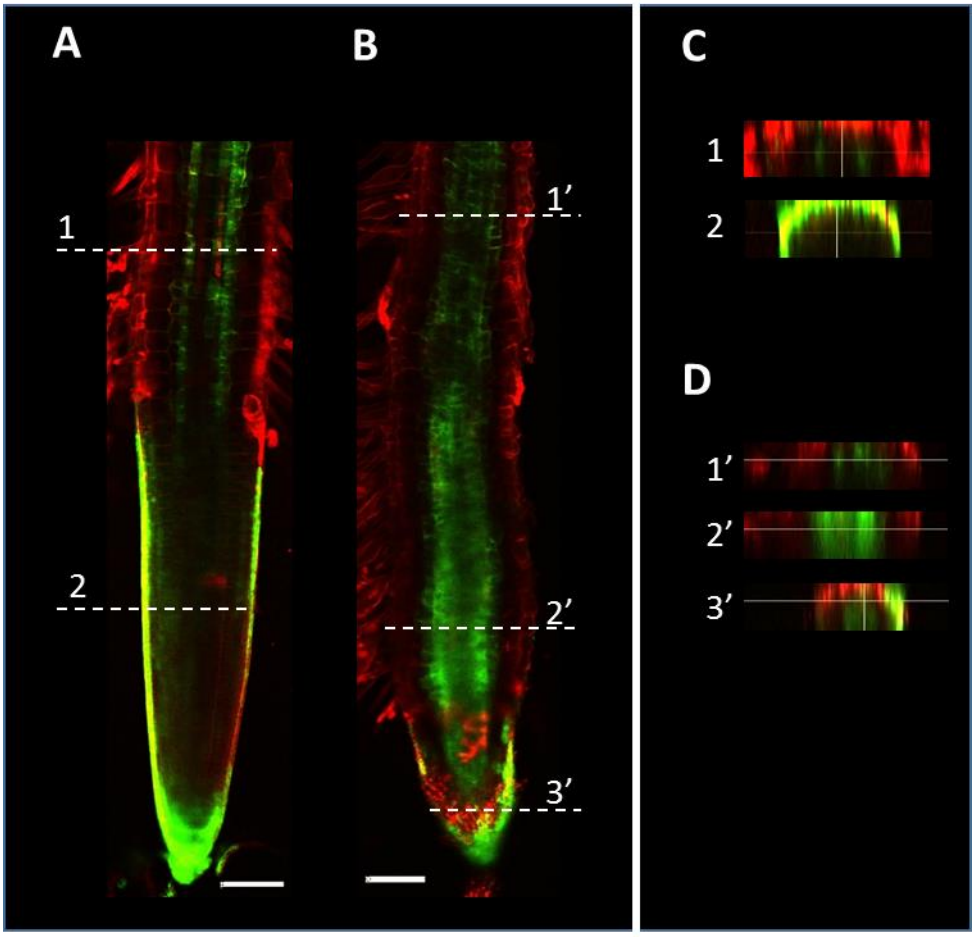


Figure 21. GFP expression in optical medial sections of *Arabidopsis thaliana* transgenic line J2672 in direct growth experiments.
Arabidopsis grown in B-sufficient (A, C) or in B-deficient (B, D) conditions during 7 days. (A, B) Root organization at medial optical section. (C, D) Orthogonal view at positions indicated in (A, B). Scale bars: 50 µm. Scale bars: 100 µm.

Finally, line 2812 was used as marker of epidermis, cortex, and lateral root cap (Fig. 22). In +B plants, GFP signal appeared as expected, revealing a typical plant cell polygonal shape, and a strong signal in the transition zone, where cells started to elongate (Fig. 22A, B). In –B plants cell length did not change, indicating poor cell elongation, polygonal structure was not so evident, and the GFP signal appeared close to the root tip, coinciding with the development of root hairs also close to the root apex (Fig. 22C, D).

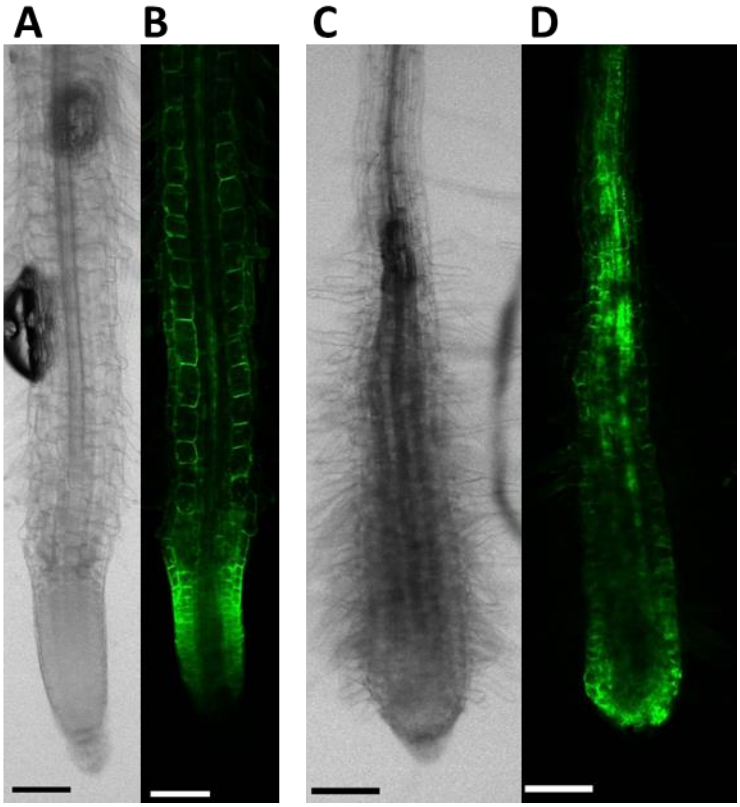


Figure 22. *Arabidopsis thaliana* transgenic line J2812 at day 7 after germination in direct growth experiments.
Arabidopsis grown in B sufficient (A, B) or B deficient media (C,D) were photographed at 7 days post germination. Bright field signal (A, C) and GFP signal (B, D) were acquired. Scale bars: 100 µm.

To confirm that some of the above results are not due to the long term exposure to boron deficient conditions, we transferred lines Q0990 (Fig. 23, Suppl. Fig. 2 and 3) and J0121 (Fig. 24) from B sufficient to B deficient plates, and then both lines were photographed at day 3 post-transference.

Q0990 B sufficient plants maintained the previous mentioned regular root structure, with the cells from QC and the vascular parenchyma expressing GFP (Fig. 23A, C, and Suppl. Fig. 2). However, Q0990 B deficient plants showed the typical B deficient symptoms as root swelling, or root hairs appearing in small cells close to the root tip (Fig. 23B, D, and Suppl. Fig. 3). Furthermore, as described in the direct growth assays, the inner root tissues were not well defined likely due to the accumulation of some substance in the cortex/endodermis, which somehow prevents PI staining and masked GFP signal in –B plants (Fig.23B, C and Suppl. Fig. 3).

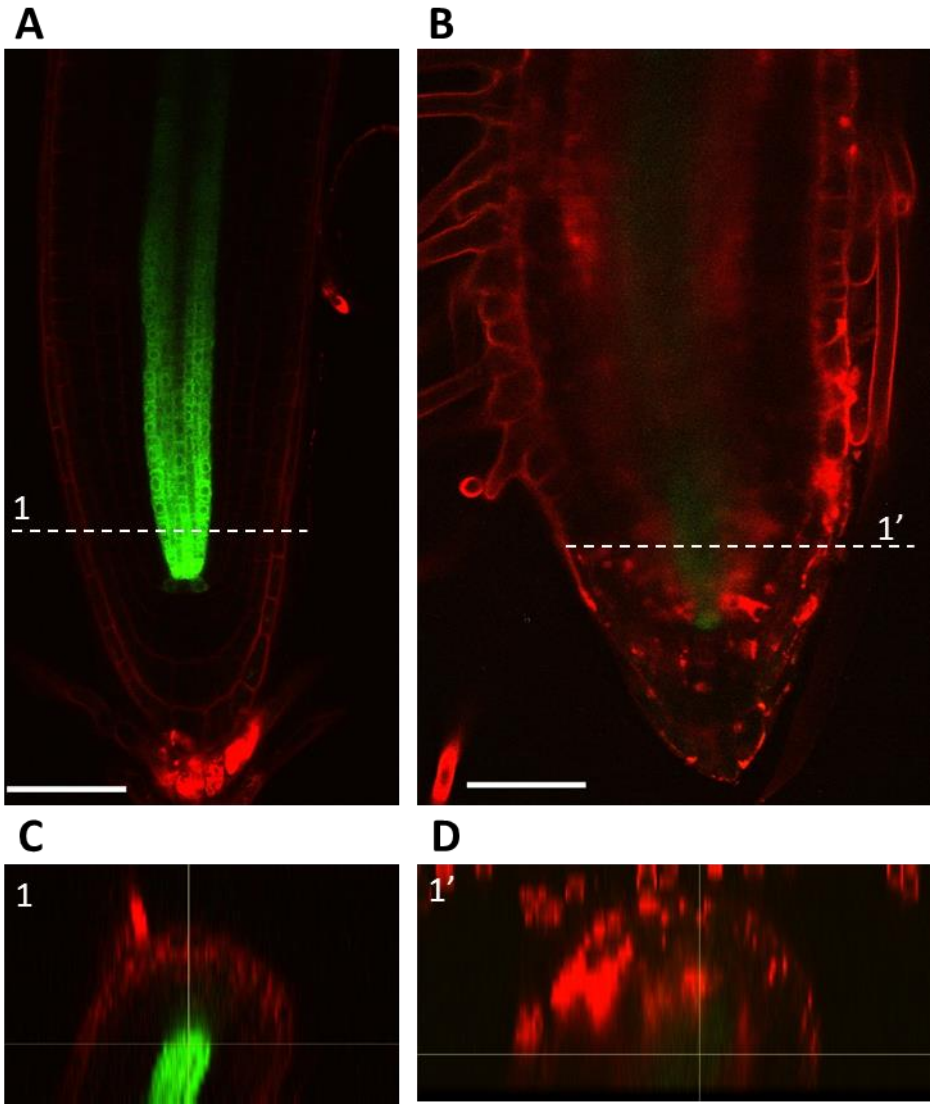


Figure 23. GFP expression in optical medial sections of *Arabidopsis thaliana* transgenic line Q0990 in transference experiments.
Arabidopsis plants grown in B sufficient conditions during 5 days were transferred to B sufficient (A, C) or B deficient (B, D) media and photographed at 3 days post transference (dpt). (A, B) Root organization at medial optical section. (C, D) Orthogonal view at positions indicated in (A, B). Scale bars: 50 µm.

In transference experiments with line J0121, a stronger signal in B deficient plants when compared with sufficient ones was detected. Also the expression of GFP revealed thicker pericycle strands 3dpt in –B plants, and the labeling started near the root tip (Fig. 24B, D).

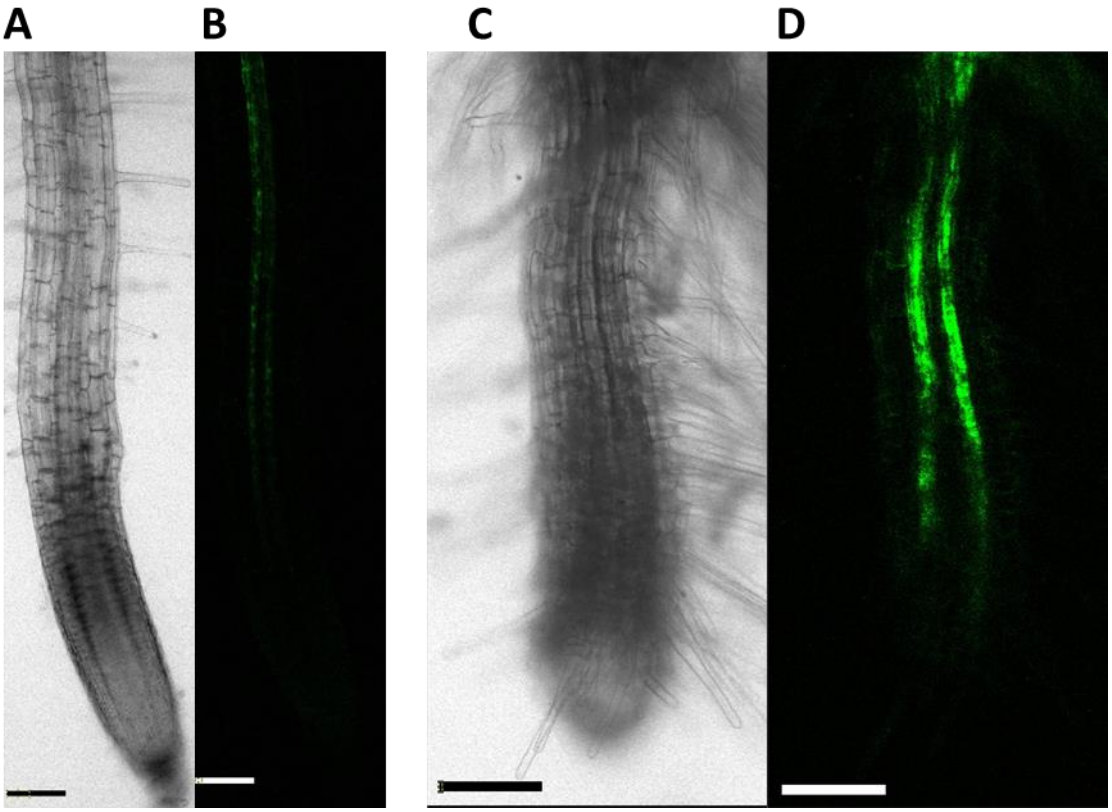


Figure 24. GFP expression in optical medial sections of *Arabidopsis thaliana* transgenic line J0121 in transference experiments.
Arabidopsis plants grown in B sufficient conditions during 5 days were transferred to B sufficient (A,B) or B deficient media (C,D) and photographed at 3 days post transference (dpt). Bright field signal (A, C) and GFP signal (B, D) were acquired. Scale bars: 100 µm.

4.2.4. Ectopic lignification and callose deposition under low boron levels.

Although previous reports have shown defects in cell wall composition in response to B deficiency, to date, deposition of polymers as callose or lignin have not being investigate in *Arabidopsis*. In Fig. 25 we observed numerous callose deposits in cotyledons and hypocotyls of plants grew in B deficient or low B conditions (0.01 and 0.1 µM H_3BO_3), whereas in the same conditions the number of deposits found in root was not so high.

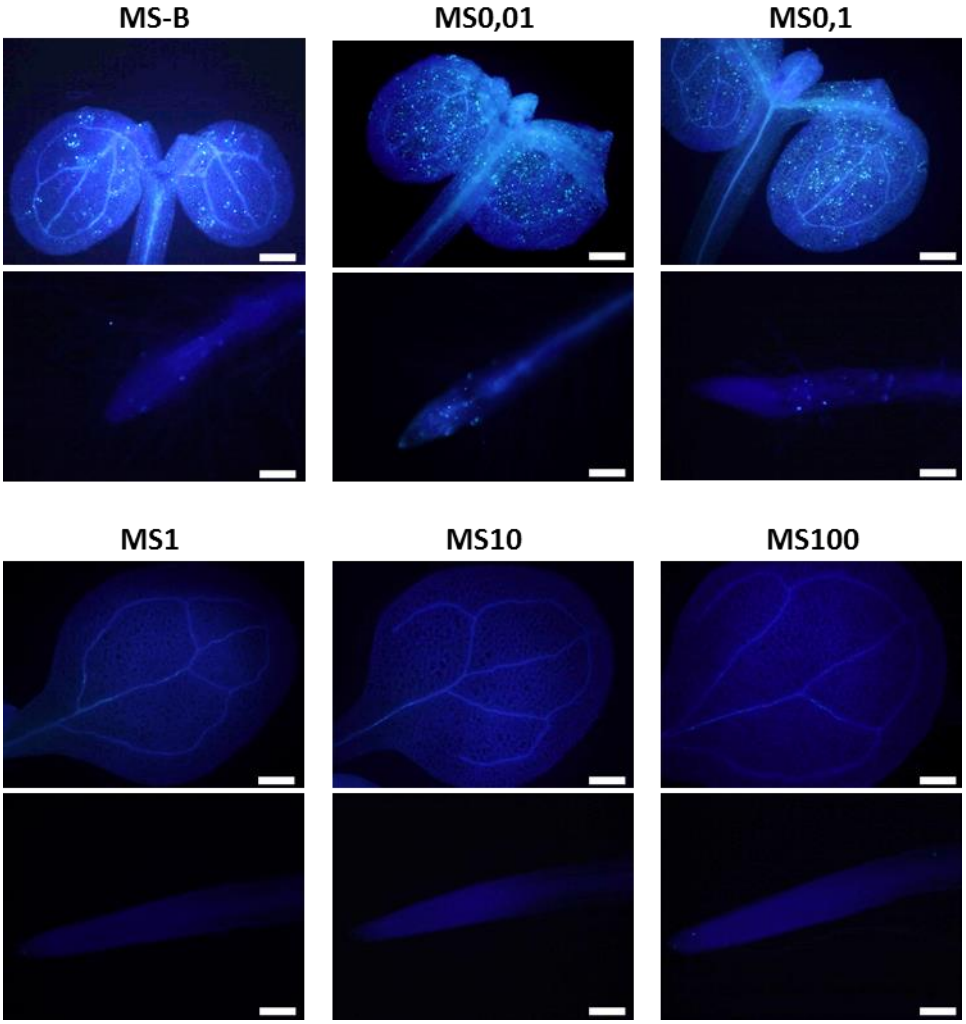


Figure 25: B deficiency causes callose accumulation in cotyledons but not in roots of *Arabidopsis thaliana*.
Callose deposits were detected with Aniline Blue staining and observed under fluorescence microscopy. Seedlings were grown on semisolid plates containing ½MS medium without (-B) or with the indicated boron concentration (as µM H_3BO_3) during 5 days post germination. Scale bar represents 200 microns in cotyledons (top) and 100 microns in root images (bottom).

Lignin was normally present in secondary cell wall of specific tissues: Caspary band in endodermis and xylem vessels. These distribution was observed under B sufficient conditions ([H_3BO_3]>1 µM) (Figs 26, 27, 28). By contrast, in B deficient plants (MSm-B) we observed ectopic presence of lignin in cotyledons, hypocotyls, and roots. The distribution of lignin in hypocotyls occurred as a patchy pattern, usually surrounding vascular tissue (Figs. 26 and 27). Ectopic lignification symptoms in cotyledons were observed in concentrations up to 0.1 µM of H_3BO_3, and in hypocotyl t in concentrations up to 0.01 µM of H_3BO_3 (Fig. 26). In roots, lignin was observed in B deficient plants as

a continuum thick layer in the endodermis (but not in the epidermis or cortex cells) which started in the elongation/differentiation zone (Figs. 26, 28).

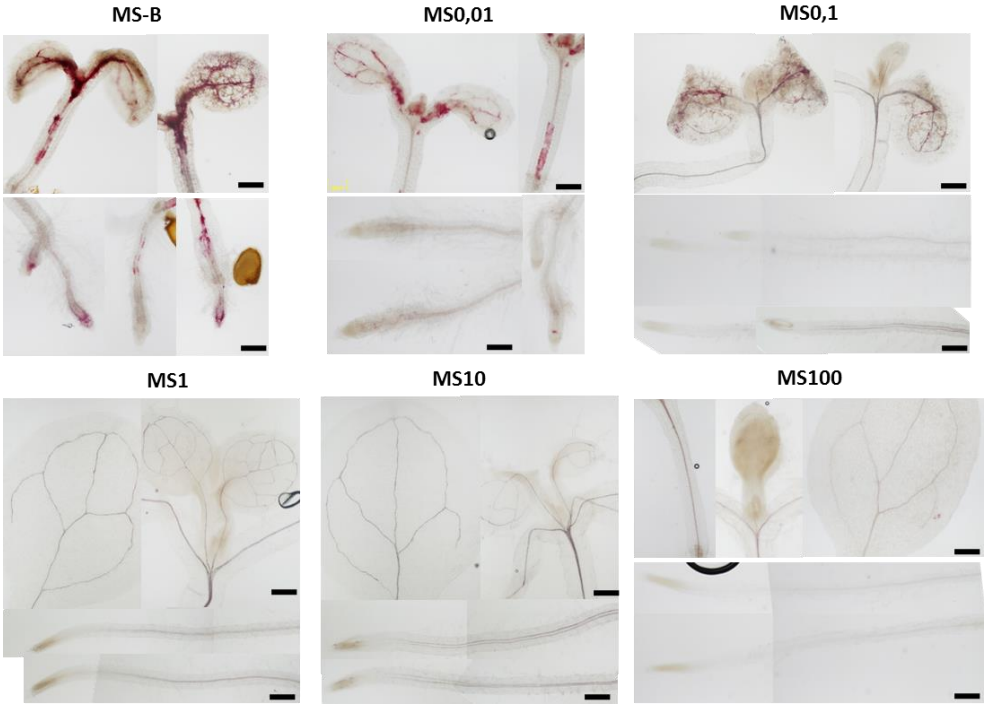


Figure 26. Ectopic lignification occurs in B-deficient and low B plants of *Arabidopsis thaliana* 5 days after germination.
Lignin was detected as a red signal using Phloroglucinol staining. Seedlings were grown on semisolid plates containing ½MS medium without (-B) or with the indicated boron concentration (as µM H_3BO_3) during 5 days post germination. Scale bar represents 200 microns in cotyledons (top) and 100 microns in root images (bottom).

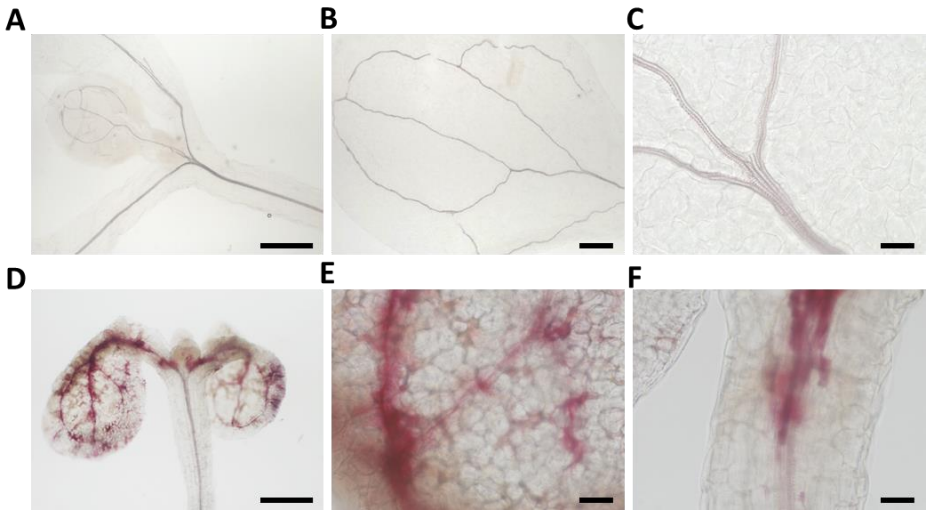


Figure 27. Details of ectopic lignification occurring in shoots and cotyledons of B-deficient *Arabidopsis thaliana*.
Lignin was detected as a red signal after Phloroglucinol staining. Seedlings were grown on semisolid plates containing ½MS medium with boron (A, B, C) or without boron (D, E, F) during 5 days post germination. Scale bar represents 400 microns in (A, D); 100 microns in (B), and 50 microns in (C, E, F). Red arrowheads highlight protoxylem, black arrowheads highlight metaxylem. (A,D) Shoot meristem; (B, C, E) Cotyledon details; (F) Hypocotyl.

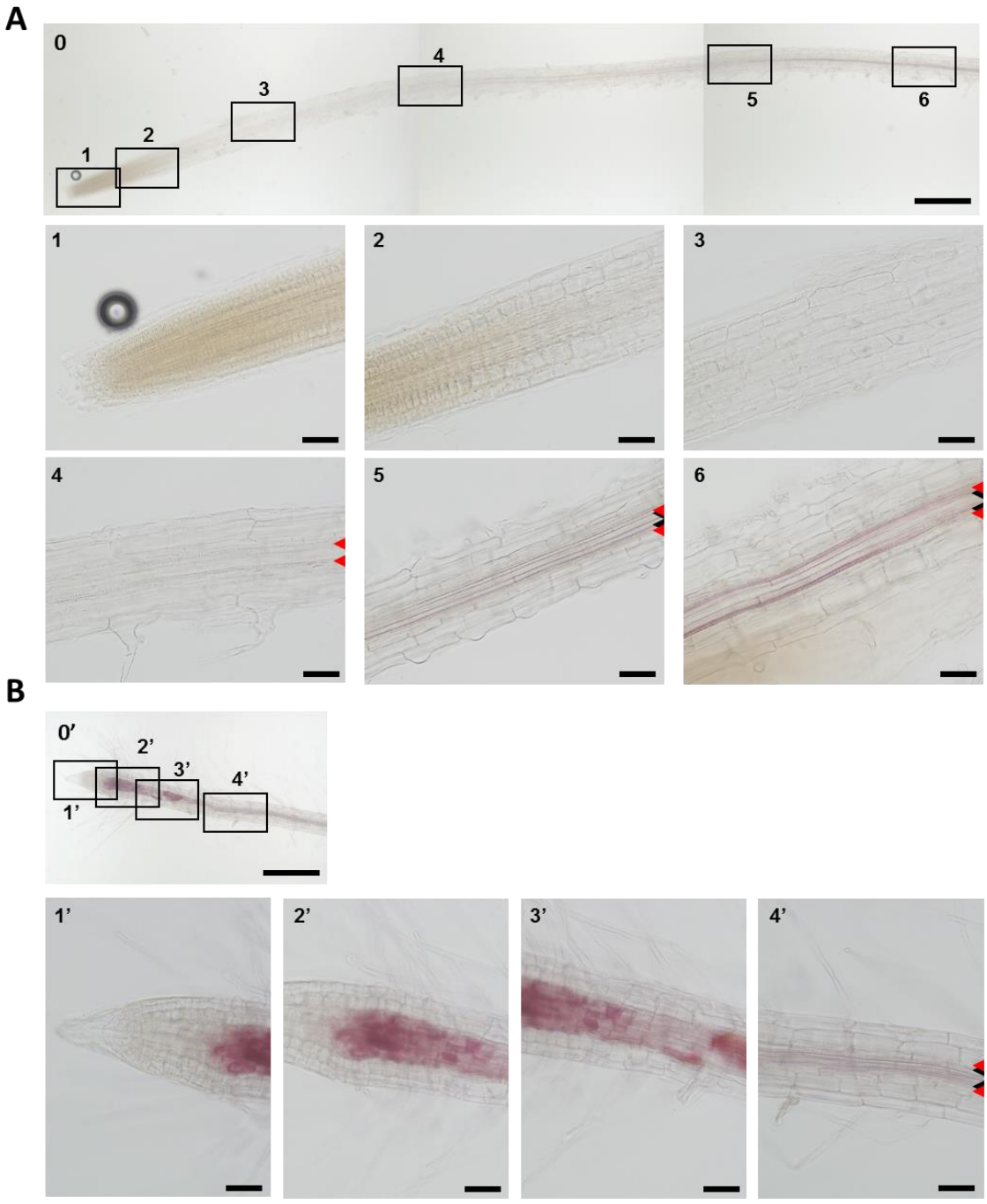


Figure 28. Details of vascular tissue and ectopic lignification in *Arabidopsis thaliana* roots. Lignin was detected as a red signal after Phloroglucinol staining. Seedlings were grown on semisolid plates containing ½MS medium with boron (A0-A6) or without boron (B0'-B04') during 5 days post germination. Scale bar represents 400 microns in 0 and 0'; and 50 microns in 1-6 and 1'-4' magnifications. Red arrowheads highlight protoxylem, black arrowheads highlight metaxylem.

A) 0: Overall view; 1: Root tip and division zone; 2: Transition/elongation zone; 3: Fully elongated zone; Differentiation zone; 5: Start metaxylem; 6: Fully mature root.

B) 0: Overall view; 1' Root tip; 2' and 3': "Elongation" and differentiation zone; 4': Fully elongated zone (developed before transference).

4.3. Protein and N-glycoprotein analysis in Arabidopsis plants grew with different boron levels.

Following morphological and histological characterization of B deficiency in Arabidopsis, I proceeded to analyze the effects of B nutrition on N-glycosylation during plant development, in order to test whether abnormal accumulation of N-glycoproteins is conserved in this model plant, so the accumulation of N-glycoproteins observed in B-deficient *Rhizobium*-legume root and nodules were a response to B starvation or contrarily were a response to the failure of the symbiotic interaction.

4.3.1. Beta-glucosidases are accumulated under low boron conditions.

After protein staining with Coomassie of +B and –B extracts, derived from *Arabidopsis* 3dpg and older plants, first evident changes in the protein pattern appeared at day 5 and were increased at 7dpg (Fig. 29, 30A). I observed the appearance of an overproduced extra band of approximately 65 kDa in B-deficient and low B (up to 0.1 μM of H_3BO_3) extracts (Fig. 29, band 2). This band was identified by peptide fingerprint as βGlu18 (At1g52400), a β-D-glucopyranosyl abscisate β-glucosidase described in TAIR database as a protein located in wound-inducible ER bodies, and required for ER body organization. Therefore, overproduction of this protein under B deficiency could point to a failure of ER dynamic and function, and hence to abnormal N-glycosylation.

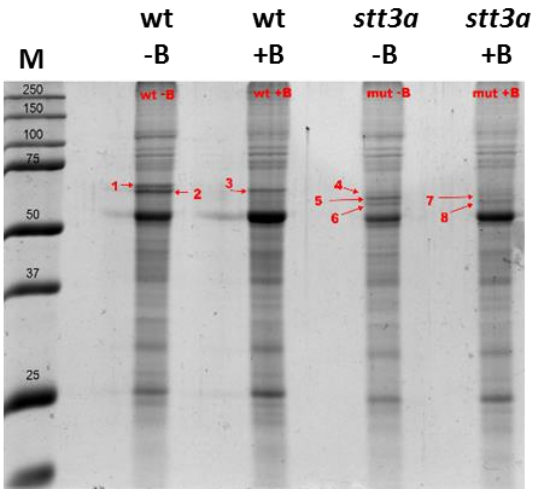


Figure 29. ER body-resident N-glycosylated β-glucosidases are induced in B deficiency. Analysis of total proteins extracted from *Arabidopsis thaliana* wild-type and *stt3a* mutant seedlings 7 days post germination (dpg) in B deficient media (-B) or control conditions (+B). Proteins were subjected to SDS-PAGE under reducing conditions and stained with Coomassie for total protein. Differently expressed bands 1 to 8 were excised for protein identification by peptide fingerprint. Proteins identified are summarized in Table X.

Table 7: ER body-resident N-glycosylated β-glucosidases are induced in B deficiency. Proteins identified by MS peptide fingerprint in bands excised from gel of Fig.29.

Band #	Name	Description	Uniprot ID	TAIR ID	Score	N-glyco sites
1	AtBG1, AtBGLU18	Beta-D-glucopyranosyl abscisate beta-glucosidase	Q9SE50	AT1G52400	570	3
2	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	757	3
3	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	394	3
4	AtBG1, AtBGLU18	Beta-D-glucopyranosyl abscisate beta-glucosidase	Q9SE50	AT1G52400	79	3
	CPN60B1, LEN1	Chaperonin 60 subunit beta 1, chloroplastic	P21240	AT1G55490	76	-
	CNX1	Calnexin homolog 1	P29402	AT5G61790	56	1
	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	54	3
5	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	296	3
	AtBG1, AtBGLU18	Beta-D-glucopyranosyl abscisate beta-glucosidase	Q9SE50	AT1G52400	248	3
6	atpA	ATP synthase subunit alpha, chloroplastic	P56757	ATCG00120	456	-
	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	258	3
	rbcL	Ribulose bisphosphate carboxylase large chain	O03042	ATCG00490	68	-
7	atpA	ATP synthase subunit alpha, chloroplastic	P56757	ATCG00120	130	-
	CPN60A1	Chaperonin 60 subunit alpha 1, chloroplastic	P21238	AT2G28000	94	-
	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	92	3
8	atpA	ATP synthase subunit alpha, chloroplastic	P56757	ATCG00120	581	-
	BGLU23, PYK10	Beta-glucosidase 23	Q9SR37	AT3G09260	62	3

4.3.2. Boron deficiency causes accumulation of N-glycoproteins and UPR but not hypo-glycosylation of model N-glycoproteins.

In Arabidopsis there is two approaches to analyze if a mutant or a specific treatment alter N-glycosylation. As previously performed in legume nodules, I started with an overall study of the electrophoretic protein profile, and then analyzed N-glycosylation on specific proteins using a specific antibodies and observing how it changes its electrophoretic mobility in a denaturing gel.

Firstly, N-glycosylation pattern of *Arabidopsis* plants grown in different B conditions during 3, 5 or 7 dpg was analyzed (Fig. 30B, C, D). In suboptimal levels of B (minor than 100 μM of H_3BO_3) both MRNGPs and Fucose-containing CNGPs tend to accumulate as early as 3dpg (although differences were still not significant). The accumulation of these MRNGPs and Fucose-containing CNGPs was significantly different in low B conditions (from -B to 0.1 μM of H_3BO_3) 5 and 7 dpg (Fig. 30B, C). By contrast, patterns of Xylose-containing CNGPs were not significantly altered at day 3 and 7 after germination, although was accumulated at day 5 with the different B treatments (Fig. 30D).

Protein Disulfide Isomerase (PDI) is an ER-resident N-glycoprotein involved in protein folding, with two N-glycosylation sites which according to the literature are occupied by MRNGs. Using an antibody against PDI, this protein served us as a reporter to analyze the effect of B deficiency on the N-glycosylation process. Fig. 30E shows that in extracts derived from any B concentration appeared only one PDI band corresponding to the glycoprotein with the two N-glycosylation sites occupied. That means that this N-glycoprotein was not hypo-glycosylated in response to B nutrition.

Although glycosylation of PDI was apparently not altered, it was accumulated in low B treatments (-B to 0.1 μM of H_3BO_3). This suggests that B deficiency could lead to an incorrect protein folding/processing (Fig. 30E). To test this hypothesis, we analyzed the ER-resident chaperonin Binding immunoglobulin Proteins (BiP) (BiP1, At5g28540; BiP2, At5g42020; BiP3, At1g09080), known to be induced during Unfolded Protein Response (UPR). As Fig. (30F) shows, BiP was also accumulated in low B conditions, suggesting that B deficiency is altering protein folding/processing and induced responses similar to UPR.

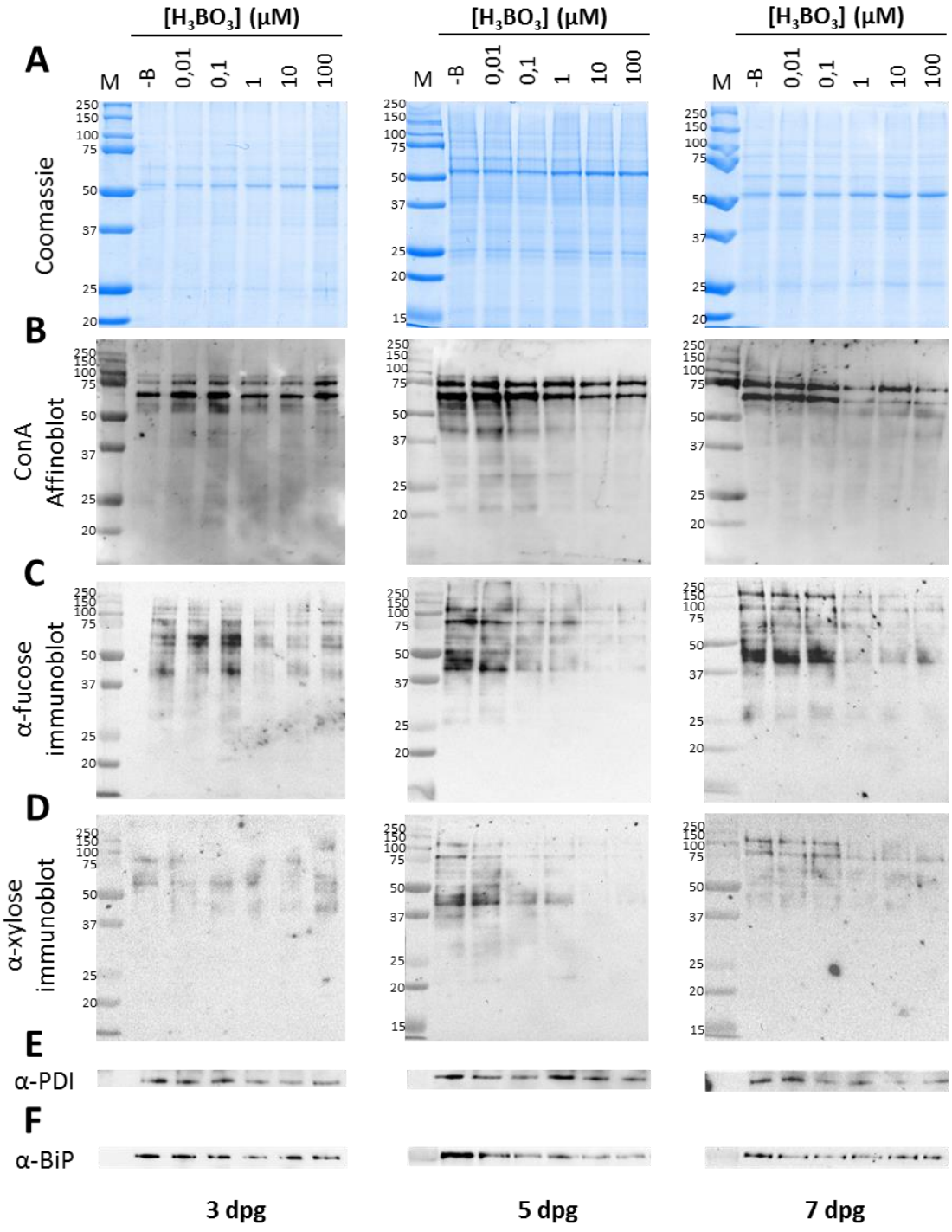


Figure 30. Temporal evolution of protein and N-glycoprotein pattern of *Arabidopsis thaliana* under different B conditions

Analysis of total proteins extracted from *Arabidopsis thaliana* grew at indicated days post germination (dpg). Proteins were subjected to SDS-PAGE under reducing conditions, stained with Coomassie for total protein (A) or transferred to blots which were analysed using: B) the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans; C) anti-fucose or D) anti-xylose antibodies, which recognize complex N-glycans with a core α1,3-fucose and β1,2-xylose residues respectively; E) anti-PDI model N-glycoprotein;F) anti-BiP.

Secondly, I analyzed N-glycosylation pattern in separated extracts from both root and shoot (hypocotyl and cotyledons) isolated from Arabidopsis plants grew 7 dpg with different B concentrations at 7 dpg (Fig. 31). A band of approximately 65 kDa (the same electrophoretic mobility than the previously identified βGlu18) was observed only in shoots of plants grew in low B conditions (-B to 0.1 μM of H_3BO_3) (Fig. 31A). MRNGPs accumulation was more evident in shoots than in roots, and a decreased intensity of labelling was observed at B concentrations optimal for growth (Fig. 31B). Fucose-containing CNGPs also accumulated in low B treatments, although immunolabelling seemed to be more intense in root extracts (Fig. 32C). However, CNGPs containing Xylose were not significantly accumulated, in any of the B treatments (Fig. 32D).

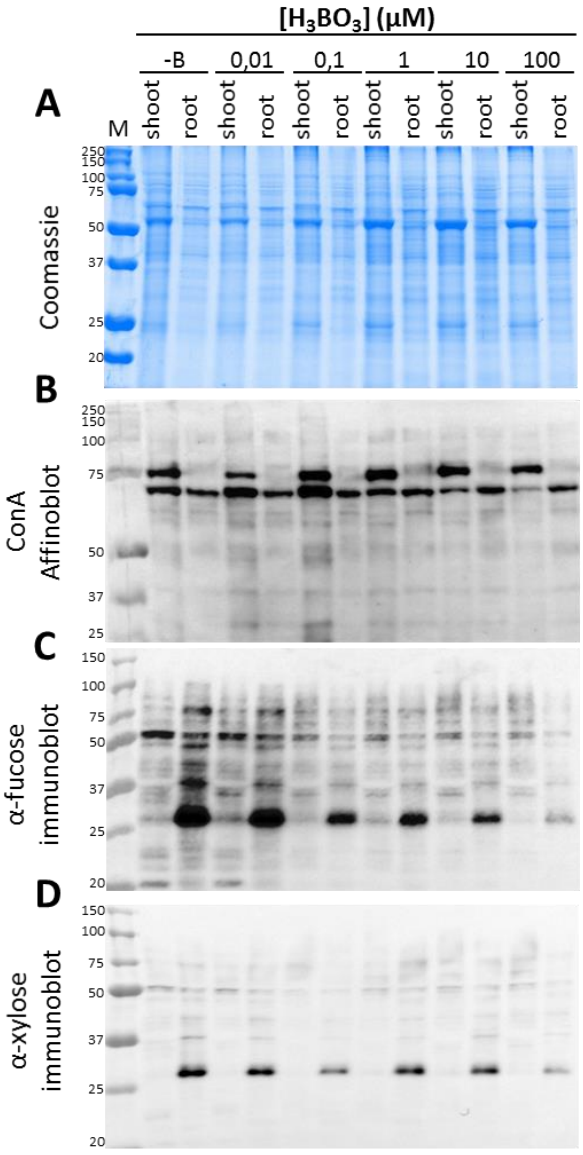


Figure 31. N-glycosylation changes occur in both roots and shoots of *Arabidopsis* seedlings
Analysis of total proteins extracted 7 days post germination from plants grew with the indicated concentrations of B. Proteins were subjected to SDS-PAGE under reducing conditions, stained with Coomassie for total protein (A) or transferred to blots which were analysed using: B) the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans; C) anti-fucose or D) anti-xylose antibodies, which recognize complex N-glycans with a core α1,3-fucose and β1,2-xylose residues respectively.

4.3.3. N-glycosylation pattern in the mutant requiring high boron, *bor1-1*.

To gain more evidences about the causes of N-glycoprotein accumulation during plant development with low B levels, I decided to analyze N-glycoprotein pattern in *bor1-1* mutant, which is impaired in boron xylem loading that results in a minor translocation of the micronutrient to the aerial part, and in a higher requirement of B to reach a normal growth.

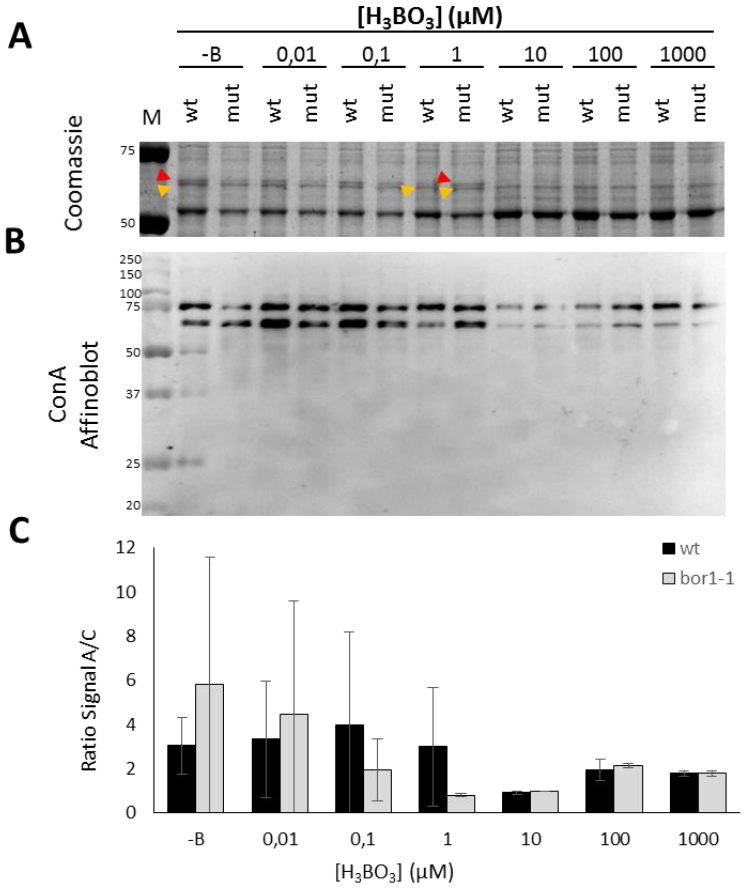


Figure32. N-glycosylation changes occur in both roots and shoots of *Arabidopsis* seedlings Analysis of total proteins extracted 7 days post germination from plants grew with the indicated concentrations of B. Proteins were subjected to SDS-PAGE under reducing conditions, stained with Coomassie for total protein (A) or transferred to blots which were analysed using: B) the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans; C) anti-fucose or D) anti-xylose antibodies, which recognize complex N-glycans with a core α1,3-fucose and β1,2-xylose residues respectively.

The Coomassie stained of protein profile showed that βGlu18 was present in concentrations of boric acid up to 1 µM in in *bor1-1* mutant, contrasting to wild type plants in which BGlu18 appeared only at concentrations of B minor than 0.1 µM (Fig. 32A, red arrow). Moreover, the affinoblot revealed that *bor1-1* mutant showed the same

tendency to accumulate MRNGPs under low B levels, similar to wild type plants, and with no significant differences between those and the mutant line (Fig. 32B, C).

4.3.4. Effects of boron nutrition on N-glycosylation mutants.

To finish this particular study, I analyzed growth behavior, root development, and MRNGP patterns in relation with B nutrition in several *Arabidopsis m*utants impaired in N-glycan synthesis (*alg3* and *alg10*), OST (*stt3a*), and N-glycan processing (*mns12*, *mns123*, *cgl1* and *gntI*).

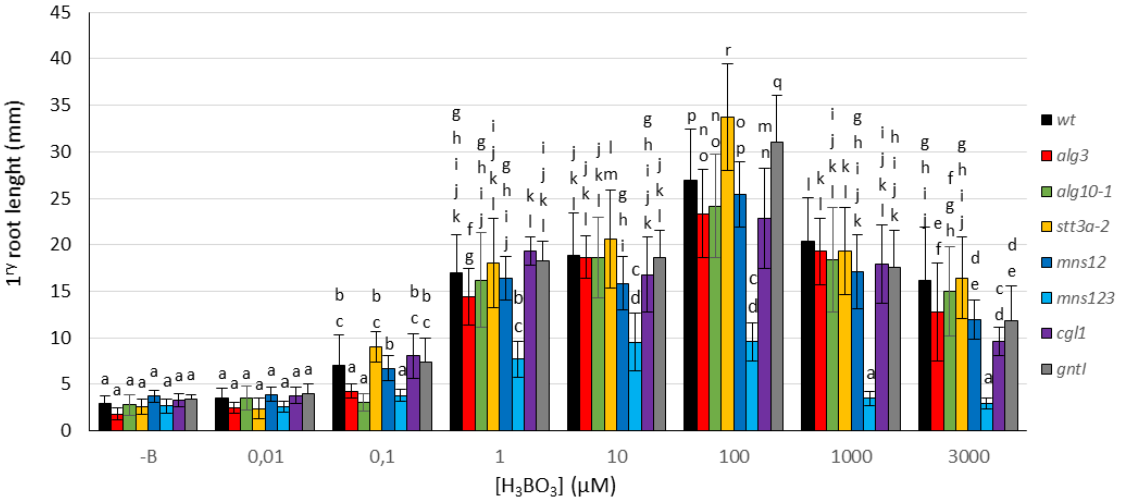


Figure 33. Root growth of Arabidopsis wild type and N-glycosylation mutants under different boron levels.
Seedlings were grown on ½MS medium for 7 days (16-h light/8-h dark) with the indicated concentrations of B, photographed, and measured using FIJI. Mutants are described in Table 3 of Material and Methods section and in the text of Results section.
Different letters indicate statically significant differences (p<0,05).

According to literature, of all mutants used in this study, only mutant *mns123* is impaired in growth and shows root swelling phenotype under control conditions (Liebminger et al., 2009). When I grew these mutants in a gradient of B, a reduced growth was observed in all treatments, excepting in B deficient conditions and in 0.01 µM H_3BO_3, concentrations that reduced growth in all other lines (Fig. 33). Mutants *alg3* and *alg10* showed a reduced growth compared with wt in low (0.1 µM) and in optimal (100 µM) concentrations of H_3BO_3. Moreover, the other mutants involved in N-glycan modification (*mns12*, *cgl1* and *gntI*) were more sensitive than wt plants to B toxic concentrations (3000 µM of H_3BO_3) (Fig. 33).

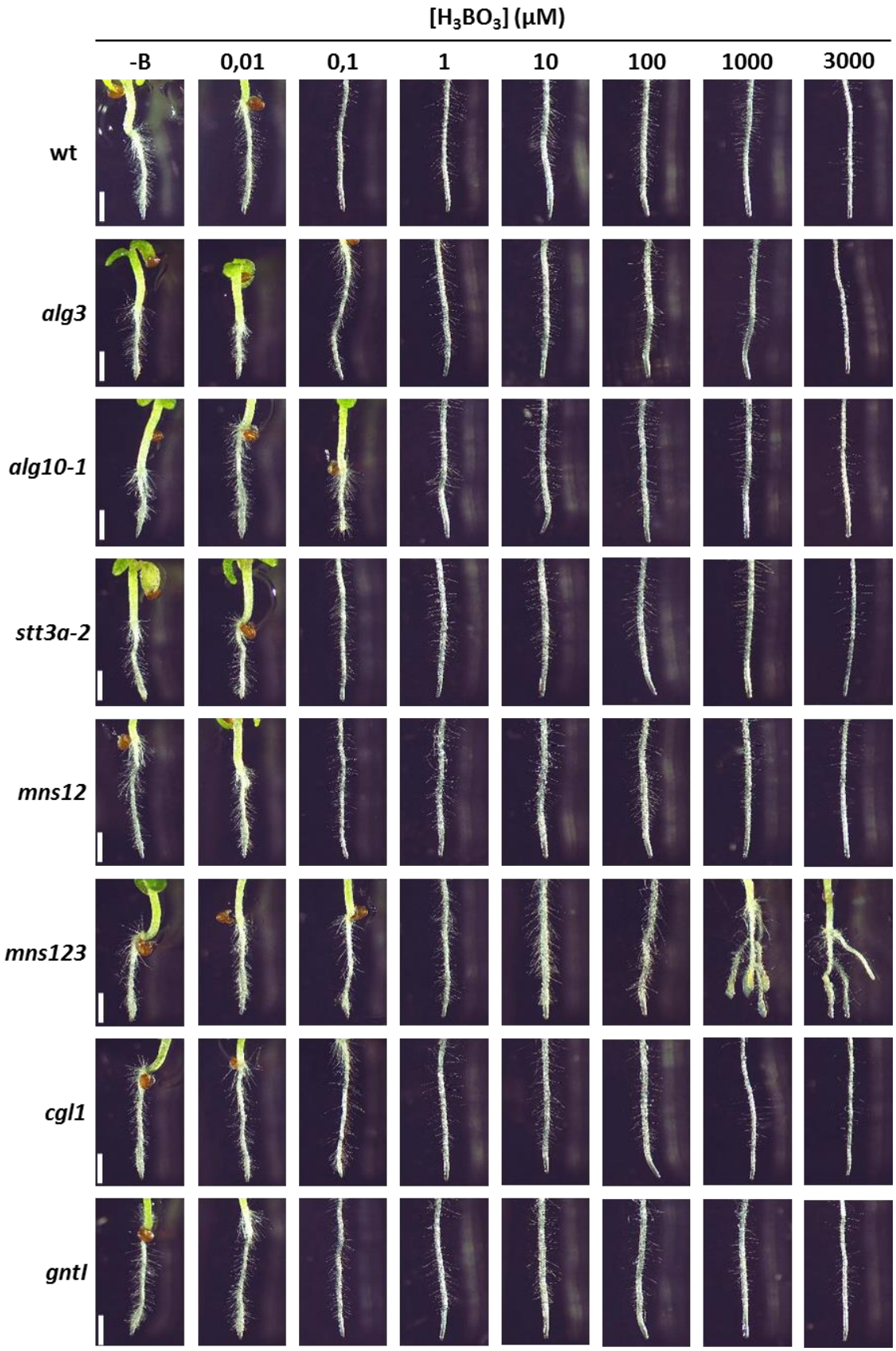


Figure 34. Root morphology of *Arabidopsis* wild type and N-glycosylation mutants
Root tips of wild-type and N-glycosylation mutants *alg3-2, alg10-1, stt3a-2, mns12, mns123, cgl1, gntI* seedlings grown for 7 days on ½MS medium with different B levels. Scale bar represents 1 mm.

Following observation of roots under stereoscopic microscope we found that all lines showed hairy phenotype and root swelling in B deficient conditions. Also, toxic concentrations of B (>1000 μM of H_3BO_3) reduced the number of emerged root hairs in all plants, concomitant to the growth inhibition (Fig. 34). Mutant *mns123* showed a particular behavior: at any B concentration assayed, root hair emergence always occurred close to the root tip. At concentrations of boric acid higher than 1000 μM, lateral roots were developed, and both primary and lateral roots accumulated some brown substances and presented swollen tips (Fig. 34).

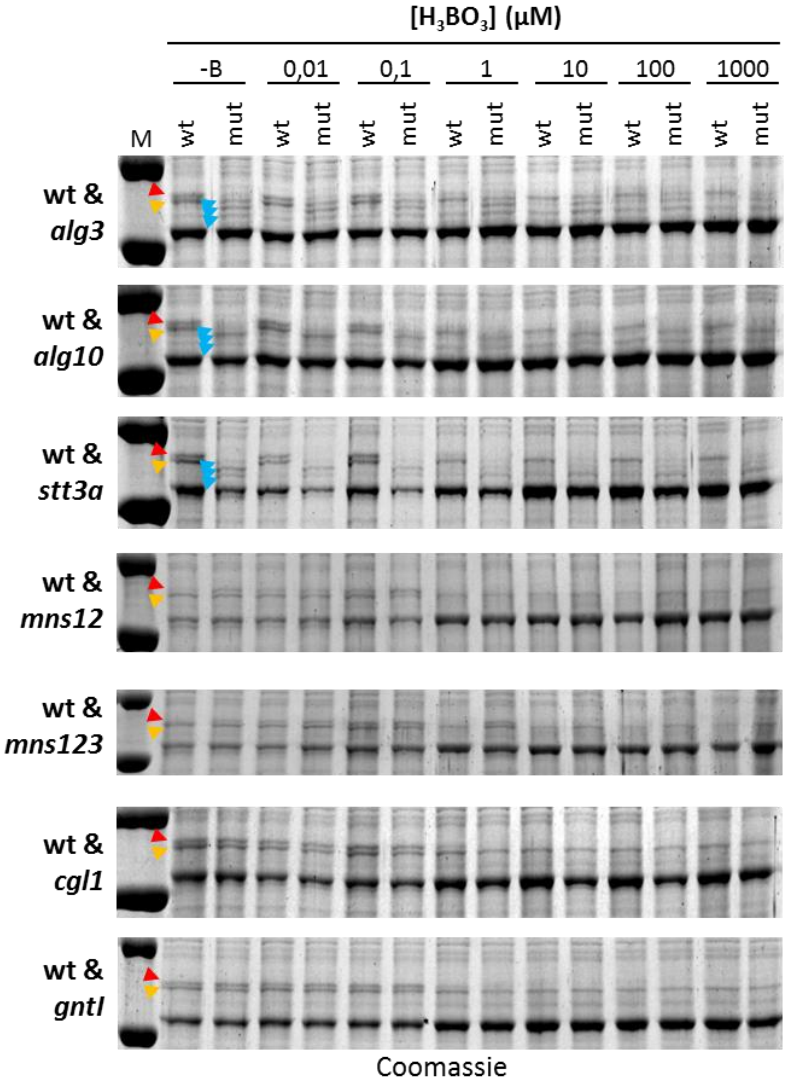


Figure 35. Changes in protein pattern in *Arabidopsis* N-glycosylation mutants.
Analysis of total proteins extracted from wild-type (wt) and N-glycosylation mutants (mut) *alg3-2, alg10-1, stt3a-2, mns12, mns123, cgl1,* and *gntI* seedlings at day 7 post germination (dpg). Proteins were subjected to SDS-PAGE under reducing conditions and stained with Coomassie for total protein. Yellow arrowhead indicates βGlu23 protein which is presents in wt in all B concentration. Red arrowhead indicates βGlu18 protein, presents in low B conditions in wt and mutants *mns12, mns123, cgl1 and gntI*. Blue arrowheads indicate some fast migrating bands, identified in Fig. 29 and Table 7.

Finally, we analyzed protein and Mannose Rich N-glycoprotein pattern and glycosylation of PDI in mutants impaired in N-glycosylation.

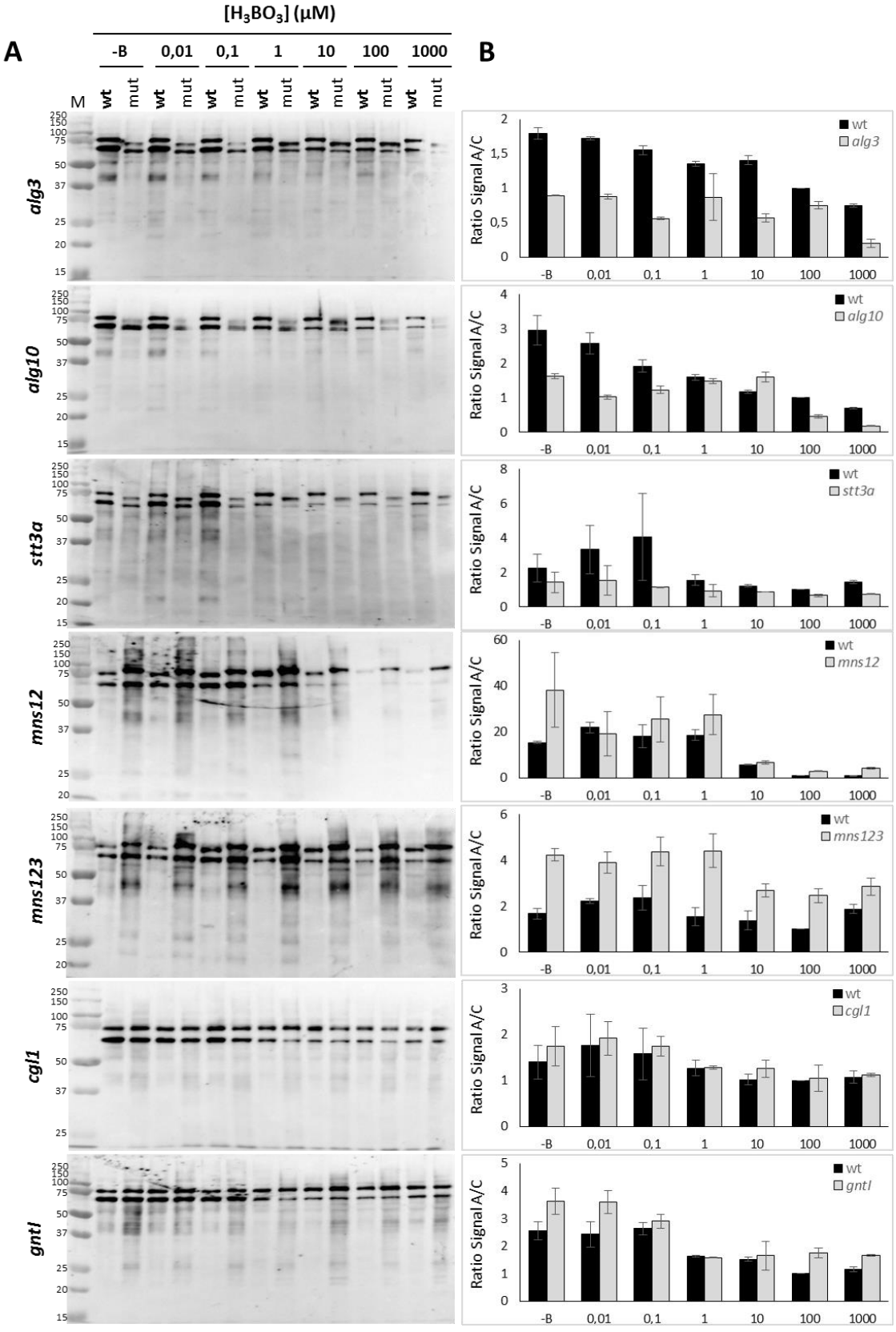


Figure 36. Boron diminishes mannose rich N-glycoproteins in both wild type and mutants of Arabidopsis in N-glycosylation pathway.
Analysis of total proteins extracted from wild-type and N-glycosylation mutants *alg3-2, alg10-1, stt3a-2, mns12, mns123, cgl1,* and *gntI* seedlings at day 7 post germination (dpg). Proteins were subjected to SDS-PAGE under reducing conditions, transferred to blot and detected using the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (A). B) Semiquantification of MRNGPs relative to total protein is summarized.

As observed in Fig. 35, protein pattern is altered in mutants *alg3*, *alg10* and *stt3a*. Specifically was observed that band corresponding to βGlu18 and βGlu23 disappeared in those mutants (Fig. 35, red and yellow arrowheads), but at the same time appeared some bands with a higher electrophoretic mobility (Fig.35, blue arrowheads), which could correspond to hypo-glycosylated forms of βGlu18 and βGlu23. To confirm that hypothesis I identified the new bands appearing in *stt3a* background by peptide fingerprint. Although there was other contaminant proteins, was confirmed that βGlu18 and βGlu23 were present in B-deficient *stt3a* plants whereas in B-sufficient *stt3a* plants only βGlu23 was identified (Fig. 29 and Table 7), suggesting that both proteins were not correctly n-glycosylated in mutant *alg3*, *alg10* and *stt3a*. MRNGP pattern was also altered in *alg3*, *alg10* and *stt3a* mutants, showing some fast migrating bands that coincided with the previously described N-glycosylation pattern of these mutants (Fig. 36A). Mutants *mns12* and *mns123* also had a different profile, but in this case some proteins showed a low migration compared with wt (Fig. 36A). Boron nutrition did not affect those changes in electrophoretic motility. When we performed a semi quantitative analysis of band intensity in the different B treatments, mutants *mns12*, and *gntI* followed a similar behavior than wild type plants, namely, that plants grew under low B conditions accumulates MRNGPs (Fig. 36B). Accumulation of MRNGP at low B concentrations in mutants *alg3*and *alg10* was lower than in wt, and *stt3a* did not showed significant differences of band intensities at any B concentration. Finally, *mns123* accumulated more N-glycoproteins than the wt in all B treatments (Fig. 36B).

To complete this study, we analyzed the N-glycosylation status of PDI. The appearance of fast migrating bands indicated that PDI was hypo-glycosylated in *alg3* and *alg10* backgrounds, as previously indicated, and any B concentrations used in this study alleviate or aggravate the hypo-glycosylation defects of these plants (Fig. 37). In the other analyzed mutants, PDI appeared correctly glycosylated (Fig. 37).

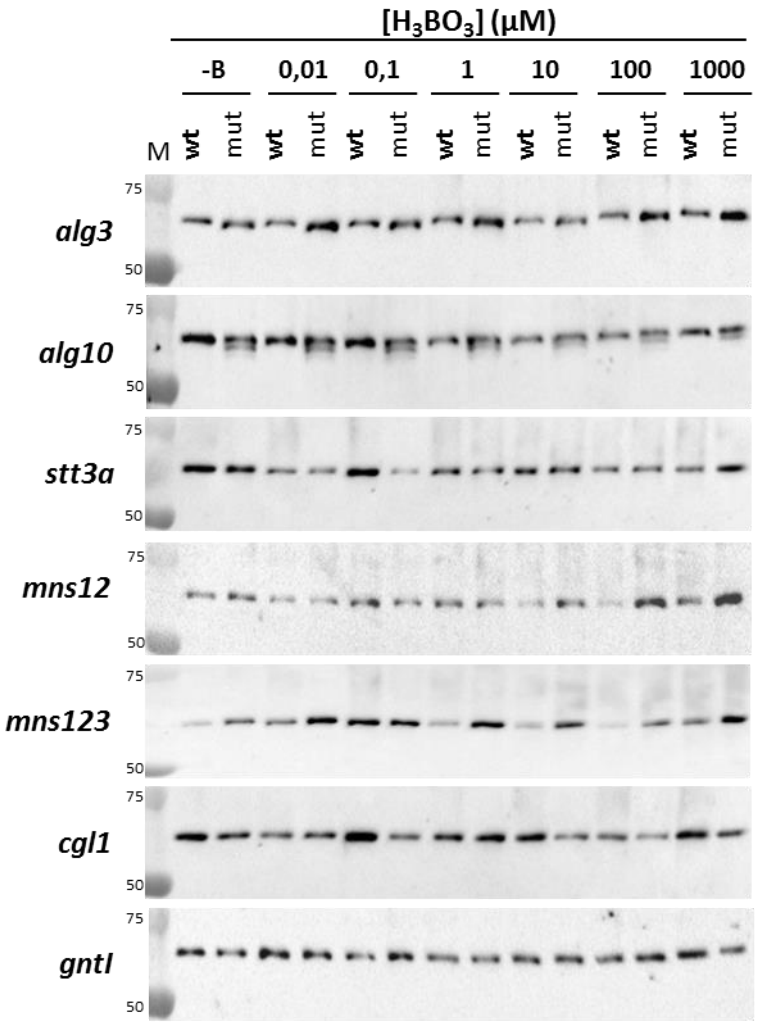


Figure 37. Boron does not alter N-glycosylation of model N-glycoprotein Protein Disulfide Isomerase (PDI) in N-glycosylation mutants of Arabidopsis.
Analysis of total proteins extracted from wild-type and N-glycosylation mutants *alg3-2, alg10-1, stt3a-2, mns12, mns123, cgl1,* and *gntI* seedlings at day 7 post germination (dpg). Proteins were subjected to SDS-PAGE under reducing conditions, electroblotted and probed with anti-PDI. The B treatments sometimes influenced the intensity of labelling but did not rescue the hypoglycosylation defects, observed by the appearance of fast migrating bands in mutants *alg3-2* and *alg10-1.*

4.4. Drug treatments can alleviate symptoms produced by boron deficiency or phenocopy boron-deficiency in boron sufficient plants.

To test if some of the described B deficiency responses (like hairy phenotype and root swelling, ectopic lignification, and accumulation of N-glycoproteins) are primary or secondary effects of B deficiency (i.e. derived from alterations of hormone balance, endomembrane disorganization/failure, etc.), I treated both B-sufficient and B-deficient plants with different drugs and analyze those morphological and cytological parameters.

4.4.1. ACC/ethylene signaling pathway is necessary for development of hairy roots under boron deficiency, but does not explain root swelling, ectopic lignification nor accumulation of N-glycoproteins.

As mentioned above (section 4.2.2.), B deficiency caused a reduction in cell length similar to that produced by ACC treatments (ethylene precursor) in both B sufficient and B deficient plants (Fig. 13). Therefore, I analyze if ACC (ethylene precursor) or Ag^+ (ethylene receptor antagonist) treatments can alleviate/aggravate some of the above mentioned symptoms produced by B deficiency in *Arabidopsis*.

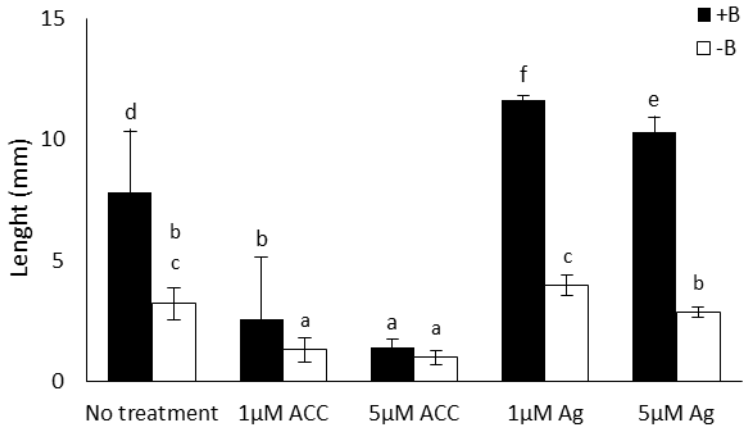


Figure 38. Growth arrest under B deficient conditions are alleviated with Ethylene antagonist (Ag) and aggravated with ACC.
Arabidopsis plants grew in B sufficient media during 5 days were transferred for 3 days to the indicated +B or –B media supplemented with the specified treatments. At the end of the experiment we measured root length after transference. Different letters indicate significant differences (α=0,05)

In B-sufficient plants, treatment with ACC caused growth inhibition (Fig. 38) and induced a hairy phenotype (Fig39), whereas in B-deficient plants, treatment with ACC resulted in an even more reduced growth and in also aggravated hairy phenotype (Figs. 38 and 39). Treatment with silver ions stimulated growth in B sufficient plants but was unable to rescue growth or prevent root swelling caused by B deficiency (Fig. 38 abd 39). Ag^+ ions were able only to reduce the hairy phenotype in B deficient plants. (Fig. 39).

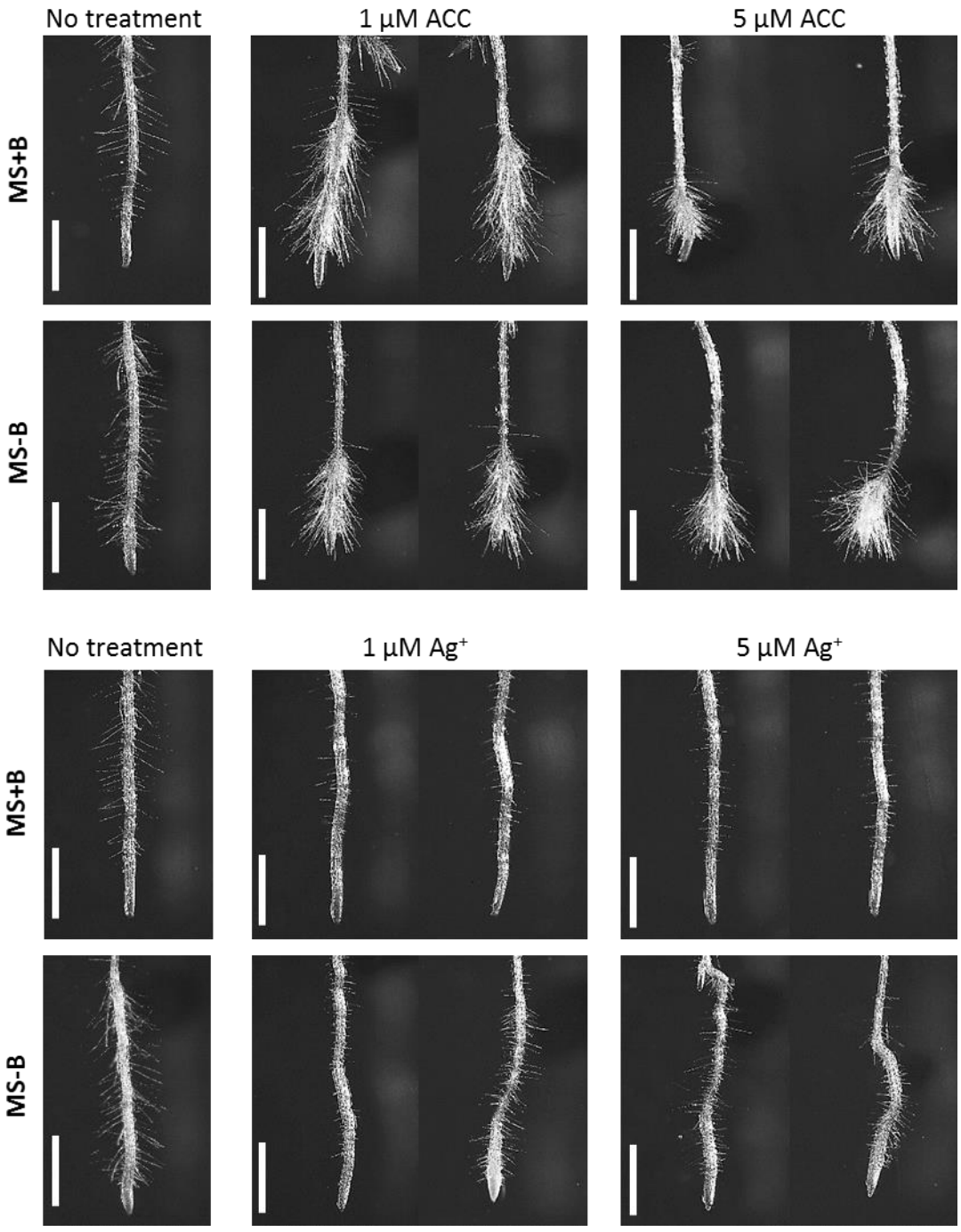


Figure 39. Ethylene/ACC signalling pathways are involved in B deficiency response. Arabidopsis plants grew in B sufficient media were transferred to the indicated +B or –B media supplemented with the specified treatments and photographed 3 days post-transference. ACC induced a hairy phenotype in both B sufficient and deficient plants. Ag^{+} partially alleviates hairy phenotype, but not root swelling. Bar indicates 1 mm.

ACC treatments did not trigger lignification in control plants (MSm+B), and Ag^{+} treatment did not alleviate ectopic lignification in B deficient plants (Fig. 40), suggesting than lignification is an ACC/ethylene independent response.

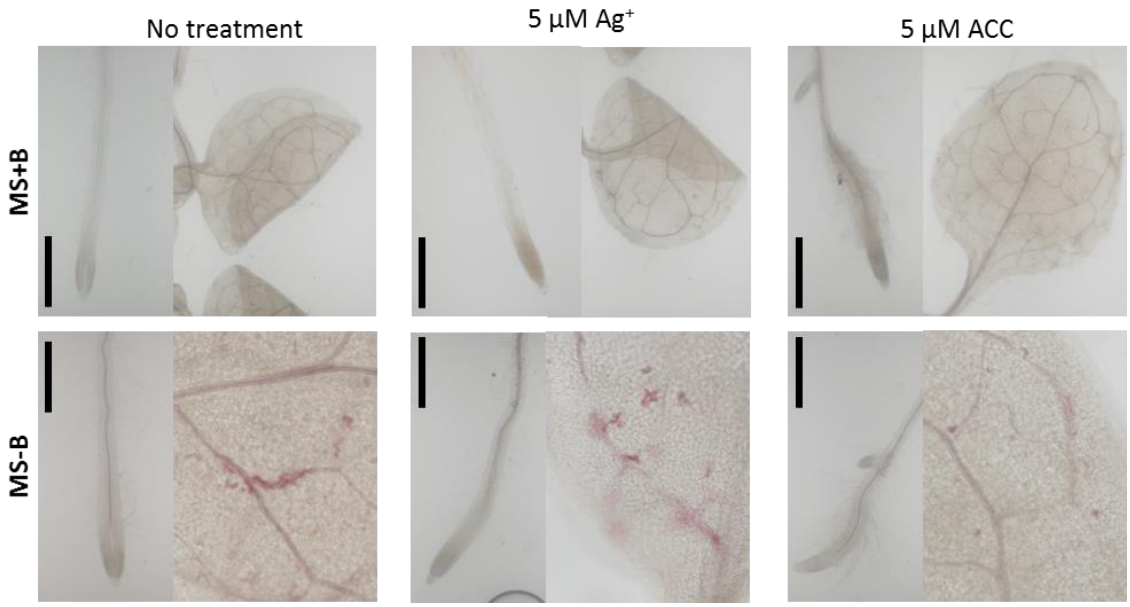


Figure 40. Ethylene signalling pathways were not involved in lignification response occurring after transference to B deficiency.
Arabidopsis grew in control media during 5, when was transferred to B sufficient (+B) or B deficient (-B) supplemented with the different treatments for 3 days. Then seedlings were stained with Phloroglucinol and observed under microscope. Lignin deposits were observed as red signal.

Finally, I analyzed MRNGPs in B-sufficient and B-deficient plants treated with ACC and Ag^+ ions (Fig. 41). As previously shows, exposure to B deficiency conditions during 3 days caused a significant accumulation of MRNGPs when compared to control conditions. ACC treatments led to an increase in the amount of MRNPGs in both control and sufficient plants (Fig. 41). Meanwhile, Ag^+ treatment has opposite effects in both B conditions: in +B plants increased the amount of MRNGPs but in –B plants led to a reduction of the amount of N-glycoproteins, especially de 5 µM Ag treatment (Fig. 41).

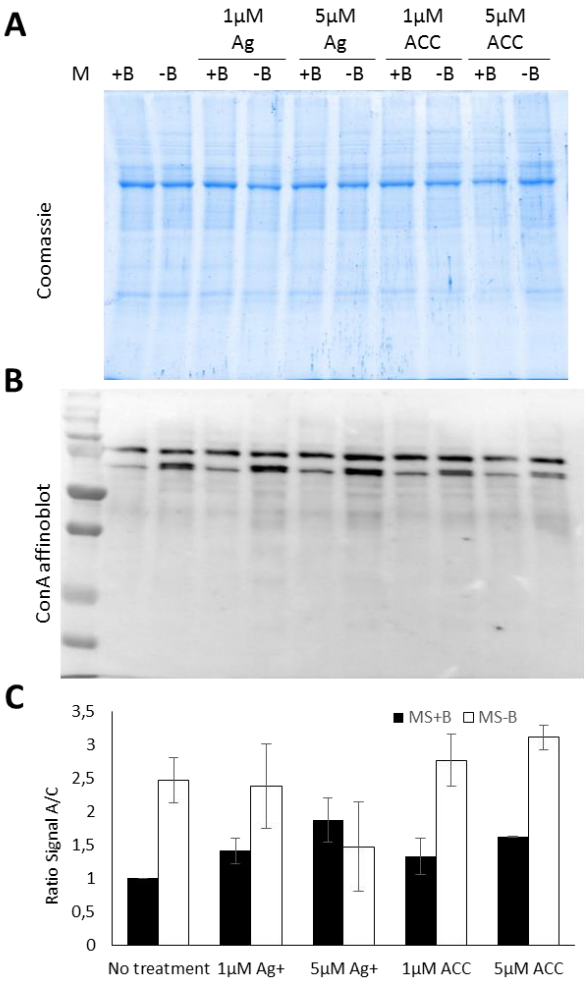


Figure 41. Ethylene agonists and antagonists do not alter Mannose Rich N-glycoprotein pattern.
Arabidopsis plants grew in B sufficient media were transferred to the indicated media supplemented with the specified treatments. Plants were harvested 3 days post-transference. Proteins were extracted, separated electrophoretically and detected using: (A) Coomassie staining and (B) ConA affiniblot. C) Semicuantification of MRNGPs relativized to total protein. +B: B-sufficient plants; –B: B-deficient plants.

4.4.2. Drugs disrupting the endomembrane systems led to growth inhibition, root swelling, lignification and accumulation of N-glycoprotein, comparable to boron deficiency response.

In an attempt to connect the symptoms occurring in B-deficient plants with a hypothetical function of the micronutrient in the endomembrane system, I use several drugs which disrupted glyco/protein synthesis and/or secretion. DMSO was included as control and behave as non-treated plants (Figs. 42, 43, 44, 45, 46).

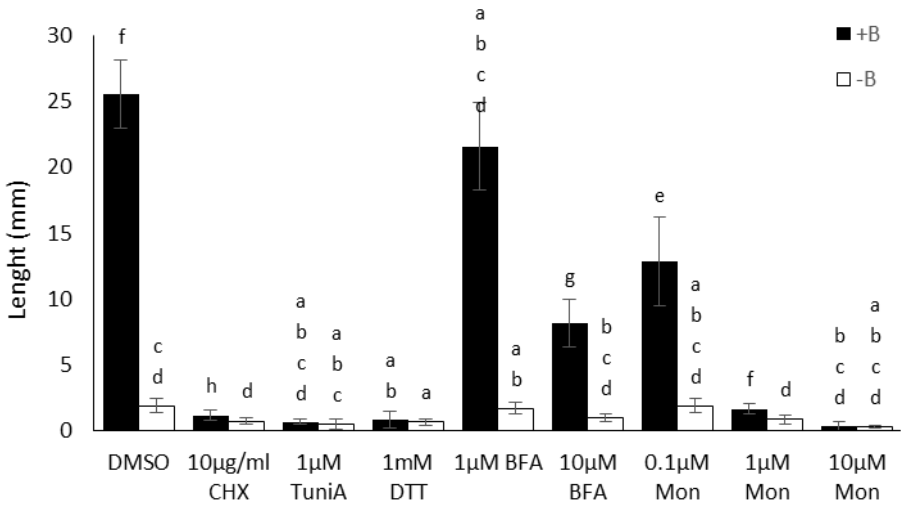


Figure 42. Treatments altering protein/glycoprotein synthesis and endomembrane trafficking alter growth in B-sufficient but not in B-deficient plants of Arabidopsis.
Plants grew in control media during 5 days were transferred to B-sufficient (+B) or B-deficient (-B) plates supplemented with the indicated treatments for 3 days. Then we scanned plates, and root growth after transference was measured. Different letters indicates significant differences (α=0,05). Treatments: BFA, Brefeldin A; CHX, Cycloheximide; DTT, Dithiothreitol; Mon, Monensin; Tuni A, Tunicamycin A.

Cycloheximide (CHX; a common used protein synthesis inhibitor) and tunicamycin A (TuniA; a nucleoside antibiotic which interferes with N-glycan synthesis, causing inhibition of N-glycoprotein synthesis and several associated stresses as UPR and apoptosis) caused such alterations in Arabidopsis seedlings that there were no differences of growth between +B and –B treatments in the presence of these chemical compounds. When plants were transferred to B-sufficient or B-deficient plates supplied with 10 µg/ml of CHX in both +B and –B growth was completely aborted (Fig. 42), roots were hairless and developed an agravitrotopic phenotype (Fig. 43). However we did not detect ectopic lignification (Fig. 44) nor accumulation of N-glycoproteins or changes in glycoprotein pattern (Fig. 45). TuniA treatments caused growth arrest (Fig. 42), induction of lateral roots, defects in root hair elongation and the appearance of some brown coloration in the root meristem (Fig. 43). We observed also ectopic lignification in both +B and –B plants (Fig. 44), but not differences in the N-glycoprotein pattern (Fig. 45).

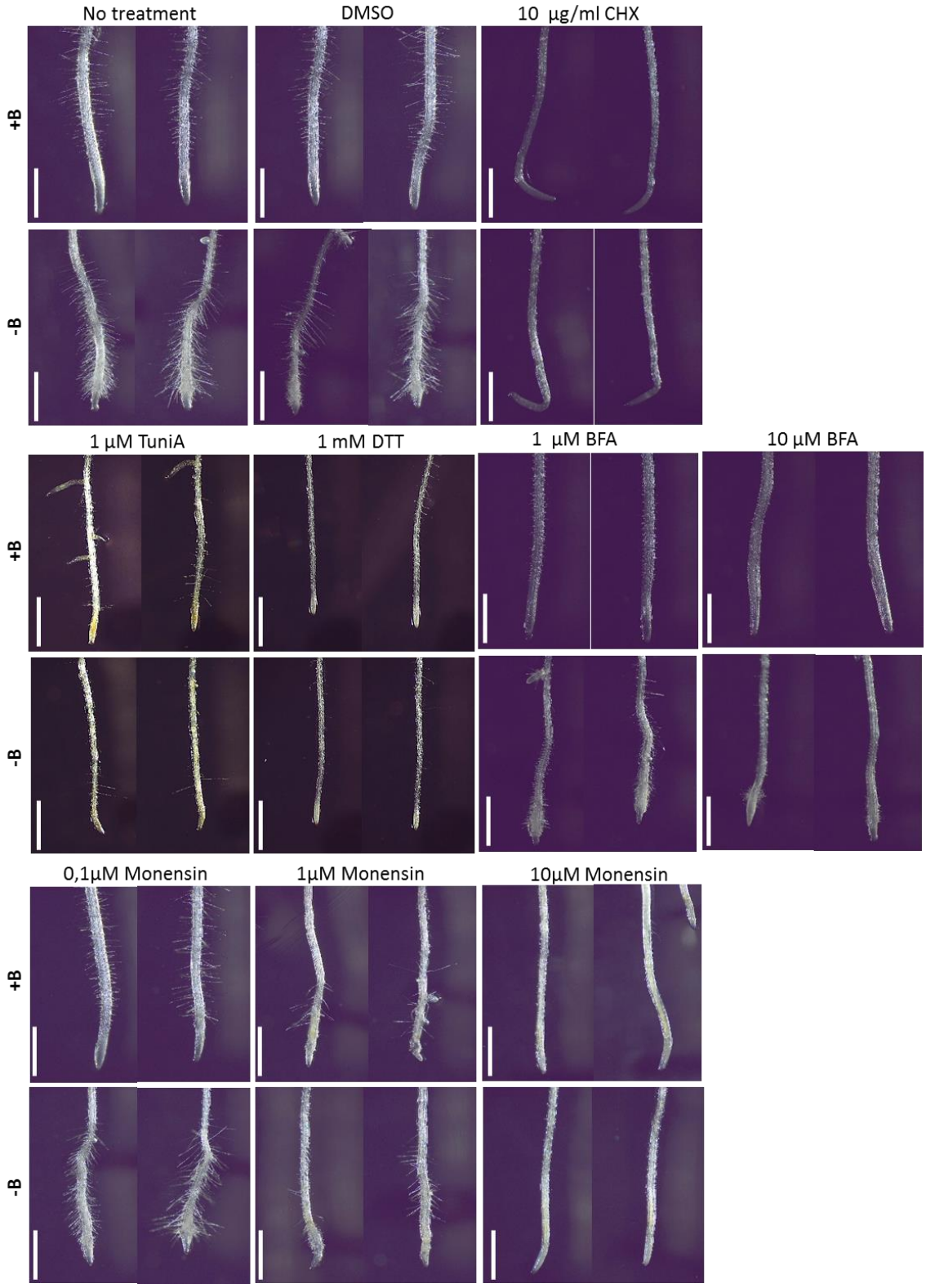


Figure 43. Hairy phenotype in response to B deficiency requires synthesis of proteins, glycoproteins and endomembrane trafficking.

Arabidopsis grew in control media during 5 days and then was transferred to B sufficient (+B) or B deficient (-B) supplemented with the different treatments for 3 days and photographed. Scale bar indicates 1 mm. Treatments: BFA, Brefeldin A; CHX, Cycloheximide; DTT, Dithiothreitol; Mon, Monensin; Tuni A, Tunicamycin A.

DTT, a reductive agent commonly used to induce ER stress, caused in both +B and –B plants growth inhibition (Fig. 42), appearance of hairless root phenotype (Fig. 43) and accumulation of lignin (Fig. 44). Overall protein pattern of B-sufficient and B-deficient DTT-treated plants was similar except that -B DTT-treated seedlings did not showed the presence of βGlu18 protein (~65 kDa) (Fig.45A). Finally, the amount of N-glycoproteins and their pattern changed in DTT treated plants, in both +B and –B plants (Fig. 45B, C).

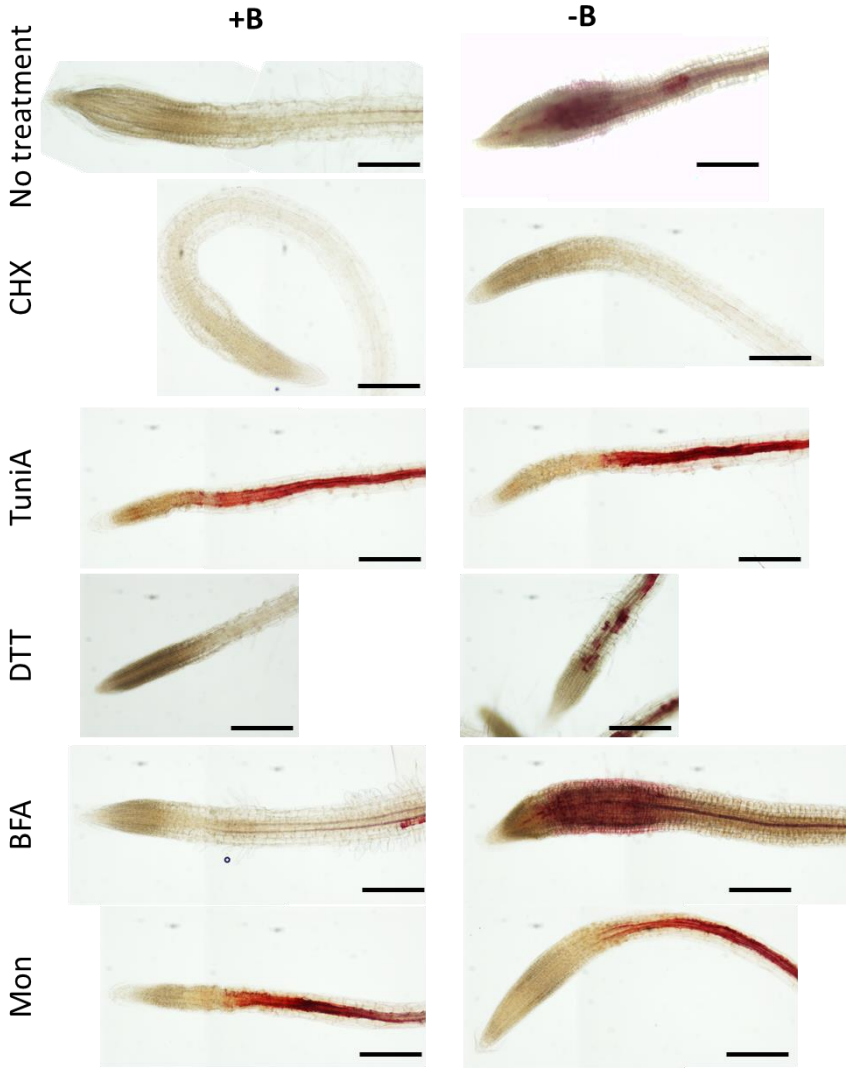


Figure 44. Treatments altering synthesis or secretion of N-glycoproteins caused an ectopic lignification phenotype.
Arabidopsis grew in control media during 5, when was transferred to B sufficient (+B) or B deficient (-B) supplemented with the different treatments for 3 days. Then seedlings were stained with Phloroglucinol and observed under microscope. Lignin deposits were observed as red signal. Scale bar represents 100 microns. Treatments: BFA, Brefeldin A; CHX, Cycloheximide; DTT, Dithiothreitol; Mon, Monensin; Tuni A, Tunicamycin A.

Brefeldin A (BFA) and Monensin (Mon) are known trafficking inhibitors. BFA is a fungal macrocycle lactone which inhibits the formation of COPI vesicles leading to two subsequent processes: a) the fusion between ER and Golgi Apparatus (GA); b) the appearance of BFA compartment (merging of *trans*-Golgi vesicles, TGN, and endocytic vesicles). On one hand, BFA addition to B-sufficient plants, partially inhibited root growth (Fig. 42), caused appearance of hairless roots (Fig. 43), did not affect lignin deposition (Fig. 44) nor N-glycoprotein pattern (Fig. 45A, B) but led to an accumulation of MRNGPs (Fig. 45B, C). In the other hand, in B-deficient BFA-treated plants growth arrest was comparable to not treated –B plants (Fig. 42); root hair length was diminished, although root hairs still emerged close to the root meristem and root swelling remained as in –B plants (Fig. 43 and Fig. 44). Lignification pattern is also comparable to those

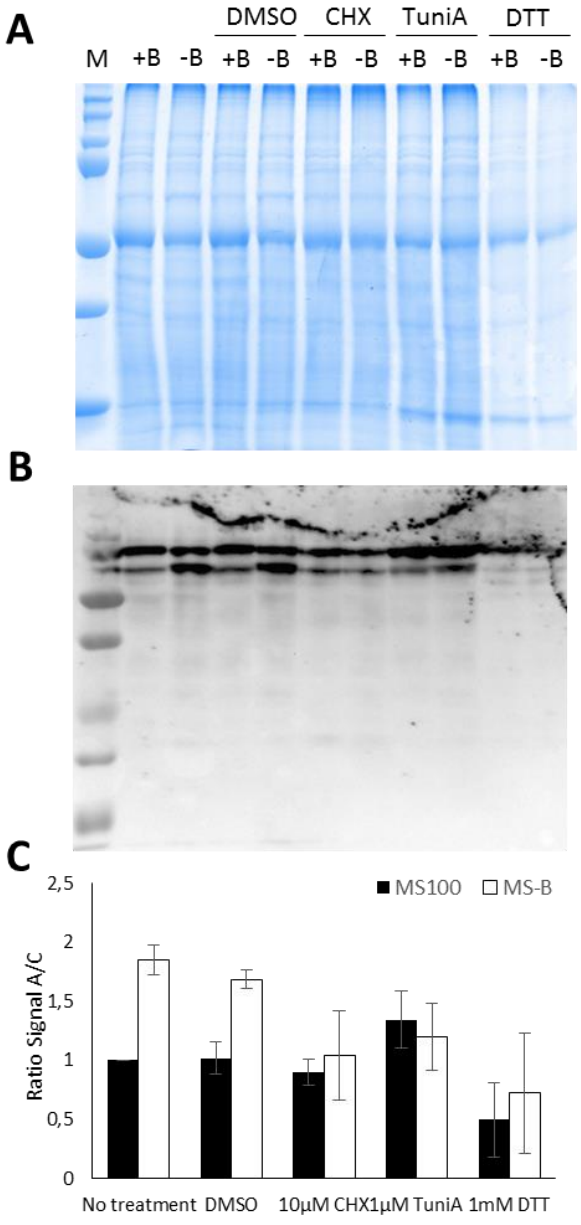


Figure 45. Treatments altering glyco/protein synthesis alter Mannose Rich N-glycoprotein pattern. Analysis of total proteins extracted from Arabidopsis plants transferred to B sufficient (+B) or B deficient (-B) plates supplied with the different treatments for 3 days. Proteins were subjected to SDS-PAGE under reducing conditions, stained with Coomassie for total protein (A) or electroblotted to nitrocellulose and analysed using the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (B). C) Semiquantification of MRNGPs relative to total protein. Treatments: CHX, Cycloheximide; Tuni A, Tunicamycin A; DTT, Dithiothreitol.

observed in –B not treated plants (Fig. 44). From the analysis of N-glycoproteins in -B BFA-treated plants we observed an increased accumulation of MRNGP signal, although the pattern was similar to that of not treated plants (Fig. 46).

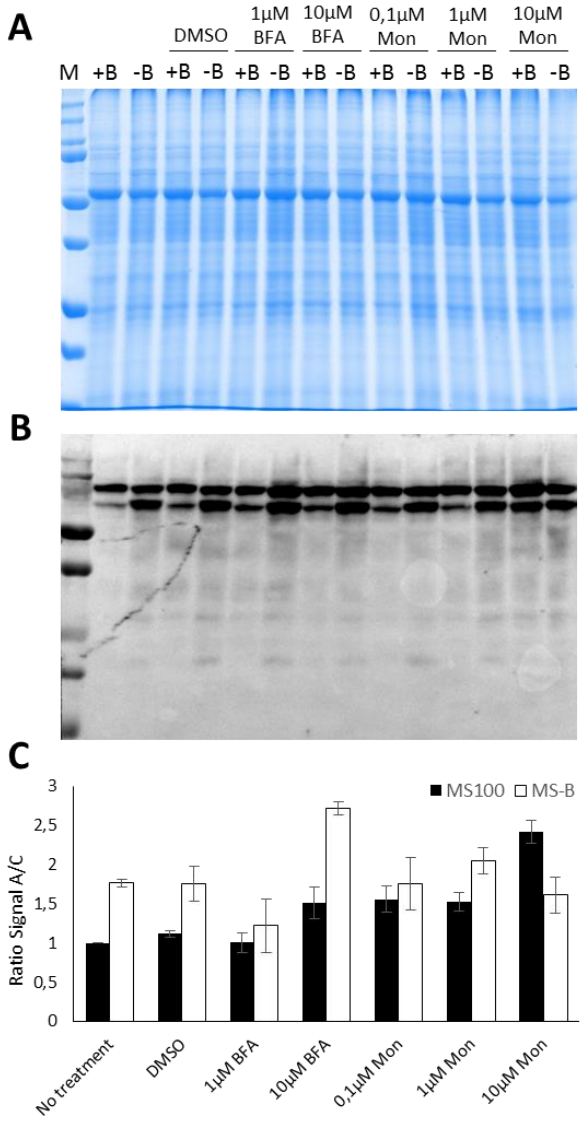


Figure 46. Treatments altering endomembrane trafficking produce N-glycoprotein accumulation.
Analysis of total proteins extracted from Arabidopsis plants transferred to B sufficient (+B) or B deficient (-B) plates supplied with the different treatments for 3 days. Proteins were subjected to SDS-PAGE under reducing conditions, stained with Coomassie for total protein (A) or electroblotted to nitrocellulose and analysed using the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (B). C) Semiquantification of MRNGPs relative to total protein.

Treatments: BFA, Brefeldin A; Mon, Monensin.

Last experiment was performed using monensin, a monovalent ion-selective ionophore which impairs vacuolar secretory pathway of soluble proteins likely due to acidification of the TGN, and which finally results in swollen Golgi cisternae. In B sufficient seedlings, increasing Mon concentration caused growth inhibition (Fig. 42). At dosses 0.1 and 1µM, monensin caused in +B plants defects in root meristem, including an irregular hairy phenotype, development of lateral roots very close to the root meristem and some browning of the root tip (Fig. 43). B-deficient plants treated with 0.1 µM of monensin were indistinguishable from not treated plants (Fig. 42), but at higher doses of monensin (1 µM) root hair elongation was altered and browning of root hairs was also evident (Fig.

43). The highest amount of monensin (10 μM) altered both +B and –B plants in a similar way: growth arrest, hairless phenotype and accumulation of some brown substance in the meristem (Fig. 42 and Fig. 43). This concentration of monensin also induced lignification (Fig. 44) and led to an accumulation of MRNGs in +B but not aggravated it in –B plants (Fig. 46).

5. Discussion

To date the only confirmed function of B in plants is the dimerization of RGII, which determine cell wall porosity and strength (Findeklee and Goldbach, 1996; Fleischer et al., 1999; Matoh et al., 2000; Ryden et al., 2003). However the pleiotropic effects found in B deficient plants, including those reported herein, although could be properly explained by a structural alteration of RGII, suggest other possible functions of B (Goldbach, 1997; Goldbach et al., 2000; Matoh and Kobayashi, 2002; Goldbach et al., 2002; Pérez et al., 2003; Bolaños et al., 2004; O'Neill et al., 2004; Goldbach et al., 2007; Goldbach and Wimmer, 2007; Bar-Peled et al., 2012; Reboul and Tenhaken, 2012; González-Fontes et al., 2013; González-Fontes et al., 2014; Funakawa and Miwa, 2015). Moreover, some of the defects of root development resemble those described in living organisms devoid of RGII, like animals (Stangoulis and Reid, 2002; Bolaños et al., 2004; Goldbach et al., 2007; Goldbach and Wimmer, 2007). Combining the knowledge about B nutrition generated during more than 100 years with the last high-throughput approaches, I propose a holistic view of B nutrition where several B-deficiency symptoms described in the present work could be explained by the combined activation of several signaling pathways which would include, at least, ACC/ethylene, auxin/cytokinin ratio and wound/Cell Wall Damage (CWD) response. In this scenario B deficiency would trigger by an uncertain mechanism numerous signaling pathways, likely involving N-glycans, which could be in the basis of the late pleiotropic symptoms reported in the B deficiency response. Specifically ACC/ethylene would control the reported inhibition of cell elongation and “hairy” phenotype (Figs. 13, 38- 41); Auxin/cytokinin ratio would explain changes in cell differentiation and decrease in cell division (Figs. 14, 15, 18-24); and Jasmonic Acid (JA)-dependent wound/CWD response would be controlling accumulation of specific proteins, callose and ectopic lignification (Figs. 25-28, 40 and 44). By contrast, accumulation of N-glycoproteins under B deficiency, both in legume roots and nodules (Figs. 6 and 7), and in shoots and roots of Arabidopsis (Figs. 30-32), seems to be directly related with a failure in endomembrane trafficking (Fig. 45 and 46), although ethylene also modulates the intensity of the stress.

In the next pages of this discussion section I will compare the B deficiency phenotype with the phenotype observed in mutants altered in B homeostasis or RGII synthesis. Finally I propose a model where changes in RGII, or other recently described B ligands

as glycolipids and glycoproteins, would connect molecule structure with signaling functions.

5.1. Previous consideration about boron requirements in *Arabidopsis* compared with other plants and organisms.

Arabidopsis thaliana has been used in several studies of B nutrition (Noguchi et al., 1997; Noguchi et al., 2000; Takano et al., 2001; O'Neill et al., 2001; Yu et al., 2001; Takano et al., 2002; Noguchi et al., 2003; Takano et al., 2005; Miwa et al., 2006; Takano et al., 2006; Zeng et al., 2007, 2008; Camacho-Cristóbal et al., 2008; Tanaka et al., 2008; Kocabek et al., 2009; Kato et al., 2009; Pang et al., 2010; Kasajima et al., 2010; Camacho-Cristóbal et al., 2011; Kasai et al., 2011; Martín-Rejano et al., 2011; Sakamoto et al., 2011; Tanaka et al., 2011; Stoces et al., 2012; Yoshinari et al., 2012; Oiwa et al., 2013; Tabata et al., 2013; Gruber et al., 2013; Yamauchi et al., 2013; Miwa et al., 2013; Kasai et al., 2014; Miwa et al., 2014; Abreu et al., 2014; Uehara et al., 2014; Xu et al., 2015; Shimotohno et al., 2015; Camacho-Cristóbal et al., 2015; Li et al., 2015), however previous studies have not characterized the optimal concentration. In this work I found that 100 μM boric acid was the optimal boron concentration for growth in this experimental conditions, hence it was chosen as control treatment in all the experiments (Fig. 10). According to previous reports at 100 μM boric acid, B homeostasis is being sustained by passive diffusion and would not require the coordinated presence of *NIPs* or *BORs* transporters (Noguchi et al., 1997; Noguchi et al., 2000; Takano et al., 2001; Noguchi et al., 2003; Takano et al., 2004; Takano et al., 2005; Takano et al., 2006; Tanaka et al., 2008; Takano et al., 2010; Kasai et al., 2011; Tanaka et al., 2011; Yoshinari et al., 2012; Miwa et al., 2013). Also, from this growth curve emerged that *Arabidopsis* presented a wider range of optimal growth (between B deficiency and toxicity) than any other plant described to date, and the optimal value is slightly higher than those described for other dicots.

Both B deficiency and B toxicity decrease root growth (Figs. 10, 11 and 12), as previously described (Takano et al., 2006; Martín-Rejano et al., 2011; Aquea et al., 2012; Gruber et al., 2013). However hypocotyl elongation was promoted by B toxicity in both light and dark conditions (Fig. 11), even when plants are suffering shoot chlorosis. According to Fellner's group, light receptors and auxins influence hypocotyl elongation

under increasing amounts of B (Kocabek et al., 2009; Stoces et al., 2012) but did not prevent elongation itself, so the primary cause is probably a vacuolar compartmentalization of B with the subsequent increase of turgor pressure, as those described when the tonoplast aquaporin *TIP5;1* is overexpressed (Pang et al., 2010; Reid, 2014). Further studies will be required to confirm if this higher elongation under B toxicity is based on signaling, cell wall softening, or increase of turgor pressure in vacuoles; and if the last its true, what transporters are controlling this vacuolar compartmentation.

5.2. Boron deficiency alters root growth and morphology through the combined effect of different signaling pathways.

Mineral unbalances are on the most common stresses for plants, because the heterogeneous distribution of nutrients in soils. Recently, changes in root architecture in response to such nutrient limitation have been received a lot of attention, and slowly the molecular mechanisms are being unraveled for both macro and micronutrients, which furthermore open interesting biotechnological options to develop better adapted crops (López-Bucio et al., 2003; Gruber et al., 2013; Aibara and Miwa, 2014; Kellermeier et al., 2014; Giehl et al., 2014). In the case of boron those studies are still premature, although some progress has been made (Gruber et al., 2013).

Previous work in *Arabidopsis* revealed as B deficient symptoms root growth arrest (Martín-Rejano et al., 2011a), diminution in cell elongation (Martín-Rejano et al., 2011; Camacho-Cristóbal et al., 2015), and necrosis/cell death (Oiwa et al., 2013). Also, several mechanisms/pathways involved in the response to B in plants, including above mentioned changes in gene expression (Camacho-Cristóbal et al., 2008; Kasajima et al., 2010; Oiwa et al., 2013; Quiles-Pando et al., 2013), induction of ethylene signaling (Martín-Rejano et al., 2011; Camacho-Cristóbal et al., 2015), accumulation of ROS (Koshiba et al., 2009; Oiwa et al., 2013; Camacho-Cristóbal et al., 2015) and cytosolic Ca^{2+} (Koshiba et al., 2010; Redondo-Nieto et al., 2012; Quiles-Pando et al., 2013; González-Fontes et al., 2014), and changes in auxin balance (Tang and De la Fuente, 1986a, 1986b; Wang et al., 2006; Martín-Rejano et al., 2011; Stoces et al., 2012; Camacho-Cristóbal et al., 2015; Li et al., 2015) have been proposed. In this work I described in *Arabidopsis* other additional symptoms, some of them previously described in other plant species: inhibition of cell

division (Sommer and Sorokin, 1928; Whittington, 1957, 1959; Cohen and Albert. 1974; Moore and Hirsch, 1983), root swelling (Warington, 1923; Sommer and Lipman, 1926; Brenchley & Warington, 1927; Sommer and Sorokin, 1928; Johnston and Dore, 1929; McHargue & Calfee, 1933; Neales, 1960; Dear and Aronoff, 1964), accumulation of callose (Van den Verter and Currier, 1977), and ectopic lignification (____). And for the first time, I reported an accumulation of N-glycoproteins in –B plants.

5.2.1. Ethylene-dependent inhibition of cell elongation.

The inhibition of cell elongation has typically been considered the distinctive symptom of B deficiency (Odhnoff, 1957; Albert & Wilson, 1961). My measurements coincide with previous reports in *Arabidopsis* (Camacho-Cristóbal et al., 2015), showing that B deficiency reduces cell elongation within hours (Fig. 13). In this case I observed a quick decrease of cell elongation in both B-sufficient and B-deficient plants 3 hours after transference of seedlings, probably caused by a mechanical stress during handling. After that, cell elongation was recovered in B sufficient plants, whereas in B deficient plants went down until a minimum value (~40 μm), which is the same cell length observed in previous B deficient studies (Camacho-Cristóbal et al., 2015), or in treatments which inhibit cell elongation as the ethylene precursor ACC (Fig. 39; De Cnodder et al., 2005; Tsang et al., 2011). The similitudes between ACC treated plants and B deficient plants, which goes from inhibition of cell elongation to the development of a hairy phenotype (Fig. 13, 38, 39), suggested that B deficiency response could involve ethylene signaling as recently proposed (Martín-Rejano et al., 2011; Camacho-Cristóbal et al., 2015).

Ethylene was first connected with B nutrition in *Arabidopsis* in a genomic study where mutant *b26-6/ctr1-16* showed a high requirement of B for elongation (Tabata et al., 2013). In this screening, the point mutation was located in the gene *CONSTITUTIVE TRIPLE RESPONSE1 (CTR1)*, a negative regulator of ethylene response which, when mutated, phenocopied ethylene treated plants (Kieber et al., 1993). B deficient phenotype resembles the phenotype of ACC/ethylene treated plants and complementary, some effects of B deficiency were suppressed with ethylene antagonists like Ag^+ (Fig. 13, 38, 39, 41; Camacho-Cristóbal et al., 2015), AIB and AVG (Camacho-Cristóbal et al., 2015), and in *ein2-1* mutant background (Martín-Rejano et al., 2011). An increase of ethylene in B deficient plants was confirmed by reporter lines *EBS:GUS* (Suppl. Fig. 4; Martín-Rejano et al., 2011) and *ACS11:GUS* (Camacho-Cristóbal et al., 2015). It has been

reported that ethylene signaling is followed by an increase in ROS (Tsang et al., 2011) and auxin (Růžička et al., 2007; Swarup et al., 2007; Tsang et al., 2011), which explain the oxidative crosslinking of cell wall polysaccharides and the rapid inhibition of cell elongation respectively. The induction of ROS after B deprivation have been confirmed independently using different probes (Oiwa et al., 2013; Camacho-Cristóbal et al., 2015), and confirmed because DPI (a NADPH-oxidase inhibitor) restored cell elongation in –B plants (Camacho-Cristóbal et al., 2015). In addition, inhibition of cell elongation produced by ACC/ethylene treatments would be suppressed by ROS scavengers (KI), or inhibiting the ability of HydroxyProline-Rich Glycoproteins (HGRPs) to mediate the oxidative crosslinking using tyrosine (as a substrate competitor) or 3,4-DL-Dehydroproline (DHL, inhibits proline hydroxylation and therein O-glycosylation of HGRPs) (De Cnodder et al., 2005; Tsang et al., 2011). Some of these treatments resulted in the restoring of cell elongation in B-deficient plants (Suppl. Fig. 6; Abreu et al., unpublished). Moreover, B-deficiency rescued *rhd2* mutant (Bassil et al., 2005) which also support that ROS (induced or not by ethylene) are involved in B deficiency response.

Overall results suggest that early inhibition of cell elongation observed in B deficient plants is mediated by ethylene signaling (Fig.47) and not directly produced by a failure in RGII synthesis, because the phenotype is not observed neither in some mutants backgrounds (*ein2-1; aux1-22;* Martín-Rejano et al., 2011) or plants treated with ethylene agonists (Fig. 13, 38, 39, 41, Suppl. Fig. 6; Camacho-Cristóbal et al., 2015). Further studies are required to identify the "signal" produced by B deficiency and the receptors of such signal which would activate ACC/ethylene production (discussed below). Some of the symptoms observed in B deficient plants, as inhibition of cell division and disappearance of quiescent center, seem ACC/ethylene independent: on one hand, ethylene was not directly connected with cell division (Takatsuka and Umeda, 2014), and although recently some evidences suggest that this hormone also regulates inhibition of cell division (Street et al., 2015), his data suggested that the action of this hormone on cell division and endocycle involve other hormones as auxin and cytokinin; on the other hand, ACC/ethylene treatments induce multiplication rather than extinction of the QC (Ortega-Martínez et al., 2007). These symptoms observed under B deficiency should require other signaling pathways (discussed below).

5.2.2. Cell division/differentiation dependence of auxin/cytokinin ratio

As mentioned above, cell division has been reported as a B deficiency symptom in several plants (Sommer and Sorokin, 1928; Whittington, 1957, 1959; Cohen and Albert. 1974; Moore and Hirsch, 1983), but not in *Arabidopsis* (Martín-Rejano et al., 2011). In this work I found that B deprivation stopped root growth before than hypocotyl elongation in transference experiments (Fig. 12) suggesting that root division is also altered under B deficiency. To confirm those results, expression of the reporter line *pCYCB1;1:GUS* was analyze, which is expressed in cells entering mitosis, was analyzed. In contrast with previous reports using the same reporter line (Martín-Rejano et al. 2011), I found a progressive diminution of cell division under B deficiency in both direct growth (Fig. 14) and transference experiments (Fig. 15A). In fact, in transference experiments I observed that cell division was inhibited 9 hours post-transference, which coincides with the time in which cell elongation was reduced to its minimum length (Fig. 13, 15A). Further evidences come from the fact that boron resupply rescued cell division before than cell elongation (Fig. 15B).

Transition from cell division to cell differentiation requires cell elongation as a prerequisite for a correct cell differentiation. In this context, characteristic features of differentiated cells only appeared in fully elongated cells (Dolan et al., 1993, Fig. 13B, 17A). However, in B deficient plants was observed that although cells did not elongate, they showed morphological features indicating cell differentiation as the development of root hairs or the appearance of lignified protoxylem, at positions close to the root tip (Fig. 13-24). In this Thesis I used enhancer trap lines like those developed by Dr. Hasselof to track cell fate and cell differentiation. Gal4-GFP lines used in this study presented different locations, in both longitudinal and radial axis. In summary, GFP associated with well differentiated tissues (J1092, J0121, J2672 and J2812) is altered in B-deficient plants and appear at proximal positions from the root tip (Figs. 18, 20-22, 24), whereas GFP signal associated with fated tissues (Q0990) is not clearly altered by B deficiency (Fig. 19, 23).

To my knowledge no other nutrient unbalance nor abiotic stress has been reported to cause such drastic impairment of cell division, elongation and differentiation as the described here for B starvation (López-Bucio et al., 2003; Sánchez-Calderón et al., 2005; Dinneny et al., 2008). Among the signals which can produce such reorganization of cell

division and cell differentiation would not be ethylene, because treatments which rescued cell elongation in B deficient plants (as ethylene antagonists or ROS scavengers) do not rescue root growth (Fig. 38, 39; Suppl. Fig. 6). Other hormones as auxin, cytokinin, gibberellic acid or brassinosteroids, whose balance in the transition zone is controlling the division/differentiation rate would be therefore affected by B deficiency (Dello Ioio et al., 2008; Moubayidin et al., 2009; Perilli and Sabatini, 2010; Moubayidin et al., 2010; Bishopp et al., 2011; Muraro et al., 2013; Moubayidin et al., 2013; Takatsuka and Umeda, 2014; Schaller et al., 2015; Chandler and Werr, 2015).

A connection between auxin homeostasis and B nutrition has been claimed previously, mainly associated with a defect in IAA synthesis and transport (Eaton, 1944; MacVicar and Tnottingham, 1947; Galston and Dalberg, 1954; Shkol'nik et al., 1964; Jaweed and Scott, 1967; Bohnsack and Albert, 1977; Hirsch and Torrey, 1980; Hirsch et al., 1982). In *Arabidopsis* a first connection between boron and auxin balance was demonstrated using the reporter *DR5:GUS* line suggesting that auxin is accumulated under B deficiency conditions at 4 days after transference (Martín-Rejano et al., 2011). In the same work, root growth was partially restored in mutant *aux1-22*, however these authors did not found changes in cell division (Martín-Rejano et al., 2011). In further studies an auxin increase was reported as part of the ethylene response, because PEO-IAA (an auxin transport inhibitor) rescued defects in cell elongation (Camacho-Cristóbal et al., 2015). Contrarily, Li and collaborators (2015) reported a decrease of auxin in the meristem (using the DII-Venus reporter line) after 1 week growing in low B conditions, which was parallel to a reduction of PIN1:GFP signal (Li et al., 2015). Mutant *pin2/eir1-1* shows a reduced meristem under control conditions, but also a drastic reduction in meristem size under B-deficient conditions, suggesting that the mutant is more sensitive to low B levels than wt plants or other *PIN* mutants. These results suggest a direct effect of *PIN* auxin transporters in the maintenance of root meristem under B deficient conditions (Li et al., 2015). The contradictory increase (within hours; Martín-Rejano et al., 2011) and decrease (within days; Li et al., 2015) of auxin, could be integrated in a temporal and spatial fashion: ethylene would temporarily led to auxin increase at the elongation zone so collaborating in inhibition of cell length. Then, long exposure to B deficient conditions decreased auxin in the cell division zone, which would reduce the cell division rate (Fig. 47).

These results suggest that if ethylene is controlling inhibition of cell elongation in B deficient plants, auxin could partially explained inhibition of cell division (Fig. 47). However cell division is intimately and indissolubly coupled with cell elongation/differentiation, and in root these processes are mainly regulated by the auxin/cytokinin balance (Dello Ioio et al., 2008; Moubayidin et al., 2010; Moubayidin et al., 2013; Muraro et al., 2013; Takatsuka and Umeda, 2014; Schaller et al., 2015). Our group have focused their investigations in these topics and have obtained some preliminary results reinforcing this hypothesis. In one hand, long term exposure to B deficient conditions would lead to an alteration of whole hormonal pathways, for example causing an inhibition of the *AHK4/CRE1/WOL* gene (Abreu et al., 2014). In the other hand, mutants *arr1-4*, *arr1-4 arr12-1* and *cre1-1*, which usually present a large meristem because a delayed differentiation (Dello Ioio et al., 2008; Moubayidin et al., 2010), are less susceptible to low B levels (Gálvez et al., personal communication). On the other hand, ARR1 protein, a type B-cytokinin response regulator involved in meristem configuration, is early expressed and ectopically localized in B deficient plants (Poza et al., personal communication), in contrast with the precise location in transition zone at day 5 post germination described in control plants (Dello Ioio et al., 2007; Dello Ioio et al., 2008; Moubayidin et al., 2009; Moubayidin et al., 2010).

Overall, I propose a model where B deficiency is somehow increasing ethylene which led to a decrease in cell elongation; altering the auxin/cytokinin ratio through both a decrease in auxin and an increase in cytokinin, which would result in an increase of ARR1, which is controlling expression of *SHY2*, which then would mediate the repression of *PIN1* and hence to auxin depletion (Fig. 47). These changes in auxin/cytokinin would explain the simultaneous decrease in cell division and early differentiation observed in B deficient plants.

5.2.3. Unknown mechanisms are controlling cell quiescence exhaustion and root swelling.

In my experimental conditions, B deficiency produced a determinate developmental program in the *Arabidopsis* root (Fig. 10-16). So, in addition to the reduction in cell elongation (Fig. 13), the decrease in cell division (Fig. 14, 15), the early differentiation and the swollen roots (Fig. 13-24), in B deficient plants, the meristem is consumed and

finishes with the disappearance of the quiescent center (Fig. 16). In phosphate deprivation, which showed a similar response to B deficiency, QC disappearance precedes changes in cell elongation and division (Sánchez-Calderón et al., 2005). However, in B deficiency QC disappearance seems to follows altered cell elongation and division, because the extinction of the QC occurred at day 5 after germination (Fig. 16), when the root growth was almost completely arrested at day 3 (Fig. 10, 11, 14). Based on this observation, seem more likely that QC disappear by a consumption of stem cells due to the reorganization of the meristem under B deficient conditions, and/or by an indirect role of the micronutrient in the signaling pathways controlling QC maintenance (Lee et al., 2013; Drisch and Stahl, 2015).

Root swelling is a common response to B deficiency (Warington, 1923; Sommer and Lipman, 1926; Brenchley & Warington, 1927; Sommer and Sorokin, 1928; Johnston and Dore, 1929; McHargue & Calfee, 1933; Neales, 1960; Dear and Aronoff, 1964), not associated with any other mineral nutrient starvation. However this phenotype is frequently observed in plants with an altered synthesis of cell wall components, for example in plants altered in RGII synthesis (O'Neill et al., 2001; Reboul et al., 2012; or plants treated with compounds which alter RGII synthesis (Smyth et al., 2013; Villalobos et al., 2015; Dumont et al., 2015), plants impaired in cellulose synthesis (Nicol et al., 1998; Lane et al., 2001; Williamson et al., 2001; Caño-Delgado et al., 2003; Roudier et al., 2005). Some mutants altered in N-glycosylation (Kang et al., 2008; Liebminger et al., 2009) or treatments blocking it (Kang et al., 2008; Liebminger et al., 2009), as well as mutants in AGP synthesis (Hengel and Roberts, 2002; Albenene et al., 2014; Basu et al., 2015), mutants altered in microtubule organization (Baskin et al., 1994; Sugimoto et al., 2003; Zhu et al, 2015) showed swollen roots, probably because to some extend they participate in the synthesis or delivery of CW components. As consequence of those failures, it was proposed an incorrect organization of the cell division plane (transversal instead of longitudinal) which leads to swollen root, or a radial expansion of cells produced by the incorrect deposition of the cell wall. In *Arabidopsis*, B deficiency causes the swelling of roots (Fig. 13B, 14- 24, 26, 28). This phenotype coincides with the accumulation of some opaque substances in B deficient plants (Fig. 15B, 17B, 18B, 19B, 20B, 21B, 23B) which prevented a more detail analysis of the swollen roots. At the microscope we could not observe the formation of extra cell layers in the swollen root, but we detected that the cells of the epidermis and the cortex are thicker in B deficient

plants (Fig. 17B, 28B), which would fit with an abnormally enlarged lateral cell expansion more than with an increase in the number of cells or tissue layers (Hauser et al., 1995). Enhancer-trap line Q0990 suggest that fate decisions stele are not affected by B deficiency (Fig. 20, 23), but according to lines J0121 and J2672 (Fig. 20, 21, 23) become fully differentiated close to the root tip. Additionally, endodermis (J2672) and cortex (J2872) reporter lines showed a thicker signal in the swollen root (Fig. 21, 22). Sommer and Sorokin (1928) proposed a model where root swelling observed under B deficienct conditions would be caused by hyperplasia of plerome (vascular cambium) and hypertrophy of periblem (cortex cells). Our results support the hyperplasia of periblem (cortex, endodermis) but not fully support the hypertrophy of vascular bundle. In this case, *Arabidopsis* root swelling seems to be a consequence of the weakness of the cell wall rather than a signaling mediated phenotype. Nevertheless, root swelling has been associated with several mutants in ethylene, brassinosteroids and jasmonic acid (JA) (Arabidopsis Hormone Database, http://ahd.cbi.pku.edu.cn/; Jiang et al., 2010), therefore further studies using hormone agonist or antagonist as well as mutants in this signaling pathways would clarify a primary or secondary role of hormones in the swelling of roots.

Further studies are required to unravel the B deficiency response, the signal cascades controlling cell elongation, cell division, cell differentiation and cell quiescence, as well as the interaction between these signaling processes in the overall root and plant development (Fig. 47, dashed lines). It would be interesting to perform a more detailed temporal analysis, so we can reconstruct early and late B deficiency responses. As I will discuss later, also might be essential to stablish whether is free boric acid, dRGII-B or other unknown ligands/processes that are activating the above mentioned signaling pathways which led to such dramatic changes in the root organization observed under B deficiency (discussed below).

5.3. Wound/Cell Wall Integrity (CWI) response in boron deficiency.

As stated in the introduction section, boron deficiency symptoms have been usually linked with cell wall, because B abundance on this compartment and because the only described B ligand in plants is the cell wall pectin RGII (Stevenson et al., 1988; Ishii and Matsunaga, 1996; Kobayashi et al., 1996; Matoh et al., 1996; O'Neill et al., 1996; Kaneko et al., 1997). During many years cell wall had been considered mostly a passive structure, without any signaling function, however recent evidences changed such paradigm, based

on the ability of plants to perceive changes in the composition and structure of the cell wall when it is incorrectly synthesized or altered due to the action of pathogens or herbivores, and based also in the description of different cell wall receptors (Pilling and Höfte, 2003; Humphrey et al., 2007; Seifert and Blaukopf, 2010; Steinwand and Kieber, 2010; Hamann and Denness, 2011; Benatti et al., 2012; Hamann, 2012; Engelsdorf and Hamann, 2014; Le Gall et al., 2015; Nishitani and Demura, 2015). Now, with our current understanding of cell wall we can reinterpret several symptoms of B deficiency, as the accumulation of polyamines and phenolic substances, and also the changes in root morphology in relation with signaling pathways activated in response to structural or compositional changes in the cell wall structure.

Callose is an amorphous, high–molecular weight β-(1,3)-glucan polymer, usually synthesized at the cell plate, but also at sites of pathogen attack (biotic stress) or wounding (abiotic stress) (Chen and Kim, 2009; Luna et al., 2011; Ellinger and Voigt, 2014). Recently, it has been reported that callose accumulation also plays an important role during root development because a regulated deposition at plasmodesmata controls intercellular movement of metabolites and signaling molecules (Vatén et al., 2011). Several reports have described ectopic accumulation of callose under B deficient conditions in both pollen (Yang et al., 1999; Wang et al., 2003) and vegetative organs (Van de Venter 1977; Shimomura, 1982), however in the assays presented in this thesis the accumulation was mainly localized in the aerial part (Fig. 25). These results would suggest that there are tissue-specific responses, so a more detailed analysis of the B deficiency response is shoot is necessary. Callose synthesis seems to be activated by pathogen-associated molecular patterns (PAMPs) or endogenous elicitors from pathogen- or herbivore-damaged plant tissues as oligogalacturonides (OG) (Ferrari et al., 2013). Furthermore, salicylic acid (SA), JA and ROS seems to reinforce their synthesis (Yi et al., 2014). As mentioned above, accumulation of ROS has been described in B deficient *Arabidopsis* plants (Oiwa et al., 2013; Camacho-Cristóbal et al., 2015), and there is also evidences suggesting that SA responsive genes were induced under B deficiency (Koshiva et al., 2010; Oiwa et al., 2013), so we could propose a model in which B deficiency somehow activates some wound/pathogen-like response responsible of the accumulation of callose.

A link between boron and lignification was reviewed by Lewis (1980a, b), which suggested that the micronutrient was required for land colonization due to a specific requirement of the nutrient during phenol/lignin metabolism. This hypothesis was based on previous literature which describes an increase in polyphenols and lignin-like polymers under B deficiency (Watanabe et al., 1961; 1964). Several research groups still reporting changes in phenolic compounds in B deficient plants (Pfeffer et al., 1998; Camacho-Cristóbal et al., 2002, 2004; Dordas and Brown, 2005; Liakopoulos and Karabourniotis, 2005; Stavrianakou et al., 2006; Rummukainen et al., 2007), however the biochemical/molecular reasons of these accumulation have received little attention in late literature (Cakmak and Römheld, 1997; Cara et al., 2002). During this work I described an ectopic accumulation of lignin in Arabidopsis plants grew in B-deficient or low B media (Fig. 26-28, 40, 44). Such accumulation is more obvious in hypocotyls and cotyledons than in roots, where symptoms of lignin accumulation were only observed under B deficiency (Fig. 26). Ectopic lignification observed in B deficient roots resemble the phenotype of mutants or drug treated plants with an altered synthesis/deposition of CW components (cellulose (Caño-Delgado et al., 2000, 2003; Bischoff et al., 2009; Denness et al., 2011), N-glycoproteins (Fig.44; Nozaki et al., 2012), as well as other cell wall components (Hayashi et al., 2008), which are usually accompanied of other B deficiency associated phenotypes as growth arrest and root swelling. Such ectopic lignification seemed linked with the activation of a wound/cell wall damage-like response (Caño-Delgado et al., 2003; Denness et al., 2011) instead of a biochemical role of B in critical enzyme activities as previously described for Phenylalanine-Amonio Lyase (PAL) or Polyphenoloxidase activity (PPO) (Pfeffer et al., 1998; Camacho-Cristóbal et al., 2002).

According to Denness and collaborators (2011), ectopic lignification is activated through some cell wall perception proteins and would require JA, Ca^{2+} signaling, and ROS production. As mentioned above, intracellular Ca^{2+} and ROS are induced in response to B deficiency in *Arabidopsis* (Quiles-Pando et al., 2013; Oiwa et al., 2013; Camacho-Cristóbal et al., 2015) and in other plants (Koshiba et al., 2009; 2010), but we have no conclusive evidences demonstrating an activation of JA in response to B deficiency. The only available data in that direction come from previous gene expression studies where an induction of *AOX* and *PAL* genes in B deficient conditions was observed in tobacco BY2 cells (Koshiba et al., 2010) and *Arabidopsis* (Oiwa et al., 2013).

In this study I reported the induction under low B of a protein identified as β-Glu18 (Figs. 29, 30; Table 7). *β-GLU18* encodes a wound-inducible ER-body resident protein involved in hydrolysis of inactive glucose-conjugated abscisic acid (ABA) (Kato-Noguchi et al., 2009). Moreover the protein is critical in recruiting other proteins to the *Brassicales*-specific ER compartment ER-body (Yamada et al., 2009; Nakano et al., 2014). Because this protein is induced by wounding, the presence of β-GLU18 in low B conditions reinforced the idea that JA is activated in response to B deficiency, and because β-Glu18 enzymatic activity would release ABA, maybe this hormone also could play a role in B stress. So far, ABA was never considered as signaling molecule involved in the response to B deficiency but in B toxicity response (Aquea et al., 2012). Because typical ABA responses, as water transport and transpiration impairment, were reported in long term exposure to B deficiency (Wimmer and Eichert, 2013) and because ABA signaling could alter root development (Takatsuka and Umeda, 2014), future experiments should explore the role of ABA in B deficiency response, including the analysis of root morphology and gene expression using chemical inhibitors, as fluridone, or mutants in this signaling pathway.

A final evidence supporting the claim of a JA or SA-mediated B-deficiency response came from the accumulation of polyamines in B deficient plants (Camacho-Cristóbal et al., 2005). Accumulation of polyamines has been connected with wound-response and pathogen-response signal (Walters, 2003; Liu et al., 2015). Also, it is known that the accumulation of polyamines, specially spermidine and putrescine was associated with JA in other nutrient deficiencies as K^+ deficiency, which coincides with an upregulation of arginine decarboxylase by JA (Pérez-Amador et al., 2002; Urano et al., 2003).

Overall data suggest that symptoms of B deficiency are connected with cell wall defects, and which jasmonic acid and/or salicylic acid could be induced in response to B deficiency, mediating a wound/CWD-like response which would explain the accumulation of callose, lignin and polyamines, and the induction of β-GLU18 protein (Fig. 47). To confirm this hypothesis we should measure jasmonic acid, salicylic acid and their intermediaries at different times after transference to B deficient media, or complementary use reporter lines as *35S:JAZ9:GUS* for Jasmonic acid (Monte et al., 2014). Another interesting approach would consist in analyze some B-deficiency

"specific" responses (growth arrest, inhibition of cell elongation/division/differentiation, induction of B transporter or other B responsive genes) with agonist and antagonists of JA, SA, ROS, and Ca^{2+} signaling pathways, as well as mutants in those signaling pathways.

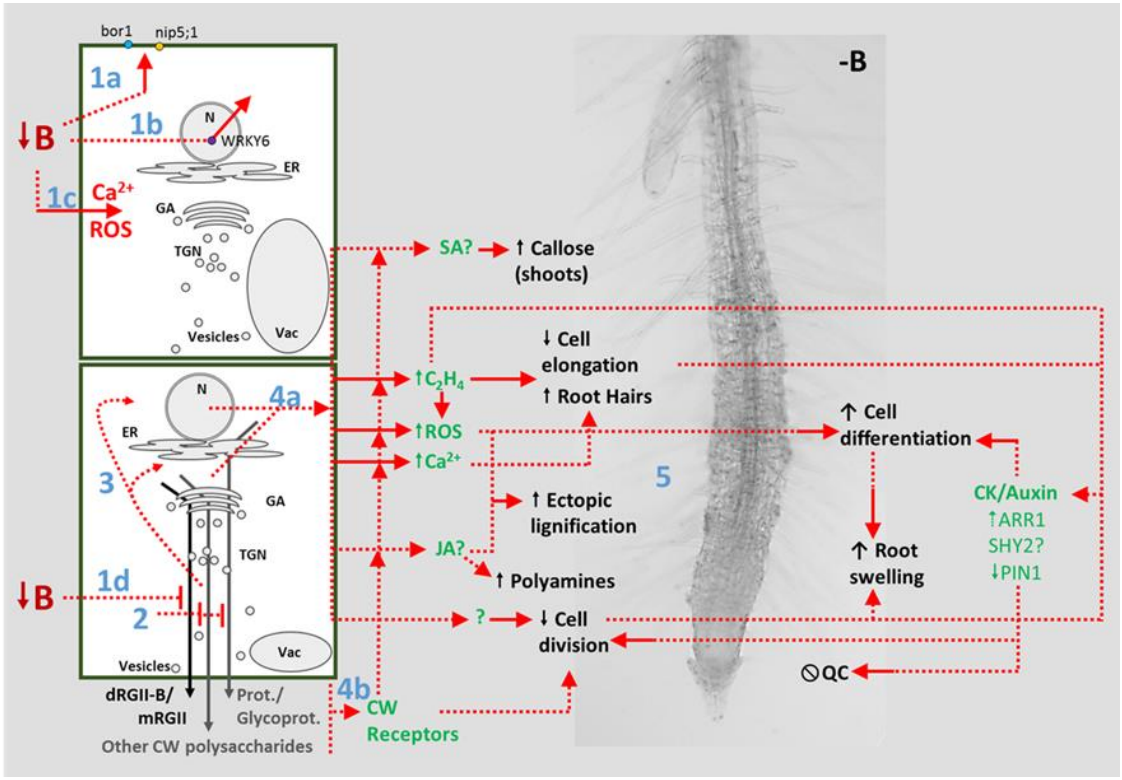


Figure 47. Proposed model integrating known B deficiency responses and potential signalling cascades.

Rhamnogalacturonan II (RGII) is synthesized at Golgi Appartus (GA) and secreted in vesicles, probably in parallel with glyco/proteins and other cell wall polysaccharides as hemicelluloses. B deficiency leads to changes in B transporters *NIP5;1* and *BOR1* (1a), *WRKY6* transcription factors (1b), and to increase in cytosolic Ca^{2+} and ROS (1c) by still unknown mechanisms.
B deficiency also results in decrease of dRGII-B formation which could alter its secretion (1d). In our model we propose that this would alter overall endomembrane trafficking (2) which would explain the accumulation of glyco/proteins and induce signalling cascades to nucleus and other cell compartments (3). An intracellular (4a) or Cell Wall dependant (4b) pathway would lead to the activation of different signalling cascades: Ethylene (C_2H_4), ROS, Ca^{2+}, salicylic acid (SA) and jasmonic acid (JA), Cytokinin (CK)/Auxin ratio, or other pathways (?) which would directly regulate cellular processes (5): Cell elongation, cell division, cell differentiation, root swelling, quiescent center (QC), or accumulation of callose, lignin and polyamines.Solid lines represent known responses, whereas dashed line indicates postulated signalling pathways. ER, Endoplasmic Reticulum; GA, Golgi Apparatus; N, Nucleus; TGN, *Trans* Golgi Network; Vac, Vacuole.

5.4. An integrative model of boron deficiency response: from ligands to signaling pathways determining boron deficiency symptoms.

One intriguing topic in B nutrition is the diverse chemical structure and biological functions of the known B ligands: linear polyketides as boromycin, tartrolon and related antibiotic ionophores; the furanose ring in AI2; aromatic polyketides as borolithochromes; carboxylic and cathecol type of siderophores; and complex polysaccharides as RGII. None of these B ligands is evolutionary conserved, so it is difficult to support a universal requirement of boron for life based on such diversity of ligands. However, there are molecules containing *cis*-diol residues with the potential to interact with B highly conserved in life. Among them we found ribonucleotides, monosaccharides and glycoconjugates (glycolipids, glycoproteins, or other molecules conjugated with glycan moieties), whose potential to interact with B has been demonstrated *in vitro* and/or *in vivo* (Ralston and Hunt, 2001; Kim et al., 2003, 2004; Sparbier et al., 2005, 2006; Wimmer et al., 2009; Abreu et al., 2012; Martin et al., 2013; Nishikaze et al., 2013; Lacina et al., 2014; Li et al., 2014; Voxeur and Fry, 2014).

Another unsolved question in B nutrition is how to connect the primary function of B with the signaling pathways proposed above that would mediate the B deficiency response. As mentioned, the description of cell wall receptors open the possibility that plants could perceive dRGII-B itself or through its effect in the mechanical properties in the cell wall. This cell wall receptors would elicit several signaling pathways which would explained the B deficient symptoms. This hypothesis would satisfy symptoms of low B in organisms without RGII and even devoid of cell walls, as bacteria or animal, because although RGII is not present, comparable mechanism also exist in those organism. Another possibility is that some glycolipids or glycoproteins playing signaling functions in plants, and beyond, would interact with B or boron ligands, and this would affect its function or directly report B levels. All these alternatives would be discussed in the next pages.

5.4.1. Similitudes between boron deficiency response and RGII defects.

The importance of the dimerization of RGII was confirmed *in vivo* in different studies, with B playing a role in the mechanical strength of the cell wall and the pore size (Findeklee and Goldbach, 1996; Fleischer et al., 1999; Matoh et al., 2000; Ryden et al.,

2003). However, during many years RGII itself failed to explain other pleiotropic effects of B deficiency. Nevertheless, new information about RGII location, synthesis, and responses when this polysaccharide is altered, merits a revaluation of its relevance in the response to B nutrition in plants. For that, it would be interesting to compare B deficiency responses with those responses observed when the polysaccharide is incorrectly synthesized, and to integrate them with our current understanding of the pathways controlling root development and the description of a cell wall perception mechanism. Below, we discuss, in the context of our current investigation and the information present in the literature, as well as several misconceptions.

As mentioned in the introduction RGII was assumed to be localized in the primary cell wall, so it was expected that a failure in RGII dimerization would affect cell elongation, because the process requires that a high synthesis of cell wall components takes place. Several report confirmed the absence of RGII in the QC region, however the pectin appears in the division zone and the signal detected with an anti-RGII antibody become stronger in the elongation zone (Williams et al., 1996; Matoh et al., 1998; Bárány et al., 2010). In that sense, RGII has been found also in nascent cell plates (Baluska et al., 2002, 2005; Dhonuske et al., 2006), which suggests that a failure in RGII dimerization could alter cell division. Based on this work (Figs. 14, 15), dRGII would be even more crucial during cell division than during cell elongation, because inhibition of cell elongation can be rescued by treatments with exogenous substances, whereas cell division is not. This would also fit with the results previously described by our group in rhizobia-legume symbioses forming indeterminate nodules, where cell division and endoreduplication regulates the size of the nodule (Reguera et al., 2009). The RGII presents in cell plates was considered dRGII-B whose origin was the cell wall, because it was also accumulated in BFA compartments when plants were treated with such compound (Yu et al., 2002; Baluska et al., 2002, 2005; Dhonuske et al., 2006). However several evidences suggest that those results were misinterpreted by the authors because the antibody used recognize monomeric and dimeric RGII (Matoh et al., 1998), and because the BFA treatments not only prevent endosome recycling but also secretion of newly material, so these pathways coincides in the TGN/EE compartment (Driouich et al., 1993; Lam et al., 2009; Viotti et al., 2010; Worden et al., 2012). In that way, it should be carefully reinterpreted previous results (Baluska et al., 200; Yu et al., 2002; Baluska et al., 2005; Dhonuske et al., 2006; Pagnussat et al., 2012), which could suggest a cell wall origin of endocited pectins, but

which is not fully confirmed. Those results, based on the accumulation of RGII, and other pectins, on BFA compartments are compatible with an incorrect secretion of these polysaccharides and directly connected with an object of controversy in B nutrition, namely: is dimerization of RGII occurring *in muro* or intracellularly at Golgi apparatus, TGN or secretion vesicles? During several years the paradigm accepted supported the *in muro* hypothesis, however the evidences provided to date are compatible with both hypothesis. To confirm an apoplastic or intracellular dimerization of B we propose to use a similar methodology as the used by Chormova and collaborators (2014a) but using secretion inhibitors as Concanamycin A, Brefeldin A among others (Driouich et al., 1993; Zhang et al., 1993; Satiat-Jeunmaitre et al., 1994; Robinson et al., 2008). Alternatively, we can isolate cell wall and vesicles, and after precipitate cell wall and degrade with polygalacturonase enzyme we could analyze the mRGII/dRGII-B ratio in those compartments. As a complementary approach, would be interesting to repeat the *in* vivo dimerization assay (Chormova et al., 2014a) with protoplasts instead of mature cells, to make accessible the labelled RGII to the cell wall-plasma membrane interface where nascent polysaccharides are deposited. The relevance of this finding is to clarify whether perception of a failure in RGII dimerization would be perceived as a defect in cell wall or perceived intracellularly, before the changes in cell wall structure.

Several genes involved in RGII synthesis have been described in *Arabidopsis*, tobacco and tomato: GDP–D-mannose-4,6-dehydratase, *MUR1* (Reiter et al., 1997; O'Neill et al., 2001); pectin glucuronyltransferase 1, *NpGUT1* (Iwai et al., 2002; Iwai et al., 2006); UDP-D-apiose/UDP-D-xylose synthase, *NbAXS1* (Ahn et al., 2006); UDP-rhamnose synthase, *RHM1* (Diet et al., 2006); (1,3)-a-D-xylosyltransferases, *RGXT1*, *RGXT2*, *RGXT3* (Egelund et al., 2006, 2008); Kdo-8-P synthase, *AtKDSA1* and *AtKDSA2* (Delmas et al., 2008); CMP-KDO synthetase, *CKS* (Kobayashi et al., 2011); GDP-D-mannose 3,5-epimerase, *GME* (Gilbert et al., 2009; Voxeur et al., 2011, 2012); RGII xylosyltransferase, *MGD4* (Liu et al., 2011); UDP-glucose dehydrogenase, *UGD2*, *UGD3* (Reboul et al., 2011); syalyl transferase-like protein, *SIA2* (Dumont et al., 2014). According to their defects we can cluster them in two groups: a) those altered in the synthesis of a monosaccharide or a sugar nucleotide which present the same amount of RGII but with an altered structure; and b) those with a reduced synthesis of RGII. In group a) are included mutants or transgenic lines in genes *NbAXS1*, *CKS*, *GME*, *KDSA1*, *KDSA2*, *MGD4*, *MUR1*, *RGXT1-3*, *RHM1* and *SIA2*. In this group is also included two

monosaccharide analogs, 2-dehydroxy-Kdo (Smyth et al., 2013), 2-Fluoro-L-Fucose (Dumont et al., 2015; Villalobos et al., 2015), with impaired RGII synthesis. In group b) only mutant *uge2,3* has been described (Reboul et al., 2011). This gene is altered in the enzyme UDP-Glucose dehydrogenase (*UGD)*, which catalyzes the conversion of UDP-Glu in UDP-Glucuronic acid (UDP-Glc). This mutant has a reduced pool of UDP-Glc, UDP-Xyl, and UDP-Api which results in a decrease of RGII, without any defect in its structure, but with minor structural changes in other polysaccharides containing glucuronic acid, xylose or apiose as RGI and XG (Reboul et al., 2011). As a secondary response, authors found in *ugd2,3* mutant defects in the deposition of other polysaccharides as galactomannan, which probably are induced as compensatory mechanism (Reboul et al., 2011). Both mutants of group a) and group b) showed a drastic decrease in growth, hairy phenotype and root swelling, all known B deficiency responses. Of mutants clustered in group a), which showed changes in the RGII structure that diminish the formation of dRGII-B, addition of solutions containing mM concentrations of boric acid partially rescued growth in *mur1-1* (in shoots, O'Neill et al., 2001; but not in roots, van Hengel et al., 2002); *GDE* RNAi-silencing line (Voxeur et al., 2011) and *mgd4* (Liu et al., 2011); and those caused by the use of non-metabolic monosaccharides (Smyth et al., 2013; Dumont et al., 2015; Villalobos et al., 2015). In contrast, exogenous addition of B could not rescue growth arrest nor cell wall swelling in mutant *ugd2,3* because low dRGII-B amounts were due to a minor synthesis of RGII itself (Reboul et al., 2013).

In *Arabidopsis* thaliana boron transporter mutants *nip5;1-1*, *nip6;1-1*, *bor1-1* and *bor2-1* also can be rescued by increasing boron in nutrient solution (Takano et al., 2002; 2006; Tanaka et al., 2008; Miwa et al., 2013). However, only in *bor1* and *bor2* allelic mutants have been reported an analysis of RGII dimerization, which showed a decrease of dRGII-B under low B conditions, when the function of these transporters are crucuial (Noguchi et al., 2003; Miwa et al., 2013). In the case of *BOR2*, because its location in inner epidermis and because the mutant show strong defects in cell elongation, this transporter was connected with RGII dimerization, in a model where BOR2 would mediate the extrusion of B to the apoplast if the RGII dimerization occurs *in muro* as assumed until now, but also opens the possibility that BOR2 located at TGN/EE compartment could transport B to the secretion vesicles if the dimerization of RGII occurs intracellularly (Miwa et al., 2013), as recently proposed (Chormova et al., 2014a, b).

shv3 slv1 double mutant, encoding two glycerophosphoryl diester phosphodiesterase-like proteins of unknown function, showed impairment in cell wall synthesis, including RGII dimerization (Hayashi et al., 2008). Although the molecular/biochemical function of these genes are unknown, the recent description of GIPC-B-RGII complex, where sphingolipids would act as B donor in RGII dimerization reaction (Voxeur and Fry, 2014) open the possibility that *SHV3 SLV1* would participate in an enzymatic boration mechanism. Although speculative, it is interesting to note that this mutant accumulates lignin ectopically, so also could be argued that ectopic lignification occurring in B deficiency conditions could be explained by an incorrect RGII dimerization. To date none study has explored in detail the behavior of mutants altered in RGII synthesis on cell division, cell elongation or cell differentiation (Reiter et al., 1997; O'Neill et al., 2001; Iwai et al., 2002;; Iwai et al., 2006; Ahn et al., 2006; Diet et al., 2006; Egelund et al., 2006, 2008; Delmas et al., 2008; Kobayashi et al., 2011; Gilbert et al., 2009; Voxeur et al., 2011; Liu et al., 2011; Reboul et al., 2011; Dumont et al., 2014). Although phenotypically some similitudes between B deficiency symptoms and the phenotype of mutants were found: growth inhibition, root swelling, incorrect organization of the root meristem, increase in cell wall thickness or burst of pollen tubes. It would be therefore interesting to analyze whether RGII mutants show changes similar to those reporter in this work, including cell division (using *pCYCB1:GUS* construction), cell elongation (using LEH parameter), cell differentiation (using enhancer-trap lines), secondary responses as accumulation of callose or lignin, or changes in N-glycoprotein pattern, as well as the expression/location of B transporters or the expression of B responsive genes. The development of *mur1-1* inducible mutant (Bienert et al., personal communication), or the use of non-metabolic monosaccharides which compete or alter RGII structure as 2β-Deoxy-Kdo (Smyth et al., 2013) and 2-Fluoro-L-Fucose (Dumont et al., 2015; Villalobos et al., 2015) are also interesting approaches. I would like to highlight the recent work of Dumont and collaborators (2015), using the non-metabolizable fucose analog 2-Fluoro-L-Fucose. This compound interferes in the synthesis of all fucose-containing polysaccharides or glycoconjugates. In that sense the authors confirmed that addition of 2-Fluoro-L-Fucose altered RGII dimerization, that B partially rescue such response, and combining 2-Fluoro-L-Fucose with several mutants impaired in N-glycosylation or xyloglucan synthesis (other fucose-containing compounds), the authors proposed that the defects observed in root growth, cell elongation and cell differentiation are associated

with RGII dimerization (Dumont et al., 2015). Future work using this chemical treatments would clarify the importance of the polysaccharide during root development, the differences/similitudes between boron deficiency response and the molecular mechanism involved in B deficiency.

5.4.2. Potential pectin receptors: linking structure and signaling.

The synthesis and assembly of cell wall components is highly regulated in specialized cell structures as root hairs or pollen (Gu and Nielsen, 2013), or during high elongation processes as skotomorphogenesis (development in darkness) (Gendreau et al., 1997). Also, when a cell wall component is incorrectly synthesized, compensatory responses are activated (Wolf et al., 2012; Le Gall et al., 2015). Also, the cell wall plays a critical role in perception to both biotic (herbivores, biothrophes and necrotrophs) and abiotic stresses (salinity, wound…), based on the changes that a specific stress produces in the mechanical properties and/or chemical composition of the cell wall (Malinovsky et al., 2014; Miedes et al., 2014; Le Gall et al., 2015; Tenhaken, 2015).

The description of several families of cell wall receptors creates a framework to connect cell wall structure/composition with the activation of specific signaling cascades. To date six main families of cell wall sensors have been described: *Wall-Associated Kinases (WAKs)*, Pro-Rich Extensin-Like Receptor Kinase (PERKs), *Catharanthus roseus*-Like Receptor-Like Kinase 1-Like (CrRLK1L), Leu-Rich Repeat Receptor Kinases (LRR-RLKs), Leguminous L-Type Lectin RLKs (L-lectin RLKs) and finally mechanosensitive ion channels (Perret et al., 1999; Kohorn, 2001; Anderson et al., 2001; Becraft, 2002; Kohorn and Kohorn, 2012; Engelsdorf and Hamann, 2014). However, none of them has been confirmed as a RGII sensor. The closest candidates would be *WAK*s, which are tightly associated with cell wall, and specifically with the pectin fraction (He et al., 1996; Perret et al., 1999; Anderson et al., 2001; Wagner and Kohorn, 2001; Kohorn et al., 2006a). According to UniProt database, WAK1 predicted molecular weight is ~81 kDa, and in this estimation are not included the increase of molecular weight that would occur if the N- and O- glycosylation sites are occupied. However, when released with an extraction buffer containing SDS and DTT or by a polygalacturonase treatment, the protein migrated as a ~68 kDa or ~67 kDa zone respectively, suggesting that the protein is somehow processed or that is linked to negative charged molecules which would increase its electrophoretic mobility (Anderson et al., 2001; Kohorn et al., 2006a).

Because pectinase-released WAK1 was immunoreactive either to JIM5 and JIM7 antibodies, suggesting the presence of low-methyl esterified HG and high methyl-sterified HG respectively, the second option seems to be true (Kohorn et al., 2006a). It was also tested the presence of other pectin epitopes as (1-4)-β-galactan or (1-5)-α-L-arabinan (Kohorn et al., 2006a); *in vitro* studies demonstrated that WAKs also interacted with Ca^{2+} crosslinked homogalacturonan and oligogalacturonides (and not its monomers) (Decreux and Messiaen, 2005; Decreux et al., 2006; Kohorn et al., 2006a, 2009), and finally other authors reported the existence of a complex between WAK1 and a glycine-rich protein (GRPs) (Park et al., 2001). *In vivo* WAK1 co-localized with JIM5 (HG) in cell wall, but the protein also showed co-localization with Golgi markers (SYP31 and SYP41) but not with FM4-64, suggesting that it is not present in endosomal compartments (Kohorn et al., 2006a). In the same work, the authors demonstrate that the complex WAK1-pectins is occurring within cells, the complex occurred in the *mur1-1* mutant although with a different electrophoretic mobility and a faster secretion rate in the mutant (Kohorn et al., 2006a). The other cell wall receptor interacting with pectin was PERK4 (Bai et al., 2009a), a member of the proline-rich extensin-like receptor-like kinases family (Nakhamchik et al., 2004). As WAKs, PERK4 was released from the cell wall by treatments under denaturing reducing conditions or by the action of pectolyase (Bai et al., 2009a). PERK4 seems to control root growth in response to ABA, perturbing Ca^{2+} homeostasis, and modulating gene expression (Bai et al., 2009a, b), but the mechanisms are still non described.

So far, nobody has tested if WAKs bind to RGII, so we cannot rule out the possibility that RGII could be directly perceived by WAKs. If RGII is not a direct ligand, there is still the possibility that WAKs would recognize RGII defects through the polymer formed in plants by the covalent crosslinking of the three different pectin domains (HG, RGI and RGII) (Vincken et al., 2003), which would explain the abnormal secretion and electrophoretic mobility of WAK in the mutant *mur1-1* (Kohorn et al., 2006a). Future experiments are necessary to confirm the hypothesis that the incorrect RGII dimerization occurring in B deficiency triggers *WAK*-dependent signaling pathway. Evidences supporting this hypothesis are that addition of RGII to protoplast caused a *WAK2*-dependant induction of invertase (Kohorn et al., 2006b; 2009). If isypothesis is true, then the activation of *MAPK3* and *MAPK6* previously reported in B deficiency (Kohorn et al., 2009, 2012), could be explained by the action of WAKs. Also *PERK*-dependent signaling

pathways would be interesting targets in B deficiency response if ABA signaling is involved in B deficiency response, as we proposed above. In this case, an involvement of ABA *PERK*-mediated signaling pathway (Bai et al., 2009a, b) would explain the transcriptional repression of cell wall enzymes (Camacho-Cristóbal et al., 2008).

5.4.3. N-glycoproteins as potential boron ligands.

Several authors argued that boron could interact with other molecules containing *cis*-diol groups present in all or at least most living organisms, and that those interactions would explain some of the pleiotropic symptoms observed under B deficiency (Dembitsky et al., 2002; Bolaños et al., 2004; Reguera et al., 2010b). Nowdays there is *in vitro* and/or *in vivo* evidences that B is able to interact with nucleotides (Ralston and Hunt, 2001; Kim et al., 2003, 2004), glycolipids (Abreu et al., 2014b; Voxeur and Fry, 2014) and glycoproteins (Wimmer et al., 2009; Reguera et al., 2010a; Chormova and Fry, 2015). Among these potential interactors, glycoproteins are particularly interesting because they play very diverse functions, from structural proteins to enzymes or receptors. To date, the glycoproteins identified or proposed as B ligands play a structural role: in one hand our group described through immunoprecipitation assays a B-dependent complex constituted by RGII and an ArabinoGalactan Protein Extensin-like (AGPE) (AGPE-B-RGII) which would strength glycoprotein matrix present in the lumen of infection threads (Reguera et al., 2010a). In the other hand, the complexes recently proposed between RGII and non-natural (polylisine or polyhistidine) or natural (Hydroxyproline-Rich Glycoproteins, HPRGs) polycations would act as intermediaries in the formation of dRGII-B (Chormova and Fry, 2015).

Wimmer and collaborators (2009) isolated several plasma membrane glycoproteins in *Arabidopsis* using PBA cromatography, including several ATP synthases, β-glucosidases, myrosinases and luminal-binding protein (BiPs). During the development of this thesis I found the induction of βGlu18 (Fig. 29, 30) which is forming part of the ER bodies in *Arabidopsis* with the potential B ligands PYK10 (Wimmer et al., 2009). According to its work, BiPs would be also B ligands, and although it was not already identified using our AAC method, several BiP isoforms appeared in B deficient root and nodules (Fig. 8), and also were accumulated under low B conditions in *Arabidopsis* (Fig. 30). Besides AGPE our group described some other B ligands in the rhizobia-legume

symbiosis using Amberlite IRA 7-43 affinity chromatography, which were principally involved in O_2 protection (Reguera et al., 2010c). In the present work I used a similar approach with Amberlite to analyze qualitatively if glycoproteins presented a high affinity for B, as suggested by Wimmer and collaborators (2009). We identified several proteins and MRNGPs interacting with Amberlite IRA 7-43, but any CNGP (Fig. 9). These differences are probably related with the low detection limits of the anti-Fucose and anti-Xylose antibodies (Fig. 9C, 9D), therefore we still do not discard the presence of CNGP as potential B ligands. Importantly, we found proteins and MRNGPs in both retained and no retained fraction, suggesting that the presence of the N-glycan itself do not make the protein a potential B ligand, but may be some steric/enzymatic mechanisms which determine the "boration" or not of a specific protein.

In this work I did not include O-glycoproteins, because the lack of methodologies to analyze it, however in future experiments my group would bypass this gap using total glycoproteins staining kits. The number of potential B ligands would increase in the next years, with the use of affinity methods as the Phenylboronic Affinity (PBA) chromatography (Wimmer et al., 2009) or the Amberlite IRA 7-43 Affinity Chromatography (AAC) (Reguera et al., 2010c), which offer the opportunity to identify complementary ligands.

Our group is currently looking for more powerful detection techniques in order to identify potential B ligands. New proteins forming one side B complexes in both rhizobia-legume symbiosis and *Arabidopsis* will be identified, enriching this fraction through an Amberlite IRA 7-43 Affinity Chromatography step, coupled offline with a medium/long gradient LC-MS/MS. With these data we expect to identify potential B ligands and explore their potential functions. Also, once identified the potential interactors, the presence of B should be validated by ICP-MS or NMR. The existence, nature and stoichiometry of the existing complex would be analyzed later, using for example co-immunoprecipitation or fluorescence co-localization.

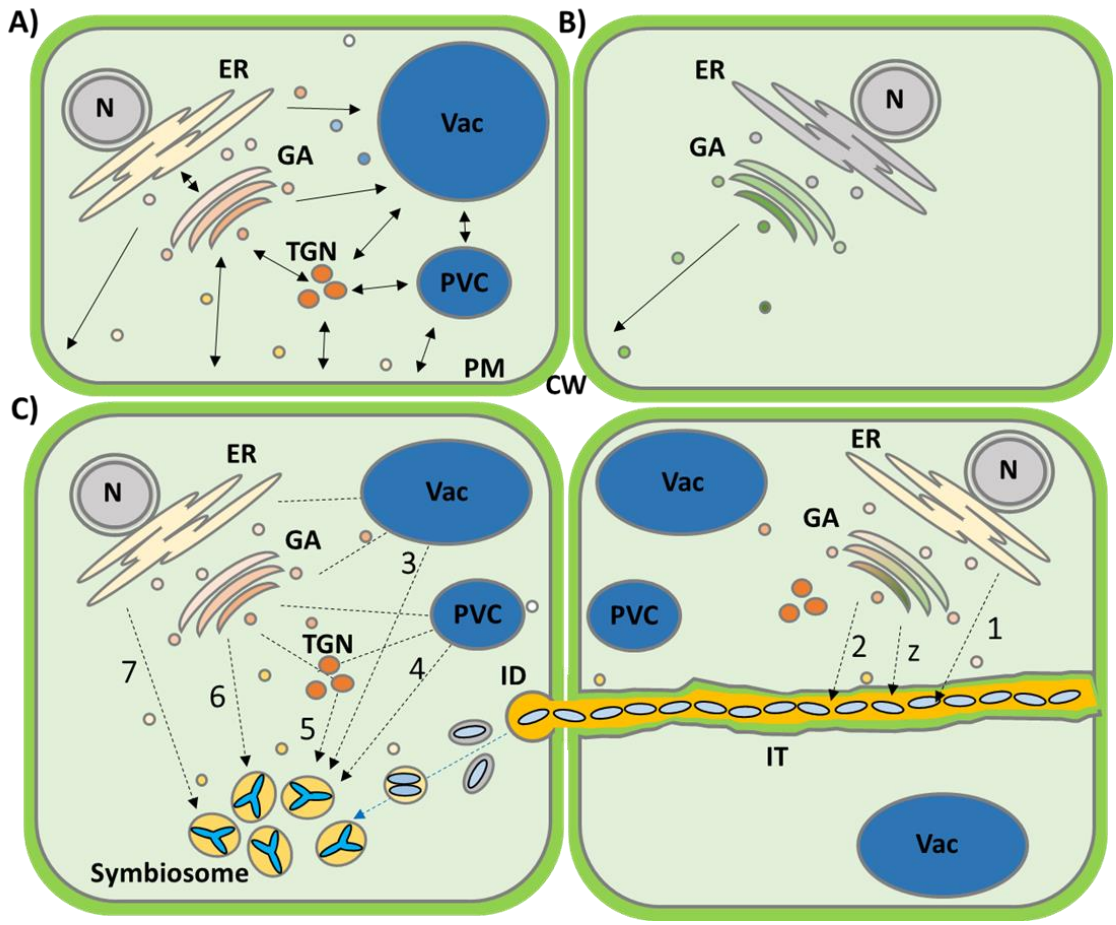


Figure 48. Schematic model of glyco/protein and polysaccharide secretion pathways rhizobia-legume symbiosis forming indeterminate nodules.

A) Protein trafficking includes different pathways, connecting almost every organelle and compartment in mono or bidirectional ways. B) Several plant cell wall components (pectins, hemicelluloses) are synthesized at GA and secreted in vesicles to CW. C) Potential pathways for the secretion of proteins, glycoproteins, and polysaccharides to infection thread (right panel) or to symbiosomes (left panel) in in rhizobia legume symbiosis. Proteins and glycoproteins could be secreted 1) From ER to IT lumen; 2) From GA to IT lumen; 3) From Vac to Symbiosome; 4) From PVC to symbiosome; 5) From TGN to symbiosome; 6) From GA to symbiosome or 7) From ER to symbiosome. Polysaccharides would be synthesized in GA and secreted to IT lumen, z).

CW, Cell Wall; ER, Endoplasmic Reticulum; GA, Golgi Apparatus; IT, Infection Thread; ID, Infection Drop; N, nucleus; PM, Plasma Membrane TGN, *Trans*-Golgi Network; Vac, Vacuole.

5.4.4. Accumulation of N-glycoproteins under low boron conditions: a mechanical consequence of boron deficiency or a signaling response?

For the first time, I reported the accumulation of N-glycoproteins under B deficiency conditions in both types of rhizobia-legume symbioses (Figs. 6, 7). Two causes could explain this accumulation: an incorrect sorting or delivery of glycoproteins to their final compartments which would lead to a general accumulation of N-glycoproteins versus a

failure in correct N-glycoprotein synthesis, accompanied by an overexpression of such proteins. Both hypotheses have been previously proposed to explain the absence of Hydroxy-Proline Rich Glycoproteins in B deficient nodules (Bonilla et al., 1997), the incorrect synthesis of the symbiosomal PsNLEC1 glycoprotein (Bolaños et al., 2001), the accumulation of glycoproteins showing some antigenicity to RGII antibody (Redondo-Nieto et al., 2007), or to explain the accumulation of AGPE in –B nodules (Reguera et al., 2010a). During symbiosis establishment, there is a massive synthesis of glycoproteins, which are critical for the formation of the infection threads and the symbiosome development (Brewin, 2004), and always appear new secretory pathways to fed the requirements of the nascent subcellular compartments (Wang et al., 2010). Not only those particular studies, but the abnormal N-glycoprotein accumulation reported therein would explain why B is so important during the symbiosis, and why the most sensitive symbiotic stages to B deprivation are infection and symbiosome maturation (Fig. 48).

Although the legume-rhizobia symbiotic root nodule is a good model for studies of organogenesis in plants, it has the limitation of being controlled by both plant and bacteria signaling mechanisms that difficult the unraveling of early effects primarily due to nutritional stress. Development of a pre-nodule takes one week, therefore the high accumulation of N-glycoproteins under B deficiency could be interpreted as a secondary effect due to the long exposure time to B deficient conditions. Actually, not all bands which are accumulated (Fig. 6) correlates with potential B ligands identified by the Amberlite IRA 7-43 affinity chromatography (Figs. 9) suggesting that the accumulation of N-glycoproteins can be an unspecific effect of the micronutrient deprivation. Therefore, rhizobia-legume symbiosis is not a suitable model to explore the hypothesis of overall N-glycan accumulation due to B deficiency, because the studies of N-glycosylation pathways and transport of proteins and glycoproteins during the symbiotic process are still poor, and mainly because legume development is not amenable to short term B deficiency experiments. For these reasons we moved to *Arabidopsis*, a best known model which allow us to easily perform transference experiments and short time exposures to B deficiency.

After the phenotypic characterization of B-deficient Arabidopsis, that we considered essential, we confirmed that, as in the legume nodule model, *Arabidopsis* plants grew in B deficient or low B media showed a clear accumulation of both Mannose-Rich and

fucose-containing Complex N-glycoproteins, although only some light changes in xylose-containing Complex N-glycoproteins (Figs. 30, 31). However, B deficiency did not alter the N-glycosylation of model glycoproteins as PDI (Fig. 30E), nor rescue the hypo-glycosylation phenotype observed in the mutants *alg3* and *alg10* (Fig. 37). Furthermore, the βGlu18 and βGlu23 proteins identified, which have three N-glycosylation sites (Matsushima et al., 2003), were correctly glycosylated in wt plants (independently of the amount of B) but not in the mutants *alg3*, *alg10* and *stt3a* (Fig. 29, 35; Table 7). These results, although would be complemented with the analysis of the glycosylation state of other model N-glycoproteins as TGG1 (Ueda et al., 2006) or BRI1 (Mora-García et al., 2004) or, concur with the abnormal secretion hypothesis rather than with the failure of synthesis.

The *mur1-1* mutant, besides the changes in RGII previously described, showed defects in other polysaccharides and glycoconjugates as N-glycoproteins. Specifically, *mur1-1* showed an accumulation of MRNGPs and Xylose-containing CNGPs (Rayon et al., 1999), which were not observed in mutants lacking the Golgi fucosyltransferase (FucT AB) which catalyze addition of a α1-3fucose to the N-glycan (Strasser et al., 2004). Indeed, of the mutants used in this study only *mns123* showed growth arrest and other symptoms observed in B deficiency as root swelling and accumulation of MRNGPs (Liebminger et al., 2009). The others (*alg3, alg10, stt3a, mns12, cgl1* and *gntI*) were apparently indistinguishable of wild type plants when grew in control conditions, although their glycoproteins lacked some N-glycans (Koiwa et al., 2003; Strasser et al., 2005; Frank et al., 2008; Henquet et al., 2008; Kang et al., 2008; Liebminger et al., 2009; Farid et al., 2010; Kajiura et al., 2010). In that sense, the more obvious difference between *mns123* and the other mutants is that the mutation in *mns123* impedes the progression of proteins along the secretion pathway because an incorrect removal of some mannose residues lead to an accumulation of these proteins in the ER, and the subsequent activation of UPR (Unfolded Protein Response) and ERAD (ER-Associated Degradation) responses (Liebminger et al., 2009). Indirect evidences, as the accumulation of BiP under B deficiency (Fig.30F) suggest that UPR could be activated, although a direct confirmation at molecular level, for example analyzing gene expression of BiP, PDI and other responsive genes is still needed (Liu and Howell, 2010; Iwata and Koizumi, 2012; Fanata et al., 2013).

According to the current evidences, there are proteins, glycoproteins, and polysaccharides sharing the secretion pathway as well as the machinery involved in the formation of the vesicles, and the cargo recognition. Accumulation of both actin and tubulin was reported after few minutes of B deficiency (Yu et al., 2001). This fits with ultrastructural cell changes described under B deficiency including a fast intracellular accumulation of swollen vesicles (Kouchi and Kumazawa, 1976). Although the nature and content of such vesicles is unknown, they are quite similar to those observed in mutant *ugd2,3*, altered in the synthesis of RGII (Reboul et al., 2011); in the mutant *fra1*, altered in a kinesin protein contributing to microtubule-mediated trafficking of cell wall components (Zhu et al., 2015); or those observed in plants treated with inhibitors in the secretion machinery as brefeldin A, concanamycin A or monensin (Driouich et al., 1993; Zhang et al., 1993; Satiat-Jeunmaitre et al., 1994; Robinson et al., 2008). Taken together, these results fit in a model where B is required for a correct endomembrane trafficking. Still unsolved whether the micronutrient play a primary role interacting with the cytoskeleton used for vesicles as rails o as a linker between polysaccharides/proteins contained within vesicles and the protein machinery recognizing vesicle cargo. Our group is currently looking for direct evidences using the secretion reporter line *secGFP* S76 developed by Dr. Moore (Zheng et al., 2004; Teh and Moore, 2007), but other hypotheses as that a decrease in dRGII-B would cause this failure in secretion should be tested. In that way it would be interesting to analyze the changes in N-glycosylation pattern observed in *mur1-1* under low and high B (because at mM concentration of boric acid, shoot but not root growth are rescued; O'Neill et al., 2002; Hengel et al., 2002), as well as in other RGII mutants (*ugd2,3* and *shv3 svl1*; Reboul et al., 2011; Hayashi et al., 2008) or treatments altering RGII synthesis (2β-Deoxy-Kdo and 2-Fluoro-L-Fucose; Smyth et al., 2013; Villalobos et al., 2015; Dumont et al., 2015).

Another interesting question is whether the hormone balance could alter N-glycosylation in plants. N-glycan maturation is required for normal signaling (Häweker et al., 2010; Fanata et al., 2013). Complementary, it has also been observed that exogenous cytokinin increased expression of N-glycosylation machinery (Motyka et al., 2003). Our results showed that ACC treatments produced an accumulation of MRNGPs in both sufficient and deficient plants, and that complementary Ag^{+} treatments alleviated the accumulation of MRNGPs observed in -B plants (Fig. 41). However the increase of MRNGPs in B sufficient ACC-treated plants did not reach the levels observed in –B

plants, nor –B+Ag^{+} plants showed a decrease to control levels (Fig. 41), suggesting that ethylene has influence on the accumulation of N-glycoproteins but it is not the primary cause of the N-glycoprotein accumulation on B-deficient plants. A possible explanation for this behavior could be found in that ethylene receptors, located in ER (Ju and Chang, 2012), would be activated in response to the accumulation of glycoproteins and the UPR. Also has been reported that ethylene signaling rescue the phenotype of *rhd1* mutant, encoding a UDP-glucose 4-epimerase gene (*UGE4*) which participates in the synthesis of xyloglucan, superimposing its own developmental program (Seifert et al., 2004). Although ethylene is able to hide the cell wall defects observed in mutant *rhd1*, this is not the case in B deficiency.

5.4.5. Alternative boron perception mechanisms.

As discussed above, from a top-down approach, the morphological symptoms observed in B-deficient plants could be explained by the activation of different signaling pathways, including the hormones ethylene, auxin, cytokinin and jasmonic acid, which recruit secondary signal cascades mediated by Ca^{2+} and ROS. From a bottom-up approach, there is no evidences of a direct role of B as signaling molecule, so the best estimation of the B nutritional status would be the dRGII-B/mRGII(B) ratio, which could be perceived by *WAK*s and *PERKs* as discussed in section 5.4.2). However, recently it has been described in plants the existence of other B complexes than dRGII-B, namely AGPE-B-RGII (Reguera et al., 2010a), GIPC-B-RGII (Voxeur and Fry, 2014), and probably a transitory HPRG-B-RGII (Chormova and Fry, 2015), although all of them involve a RGII monomer. GIPC and AGPE, seem to be respectively intermediaries in the synthesis of dRGII-B (Voxeur and Fry, 2014; Chormova and Fry, 2015) and structural components of the infection thread in legume nodules respectively (Reguera et al., 2010a). So, although none of them change significantly the structural role of the micronutrient, they open the possibility to other perception of B levels mechanisms which would involve not only the ratio dRGII-B/mRGII but maybe the "borated" forms of GIPC and AGPE, or the complexes between those molecules and RGII (GIPC-B-RGII and AGPE-B-RGII respectively). Based in the description of GIPC and AGPE as boron ligands, in the exploratory work of Wimmer and collaborators (2009), where they found several proteins which potentially interact with boric acid (Wimmer et al. 2009), and the results presented in this manuscript (Fig. 9), we should reject the paradigm that RGII was the only B ligand in plants. New B ligands will be identified in future, among them several

N-glycoproteins, which also will constitute potential sensors of B, and we should integrate the signaling pathways discussed above with the new ligands which will appear. In this context, B perception could also be indirectly perceived through the changes produced in the cell wall in terms of mechanical stiffness, turgor pressure or pore size, resulted of an incorrect dimerization of the RGII (Findeklee and Goldbach, 1996; Fleischer et al., 1998, 1999; Matoh et al., 2000; Ryden et al., 2003). Other families of cell wall receptors mentioned in section 5.4.2 could perceive such changes and translate them through established signaling cascades involving ROS, JA, SA, ACC/Ethylene and ABA (Engelsdorf and Hamann, 2014; Malinovsky et al. 2014, Miedes et al. 2014), leading to the downstream responses observed in B-deficient plants.

B has been also proposed to interact directly with some enzymes or their substrates/products, which would impair plant metabolism. There is evidences suggesting that boron alters carbohydrate metabolism through the enzymes 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Lee and Aronoff, 1967). Also has been proposed that B deficiency led to the accumulation of phenolic substances due to a presumptive role of the micronutrient on phenylamonio lyase (PAL) and polyphenol oxidase (PPO) enzymes (Cakmak and Röhmel, 1997; Ruiz et al., 1998; Camacho-Cristóbal et al., 2002; Cara et al., 2002). The micronutrient also would alter metabolism interfering reactions which include other *in vitro* B ligands as ribonucleotides (RNA and energy metabolism, glycoconjugate synthesis), SAM (methylation reactions), or monosaccharides (energy metabolism). Finally a role of on membrane integrity and on the activity of the H^+-ATPase has been proposed based on the ion leakage observed in B deprived plants (Goldbach et al., 1990; Roldan et al., 1992; Ferrol et al., 1993). To date, any of this "enzymatic" functions of boron has been validated identifying B in the enzyme or in intermediaries, however this functions of B in metabolism also would act as signal in B deficiency response through dedicated pathways to "nutrient perception" as TOR (Target of Rafampycin) (Henriques et al., 2014; Xiong and Sheen, 2014; Barrada et al., 2015).

At least other three mechanisms independent of those discussed above seem to operate in the regulation of B transporters: i) polar localization; ii) post-transcriptional control of *NIP5;1;* iii) Post-translational control of *BOR*s. Polar localization of B transporters is critical to control the acquisition or extrusion of the micronutrient according to the amount

of B present in the nutrient solution (Fuji et al., 2009; Miwa et al., 2010). Then, there is two independent ways to control the amount of B transporters that are finally situated in the plasma membrane. Regulation of *NIP5;1* occurred at post-transcriptional level, apparently through a small sequence in the 5'UTR region, which would regulate the stability of the mRNA depending of the B present in the cell (Tanaka et al., 2011). On the other hand, BOR1 is controlled at posttranslational level. Under high B the protein is recruited from plasma membrane, endocyted and degraded. Several key residues involved in such regulation are highly conserved, but the machinery participating still unknown (Wakuta et al., 2015). Future experiments would also clarify whether free B, pectins or other B ligands are involved, and favor or avoid the formation of the above mentioned complexes which led to NIP5;1 mRNA degradation or BOR1 endocytosis.

Finally, plants capable of synthesize polyols, and remobilize B, seem to control the abundance of polyols in response to the soil B levels (Perica et al., 2001a, 2001b; Liakopoulos et al., 2005, 2009). Although more evidences are needed, this mechanism would involve a perception mechanism in meristematic organs (where the micronutrient levels would be lower), and a signaling pathway which would involve a transport signal because synthesis of polyols would occur in B rich organs (old leaves).

5.5. Final considerations and future perspectives

Considering the work of Agulhon (1910) as the first reports, the investigations of B nutrition in plants have run for more than 100 years. Although many symptoms of B deficiency were described and a function of B was finally convincingly demonstrated, we still poorly understand the connection between B function and several responses which characterize B deficiency. In the present work, I have found that the symptoms observed in *Arabidopsis* are pretty similar to those observed in other dicotyledonous plants (including growth arrest, induction of lateral roots, and root swelling). Also I observed a dramatic disorganization of the root meristem, including inhibition of cell division and cell elongation, abnormal cell differentiation and exhaustion of quiescent center. Also, was reported the accumulation of callose and lignin. Finally, for the first time I described an accumulation of N-glycoproteins in B deficient plants, both in *Arabidopsis* and in nodules derived from rhizobia-legume symbioses. Furthermore, some of them were potential B ligands.

Taking advantage of the accumulated knowledge in model plants as *Arabidopsis* and to the phenotypes described in this work or by other authors, I propose a model to explain the morphological symptoms observed in B-deficient plants. It would be as follows: 1) B deprivation led to a decrease of dRGII-B; 2) the decrease of dRGII-B would be the signal perceived by cell wall receptors. 3) Cell wall receptors would translate it through signaling cascades into different output secondary signals: ACC/ethylene, JA, SA, Ca^{2+}, ROS…; 4) the secondary signaling pathways would control final targets, producing changes in metabolism, cell wall synthesis or in root development (Fig. 47). Other pathways recognizing indirect effects on cell wall as mechanical stiffness or turgor pressure, or recognizing other B ligands than RGII, could occur in parallel and converge in the above mentioned model.

Fast mechanisms controlling B transporters abundance probably would not involve hormonal signaling but a direct interaction between boron and the regulatory mechanism (ribosomal machinery for *NIP5;1* and endocytosis machinery for *BOR1*).

In a similar way, I propose that the accumulation of N-glycoproteins is caused by a mechanical effect of B on the secretory system. This "direct" effect of B in secretory machinery, which could be related with the disassembly of cytoskeleton observed under B deficient conditions, could be extended to eukaryotes because in eukaryotes glyco/protein synthesis and secretion pathways are highly conserved (Varki et al., 2009). This hypothesis would be further explored but would explain some previous results obtained in zebrafish, where the developmental stages which require a high synthesis of glycoproteins are the most susceptible to B deficiency (Reguera, 2009).

Future research would address the validity of the proposed model, and also would answer if B deficiency response is caused by something else than an altered RGII dimerization. For that purpose would be interesting to clarify RGII synthesis, dimerization and associated signaling cascades. Also analyze similitudes and differences in B deficiency response and defects on RGII synthesis at transcriptomic, biochemical and morphological level. Will be of extreme interest to understand the mechanisms regulating B transport at short or long distance level, because their biotechnological potential. Also would be interesting to confirm the involvement of hormones (jasmonic acid, salycilic acid, cytokinins, auxins, abscisic acid) and other signaling pathways (ROS,

Ca^{2+}, cell wall fragments) in the B deficiency response, analyzing the whole battery of B deficiency simptoms (from growth arrest, inhibition of cell elongation and cell division, root swelling, induction of specific proteins, accumulation of callose, lignin or N-glycoproteins, bRGII-B/mRGII ratios, dimerization using for that genetic and pharmacological approaches. Finally, would be interesting to clarify if there is a universal requirement of B in green plants (*Viridiplantae*) and in other eukaryotes, to confirm if accumulation of N-glycoproteins are a universal response to B deficiency, and finally explore B deficiency and toxicity symptoms in model organism where RGII is low or absent.

In my opinion, we are entering in a new "golden age" for B nutrition studies. On one side because methodologically we can generate very accurate B deficiencies, so we can analyze B requirements over the life tree, including animals, and hopefully we will answer finally the question of whether B requirement was "appearing" independently in different organisms/ kingdoms, or B requirement is conserved throughout the evolution. Also, the enrichment methodologies that we have discussed herein will allow explore the existence of other B ligands, which then would require a specific and detailed characterization. Last, but not least, in the System Biology age we will have the chance to understand the whole B deficiency response, from the role of B at cellular level, passing through the different signaling mechanisms that plants posse to maintain B homeostasis and to regulate plant architecture. Molecular and pharmacological approaches in conjunction with high-throughput methodologies will be useful for solve such questions. From a practical point of view, the knowledge of the signaling pathways operating in B deficiency response, will allow the selection of best adapted varieties or those with a better boron efficiency use. Also, if natural diversity is not sufficient, we could generate transgenic plants with improved acquisition or remobilization capabilities, or with low B requirements.

5. Conclusions

The main conclusion of this work are:

i) B deficiency causes an accumulation of N-glycoproteins in rhizobia-legume symbiotic nodules which does not correlate with the N-glycoproteins identified as potential B ligands. In legume roots and in Arabidopsis, we also observed an accumulation of N-glycoproteins, suggesting that this is a common response to B deficiency in plants. Because glycosylation of model glycoproteins was not altered by B deficiency, we propose that the accumulation should be associated with a failure in protein secretion, which fits with the accumulation of secretion vesicles in B deficient plants.

ii) Specific N-glycoproteins interact *in vivo* with B, forming one-side complexes which could be isolated with Amberlite IRA 7-43. Although the identity of these proteins is unknown, it reinforces the hypothesis that other B ligands exist in plants. Because not all N-glycoproteins formed stable complexes with B, it would support the existence of an unkown mechanism mediating "boration" on specific targets.

iii)The B deficiency response in Arabidopsis include growth arrest, root swelling, hairy phenotype, inhibition of cell division and cell elongation, cell differentiation proximal at root tip, disappearance of quiescent center, ectopic deposition of lignin and callose, induction of wound-inducible βGlu18 protein, and accumulation of N-glycoproteins. Many symptoms described in B-deficient plants coincide with symptoms observed in mutants in RGII or after treatments that alter RGII synthesis, although future comparative studies would clarified this aspects.

iv)Overall defects observed in the present manuscript in conjunction with previous data of the symptoms accompanying B deficiency, led us to divide B deficiency response in five branches, related with different signaling cascades or biological processes:

a) A quick physical effect of B on endomembrane machinery, supported by the accumulation of vesicles and the disorganization of cytoskeleton, which would cause the accumulation of N-glycoproteins.

b) In parallel, A B-mediated process involving direct interaction between proteins and/or RNA would control B transporters.

c) Activation of wound /cell wall damage-like response by uncertain mechanisms would cause the induction of ROS, Ca^{2+} unbalances, accumulation of phenols, ectopic accumulation of lignin and callose, and the induction of βGlu18.
d) Activation of ACC/ethylene signaling pathway is controlling the inhibition of cell elongation, and downstream effects as increase in auxin, ROS and Ca^{2+}. Ethylene also could play a role in the accumulation of N-glycoproteins.
e) Changes in the balance between auxin/cytokinin ratio could be relevant in controlling the decrease in cell division and early differentiation observed in B deficient plants.

Las principales conclusiones de este trabajo son:

i) La deficiencia de boro genera en los nódulos desarrollados durante la simbiosis entre rizobios y leguminosas una acumulación inespecífica de N-glicoproteínas, que no se corresponde con aquellas identificadas como potenciales ligandos de boro. Tanto en la raíz de plantas inoculadas como en la planta modelo *Arabidopsis thaliana* observamos la acumulación de N-glicoproteínas, de lo que podemos derivar que se trata de una respuesta generalizada en plantas. Dado que la glicosilación de proteínas específicas no se ve afectada por los niveles del micronutriente, la acumulación de N-glicoproteínas descrita en deficiencia de boro se debe a un fallo en la secreción de proteínas, que concordaría con la acumulación de vesículas intracelulares observada en plantas deficientes en boro.

ii) Hay varias N-glicoproteínas específicas que interaccionan *in vivo* con boro, formando complejos mono-ester que pueden ser aislados mediante la cromatografía de afinidad con la resina Amberlita IRA 7-43. Aunque no hemos identificado la identidad de esas proteínas, nuestros resultados refuerzan la hipótesis de que existen otros ligandos de boro. Sin embargo, el hecho de que no todas las N-glicoproteínas formen complejos estables con el micronutriente, sugiere la existencia de un mecanismo específico para la "boratación" de dianas específicas.

iii) La respuesta a la deficiencia de boro en *Arabidopsis* incluye varios fenotipos: detención/parada del crecimiento, engrosamiento de la raíz, fenotipo "peludo", inhibición de la elongación y la división celular, aparición de células diferenciadas en el

ápice de la raíz, desaparición del centro quiescente, acumulación de lignina y callosa, inducción de la proteína inducible por herida βGlu18, y acumulación de N-glicoproteínas. Varios fenotipos descritos en la deficiencia de boro han sido también caracterizados en mutantes o tratamiento que presentan una estructura del RGII alterada, aunque una comparación más detallada es necesaria.

iv) Los síntomas aquí descritos, en conjunción con los observados en trabajos previos, sugieren que la respuesta a la deficiencia de boro se puede dividir en cinco ramas correspondientes a distintas rutas de señalización o procesos biológicos:

a) Una respuesta rápida relacionada con un papel del boro en el sistema de endomembrana, que vendría apoyado en la acumulación de vesículas y la desorganización del citoesqueleto, y que causaría la acumulación de glicoproteínas.
b) En paralelo, la participación directa del boro a través de la interacción con proteínas y/o RNA en el control de los transportadores del micronutriente.
c) La activación por señales aún desconocidas de una ruta de señalización similar a la respuesta por herida/daño en la pared celular, que causaría la inducción de especies reactivas de oxígeno (ROS), desbalances en la concentración de Ca^{2+}, acumulación de fenoles, acumulación ectópica de callosa y lignina, y la inducción de la proteína βGlu18.
d) La activación de una ruta de señalización mediada por ACC/etileno que controlaría la inhibición de la elongación celular, implicando respuestas secundarias como el aumento en auxina, ROS y Ca^{2+}. El etileno también podría estar relacionado indirectamente con la acumulación de N-glicoproteínas.
e) Cambios en el balance el balance entre auxina y citoquinina, que disminuirían la división celular y explicarían la temprana diferenciación celular observada en la deficiencia de boro.

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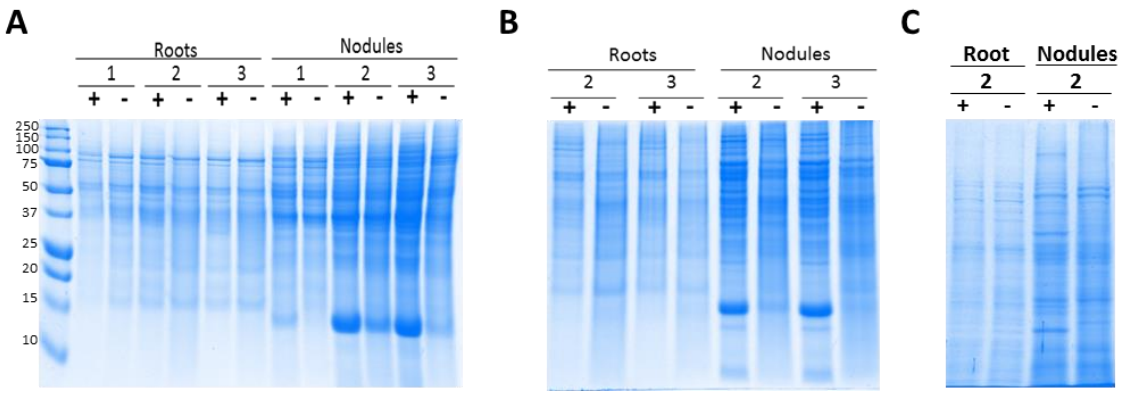
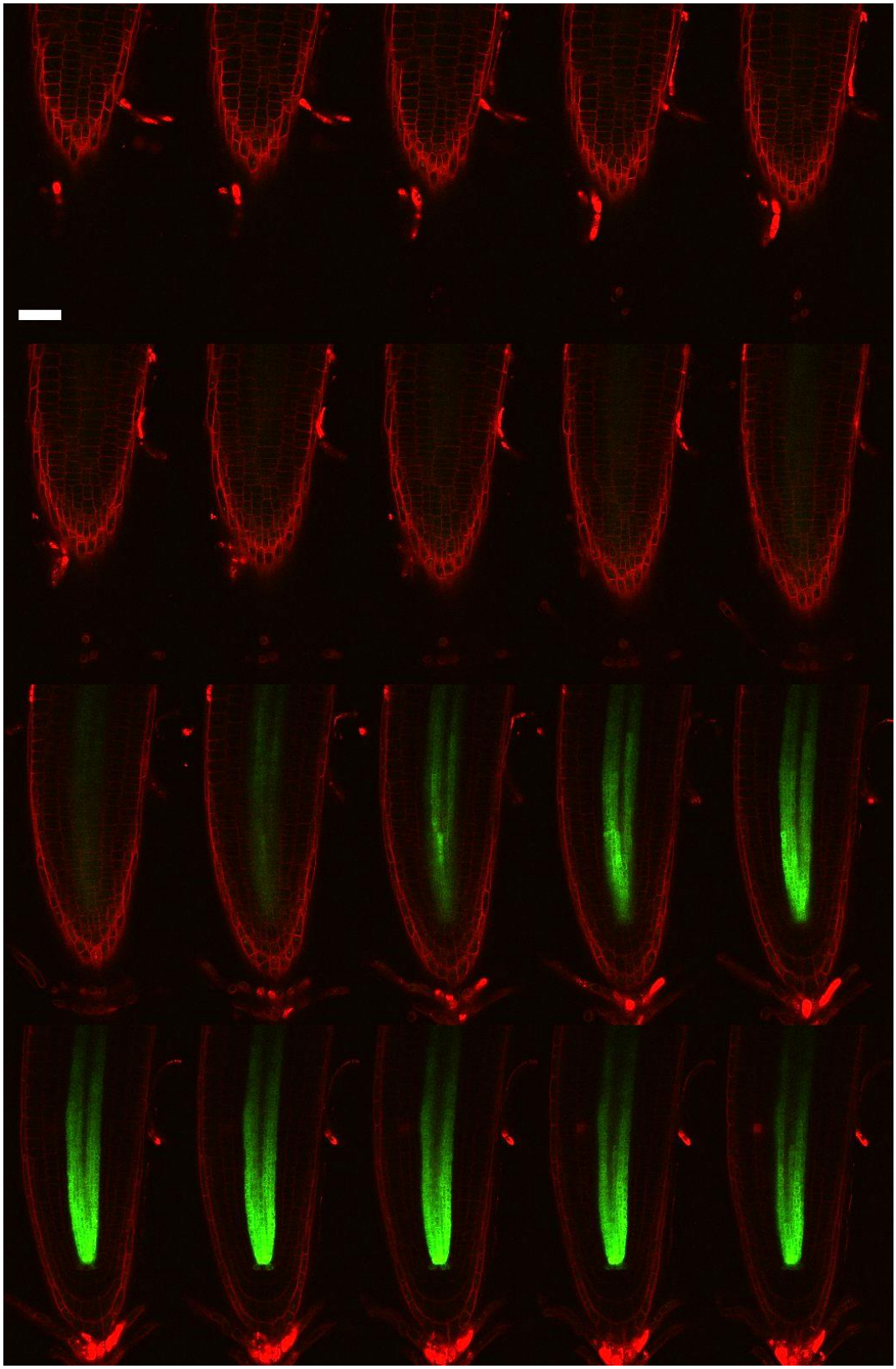
Supplementary material



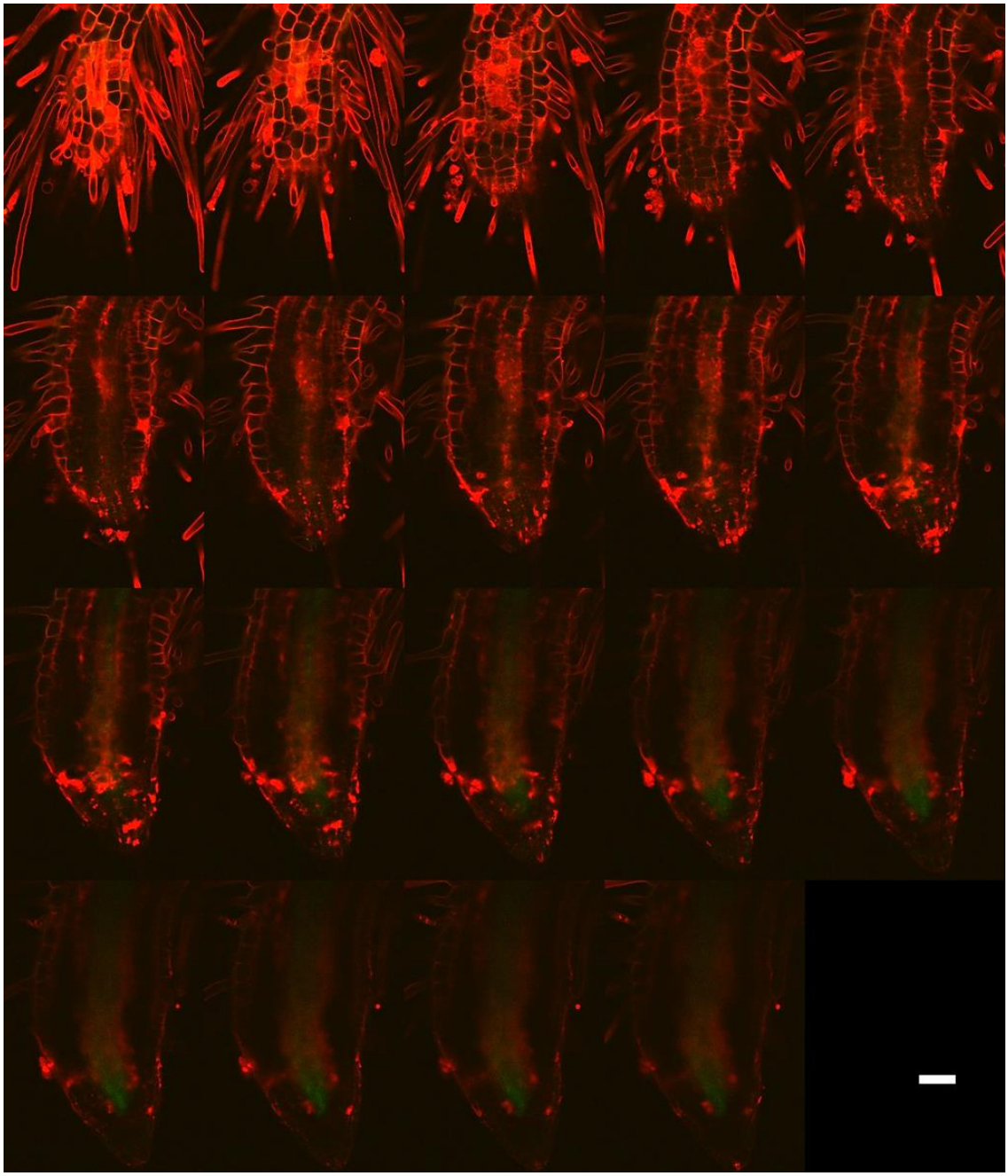
Supplementary Figure 1. Coomassie stained gel showing equilibrated loading.

Analysis of total proteins extracted from *Rhizobium leguminosarum* bv. 3841 – *Pisum sativum* (A) or *Ensifer meliloti* 1021 – *Medicago truncatula* symbiosis (B) and *Rhizobium leguminosarum* B625-*Phaseolus vulgaris* (C) grew during 1, 2, or 3 weeks after inoculation. Proteins were subjected to SDS-PAGE under reducing conditions and stained with Coomassie. (+) B-sufficient samples; (-) B-deficient samples.



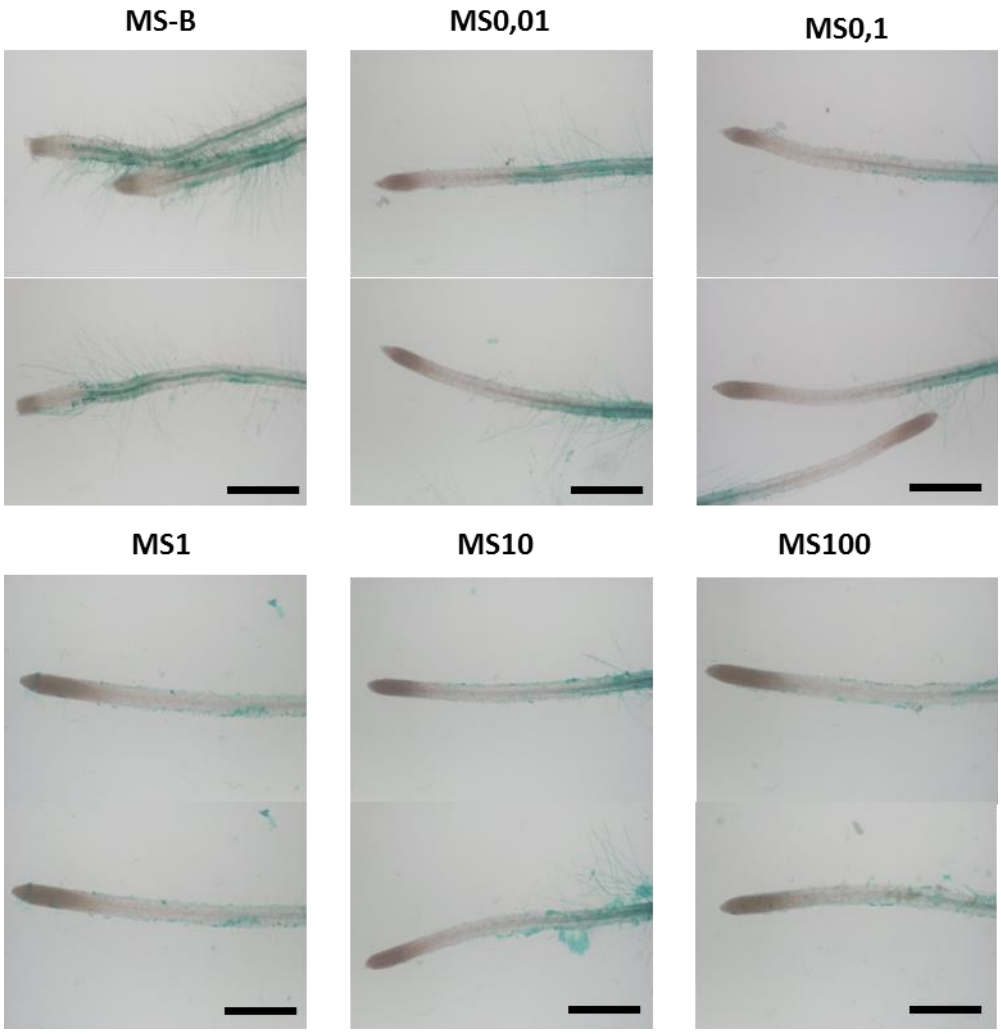
Supplementary Figure 2. Z-stack of *Arabidopsis thaliana* transgenic line Q0990 transferred to B-deficient (MS-B) media and recorded 3 days post-transference.

Arabidopsis plants grew in B sufficient conditions during 5 days were transferred to B sufficient media and photographed at 3 days post transference (dpt). Scale bar: 50 µm.



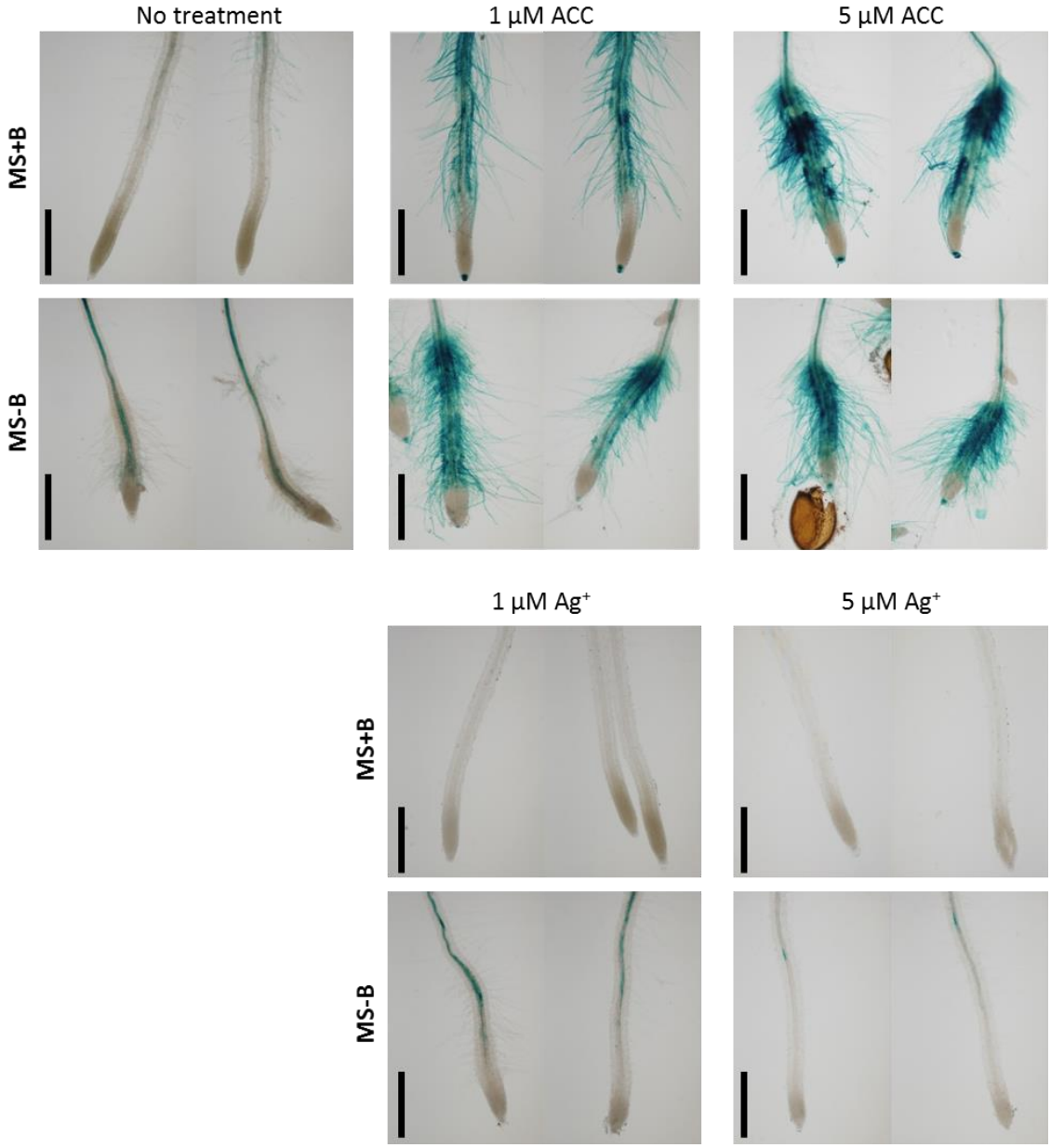
Supplementary Figure 3. Z-sack of *Arabidopsis thaliana* transgenic line Q0990 transferred to B deficient (MS-B) media and recorded 3dpt.

Arabidopsis plants grew in B sufficient conditions during 5 days were transferred to B deficient media and photographed at 3 days post transference (dpt). Scale bar: 50 µm.



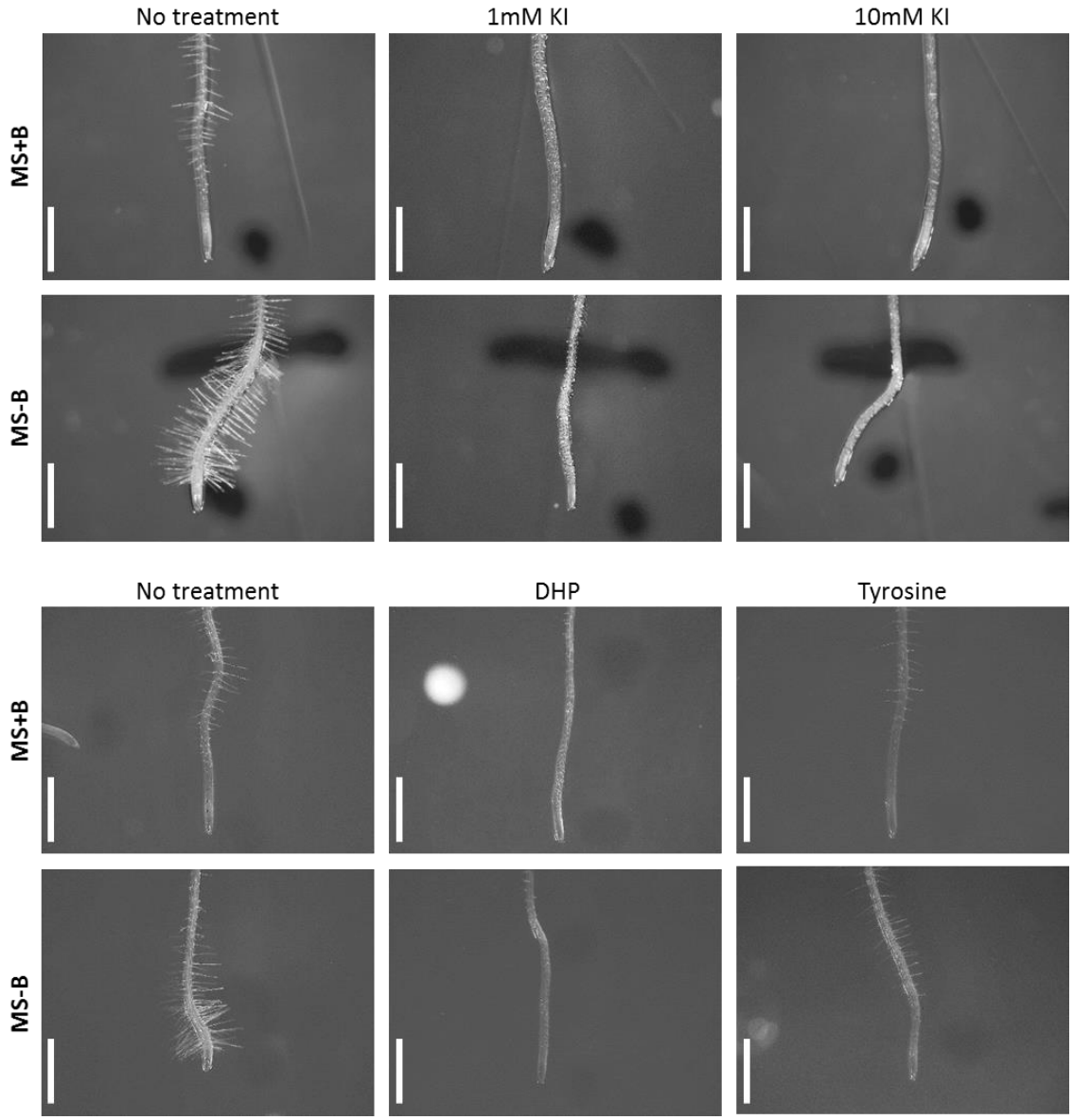
Supplementary Figure 4. EBS::GUS expression occurs close to the meristem in B-deficient Arabidopsis plants 5 dpg.

Scale bar indicates 500 µm.



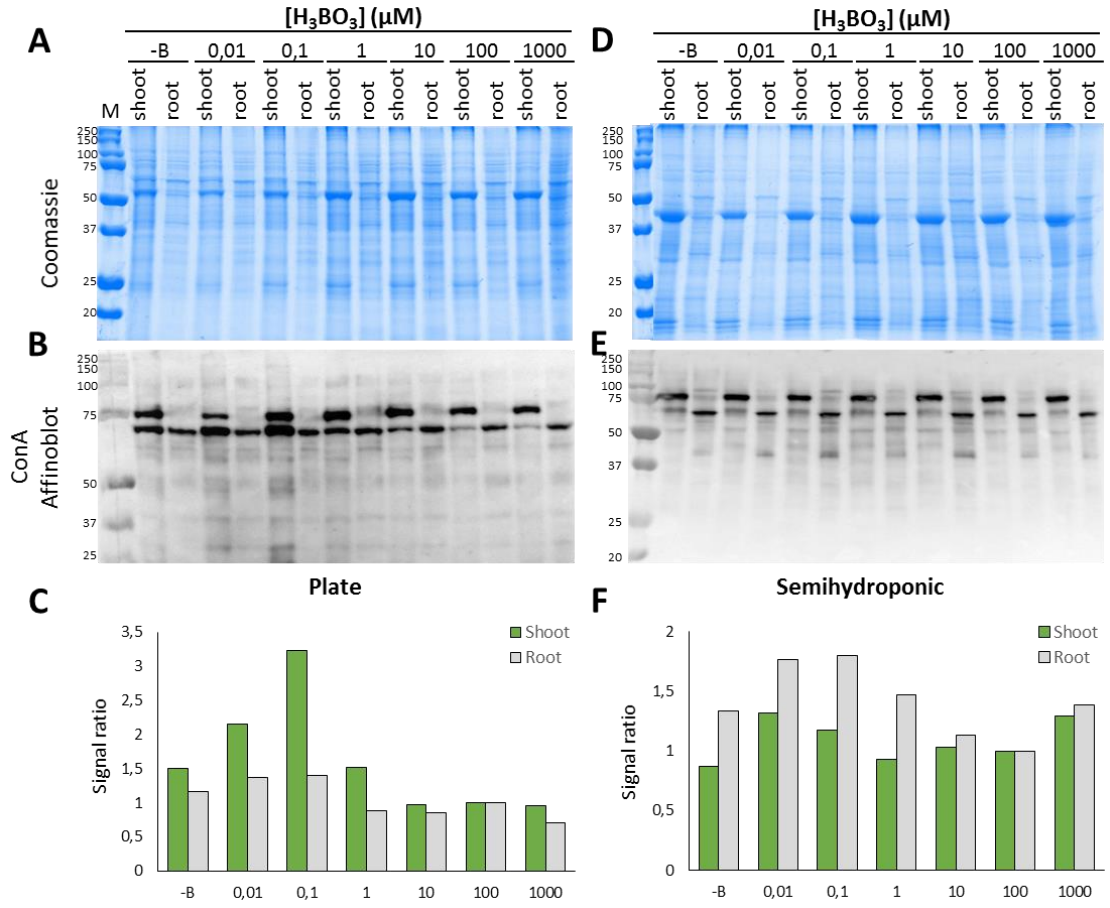
Supplementary Figure 5. Ethylene reporter *EBS:GUS* is activated by B deficiency and ethylene agonist ACC.

EBS:GUS Plants grew in B sufficient media were transferred to the indicated media supplemented with the specified treatments, GUS stained (24h) and photographed 3 days post-transference. ACC induced EBS:GUS signal, whereas Ag^{+} partially suppress EBS:GUS expression. +B: B sufficient plants; –B: B deficient plants. Bar indicates 500 µm.



Supplementary Figure 6. ROS scavenger (KI) and crosslinking competitors (DHP and Tyrosine) prevent "Hairy phenotype" but not growth arrest in Arabidopsis plants transferred to B deficiency 1 day post transference.

Bar indicates 1 mm.



Supplementary Figure 4. N-glycoprotein accumulation under low B conditions occurs is consistent under plate and semi-hydroponic growth conditions.

Analysis of total proteins extracted from wild-type (wt) plants grew in plates with the indicated concentrations of B at day 7 post germination (A, B, C) or semihydroponic system at day 21 post germination (D, E, F). Proteins were subjected to SDS-PAGE under reducing conditions, stained with Coomassie for total protein A) and D) or transferred to blots which were analysed using the lectin concanavalin A (ConA) affinoblot for Mannose-rich N-glycans (B, E). Semiquantification of MRNGPs relative to total protein is