CENTRO DE INVESTIGACIÓN Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITÉCNICO NACIONAL

UNIDAD IRAPUATO

Explorando la Función del Factor de Transcripción BOL/DRNL/ESR2 en la formación de órganos y su relación con citocininas.

Tesis presentada por:

M. en C. Yolanda Durán Medina

Para obtener el grado de:

Doctora en Ciencias

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Director de Tesis: **Dra. Nayelli Marsch Martínez**

Irapuato, Guanajuato Julio, 2018

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RESUMEN

El desarrollo de órganos en las plantas es un proceso muy importante. BOL/DRNL/ESR2 es un factor de trnanscripción de tipo AP2/ERF que se expresa en la región en donde un nuevo órgano va a desarrollarse. Se ha porpuesto a BOL como un marcador de las células fundadoras de órganos florales. Su función se ha relacionado con fitohormonas, particularmente con auxinas. Se ha propuesto que las auxinas especifican el sitio donde iniciará la formación de un órgano floral. Sin embargo la relación entre BOL y auxinas parece ser indirecta. Las citocininas son otras fitohormonas importantes para el control de la actividad meristemática y el desarrollo de órganos. La combinación entre auxinas y citocininas promueve el desarrollo de callos a partir de algunos tejidos vegetales. Un efecto de la sobre-expresión de BOL es la formación de callos en raíces de Arabidopsis. Este fenotipo entre otras evidencias sugiere una posible interacción entre BOL y la ruta de citocininas. Por tal motivo, el objetivo del trabajo en esta tesis fue realizar una exploración para obtener información que contribuya al entendimiento de la función de BOL. Esta exploración inició con la corroboración de la posible interacción de BOL con la ruta de citocininas. Como una primera aproximación se quiso saber si la pérdida de función de BOL afecta la respuesta de la planta a la aplicación exógena de citocininas. La respuesta de plantas silvestres al tratamiento de citocininas es muy fuerte en el ginece, por esta razón se usó a este órgano para evaluar la respuesta de la mutante de BOL (*drnl-2*). Con esta exploración se encontró que BOL es importante para el desarrollo del gineceo y que esta función está relacionada a AHP6 (un regulador negativo de la señalización de citocininas.

Por otra parte, para identificar a otros posibles blancos de este factor de transcripción relacionados con la ruta de citocininas se realizó un análisis de expresión diferencial utilizando el tejido aéreo de una línea inducible de BOL (*DRNL-ER*). A partir de este análisis identificamos que BOL regula la expression de *AHP6* e *IPT5*, entre otros genes relacionados con la degradación, conjugación, transporte, señalización y respuesta a citocininas. Cuando se realizaron analisis de expression más dettallados, identificamos que BOL parece estar regulando a *AHP6* e *IPT5* en el tejido vascular de diferentes órganos, lo cual se puede relacionar a su efecto en el desarrollo de callos. A partir del análisis de expression diferencial también se encontró que BOL regula la expression de genes involucrados en muchos otros procesos, tales como respuesta a varios tipos de estrés, receptores de tipo cinasas y genes involucrados con procesos clave del desarrollo tales como división celular y replicación de ADN.

ABSTRACT

The development of plant organs is a very important process. BOL/DRNL/ESR2 is an AP2/ERF transcription factor expressed in the region where an organ will develop (leaves and floral organs), However, the function of BOL in this tissue is not clear. It has been proposed as a marker of flower organ founder cells. Its function has been related to phytohormones, particularly to auxins. Auxins have been related with the specification of the site of flower organ initiation. However, the relationship between BOL and auxins seem to be indirect. Cytokinins are another phytohormone important for the control of the meristematic activity and organ development. The combination of auxins and cytokinins promotes the development of calli from some plant tissues. An effect of *BOL* over-expression is callus formation in Arabidopsis roots. This phenotype among other evidences suggests a possible interaction between BOL and the cytokinins pathway. For these reasons, the objective of the work in this thesis was to perform an exploration to obtain information that contributes to the understanding of the BOL function. This exploration began with the corroboration of an interaction between BOL and cytokinins pathway. As a first approximation we wanted to know if the BOL loss of function affects the plant response to exogenous cytokinin application. The response to cytokinin treatment in the wild type plant is very strong in the gynoecium, for this reason we used this organ to evaluate this response in the BOL mutant (*drnl-2*). With this exploration we found that BOL is important for gynoecium development and this function is related to AHP6 (a negative regulator of cytokinins).

On the other hand, in order to identify other possible targets of this transcription factor related with cytokinins pathway, a differential expression analysis using the aerial tissue of an inducible line of BOL (*DRNL-ER*) was carried out. From this analysis we found that BOL regulates the expression of *AHP6* and *IPT5*, among other genes related to degradation, conjugation, transport, signalling and response to cytokinins. When more detailed expression analyses were performed, BOL appeared to regulate *AHP6* and *IPT5* in the vascular tissue of different organs, which can be related to its effects in callus development. From the differential expression analysis, we also found that BOL regulates the expression of genes involved in many other processes such as, response to several types of stress, receptor-like protein kinases and genes involved with key processes of development such as cell division and DNA replication.

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Exploring the function of the BOL/DRNL/ESR2 transcription factor in plant organ formation and its relationship with cytokinins.

Scope of the thesis

Humans have a relatively long life cycle and during our life span we are subjected to various challenges and situations that put our survival at risk. The human body is composed of several organs and tissues that wear out as part of normal physiological functions and can be lost by disease or injury. Could you imagine a completely different reality in which the wear out and loss of organs wouldn´t imply a problem for us, because our organism has the capacity to form organs in a constant way? Unfortunately, this doesn´t happen, but there are organisms that have this wonderful characteristic. Plants are multicellular organisms that possess potential capacity for unlimited growth throughout their life cycle (Srivastava, 2003).

It´s interesting to explore this wonderful plant ability to form organs in a constant way that humans do not have. For this reason, this project was based on the study of a marker of organ founder cells (Chandler et al., 2011b). This marker is an AP2/ ERF transcription factor of *Arabidopsis thaliana* known variously as as BOLITA, DÖRNROSCHEN-LIKE, ENHANCER OF SHOOT REGENERATION2, and SUPPRESSOR OF PHYTOCROME B (BOL/DRNL/ESR2/SOB) (Martínez et al., 2006; Ikeda et al 2006; Ward et al., 2006; Chandler et al., 2007). This transcription factor from now on will be named as BOL.

BOL is expressed in restricted regions just before organ primordium emergence and its expression is maintained in organ primordia and young organs. It has been attributed several functions that have arisen mainly from observed phenotypes generated by its overexpression (i. e., *35S::ESR2-ER*) and or by gain or loss of its function (i. e., *bol-D* and *drnl-2*) (Marsch-Martínez et al., 2006; Ikeda et al 2006; Ward et al., 2006; Chandler et al., 2007; Nag et al., 2007). The *BOL* loss of function and over-expression cause severe alterations in morphology and organ development in Arabidopsis, suggesting that it plays an important role at early stages of organ development (Marsch-Martínez et al., 2006; Nag

et al., 2007). However, it is not yet known what role BOL plays during new organ development.

The *BOL* expression pattern, as well as the *BOL* over-expressing phenotypes and data from microarrays (Marsch-Martínez et al., 2006, Ikeda et al., 2006) suggest a connection of this transcription factor with plant growth regulators (PGR) or phytohormones. Auxins and cytokinins are PGR that act together to regulate organ development. Because a direct and clear relationship of this transcription factor with auxins has not been identified, the objective of this work was to understand BOL function during organ development and to determine if this function is also related to cytokinins.

In order to achieve this aim we proceeded to obtain information from the *BOL* loss of function and over-expression phenotypes. Different tissues and organs were analyzed and these analyses are organized as different chapters in this thesis. The first part of this study was to determine whether *BOL* had a function during gynoecium development (chapter II), an organ where its function had been barely studied. The question of its function in this organ arose because *BOL* is expressed during leaf primordia development, and its role in gynoecium development was interesting to evaluate, considering the hypothesis that carpels and leaves are evolutionarily related organs (Alonso-Cantabrana et al., 2007). The objective in the second part was to identify possible BOL cytokinins-related targets using vegetative aerial tissue (chapter III) and in the last part we sought to perform a global gene expression analysis in order to identify other process regulated by BOL (chapter IV).

CHAPTER I

I.1 Background

While all organs present in an adult animal are formed during embryogenesis, embryogenesis in plants results in a simple organism consisting of a hypocotyl with one or more cotyledons, the shoot apical meristem (SAM) and the embryonic root with a root apical meristem (RAM) (Figure 1.1). The rest of the organs of a mature plant are formed post-embryonically thanks to the presence of these initial meristems also called primary meristems. These primary meristems, SAM and RAM, produce the aerial and subterranean parts of plant body, respectively, whereas additional or secondary meristems may develop later in development (Figure 1.2). The secondary meristem allows the plant to grow radially and provides cells for vasculature development and for mechanical support structures (Jouannet et al., 2015).

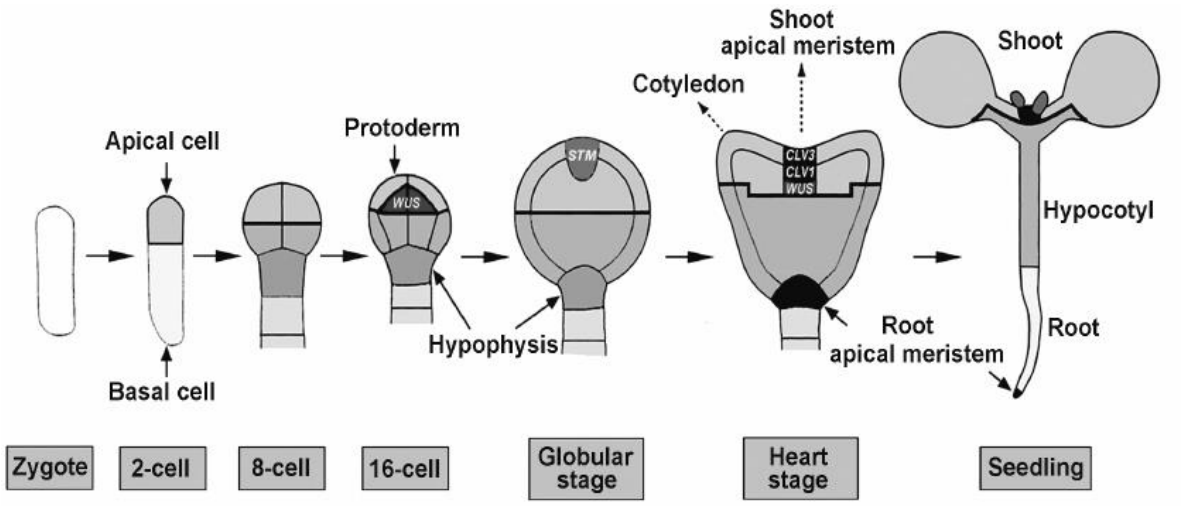

Figure 1.1 Formation of the shoot apical meristem (SAM) and root apical meristem (RAM) during Arabidopsis embryogenesis. The cotyledons and the hypocotyl are also originated during embryogenesis (Figure modified from Lee, 2014).

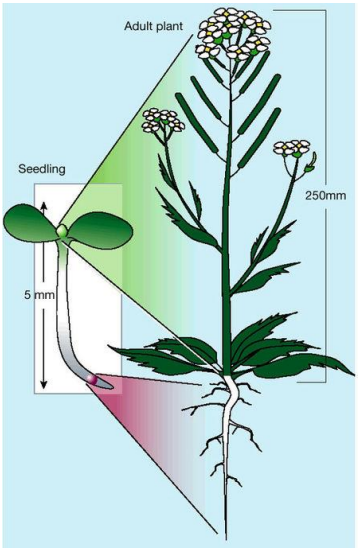

Figure 1.2 Organs and tissues present in an adult plant develop from primary meristems, SAM and RAM (Weigel and Jürgens, 2002).

I.2 ¿What is a meristem?

Meristems generate the plant body by producing cells that will become organs, such as leaves, stem, roots and flowers. This suggests that there are different types of meristems. However, in a broad sense a meristem can be defined as a pool of pluripotent stem cells (SC) which are embedded in specialized tissues and produce daughter cells that differentiate into distinct cell types (Ichihashi and Tsukaya, 2015). Some molecular developmental biologists adopt a narrower definition of meristems as proliferating tissues that maintain self-renewing stem cells (Hay and Tsiantis, 2005; Brukhin and Morozova, 2010; Miyashima et al., 2013). However this definition excludes some tissues than can also be considered as meristematic as some that are located in leaves and those that give origin to floral organs. These meristems are active only for a short time and are used in formation of those organs; sometimes they are referred to as determinate meristems (Srivastava, 2003).

I.3 Primary meristems

I.3.1 Shoot Apical Meristem

The shoot apical meristem is located at the apex of the stem. This meristem can be divided into 2 distinct histological zones: the central zone (CZ) and the peripheral zone (PZ) (Shani et al., 2006). Pluripotent cells, also called stem cells, are located in the central zone at the apex of the shoot and function as a pool of undifferentiated cells to replace organs and sustain postembryonic growth, whereas organ initiation takes place in the peripheral zone (Figure 1.3). The apical meristem of the vegetative stem is very repetitive in its activity, since it produces the same structures (leaves, lateral buds and stem tissues) over and over again. Its activity is periodic but at the same time indeterminate. In plants like Arabidopsis, this meristem begins as vegetative and later becomes a reproductive meristem, called inflorescence meristem. Instead of producing leaves, or vegetative tissue, the inflorescence meristem produces floral meristems which in turn give rise to different organs of a flower: sepals, petals, stamens and gynoecium (Fornara et al., 2010, Krizek and Fletcher, 2005).

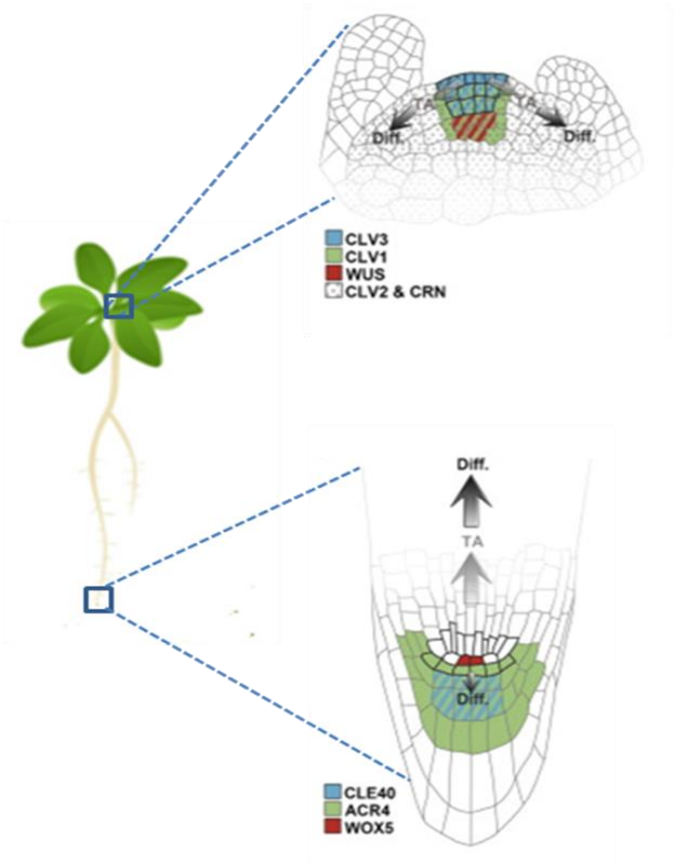

Figure 1.3 Schematic representation of the shoot and root apical meristems. Stem cells are outlined in bold. The expression domains of the main genes that control meristematic activity are color coded. TA = transit-amplifying cells; Diff. = cell differentiation (Modified figure from Stahl and Simon, 2010).

The floral meristem is a determined meristem, because it produces a definite organ number and stops its activity by producing the last organ, the gynoecium (Prunet et al. 2009; Fosket, 1993).

I.3.2 Carpel Margin Meristem (CMM)

Fruit patterning is established at the onset of gynoecium development, although different tissues arise at different stages of gynoecium development, and some of them have meristematic qualities. The gynoecium is made of two congenitally fused carpels that arise from the terminating floral meristem. It then grows to form a cylinder. Interestingly, tissue that is mechanistically similar to the SAM is conserved inside the growing cylinder (Pautot et al., 2001; Girin et al., 2009). This tissue is located along each gynoecium medial domain. It is also called the carpel margin meristem (CMM) (Reyes-Olalde et al, 2013; Wynn et al, 2011). As development continues, the CMM gives rise to the carpel marginal

tissues, which include the placenta, ovules, septum, transmitting tract, style, and stigma (Figure 1.4). On the other hand, the lateral regions of the developing gynoecium express genes that function during leaf development (Alonso-Cantabrana et al., 2007).

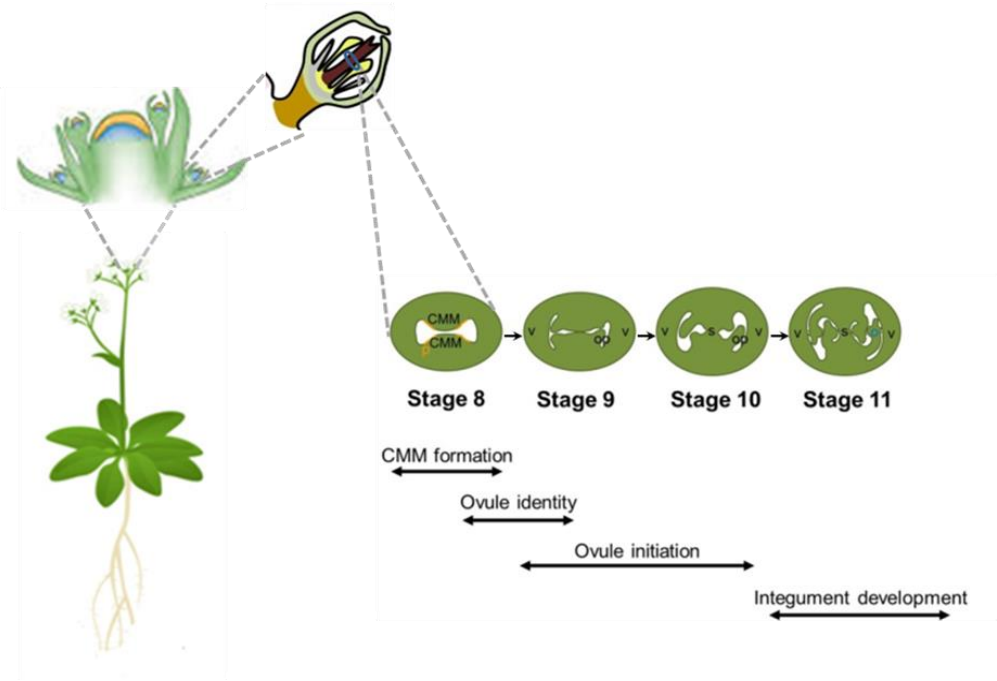

Figure 1.4 Schematic representation of carpel marginal meristem (CMM). Illustrations of gynoecium transversal sections at different developmental stages are shown. In gynoecium transversal sections, tissues and organs developed from CMM can be observed. Abbreviations: CMM, carpel margin meristem; o, ovule; op, ovule primordium; p, placenta; s, septum; v, valve. The region of the CMM where the placenta is formed is indicated with orange lines. (Modified figure from Cucinotta et al., 2014 and Denay et al., 2017).

I.3.3 Root apical meristem

In contrast to the shoot apical meristem, the root apical meristem is covered by a protective tissue known as caliptra or cap. The root apical meristem also differs from the shoot apical meristem in that it does not produce lateral organs. Instead, this meristem produces cells that will form part of the caliptra and the cells that will contribute to the growth of other

different tissues of the root axis. The lateral roots are formed by adventitious meristems that later arise in mature regions of the main root (Fosket, 1993). The root meristem has three main regions: the meristematic zone, the elongation zone and the differentiation zone. The meristematic zone contains the stem cell niche, comprising the quiescent zone (QC) which controls the surrounding stem cells (also called initial cells). After division of an initial cell, the daughter cell still in contact with the QC keeps its stem cell fate, whereas the other cell becomes a transit-amplifying cell (TA cell) and after further divisions and expansion in the elongation zone, acquires its cell fate in the differentiation zone (Figure 1.3) (reviewed in Scheres et al., 2002; Stahl and Simon, 2010).

I.4 Secondary meristems

I.4.1 Procambium and cambium

In addition to stem cells in the SAM and RAM, vascular stem cell niches, also called procambium and cambium, are present in vascular plants. This pool of stem cells, via asymmetric periclinal cell division, continuously produces xylem and phloem, the major plant vascular tissues (Figure 1.5) (Miyashima et al., 2013).

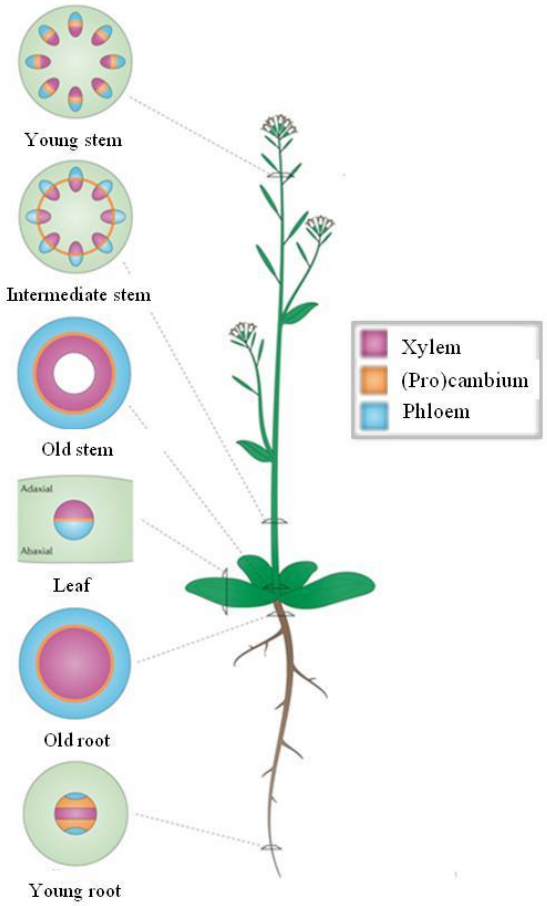

Figure 1.5 (Pro) cambium (meristematic tissue) distribution along plant architecture. This meristematic tissue is located between the vascular tissue. The exact architecture of vascular tissues differs among the individual organs of the plant (Modified figure from De Rybel et al., 2014).

The (pro) cambium contains vascular stem cells that originate from the apical meristems. Procambial cells are cytoplasmically dense, and they appear as continuous strands of narrow, elongated cells (Mähönen 2005). Whereas a proportion of procambial cells differentiate into various xylem and phloem cell types, the remaining cells persist undifferentiated as the tissue matures. The procambium in young plant organs promotes the growth of vascular tissues in the apical direction (primary growth) (Zhou et al., 2011). Later during development, these procambial cells form the vascular cambium, which is a secondary meristem that undergoes periclinal cell divisions to produce new cells on each side of the meristem (De Rybel et al., 2014). Cell division and differentiation in the cambium lead to the thickening of stems and roots and thereby increase the biomass (e. g., secondary growth). Secondary growth massively occurs especially in woody plants (Miyashima et al., 2013).

I.5 Auxins and cytokinins as important elements for cell fate acquisition

Plan growth and development depends on the continuous function of meristems, but how do meristematic tissues contribute to this process? In the shoot apical meristem it is known that as stem cells divide in the CZ, part of their progeny is pushed into the PZ where they will adopt a cell type specification (Gaillochet et al., 2015). Cell type specification is the reversible acquisition of fate by a single cell or a group of founder cells that then are activated to undergo coordinated cell division to form a primordium and then an organ (Chandler and Werr , 2015).

How can cells acquire a specific fate? Since plant cells cannot actively migrate due to their rigid walls, cells are passively displaced by cell divisions. Thus, cell fate needs to be continuously adjusted to the current position, sometimes resulting in multiple fate switches until the cell is incorporated into a specific tissue. While cells are displaced they are exposed to different biochemical signals (Gaillochet et al., 2015). Among these biochemical signals, there are plant growth regulators (PGR), also called phytohormones.

Various phytohormones directly or indirectly participate in cell fate determination and sometimes cooperate differentially in many developmental contexts (Chandler and Werr , 2015). Auxins and cytokinins have been identified as two important phytohormones that regulate cell fate in many developmental processes.

I.5.1 Auxins

Auxins are the best studied phytohormone. This phytohormone has emerged as one of the main coordinators of plant development, since it has been related to the initiation and positioning of organs, such as leaves, flowers and lateral roots. The main natural auxins is indoleacetic

acid (IAA). IAA has been implicated in many aspects of plant growth and development, as well as in defense response. This diversity of functions is reflected by the complexity in the route of biosynthesis, signalling and transport of IAA (reviewed in Schaller et al., 2005).

I.5.1.1 Metabolism, signalling and transport of auxins

The most abundant auxin, indole-3 acetic acid (IAA), is primarily synthesized in a two-step process. In the first step, tryptophan is converted to indole-3-pyruvate by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) family of transaminases (Stepanova et al., 2008; Tao et al., 2008). In the second step, indole-3-pyruvate is converted to IAA by the YUCCA family of flavin monooxygenases (reviewed in Zhao, 2014). There is evidence for other pathways of auxins biosynthesis, in particular a Trp independent pathway. The PIN-FORMED (PIN) family of auxins efflux carriers transports auxins throughout the plant in a polar manner. Auxins are transported into cells by the AUX/ LAX family of proteins. The MULTIDRUG RESISTANCE (MDR) p-glycoprotein (PGP) family of proteins also plays a role in auxins transport and likely act in concert with the PINs to regulate the distribution of auxins. Unlike the polarly localized PINs, the MDR/PGP proteins are uniformly localized around the cell (reviewed in Zazímalová et al., 2010). Auxins levels can also be regulated through conjugation. The auxins-inducible GH3 family of acyl acid amido synthetases catalyzes the conjugation of amino acids to IAA (reviewed in Ludwig-Müller 2011). The perception of auxins involves the Aux/ IAA family of transcriptional repressors, the auxins response factor (ARF) transcription factors, and the TIR1/AFB1-AFB5 F-box components of the SCF complex (Peer 2013, Salehin et al., 2015). In the presence of low levels of auxins, the Aux/ IAA proteins, together with the TOPLESS transcriptional repressor, bind to the ARFs to block their function. In the presence of elevated auxins, the Aux/ IAA proteins form a complex with the TIR1/ AFB1-AFB5 proteins, with IAA acting as “molecular glue” to hold the components of the co-receptor together. The formation of this complex results in the ubiquitination of the Aux/ IAA proteins and their subsequent degradation by the 26S proteasome, thus relieving their repression of the ARFs. The activated ARFs then modulate

the expression of a large suite of auxins-regulated genes through their binding to auxins response elements (AuxREs) (reviewed in Schaller et al., 2005) (Figure 1.6).

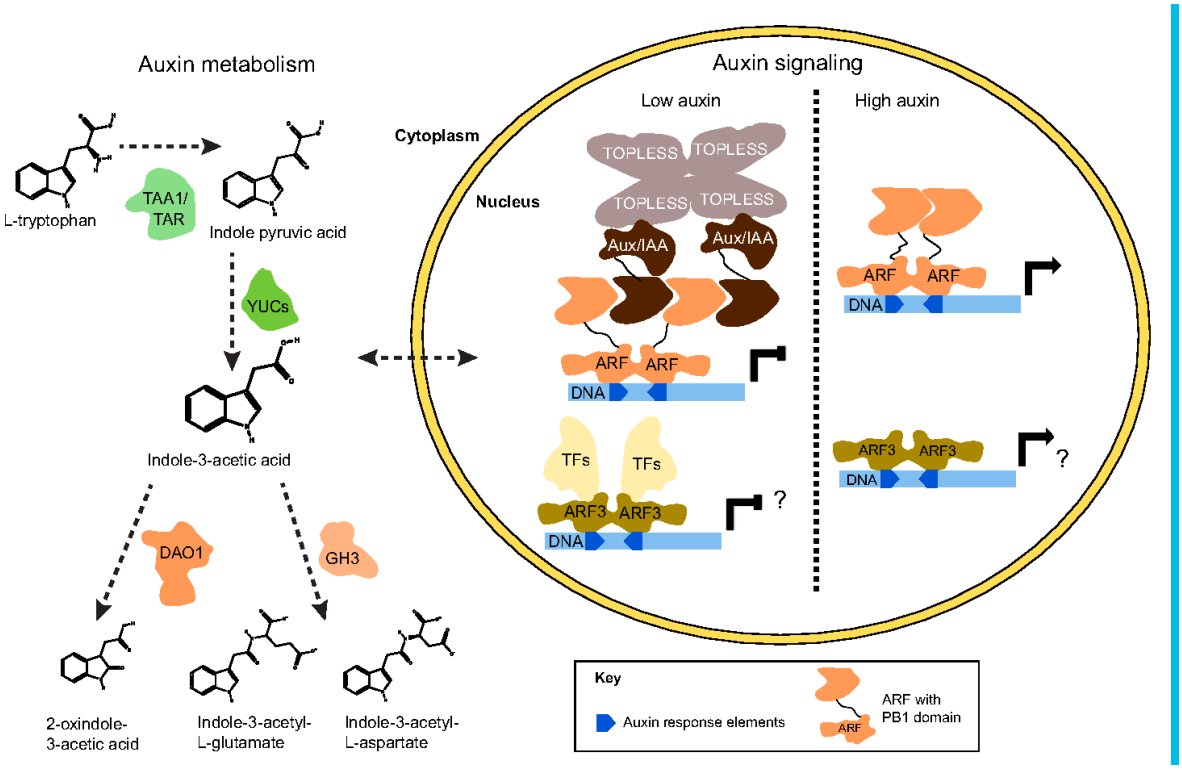

Figure 1.6 Key aspects of the auxins pathway. The main auxins biosynthetic pathway involves the enzymes TAA1/TAR and YUC. Auxins levels are also regulated by oxidation and conjugation through DAO1 and GH3. Auxins regulate transcription in the nucleus by triggering the degradation of Aux/ IAAs, thereby releasing auxins response factors (ARFs) from their repression. Different types of complexes that potentially bind to promoters of target genes are represented on the figure. Auxins can also directly regulate the interaction between ARF3 and other transcription factors (TFs), as depicted (Vernoux and Robert 2017).

The presence of auxins response maxima, as determined via reporter gene expression from the synthetic auxins responsive DR5 promoter, correlates with sites of lateral organ initiation (Heisler et al, 2005; Dubrovsky et al., 2008). This observation, as well as the

phenotypic effects of alterations in auxins signalling, biosynthesis or transport (Reinhardt et al., 2000; Cheng et al., 2006; Stepanova et al., 2008) have often led to the idea that auxins alone provide an instructive signal for organ initiation or specification (Chandler and Wer et al., 2015).

I.5.2 Cytokinins

Cytokinins (CKs) are molecules derived from adenine with a side chain attached to the amino group at position 6 of the purine ring. The naturally occurring CKss trans-zeatin (tZ), N6 (D2-isopentenyl) adenine (iP), cis-zeatin (cZ), and dihydrozeatin (DZ) are widely found in higher plant species (Hirose et al., 2008). Cytokinins were originally identified based on their ability to promote cell division in plant cells. Since the discovery, a plethora of Cytokinins biological functions have been discovered. Among these are: embryogenesis control, root and shoot meristem activity, vasculature and organ development, nodule formation, apical dominance and response to environmental stimuli (reviewed in Osugi and Sakakibara, 2015; and Zürcher and Müller, 2016). Their functions are complex and context-dependent (reviewed in Hwang et al., 2012).

I.5.2.1 Cytokinins biosynthesis and inactivation.

The beginning of cytokinins biosynthesis is the addition of a prenyl moiety from dimethylallyl diphosphate to ATP/ ADP to yield N6-isopentenyladenine (iP) ribotides. This step is catalyzed by isopentenyltransferases (IPTs) (Sakakibara, 2006). The Arabidopsis genome encodes nine *IPT* genes (*IPT1* to *IPT9*), seven of which are involved in cytokinins biosynthesis, while the other two act to modify a subset of adenine bases on tRNA (Kakimoto, 2001; Takei et al., 2001). The iP ribotides are subsequently converted to trans-zeatin (tZ)-type cytokinins by hydroxylation of the isoprenoid side chain by the cytochrome P450 enzymes CYP735A1/ CYP735A2 (Takei et al., 2004). The active forms of cytokinins are made from cytokinins ribotides by the LONELY GUY (LOG) family of cytokinins nucleoside 59 monophosphate phosphoribohydrolases (Kuroha et al., 2009) (Figure 1.7). On the other hand, the conjugation to glucose decreases the level of active cytokinins

(Bajguz and Piotrowska, 2009). Cytokinins levels are also regulated by the irreversible inactivation through cytokinins oxidases, copper-dependent amine oxidase enzymes that cleave the N6-side chains from tZ- and iP-type cytokinins (Schmülling et al., 2003; reviewed in Schaller et al., 2015).

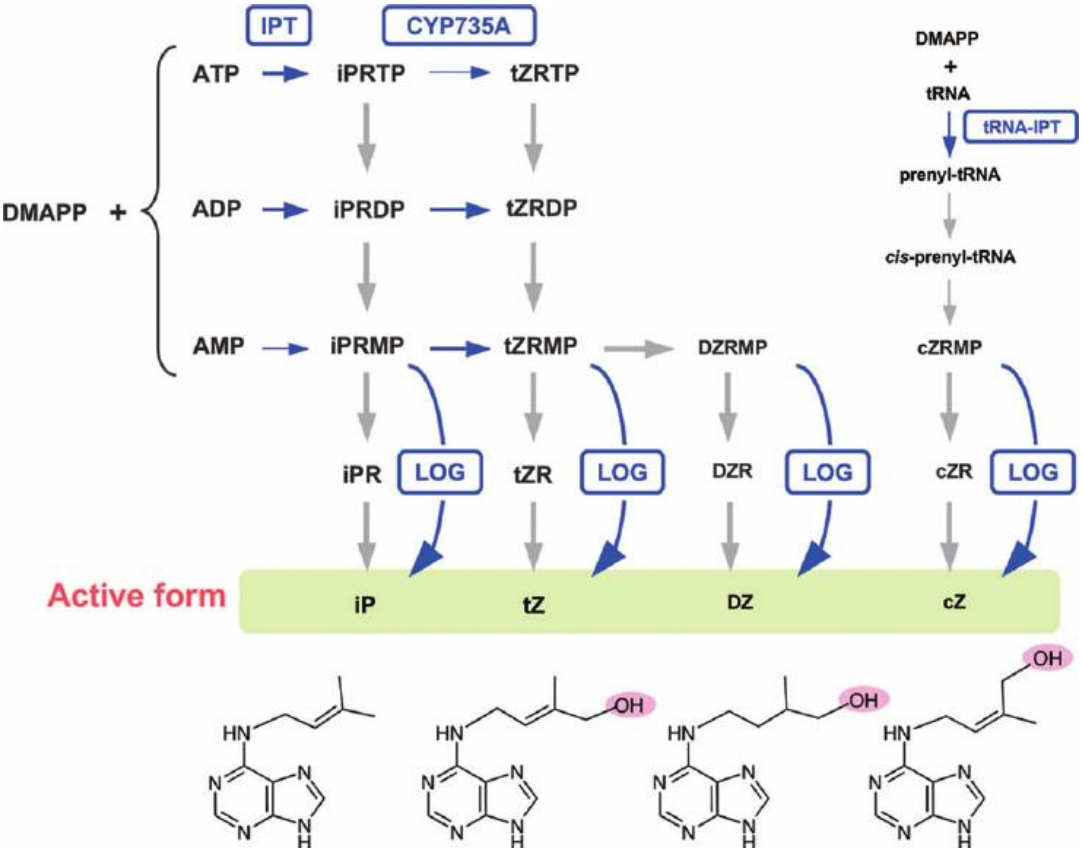

Figure 1.7 Key steps in the cytokinins biosynthesis pathway. Biosynthesis of iP-cytokinins and tZ-cytokinins is initiated by IPTs to form iP-nucleotides which can be converted to the corresponding tZ-nucleotides by CYP735As. Active free bases are produced by LOG enzymes. *cis*-Zeatin (cZ) cytokinins are synthesized in Arabidopsis exclusively by tRNA-IPTs which utilize tRNAs as prenyl acceptors. Blue arrows indicate reactions in which the genes encoding the enzymes are known, whereas grey arrows indicate those that have not been identified (Hirose et al., 2008).

I.5.2.2 Cytokinins signalling

Plants respond to CK via a two-component signalling pathway (or TCS). This pathway involves three components: a “hybrid” receptor kinase that contains both histidine-kinase and receiver domains in one protein, a histidine-containing phosphotransfer (AHP) protein, and an ARABIDOSPIS RESPONSE REGULATOR (ARR). Arabidopsis has three histidine kinase proteins: AHK2, AHK3 and CRE1/WOL/AHK4 that contain a conserved cytokinins binding CHASE domain, a histidine kinase domain, and a receiver domain (West and Stock, 2001). In this multistep pathway, the phosphate is transferred from His to Asp. The AHPs are the downstream targets of the AHK receptors and act as intermediaries in the transfer of the phosphate to the downstream response regulators (ARRs). There are five AHPs in Arabidopsis (AHP1-AHP5) with a positive function in cytokinins signalling (Hutchison et al., 2006; Hutchison and Kieber, 2007). In contrast, a sixth AHP (AHP6), without the His target of phosphorylation, negatively regulates cytokinins responsiveness, via an inhibition of the phosphotransfer reaction among the functional two-component elements (Mähönen et al., 2006).

The downstream targets of the AHPs, the ARRs, fall into two main classes, type-A and type-B ARRs. In both classes, an N terminal receiver domain harbors an Asp residue that is the target of the phosphate transfer. The type-B ARRs are transcription factors that contain a DNA binding Myb domain. The protein levels of at least a subset of the type-B RRs (ARR1, ARR2, and ARR12) are regulated by the KMD family of F-box proteins. Type-A ARRs lack a DNA binding domain and negatively regulate cytokinins signalling (reviewed in Schaller et al., 2015). Other proteins, such as the cytokinins response factors (CRFs), have also been shown to interact functionally with this pathway. CRFs were placed downstream in the signalling pathway of AHPs and probably function in parallel with the type-B ARRs in their action on cytokinins-regulated targets (Figure 1.8). CRFs are members of the AP2/ ERF family of transcription factors, containing a single AP2– DNA binding domain (Rashotte et al., 2006).

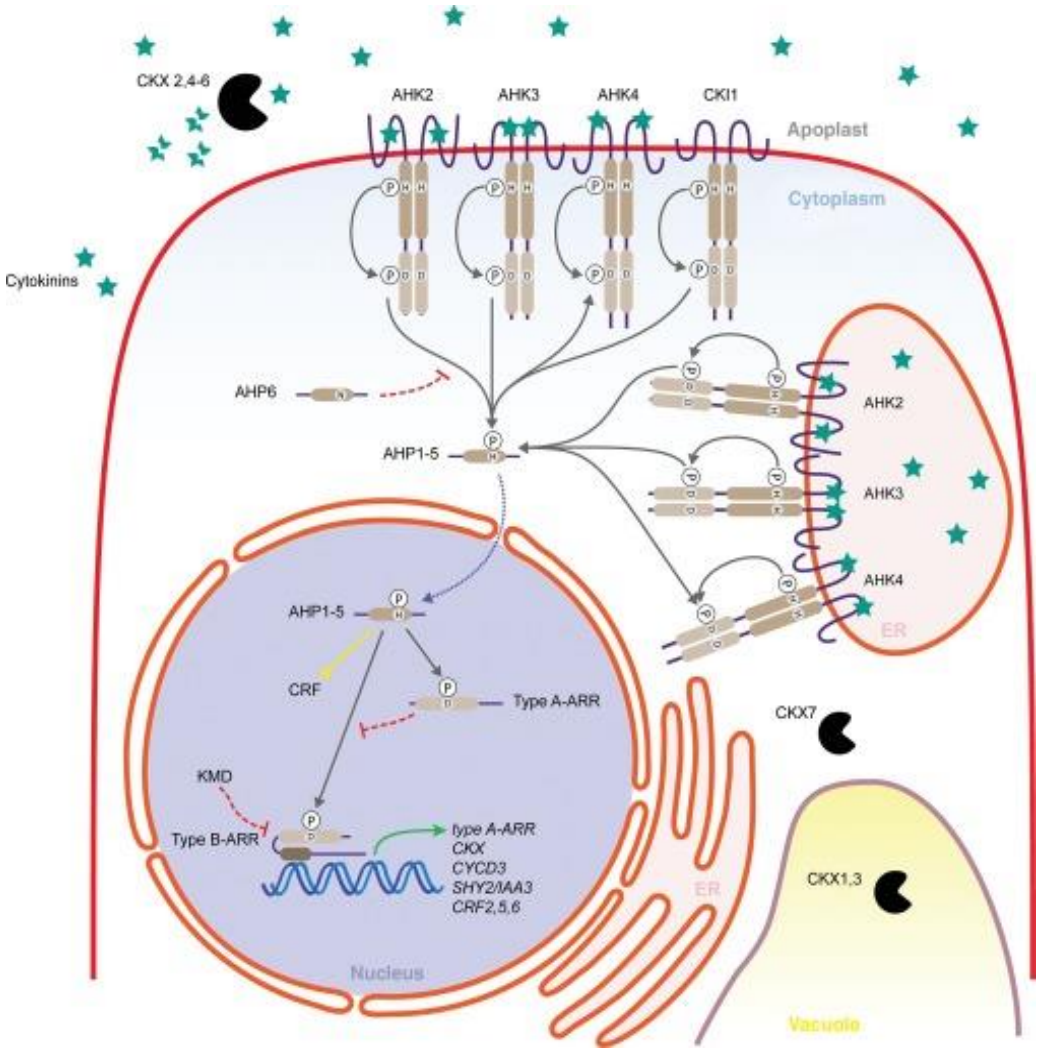

Figure 1.8 Model depicting cytokinins signal transduction pathways. Cytokinins are perceived by AHK receptors present in the membrane. CKXs in the cytosol and apoplast regulate cytokinins levels. The cytokinins signal is amplified by phosphorylation events that promote the activation of AHP proteins. AHP proteins transfer the phosphate group to type A or B ARR proteins (Zurcher and Müller, 2016).

I.6 Developmental context of cytokinins-auxins crosstalk

Auxins and cytokinins have a dynamic interaction. This interaction is tissue-or context-specific and depending on the circumstances, it can be antagonistic or supportive to confer distinct cell fates. There are many examples about how auxins and cytokinins interaction promote cell fate acquisition in different tissues. However, some of the best characterized

have been described in the root (Chandler and Werr, 2015). A description of relevant examples of the latter follow:

Root-pole specification during embryogenesis: One example of a single-cell fate determination is when, after the division of the hypophysis (the embryo upper suspensor cell), the upper and lower daughter cells are re-specified to generate two distinct stem cell pools. The upper, lens-shaped cell generates the quiescent centre, and the lower cell, the columella stem cells. To acquire a different cell fate, the upper lens-shaped cell maintains cytokinins signalling and low auxins response, while the lower cell shows high auxins response, which transcriptionally up-regulates the A-type negative cytokinins ARR7 and ARR15 (Figure 1.9) (Müller and Sheen 2008; reviewed in Chandler and Werr 2015).

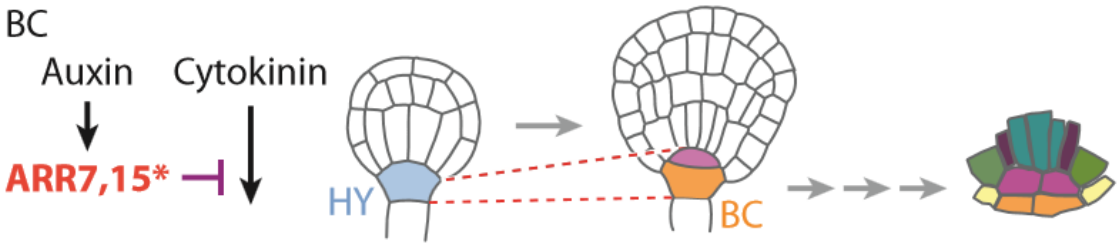

Figure 1.9 Root stem cell niche determination. Induction of *ARR7* and *ARR15* by auxins attenuate cytokinins output in the basal cell of the embryonic root. This auxins-dependent suppression of cytokinins output in the basal cell (BC) lineage after asymmetrical division of the hypophysis (HY) is required for correct establishment of the root meristem, as shown on the right, with different colors denoting the distinct stem-cell fates and precursor cells (Hwang et al., 2012).

Lateral root initiation: Lateral root development involves *de novo* meristem establishment from root pericycle founder cells adjacent to the xylem poles**.** Auxins and cytokinins have antagonistic roles in lateral founder cells determination. An auxins maximum in a single or a pair of pericycle cells is sufficient for lateral root initiation. This auxins maximum can be induced by local auxins synthesis or by polar auxins transport via PINFORMED3 (PIN3) (Marhavy et al., 2013; reviewed in Chandler and Werr 2015). While CKs inhibit lateral root

initiation, ectopic CK signalling perturbs auxins partly by altering *PIN1, 3,* and *7* transcription, which affects PIN-dependent lateral root initiation (Laplaze et al., 2007, Hwang et al., 2012). Recent findings show that CK signalling-mediated by the receptor AHK4/ CRE1/ WOL and the type-B ARR2 and ARR12 also control PIN1 localization (Figure 1.10) (Marhavy et al., 2011; reviewed in Chandler and Werr, 2015).

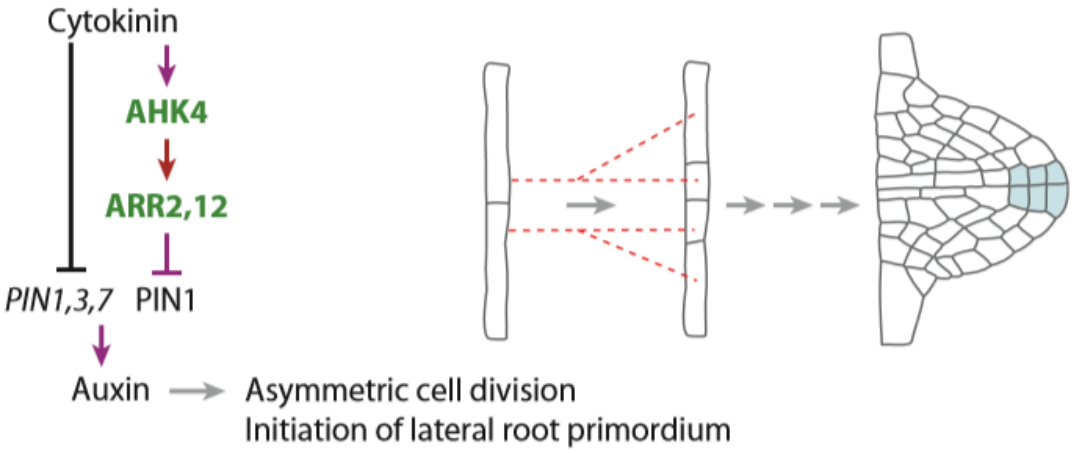

Figure 1.10 Lateral root initiation. During lateral root meristem initiation, for the asymmetric cell divisions of pericycle-derived founder cells, an auxins maximum is necessary. This auxins maximum is mediated by polar auxins transport. Cytokinins signalling repress the formation of LR by directly affecting PIN distribution patterns during early stages of organogenesis (Hwang et al., 2012).

Root vasculature specification: Cells within the xylem axis show high auxin response (Bishopp et al., 2011), while cells within phloem/(pro)cambial domains show the highest cyrokinins response (Mähönen et al., 2006; Schaller et al., 2015)**.** Inhibitory interactions contribute to maintain these domains. In the protoxylem, *AHP6* is positively regulated at a transcriptional level by auxins. AHP6 negatively regulates CK signalling in the protoxylem while PIN1 maintains a high auxins response in the protoxylem to create a file of cells with a high auxin and low CK response (Bishopp et al., 2011). Adjacent cambium cells, in contrast, have high CK and low auxins activity because the polarity of PIN7 in the lateral membranes of procambial cells predominantly directs auxins efflux into the protoxylem,

and the resulting depletion of auxins in the cambium releases the CK response from repression by AHP6 (Figure 1.11) (Bishopp et al., 2011).

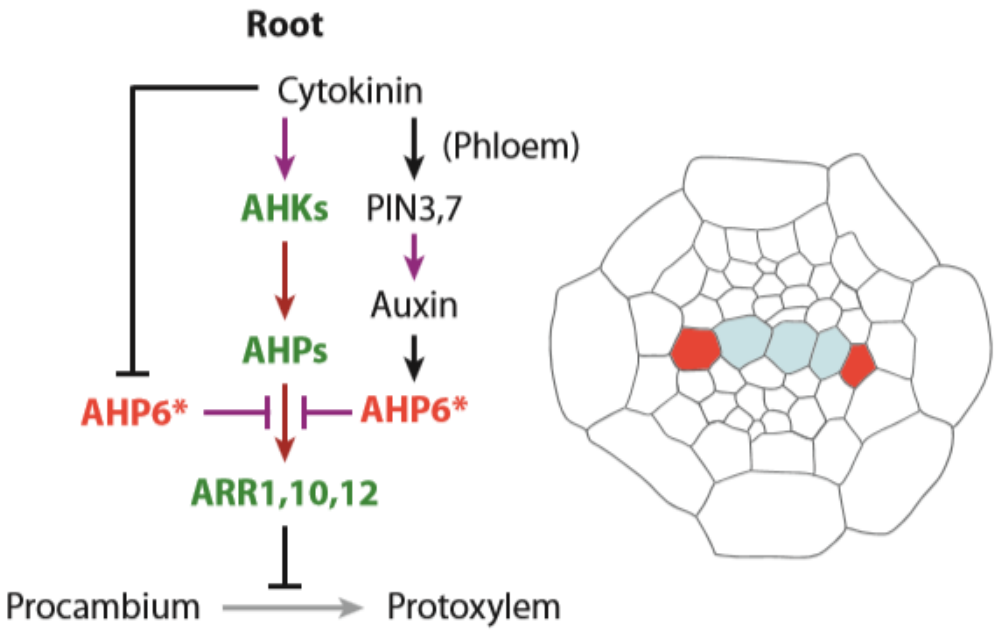

Figure 1.11 Vasculature development. During root vasculature development, CK signalling is required for maintenance of (pro)cambial cells and suppresses the expression of the CK signalling inhibitor AHP6 (red) in the (pro)cambial cells flanking the xylem axis (blue). Phloem-transported CKs direct the auxins flow into the xylem axis by modulating the distribution of PIN3 and PIN7. A high auxin level promotes expression of *AHP6* (red) at the xylem axis, which specifies the differentiation of the protoxylem (Hwang et al., 2012).

Organ initiation and phyllotaxy in the inflorescence meristem: Recent findings indicate that the timing of primordia initiation is also regulated by interplay between auxins and cytokinin signalling. Floral Meristem (FM) initiation in the Inflorescence Meristem (IM) peripheral zone is positively regulated by auxins. The AHP6 protein is induced by auxins and enriched in organ primordia and in developing flowers. The AHP6 protein produced in

primordia is then able to move to neighboring cells where it acts as an inhibitor of cytokinins signalling. AHP6 creates two contrasting domains of auxins and cytokinins between sites of successive organ initiation that allows correct temporal sequence of FM primordium initiation (Figure 1.12) (Besnard et al., 2014; Truskina and Vernoux, 2018).

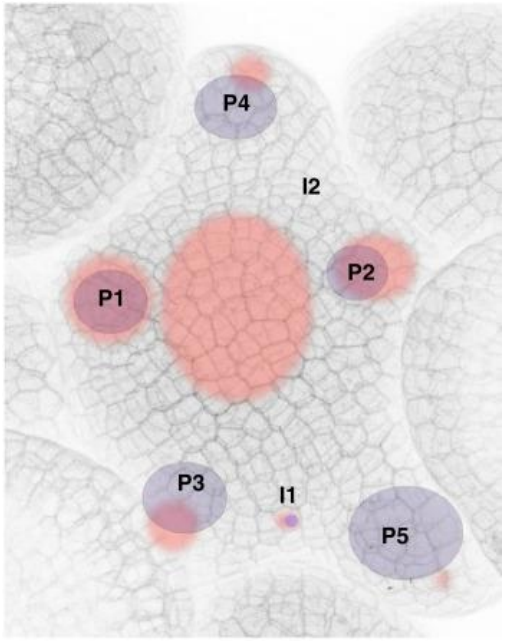

Figure 1.12 The initiation of organ primordia is regulated by auxins and cytokinins. Developing organ primordia are characterized by high auxins signalling (blue) in primordia of all stages (P1–P5). Cytokinins signalling (red) is the highest in young primodia (P1 and P2) but decreases rapidly in older primordia (P3–P5). This decrease in cytokinins signalling is due to AHP6, which is induced by auxins (Truskina and Vernoux, 2018).

I.7 BOL/DRNL/ESR2/SOB as an important transcription factor during organ development.

I.7.1 Protein structure

BOL is a transcription factor protein that has 306 amino acids (aas). This transcription factor belongs to the APETALA2 (AP2) superfamily, Ethylene Responsive Factor (ERF)

family, group VIII, subfamily B1 (Licausi et al., 2013). This superfamily is defined by the presence of the AP2/ ERF domain, which consists of about 60 to 70 aas and is involved in DNA binding. The AP2 domain was first identified as a repeated motif within the Arabidopsis AP2 protein, which is involved in flower development (Jofuku et al., 1994). The ERF domain was identified as a conserved motif in four DNA-binding proteins from tobacco, named as Ethylene-Responsive Element Binding Proteins. It was shown that the ERF domain specifically binds to a GCC box, which is a DNA sequence involved in the ethylene-responsive dependent transcription of genes (Ohme-Takagi and Shinshi, 1995). However, the AP2 and ERF domains are very similar and both are necessary and sufficient to bind the GCC-box. For this reason, this domain is now called AP2/ ERF. BOL contains one AP2/ ERF DNA binding domain and one ESR domain in the C-terminal position (Nakano et al., 2006). The ESR domain is short and weakly conserved, but G and L residues are conserved (Figure 1.13) (Chandler 2018).

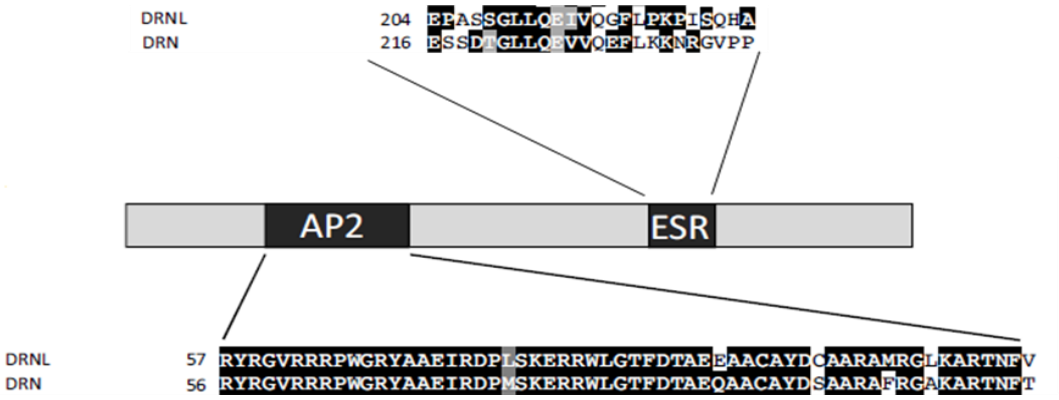

Figure 1.13 BOL protein schematic structural representation. Approximate position and relative size of the AP2 and ESR domains are represented. Alignment of the AP2 domain from BOL and its closest homolog DRN, show that the ESR domain is weakly conserved (modified figure from Chandler et al., 2018).

Although the original ERF name has been maintained, responsiveness to ethylene is not a universal feature of this protein family. It has been demonstrated that the AP2/ ERF proteins have important functions in the transcriptional regulation of a variety of biological

processes related to growth and development, as in the case of BOL, as well as in various responses to environmental stimuli (Nakano et al., 2006),.

I.7.2 BOL closest homolog

The BOL closest homolog is ENHANCER OF SHOOT REGENERATION 1 (ESR1) (Banno et al., 2001) also named DORNRÖSCHEN (DRN) (Kirch et al., 2003). For this reason, BOL has received other names such as DORNRÖSCHEN-LIKE (DRNL) (Kirch et al., 2003) or ENHANCER OF SHOOT REGENERATION 2 (ESR2) (Ikeda et al., 2006). The *DRN* and *BOL* genomic sequences do not contain introns. DRN and BOL are 91% identical in the AP2 domain, but outside the AP2 domain the similarity is much lower; overall DRN and DRNL are 31% identical (Nag et al., 2007).

I.7.3 BOL function

Evidence about BOL's transcription factor function began to emerge in 2006 from Marsch-Martinez et al. (2006), Ikeda et al. (2006) and Ward et al. (2006). These works revealed the importance of BOL during development. The BOL function has been mainly deduced from its gain of function or over-expression phenotypes.

The characterization of an Arabidopsis gain-of-function mutant was made by Marsch-Martinez et al. (2006). This mutant is small and the rosette leaves are curved downwards. This appearance makes it look like a little ball, so this dominant mutant was called *bolita-D* (*bol-D*). The defects in this mutant are due to this AP2/ ERF transcription factor, so the BOL name is derived from its characteristics. In addition to its small and curved leaves, other defects such as short petioles and stems are observed (Figure 1.14 A). Ward et al. (2006) also reported that BOL (which they named SOB [*SUPPRESSOR OF PHYTOCROME B*]) suppresses hypocotyl and petiole length. Another interesting feature produced by the BOL gain-of-function or over expression is the development of calli in

roots independently of PGR application (Figure 1.14 B) (Marsch-Martínez et al., 2006; Ikeda et al., 2006).

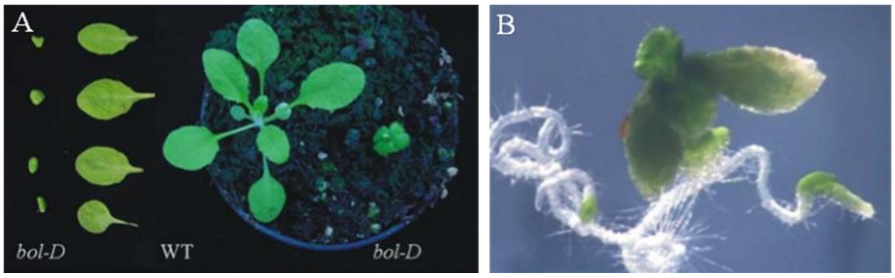

Figure 1.14 Defects during Arabidopsis development promoted by BOL gain of function and over-expression. **A)** Comparison between *bol-D* mutant and WT Arabidopsis plants. **B)** Callus development in *35S-BOL* root without the addition of PGR (Marsch-Martínez et al., 2006).

BOL and *DRN* are co-expressed throughout embryogenesis, and function redundantly in establishing basal cell division stereotypy and cotyledon boundary formation via genetic interaction with *CUP-SHAPED COTYLEDON (CUC)* genes (Chandler et al., 2007; Chandler et al., 2011a). However, despite the similarity between *BOL* and *DRN*, their spatial and temporal expression patterns are not identical throughout post-embryonic growth. The expression of *DRN* has been observed in the shoot apical meristem central zone, while *BOL* is expressed in the peripheral zone, where organ development occurs, and in leaf primordia. For this reason, *BOL* is considered a flower organ founder cell marker in Arabidopsis (Figure 1.15). This expression coincides with the location of the auxins maxima. However it has been determined that auxins-related function in this tissue is independent of BOL (Chandler et al., 2011b).

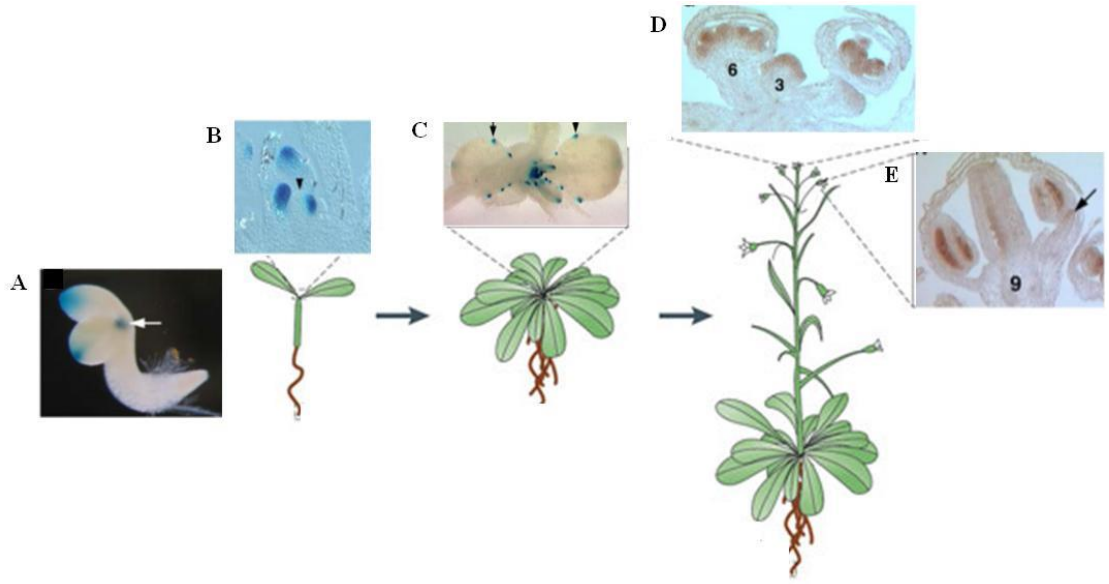

Figure 1.15 *BOL* expression in organ primordia. *BOL* is expressed broadly during the Arabidopsis life cycle. A) to C) Gus staining; D) and E) *in situ* hybridization. **A)** Prior to leaf emergence *BOL* is expressed in the shoot apical meristem (white arrow) as well as at the tips of the cotyledons. **B)** When leaves emerge, *BOL* is expressed in leaf primordia and in developing leaves. **C)** In slightly older leaves, GUS activity is detected in the hydathodes (black arrows). **D)** *BOL* RNA is detected in flower primordia and in developing anthers **(E)** (Modified from Nag et al, 2007).

In addition to the phenotypic defects observed in the *bol-D* mutant and in *BOL* overexpressing plants, there is also information about global gene expression changes in these plants. This information comes from microarrays. These microarrays were performed with *bol-D* young leaves (Marsch-Martinez et al., 2006) and root explants of DRNL-ER (BOL activity inducible line) (Ikeda et al., 2006). These microarray data link BOL with phytohormone pathways.

Apparently BOL regulates genes involved in several processes. Some putative BOL targets have been identified, among which are mainly *AHP6*, a cytokinins negative regulator (Ikeda et al., 2006) and *STY1*, indirectly involved in auxins biosynthesis (Eklund et al., 2011). However, none of these targets has been rigorously confirmed. BOL regulation

specificity towards different targets could depend on the interaction with other proteins. It has been shown that BOL and DRN interact with the class III HD-ZIP transcriptional regulators PHAVOLUTA, PHABULOSA, REVOLUTA and ATHB8. These interactions involve the PAS-like domain of the HD-ZIP proteins and the AP2 domain of BOL and DRN (Chandler et al., 2007).

Since this transcription factor was identified in 2006, additional data about BOL have been generated. Some targets have been suggested, some interacting proteins have been identified and it is known that BOL regulates, at least indirectly, the expression of genes involved with phytohormone pathways, among other processes. However, this information does not answer the questions about what is the BOL function and what mechanisms does it regulate. For this reason, we decided to perform an exploration of the BOL function. Because phytohormones, particularly the interaction between auxins and cytokinins, have been established as important elements that coordinate the organ development, and the combination of both phytohormones in *in vitro* cultures promote the development of calli (an effect promoted by BOL over-expression without the addition of both phytohormones), we were interested in such interaction. However, since the relationship of auxins with BOL has been intensively studied, but so far no direct relationship has been identified, we decided to focus our exploration on the cytokinins pathway. The objective of this exploration was to identify whether BOL is related to cytokinins and to identify molecular mechanisms that can help us understand BOL function. By obtaining more information about the function of BOL, we also hope to provide new insights in the mechanisms that guide new organ development.

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CHAPTER II

The AP2/ ERF transcription factor BOL modulates gynoecium development and affects its response to cytokinins

II.1 INTRODUCTION

In contrast to many animals, plants can make new organs post-embryonically. Stem cells produce signals to maintain a certain group of cells in an undifferentiated state with active cell division, which we call a meristem (reviewed in Gaillochet and Lohmann, 2015). Cells on the periphery of shoot and flower meristems obtain the capacity to differentiate and will develop into lateral organs. Thereby, plant growth is maintained (reviewed in Hepworth and Pautot, 2015; Taylor-Teeples, et al., 2016). Indeterminate growth may last continue over thousands of years as in the case of, for example, the immense Sequoia trees.

The formation of new organs goes hand in hand with auxins, observed in a few cells just before organ primordium emergence, which are called the organ founder cells (Reinhardt et al., 2003; Aloni et al., 2006; Aloni et al., 2003; Chandler et al., 2011b). *BOL* is an AP2/ ERF transcription factor that functions at early stages of organogenesis (Ikeda et al., 2006; Marsch-Martinez et al., 2006; Ward et al., 2006; Chandler et al., 2007). It has been attributed several functions that have arisen mainly from the observed phenotypes of overexpression (*35S::ESR2-ER*) and gain or loss of function of this gene (*bol-D* and *drnl-2*) (Ikeda et al., 2006; Marsch-Martinez et al., 2006; Nag et al., 2007; Chandler et al., 2007). It is expressed at very early stages of aerial organ formation; its expression has been characterized in detail, and it has been proposed that this gene is a marker for the flower organ founder cells in Arabidopsis (Chandler et al., 2011b).

The loss of *BOL* and *DRN* (its closest homologue) function in Arabidopsis causes cotyledon fusions, though this phenotype does not present full penetrance? (Chandler et al., 2007). Moreover, the loss of *BOL* function (*drnl-2* mutant) also causes diverse alterations in the organs of all floral whorls (Nag et al., 2007). In the reproductive organs of the

flower, these alterations are very severe in the stamens and have been well characterized. Gynoecium phenotypes have been reported to be less severe, being mostly normal but occasionally misshapen and bent (Nag et al., 2007). The valves of *drnl-2* gynoecia can be absent or asymmetric, though these defects occur at low penetrance (6%, [Chandler et al., 2011b]), while gynoecia of the triple *drnl drn puchi* (*PUCHI* is a third close homologue; Hirota et al., 2007; Karim et al., 2009) mutant do not develop valves (Eklund et al., 2011).

The pistil or gynoecium is a very important part of the flower, because it is the female reproductive system that will give rise to the fruit at a later stage of development (reviewed by Roeder and Yanofsky, 2006; Alvarez-Buylla et al., 2010). Like most angiosperms, in Arabidopsis each flower produces a gynoecium in the center. The gynoecium consists of different structures, and, in the apical-basal axis (Figure 2.1A), at the top it has a stigma with the style below, then the ovary with valves that protect the ovules, and finally the gynophore at the bottom. In Arabidopsis, the ovary is formed by two fused carpels. The floral meristem gives rise to the carpel primordia, and two congenitally fused carpels will arise and form a kind of hollow tube that during development will close at the top, followed by differentiation at the apical end, where the style and stigma will be formed. Inside the hollow tube, two meristematic regions will be formed along the side where the carpels are fused. These regions are also called the carpel margin meristem (CMM). The CMM gives rise to all the internal tissues, septum, placenta, ovules, transmitting tract, tissues that are crucial for the reproductive competence of the plant (reviewed in Bowman et al., 1999; Alvarez and Smyth, 2002; Wynn et al., 2011; Reyes-Olalde et al., 2013). As expected, transcription factors are essential for correct gynoecium development (reviewed in Ferrándiz et al., 2010; Alvarez-Buylla et al., 2010; Reyes-Olalde et al., 2013; Chávez Montes et al., 2015), but also hormones like auxins and cytokinins are important for its proper patterning and morphogenesis (reviewed in Sehra and Franks, 2015; Marsch-Martínez and de Folter, 2016). The pathways of these two hormones are connected at different levels. Both hormones are well studied, having both antagonistic and synergistic functions, which have been described for different tissues and organs (reviewed by El-Showk et al., 2013; Schaller et al., 2015). For instance, alterations in auxins signalling or biosynthesis, application of auxins, or inhibitors of auxins transport, affect the apical-basal axis of the gynoecium, i.e., the proportion of organ sizes relative to each other is affected

along this axis. One of these alterations is that valves grow at different sizes (asymmetric valves; Sessions and Zambryski, 1995; Nemhauser et al., 2000; Cheng et al., 2006; Sohlberg et al., 2006; Stepanova et al., 2008; Zuñiga-Mayo et al., 2014). The same effects are observed when exogenous cytokinins is applied (Zuñiga-Mayo et al., 2014). Interestingly, asymmetric valves have also been reported for the *drnl* mutant (Chandler et al., 2011b), suggesting that *BOL* also plays a role during gynoecium development, probably by affecting hormonal pathways. Therefore, we characterized *BOL* function during gynoecium development by studying the effects of its loss of function, following its expression during development, and exploring its connection with cytokinins.

II.2 MATERIALS AND METHODS

II.2.1 Plant materials and growth conditions

The lines used in this study were wild type (WT) ecotypes Landsberg *erecta* (L*er*) and Columbia (Col); mutants *bol-D* (Marsch-Martinez et al., 2006), *drnl-2* (Nag et al., 2007), *ahp6-1* (Besnard et al., 2014); reporter lines *BOL::GUS* (comprising 1550 nucleotides upstream the start codon; Marsch-Martinez et al., 2006), *AHP6*::*GFP* (comprising 1594 nucleotides upstream the start codon; Mähönen et al., 2006), and *TCS::GFP* (Müller and Sheen, 2008); the inducible *BOL* line *35S::DRNL-ER* (Ikeda et al., 2006; Eklund et al., 2011) also was used.

All genotypes were germinated in soil (peat moss, perlite and vermiculite 3:1:1) under long-day conditions (16–8 h, light–dark) in a growth chamber at 22°C. Two weeks after germination, the plants were transferred to a green house with a temperature range from 22 to 28°C, and natural light conditions. Day length varied in different seasons.

II.2.2 Gynoecium phenotypic analyses

Fruits were evaluated in *drnl-2* and L*er* plants, which were germinated and grown under the same conditions as in the rest of the experiments. The numbers of fruits (siliques) and

pistils that did not develop into fruits per plant were registered (n = 14 plants). Fruits were collected and classified according to their phenotype (n = 205 fruits). For the phenotypic analysis of pistils that did not develop into fruits, 199 pistils present along inflorescence stems were analyzed. Images were captured using a Stemi 2000-C microscope (Carl Zeiss).

II.2.3 *BOL::GUS* expression

β-glucuronidase staining was performed for 24–168 h at 37°C in a 2 mM X-Gluc solution (Gold BioTechnology), using established protocols (Campisi et al., 1999). *BOL* expression was observed under a DM6000B microscope coupled with a DFC420 C camera (both from Leica).

II.2.4 *AHP6::GFP* expression

To analyze the regulation of *AHP6* expression in response to BOL activity, one drop of β-estradiol or mock solution was applied per inflorescence in *DRNL-ER AHP6::GFP* plants. The β-estradiol solution contained 10 μM β-estradiol (Sigma-Aldrich) with 0.01% Silwet L-77 (Lehle Seeds). A solution containing DMSO and Silwet L-77 in the same concentration as in the β-estradiol solution was used for the mock treatment.

Transverse sections of the gynoecia were made 48 h after the treatments, according to (Reyes-Olalde et al. (2013). The sections were visualized and images were captured using a LSM 510 META confocal scanning laser inverted microscope (Carl Zeiss). Propidium Iodide (PI; at 0.01 mg/mL) was used as a counterstain. PI was excited using a 514-nm line and GFP was excited using a 488-nm line of an Argon laser. PI emission was filtered with a 575-nm long pass (LP) filter and GFP emission was filtered with a 500–550-nm band-pass (BP) filter.

II.2.5 Histological sections

Tissues were fixed in FAE (3.7% formaldehyde, 5% glacial acetic acid, and 50% ethanol) with vacuum (20 min, 4°C) and incubated for 120 min at room temperature. The material was rinsed with 70% ethanol and incubated overnight at 4°C, followed by dehydration in a series of ethanol solutions (85, 95, and 100%) for 60 min. each and embedded in Technovit® 7100 according to the manufacturer's instructions (Heraeus-Kulzer, Wehrheim, Germany). Using a rotary microtome (Reichert-Jung 2040; Leica), 10 µm thick transverse sections of L*er* and *drnl-2* inflorescences were made and stained with alcian blue (0.5% pH 3.1; Sigma-Aldrich) for 25 min and neutral red (0.5%) for 5 minutes. Micrographs were obtained using a DM6000B microscope coupled with a DFC420 C camera (both from Leica).

II.2.6 Gene expression analysis

For qRT-PCR analysis, open flowers were removed, inflorescences with only floral buds were collected, and total RNA was extracted using the Quick-RNA^{TM} MiniPrep kit (Zymo Research). The samples were treated with DNase I, included in the kit. Reverse transcription and amplification were performed using a KAPA SYBR FAST One-Step qRT-PCR Kit (Sigma-Aldrich) in a StepOne^{TM} thermocycler (Applied Biosystems). Three biological replicates and three technical replicates were included in the analysis. Target gene expression levels were normalized to *ACTIN 2*. Data was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). In the graphs, each bar (1, 2, and 3) represents one biological replicate, and error bars represent the standard error corresponding to three technical replicates of each biological replicate.

II.2.7 Cytokinin Treatments

Cytokinin treatments were performed in a similar way as described by Zuñiga-Mayo et al. (2014). The experiment was performed in greenhouse conditions with natural light in autumn, and all plants (L*er* and Col as wild type, *drnl-2* and *ahp6*) were grown simultaneously under the same conditions. The first fruits observed in the inflorescence

stem were removed, leaving only closed buds in the inflorescences. Once this was done, BAP solution drops (100 μM 6-benzylaminopurine; Duchefa Biochemie) and 0.01% Silwet L-77 (in distilled water) were applied on the inflorescences for five consecutive days. Sixteen days after treatment the gynoecia were collected and analyzed in chronological order of development. The mock solution contained Silwet L-77 and the same concentration of NaOH (0.2 mN) used to prepare the hormone solution.

II.3 RESULTS

II.3.1 Apical-basal defects in *drnl-2* mutant gynoecia

To obtain information about the role of *BOL* during gynoecium development, *drnl-2* (Nag et al., 2007) mutant gynoecia were compared to wild type gynoecia. *drnl-2* gynoecia were slightly longer and presented a broader stigma than the L*er* wild type gynoecia (Figure 2.1D). This mutant presents reduced fertility (Nag et al., 2007), and a large proportion of gynoecia do not develop fruits. Instead of elongating as a normal fertilized gynoecium converted into a developing fruit, they maintain the size of a gynoecium at stage 13 (Figure 2.1C; middle). Only about 12% of *drnl-2* gynoecia developed into a fruit in our growth conditions (Figure 2.1B), in comparison to wild type plants, where most gynoecia are fertilized and become fruits. Out of the 12% of gynoecia that converted into fruits, different altered phenotypes along the apical-basal axis of the fruits were observed, and were most evident in the ovary region. These phenotypes were classified in 4 types: wild type-like, reduced valves (RV), very reduced valves (VRV), and one valve (OV) (Figure 2.1H). An 81.5% of the total number of fruits presented a wild type-like phenotype. These fruits had symmetrical valves like those of wild type plants. The rest of the fruits presented defects in the symmetry of the valves: 5.9% had reduced valves , 2% had very reduced valves , and interestingly, the percentage of fruits with only one valve was greater than that of fruits with asymmetric valves (10.7%) (Figure 2.1F).

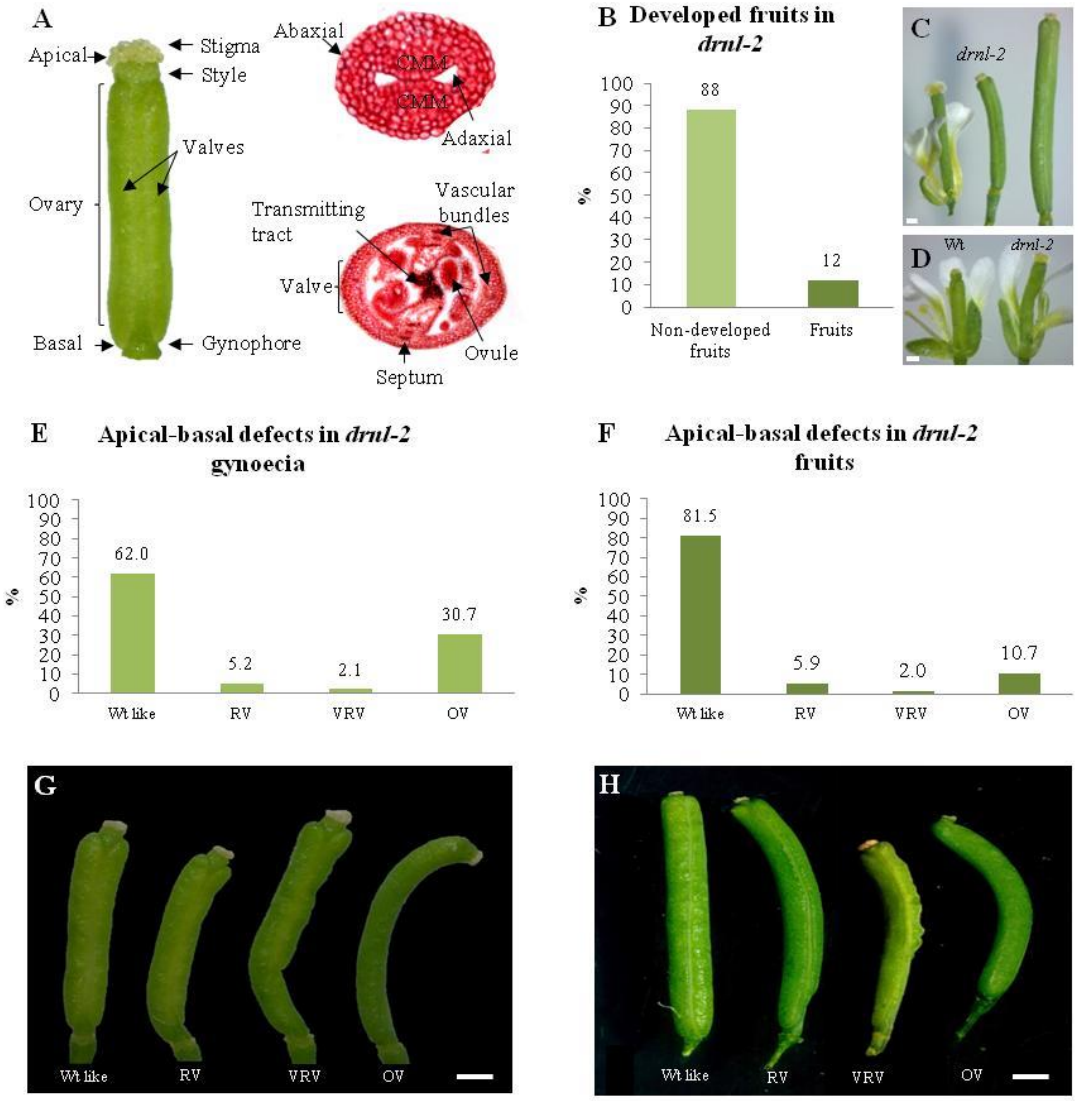

FIGURE 2.1 External defects in *drnl-2* mature gynoecia and fruits. **A)** Structures of the Arabidopsis gynoecium. **B)** Proportion of gynoecia that become fruits in *drnl-2* plants. **C)** *drnl-2* flower (left), a *drnl-2* gynoecium that did not develop into fruit (middle), and a *drnl-2* developing fruit (right). **D)** Comparison between wild type and *drnl-2* flowers at stage 13. **E), G)** Apical-basal defects in *drnl-2* gynoecia, frequencies **E)** and phenotypes **G). F), H)** Apical-basal defects in *drnl-2* fruits, frequencies **F)** and phenotypes **H)**. Scale bars: 0.2 mm in **C) and D)**; 0.5 mm in **G)**; 1 mm in **H)**.

Besides fruits, we also analyzed the phenotype of the gynoecia that stayed in the stem but did not develop as fruits (Figure 2.1C, middle). These structures also presented equivalent phenotypes as those observed in developed fruits, but the frequency of the one-valve phenotype was higher (Fig 2,1E and G). An 61.98% of these pistils presented a wild type phenotype, 5.21% had reduced valves, 2.08% very reduced valves, and 30.73% presented the one-valve phenotype.

Another phenotype that was observed in *drnl-2* fruits and gynoecia, was that some were curved, a characteristic that had also been reported by Nag et al. (2007). This curvature was more marked evident in gynoecia that presented the "one valve" phenotype (Figure 2.1G and H). On the other hand, it was interesting that in some of the gynoecia or fruits presenting "one valve", this "valve" had almost the width of two valves. While we could clearly distinguish the replum and margins of the valve on one side of the fruit or gynoecium, these structures were not visible on the opposite side. In some fruits and gynoecia it was possible to partially distinguish the presence of these structures in some regions, which appeared to be absent in others (Figure 2.2). In summary, *drnl-2* fruits presented evident external phenotypic alterations, mostly at the valves, at partial penetrance.

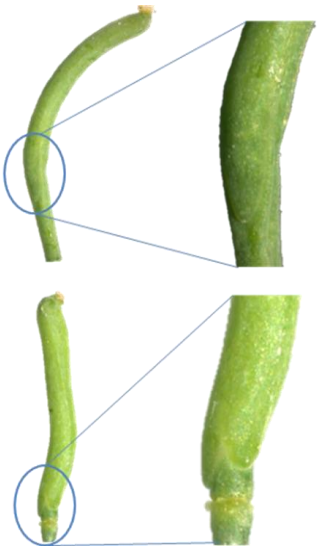

Figure 2.2. Partially fused valves in *drnl-2* gynoecia. The fusion is visible in the basal region of the gynoecium.

II.3.2 The loss of *BOL* function causes defects at different stages during gynoecium development

After observing the external phenotypes of the *drnl-2* fruits, we also examined the internal tissues of developing gynoecia, and compared them to wild type gynoecia. Histological sections allowed the visualization of the different tissues of *drnl-2* gynoecia during development. Figures 2.3A and B present sections of whole wild type and *drnl-2* inflorescences, where differences in bud development and some valve asymmetries can be observed, and are shown in more detail in 2.3 D, E, F and J. The valve asymmetries were related to the ones observed in *drnl-2* "mature" gynoecia and fruits (in total, in between 20 and 40% of them presenting defects, respectively, Figure 2.1E and F). As observed in those gynoecia and fruits, the defects observed in *drnl-2* developing gynoecia, do not appear to be 100% penetrant, and also vary in severity from pistil to pistil. Examples of altered growth were present from early gynoecium developmental stages. In Figure 2.3D, a doughnut-shaped, possibly stage 8 young *drnl-2* gynoecium can be observed, presenting a roundish central opening. In stage 8 (according to Smyth et al., 1990) wild type gynoecia normally have an oval shape, present a bowtie shaped central opening, and the valve region can be clearly identified (Figure 2.3C). Examples of valve asymmetrical growth in stage 9 and stage 10 in *drnl-2*gynoecia are presented in Figure 2.3E and F. The image in Figure 2.3F possibly corresponds to the "one valve" phenotype presented in Figure 2.1G and H), because the same structure was observed along the apical-basal axis of the gynoecium in different sections. Developmentally retarded and deformed *drnl-2* stamens, were also observed, as previously described (Nag et al., 2007). Alterations in the valves were more evident at the basal regions of the mutant fruits and gynoecia (Figure 2.2) compared to the wild type. Figure 2.3J presents an example of a fully developed gynoecium showing asymmetric valve growth at its basal region (compared to an equivalent region of a wild type gynoecium in Figure 2.3I). On the other hand, as observed in fruits and old gynoecia, many developing gynoecia presented normal valve symmetry at their middle region (Figure 2.3H, L and N, compared to wild type 2.3G, K and M). Some gynoecia also presented uneven development of their inner tissues, and examples are shown in Figures 2.3L, N, P and R, compared to their equivalent wild type counterparts in Figures 2.3K, M, O and Q.

Figure 2P (a magnification of the center of the image in Figure 2.3L) shows an example of a *drnl-2* ovary where the septum presents cell death and the transmitting tract presents the characteristic staining with alcian blue of a gynoecium at late developmental stages. However, ovule development seems to be delayed since the integuments of the ovules have not yet grown to enclose the female gametophyte, as occurs in the wild type at this point (Figure 2.3O). Figure 2.3R (a magnification of Figure 2.3N) shows the transmitting tract region of an ovary where one side presents the characteristic blue staining produced by cell death (observed as an empty space in the septum), but the other does not, which is uncommon in wild type ovaries (Figures 2.3I, M, and Q). In summary, valve defects can be detected in *drnl-2* gynoecia throughout development, and some gynoecia also present asymmetric or asynchronic development of other tissues.

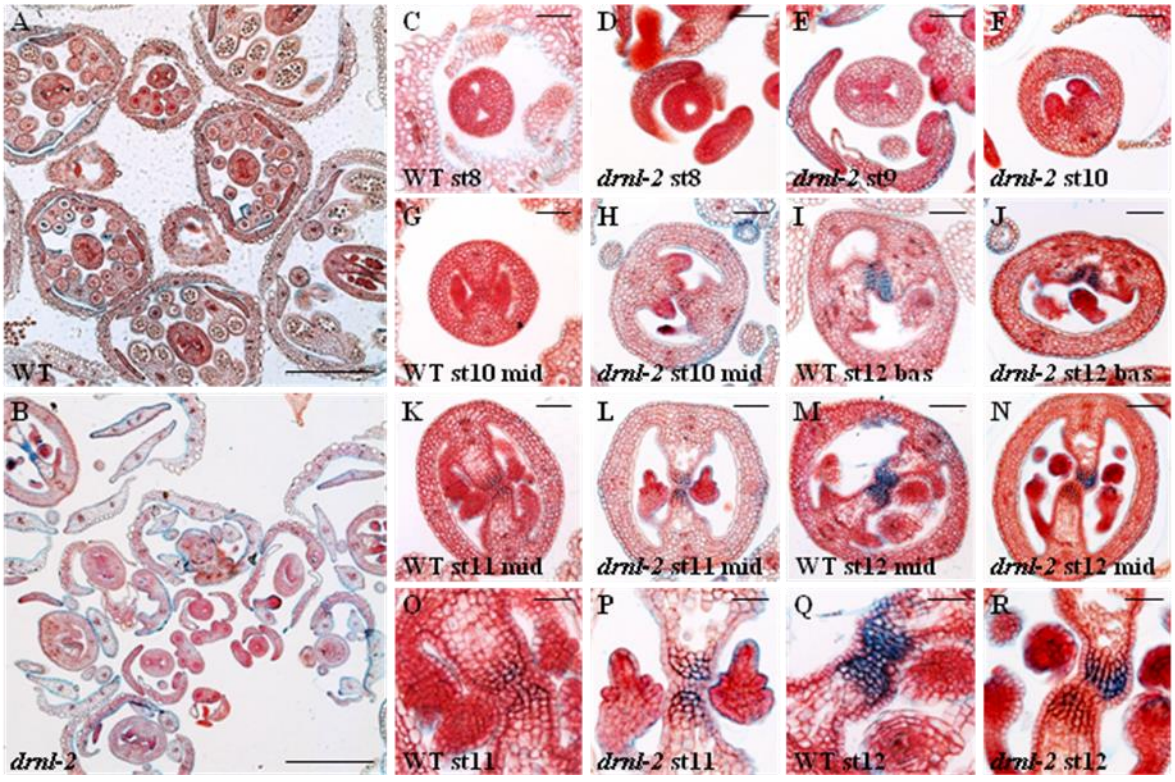

FIGURE 2.3 Cross sections of developing wild type and *drnl-2* inflorescences and gynoecia. A)-B) Cross-sections through wild type (L*er*) **A**) and *drnl-2* **B**) inflorescences. Valve asymmetries and differences in bud development can be observed. **C)-N**) Whole

gynoecia at different stages of development. Characteristic wild type early gynoecium **C**), where the two internal ridges form a "bowtie" shape. **D**) Example of an early *drnl-2* gynoecium where the two ridges are not present and a circular aperture is visible. **E)** and **F**) Examples of *drnl-2* asymmetric valve development at intermediate stages, sometimes presenting a single valve **F**). Example of a fully developed *drnl-2* gynoecium with valve asymmetry at its basal region **J**) compared to the equivalent region in a wild type gynoecium **I**). **H)**, **L)**, **N)** Examples of *drnl-2* developing gynoecia with normal valve symmetry at their middle region, compared to wild type gynoecia **G)**, **K)**, **M)**. **L)** and **N),** magnified in **P)** and **R**) Examples of asynchrony in the development of the internal tissues of almost mature *drnl-2* gynoecia at stages 11 and 12, compared to their equivalent wild type counterparts **K)** and **M),** magnified in **O)** and **Q)**. Bars: 25 μm in **O)-R)**, 50 μm in all other panels.

II.3.3 *BOL* is expressed at the prospective valves of the gynoecium

Since *drnl-2* mutants presented clear alterations in gynoecium morphology at different stages during gynoecium development, we analyzed *BOL* expression throughout this process to know whether it was also expressed at intermediate stages of development.

To determine the expression pattern of *BOL* in the reproductive tissues of developing flowers, we performed GUS staining of a *BOL::GUS* line (Marsch-Martinez et al., 2006), which revealed that GUS activity was found in developing gynoecia and stamens through different developmental stages (Figure 2.4). During floral stages 6 and 7 (Figure 2.4A; floral stages according to Smyth et al., 1990), prior to the clear differentiation of the inner meristematic outgrowths of the emerging gynoecium (CMMs), *BOL* expression was found in stamen and gynoecium primordia, as previously reported (Nag et al., 2007; Chandler et al., 2011b). Interestingly, we observed that *BOL* expression was enhanced in the regions that will later give rise to the valves. By stage 8, when the two CMMs form at the inner medial domain, GUS staining became restricted to the developing valves, at the lateral domains of the gynoecium, but expression was not detected in the epidermal cell layer. At this stage, high *BOL* expression could also be detected in the early developing stamens (Figure 2.4B). The activity of the *BOL* promoter was maintained at the valves of

developing gynoecia until stage 11 (Figures 2.4C-E), although as development proceeded, it became weaker and more restricted to the adaxial cell layers. At these stages, GUS activity was also found in stamens, mostly at the apical zone and, afterwards, throughout the anther (Figures 2.4C-E) as reported by Nag et al. (2007). At anthesis, by stage 12, *BOL* promoter activity was no longer found in the gynoecium (Figure 2.4F). However, expression was still detectable in the anthers in microspores and the tapetum (Figure 2.4F), as previously reported (Nag et al., 2007).

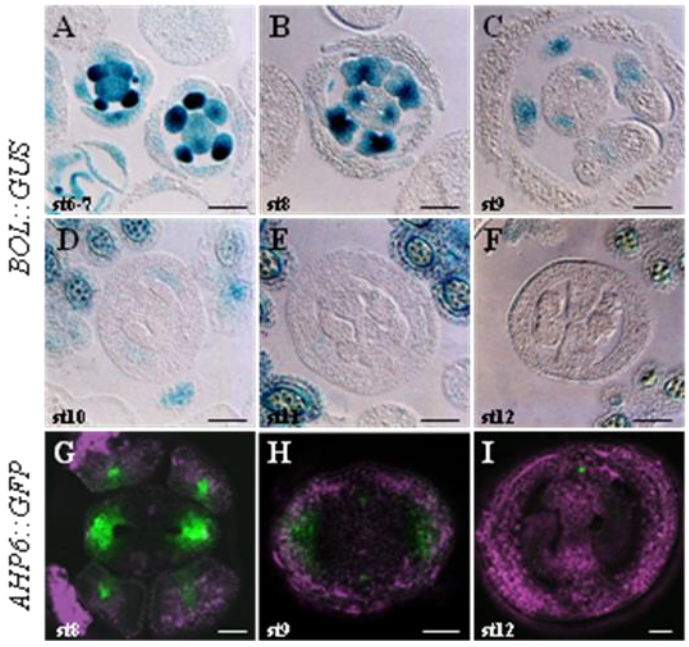

FIGURE 2.4 *BOL* and *AHP6* expression during gynoecium development. **A)-F**) *BOL* expression pattern in inflorescence transverse sections. During floral stages 6-7, the *BOL::GUS* marker expression can be detected in stamen and gynoecium primordia, slightly enhanced at the lateral region of the gynoecium primordium **A)**. By stage 8, *GUS* staining becomes restricted to the developing valves, except for their epidermal layer **B)**. During stages 9-11 **C)-E)**, *BOL* expression is found at the valves, becoming weaker and restricted to the adaxial cell layers. *GUS* activity is also found in stamens **C)** and, after that, all over the anther **D)-E).** By stage 12, *BOL* expression is no longer detected in the gynoecium, but is detectable in microspores and anther tapetum **F)**. **G)-I)** *AHP6* expression pattern in inflorescence transverse sections. In the gynoecium, *AHP6* is similarly expressed from

early developmental stages on. Similar expression in the valves at stage 8 **G)** and early 9 **H)** is shown. As *BOL*, *AHP6* is not detected in the valves of stage 12 gynoecia **I)**. Scale bars: 50 μm in **A)-F)**; 20 μm in **G)-I)**.

II.3.4 BOL can regulate *AHP6* during gynoecium development

The observed defects in the *drnl-2* gynoecia resembled to a certain extent, gynoecia of mutants affected in phytohormone transport, response, or gynoecia that have been treated with phytohormones or phytohormone transport inhibitors (Sessions and Zambryski, 1995; Nemhauser et al., 2000; Cheng et al., 2006). Auxins have an important role during gynoecium development, and previous reports have related *BOL* function to auxins (Chandler, 2008; Eklund et al., 2011), though *BOL* may be also participating in other processes. Interestingly, exogenous cytokinin treatments are also able to affect the proper establishment of the apical-basal patterning in the Arabidopsis gynoecium (Zuñiga-Mayo et al., 2014). These defects promoted by cytokinin treatments in wild type gynoecia resemble some of the alterations observed in *drnl-2* mutant gynoecia (Figure 2.1). This resemblance suggested that there could be a possible relation of *BOL* with cytokinins during gynoecium development. Therefore, we sought reported genes that participate in the cytokinins pathway and that have been found to be connected to *BOL*. One of these genes is *AHP6*, which encodes a histidine phosphotransfer protein that negatively modulates cytokinins signalling (Hwang et al., 2002; Mähönen et al., 2006). This gene has been proposed to be a possible BOL target by Ikeda et al. (2006). Like *BOL*, *AHP6* is expressed in the inflorescence meristem in the regions where floral organ primordia develop (Besnard et al., 2014;). Recently, *AHP6* was reported to be expressed at the lateral domains of the gynoecium in stages 7-9 (Reyes-Olalde et al., 2017). Therefore, we compared *AHP6* and *BOL* expression to find out whether they shared similar expression patterns during gynoecium development.

We used an *AHP6::GFP* reporter line (Mähönen et al., 2006) and observed that *AHP6* is expressed at early stages of gynoecium development, in a similar way to *BOL*. *AHP6* expression also marked the region where the valves will develop at later stages and was

maintained until stage 7. From stage 8 onward (Figures 2.4G,H), its expression began to decrease in this region, and then at stage 9, a more confined expression of *AHP6* in the valves was observed. *AHP6* expression disappeared in the valves at stage 12, but it remained in the medial vasculature (Figure 2.4I).

These analyses indicated that *AHP6* and *BOL* have very similar expression patterns. Both are expressed in the prospective valves during early stages of development. Their expression is lost in the lateral domain of the gynoecium at stage 12, when the tissue is mature. In addition, *BOL* and *AHP6* not only shared expression locations at the gynoecium, but also in other structures such as stamen primordia (Figure2.4B,G).

After this similarity in the expression patterns in the gynoecium was observed, we sought to determine whether the gain or loss of *BOL* function could affect *AHP6* expression. For this, we first analyzed the expression of *AHP6* in the gain of function *bol-D* mutant (a *BOL* activation tagging allele; Marsch-Martinez et al., 2006). When RNA obtained from whole inflorescences was assayed by qRT-PCR, the accumulation of the *AHP6* transcript was considerably increased in *bol-D*, in comparison to wild type inflorescences (Figure 2.5A). We then sought to determine whether this upregulation of *AHP6* expression through BOL was occurring at the gynoecium. For this purpose, a cross between a BOL activity inducible line (Ikeda et al., 2006; Eklund et al., 2011) to the *AHP6* reporter line (*DRNL-ER AHP6::GFP*) was performed. We observed that induction of BOL activity caused a change in the *AHP6* expression pattern in the gynoecium, detectable 48 h after the β-estradiol treatment (Figures 2.5C,D). In stage 8-9 gynoecia, *AHP6* expression was not detected in the provasculature of the medial region, but when BOL activity was induced we could observe that the expression of *AHP6* appeared in this tissue, and was slightly increased in its normal domain of expression (Figure 2.5D).

Next, we analyzed *AHP6* expression in the *drnl-2* loss of function mutant. qRT-PCR analysis was performed in mutant and wild type inflorescences, and no evident decrease in *AHP6* expression in the inflorescences of *drnl-2* could be detected (Figure 2.5B). In summary, the increased activity of BOL is able to upregulate *AHP6*, but BOL does not

appear to be a general regulator of *AHP6* expression in all tissues, possibly regulating it in very specific domains.

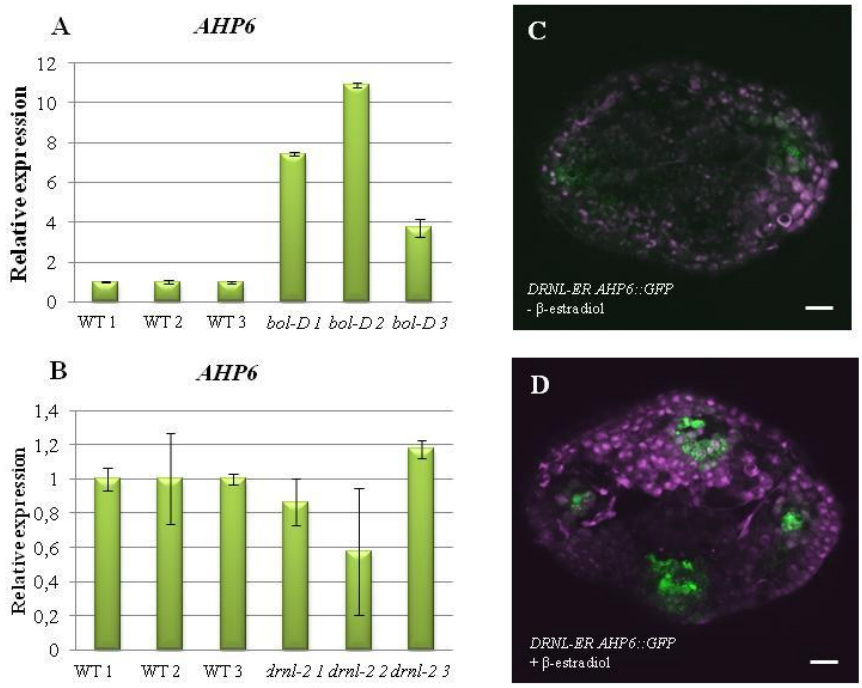

FIGURE 2.5. **Regulation of *AHP6* expression by BOL**. **A)** and **B)** *AHP6* relative expression in *bol-D* and *drnl-2* mutant inflorescences. Each bar (1, 2 and 3) represents a biological replicate, and standard error bars were calculated from three technical replicates. **C)** *AHP6* expression in *DRNL-ER AHP6::GFP* (*DRNL-ER = 35S$_{pro}$:: DRNL-ER)* gynoecia at stage 8, treated with mock solution. **D)** *AHP6* expression in *DRNL-ER AHP6::GFP* gynoecia at stage 8, induced with β-estradiol. Increased *AHP6* expression after induction of DRNL function is observed.

II.3.5 The loss of *BOL* function alters the response of the gynoecium to cytokinins

The upregulation of *AHP6* by BOL suggested that BOL could be modulating cytokinins homeostasis. To test this further, we explored whether the sensitivity of gynoecia to cytokinins was affected by the loss of *BOL* function. The application of exogenous cytokinins, besides inducing apical-basal defects, also promotes tissue proliferation in the

external medial region of the gynoecium (Marsch-Martinez et al., 2012), depending on the developmental stage at which the gynoecium receives the treatment. Because the effect of this treatment in developing gynoecia is very evident, we used it to evaluate the ability of *drnl-2* gynoecia to respond to exogenous cytokinins. We applied cytokinins to *drnl-2* inflorescences and compared the effects of the treatment to WT inflorescences treated in the same way. In parallel, considering that *AHP6* appeared to be regulated by BOL, we also applied cytokinins to the *ahp6* loss of function mutant, to compare whether its response to the cytokinin application was comparable to the response of *drnl-2*.

For this, inflorescences of *drnl-2, ahp-6,* and their corresponding WT ecotypes were treated once a day for a period of 5 days with a 100 μM BAP solution. All fruits present in the plants were removed before the beginning of the treatment. After 5 days of treatment, the flowers, pistils, and fruits were allowed to develop for 15 days more. After this period, gynoecia were detached from the stem and analyzed in chronological order from the base to the apex of the stem (i.e., from "oldest" to "youngest").

From this experiment, it became evident again that the response to cytokinins depends on the developmental stage in which each gynoecium was at the time of treatment. It should be noted that the treated gynoecia did not continue with their normal development to fruit. These gynoecia were small and showed a gradient of phenotypes as we previously reported (Zuñiga-Mayo et al., 2014; Figure 2.6). Compared to untreated gynoecia and fruits the observed phenotypes were classified into 3 categories (Figure 2.6 and Figure 2.9A), according to the developmental stage at which the gynoecia were when they were treated, and the resulting WT phenotype upon cytokinin treatment: Class I, gynoecia that were at late stages of development at the beginning of the treatment (around stage 12-13) (Figures 2.6A,D,G,J), which became short and wide after the treatment; Class II, gynoecia that were at intermediate stages of development (around stage 9-11) (Figures 2.6B,E,H,K), which presented tissue proliferation in their external medial region at the end of the treatment; and Class III, which were at early stages of development (around stage 6-8) at the beginning of the treatment, and presented apical-basal defects at the end of the treatment (Figures

2.6C,F,I,L). Both L*er* and Col WT gynoecia presented these phenotypes, though they were more severe in the Col ecotype.

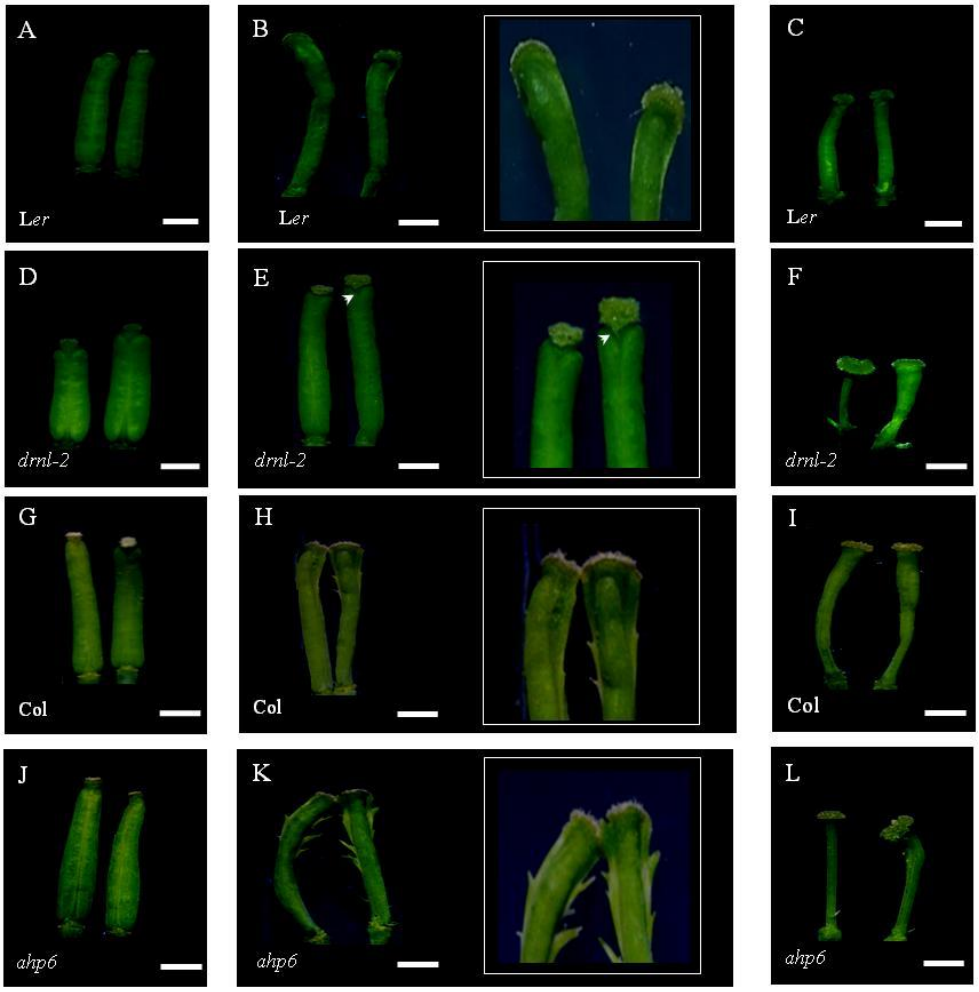

FIGURE 2.6. **Response of *drnl-2*, *ahp6*, and wild type gynoecia to cytokinin treatment (BAP). A)-C)** L*er* gynoecia. **D)-F)** *drnl-2* gynoecia. **G)-I**) Col gynoecia. **J)-L)** *ahp6* gynoecia. Gynoecia were classified in three classes according to the developmental stage in which they were when they received the BAP treatment, and the phenotype they presented after the treatment. Class I: gynoecia that were at late developmental stages, with subtle alterations in morphology; class II gynoecia that were at intermediate stages, where tissue proliferated in their external medial region, and class III gynoecia that were at early stages of development, that presented apical-basal defects as a response to BAP. **B), E), H), K)**

show magnifications of the apical region of class II gynoecia to highlight tissue proliferation in the medial region. The arrowhead **E)** highlights the absence of tissue proliferation in the medial region in comparison to the rest of the ecotypes. Scale bars: 1 mm in the non-magnified images of **A)** to **L)**.

When *drnl-2* mutant gynoecia were analyzed, it was clear that they presented an altered response to cytokinins. First, *drnl-2* gynoecia equivalent to wild type class II gynoecia did not present the evident proliferation of tissue in the external medial region that characterizes the response in wild type gynoecia (Figure 2.6E). This lack of over-proliferation indicates that these *drnl-2* gynoecia are less responsive to cytokinins. However, younger *drnl-2* gynoecia that were equivalent to class III wild type gynoecia, presented the opposite. We detected more severe apical-basal defects than those observed in treated wild type gynoecia (Figures 2.6F). These defects were so severe that some *drnl-2* gynoecia did not even develop valves, whereas in wild type plants this defect was not observed in the conditions used for this experiment (Figure 2.6F). Therefore, while older *drnl-2* gynoecia presented a reduced response, younger *drnl-2* gynoecia presented an increased response to the cytokinin treatment, compared to wild type gynoecia. These results suggest that *drnl-2* gynoecia may be more sensitive or responsive to cytokinins at early stages of development (stage 6-8), whereas at later stages (stage 9-11) mutant gynoecia are less sensitive or responsive to the external application of this phytohormone.
On the other hand, *ahp6* gynoecia appear to be more sensitive to cytokinins than wild type gynoecia at the stages analyzed, particularly young gynoecia. Both class II and class III gynoecia show a more severe response than those of Col wild type plants (Figures 2.6 H, I, K, L). Class II gynoecia develop the tissue proliferations, and this ectopic tissue was even more evident in the *ahp6* mutant than in wild type gynoecia (Figure 2.6H and K). For class III, while wild type gynoecia presented the apical-basal defects described in previous reports (reduced, very reduced, asymmetric valves, and at low frequency lack of both valves), most treated *ahp6* gynoecia presented the lack of both valves (Figures 2.6L), indicating an increase in the severity of the response.

We then compared these responses to the responses of *drnl-2* and their respectively wild type gynoecia. It became clear that the *ahp6* and *drnl-2* mutants both increased sensitivity or response of early gynoecia to cytokinins. The response of both mutants was severe and produced many gynoecia without valves. However, the effects of these mutations in the response of treated gynoecia at intermediate stages of development (Class II) was opposite, with *ahp6* increasing and *drnl-2* decreasing sensitivity or response to the treatment. We also noticed another conspicuous difference between *drnl-2* and the other genotypes (L*er* WT, Col WT, and *ahp6*): the 3 phenotype classes developed in chronological order and were easily identified in L*er* WT, Col WT, and *ahp6* gynoecia. The response per developmental stage was clear and generally uniform in these genotypes (Figure 2.7A, C, D). However, the youngest gynoecia of 3 out of 5 treated *drnl-2* inflorescences presented a mix of irregular phenotypes that did not follow any chronological or other evident order. These gynoecia presented phenotypic defects that ranged from mild and severe apical-basal defects (observed as the total lack of valves; Figure 2.7B).

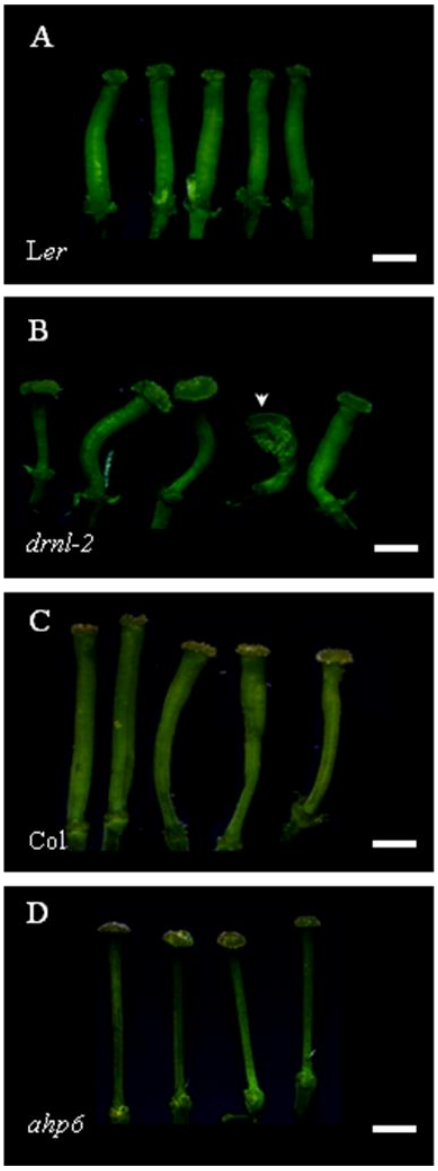

Figure 2.7. Heterogeneous response to cytokinins in *drnl-2* class III gynoecia. **B)** Treated *drnl-2* class III gynoecia. **A)**, **C)**, **D)** Homogeneous response to cytokinins in treated wild type and *ahp6* gynoecia. The arrowhead highlights a misshapen structure in *drnl-2*.

In addition, a fourth phenotype of misshaped gynoecia, where the distinct tissues could not be clearly distinguished, was observed in some *drnl-2* gynoecia, not present in the other genotypes or untreated gynoecia. These organs were not able to develop properly, and the gynoecium structure was lost (Figure 2.7B; arrowhead). This was further evidence that *drnl-2* gynoecia have increased cytokinins sensitivity or response at early stages of development.

After observing the phenotypic response of *drnl-2* gynoecia to the exogenous application of cytokinins we wanted to corroborate if the transcriptional response to cytokinins in *drnl-2* gynoecia (stages 9-10) was affected. This was analyzed using the Two Component Signalling Sensor (TCS)::green fluorescent protein (GFP) line (Müller and Sheen, 2008), which reflects the transcriptional activity of type-B response regulators. Additionally, *DRNL-ER* was also included in this analysis. The TCS signal can be visualized in the tissues of the gynoecium middle region (Figure 2.8A). When BOL activity was induced in *DRNL-ER TCS:GFP* gynoecia (Figure 2.8C) we could not observe changes in the TCS signal with respect non-induced gynoecia (Figure 2.8B). However, in the BOL loss function background (*drnl-2*), the TCS signal was strongly reduced in the gynoecium middle region (Figure 2.8D). This result could be related to the lower sensitivity of *drnl-2* gynoecia (around stages 9-11) to exogenous cytokinin application.

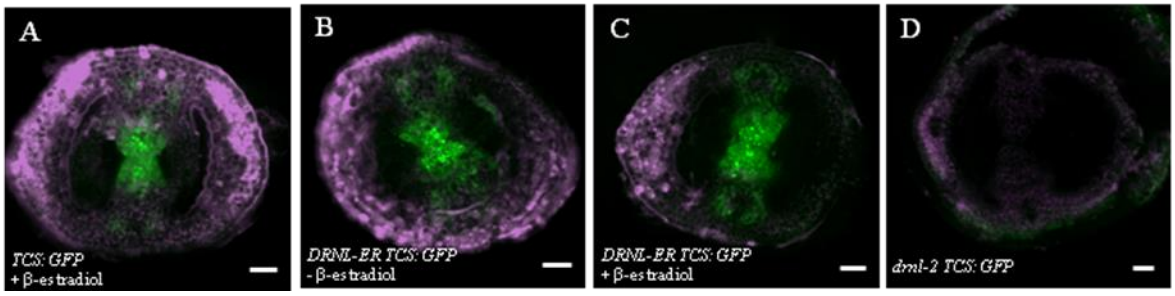

FIGURE 2.8. **Cytokinins signalling in response to BOL.** Cytokinins signalling was visualized through *TCS:GFP* sensor in *DRNL-ER* and *drnl-2* gynoecia cross sections at stages 9-10. **A)** *TCS: GFP* gynoecium with estradiol. **B)** *DRNL-ER TCS: GFP* with mock solution. **C)** *DRNL-ER TCS: GFP* with estradiol. **D)** *drnl-2* without any solution. Scale bars = 20 μM.

II.4 DISCUSSION

The loss of *BOL* function severely affects the development of sepals, petals and stamens, as initially described by Nag et al. (2007). Some defects have also been observed in *drnl-2* gynoecia and fruits, such as valve asymmetry, and more severe defects have been reported in combination with other mutations (Nag et al., 2007; Eklund et al., 2011; Chandler et al.,

2011b; Chandler and Werr, 2017). *BOL* is expressed early during organogenesis. Its expression has been observed before a primordium is histologically visible, and for this reason Chandler and colleagues have proposed it as a founder cell marker of floral organs (Chandler et al., 2011b). Most *drnl-2* gynoecia do not develop into fruits, and the most frequent defect in *drnl-2* gynoecia and fruits was the one-valve phenotype, in up to 30% of mutant gynoecia. This "one valve" may sometimes comprise the region where two normal valves would be present. The replum and valve margin that normally develop between each valve could only be detected on one side of these developing fruits. We found, also, that, beyond the founder cells and gynoecium primordium, *BOL* is also expressed at later stages during gynoecium development after the carpel primordia have been specified, and its expression gets confined to the presumptive valves as development progressed. The "one valve" mutant phenotype and *drnl-2* expression in the lateral domain is interesting considering that the different regions of the ovary of the gynoecium are thought to be molecularly similar to the Shoot Apical Meristem (SAM) and lateral organs (leaves) emerging from it (Bowman et al., 1999; Balanza et al., 2006; Ostergaard, 2009). The medial domain, where the CMM and later reproductive tissues develop (Reyes-Olalde et al., 2013), has been compared to the meristematic region, while the valves (lateral domain), have been compared to the lateral organs that initiate from the SAM.

The BOL function has been associated with lateral organ formation and impaired function causes organ fusion (at low penetrance) in cotyledons and stamens, possibly through *CUC (CUP SHAPED COTYLEDON)* genes (Ikeda et al., 2006; Nag et al., 2007; Chandler et al., 2007; Chandler et al., 2011b). Therefore, this particular "one valve" *drnl-2* phenotype may be due to a partial or total fusion of the valves (Supplementary Figure 1), and might further reflect the resemblance between valves and lateral organs. Another possibility, that cannot be discarded at present, is that some of those gynoecia arose from a single primordium, which then gave rise to a single valve with its own "medial region" on one side. It will be very interesting to further test this possibility.

Some examples of other genes expressed in the presumptive valve region in the developing gynoecium include, among others, *JAGGED*, *NUBBIN*, and members of the YABBY,

KANADI and HD-ZIP III families. They have been reported to be related to polarity or organ growth and most of their mutants present phenotypes that are different to the ones observed in *drnl-2* (Bowman et al., 1999; Kerstetter et al., 2001; Otsuga et al., 2001; Alvarez and Smyth 2002; Dinneny et al., 2004 and 2006; Roeder et al., 2006; Sundberg and Ferrandiz 2009; Nole-Wilson et al., 2010). Rather, the *drnl-2* phenotypes resemble more those caused by hormonal alterations, and at the functional level, this transcription factor has been suggested to be related to hormonal pathways (Marsch-Martinez et al., 2006; Ikeda et al., 2006; Chandler et al., 2009; Chandler et al., 2011a). An indirect connection between BOL and the auxins biosynthesis pathway through *STYLISH* activation has also been reported (Eklund et al., 2011). Moreover, coincidences between auxins maxima and organ initiation regions in the meristem periphery (Reinhardt et al., 2003; Heisler et al., 2005) have been observed. In the floral meristem, it has been suggested that *BOL* expression precedes auxins response maxima in the floral organ founder cells, and that it acts synergistically with local auxins biosynthesis and polar transport (Chandler et al., 2011b; Chandler and Werr, 2014). In the gynoecium, auxins response marker expression can be also detected as two foci, suggested to mark the two carpel primordia (Larsson et al., 2014), and this expression has been also observed in *BOL* marker lines (Chandler et al., 2011b). Mutants affected in auxins biosynthesis, transport, signalling or response (such as multiple *yucca* or *ettin* mutants) or plants treated with auxins transport inhibitors do not develop lateral organs or develop them with severe defects (Nemhauser et al., 2000; Reinhardt et al., 2000; Reinhardt et al., 2003; Heisler et al., 2005; Cheng et al., 2006). In the Arabidopsis gynoecium, these defects include the alteration of the apical-basal axis, such as valve asymmetry and reduction in valve number (Nemhauser et al., 2000; Cheng et al., 2006; Sohlberg et al., 2006), also observed in *drnl-2* gynoecia. However, it has also been suggested that auxins function, as revealed by the auxins response marker, may be independent of BOL during early floral development, because its expression is not affected in *drnl-2* (Chandler et al., 2011b).

Interestingly, cytokinin application to developing wild type gynoecia can also produce apical-basal defects (Zuñiga-Mayo et al., 2014). Moreover, altered expression of cytokinins pathway genes in mutant or altered *BOL* backgrounds has been found in global expression

analyses (Ikeda et al., 2006; Marsch-Martinez et al., 2006). One of these genes is *AHP6*, reported as a possible target of BOL (Ikeda et al., 2006). AHP6 is an important element for the correct emergence of organ primordia and the correct distribution of the primordia in the shoot apical meristem (Besnard et al., 2014). Interestingly, we observed *AHP6* expression at two lateral foci in the pre-patterned incipient carpel primordia, a similar pattern as the one described for *BOL* (Chandler et al., 2011b). The similarity of expression patterns continued through gynoecium development (Figure 2.4), and *AHP6* expression was clearly increased in *BOL* gain of function backgrounds (Figure 4). However, in inflorescences of the loss of function mutant *drnl-2,* we could not detect a clear decrease in *AHP6* expression by qRT-PCR, contrary to what we had expected. This may be because the regulation of *AHP6* by BOL is tissue-specific, restricted to very narrow domains, and the RNA used for this analysis was isolated from inflorescences and not from individual gynoecium tissues. Another possible explanation is that *drnl-2* is not a null allele (Nag et al., 2007). It is also feasable that we did not detect a strong reduction in *AHP6* expression in the loss of function background because *AHP6* is being regulated by other transcription factors such as MONOPTEROS/ AUXIN RESPONSE FACTOR 5 (MP/ ARF5; Bishopp et al., 2011; Besnard et al., 2014). However, we confirmed that the increased expression and inducible activation of BOL was able to increase the expression of *AHP6* in the gynoecium, possibly reflecting what occurs in the lateral domain, and maybe during the earliest stages of organ formation. Based on this, we expected an increase in the sensitivity to exogenously applied cytokinin in *drnl-2* gynoecia. Indeed, we found that young gynoecia showed greater sensitivity to exogenously applied cytokinin. However, we also observed that gynoecia at later developmental stages appeared to be less sensitive to this phytohormone. The increase in sensitivity in *drnl-2* young organs was revealed by more severe apical-basal defects in comparison to wild type. Furthermore,, in many *drnl-2* gynoecia valves were not observed. This type of response was similar to that observed in *ahp6* gynoecia. Interestingly, some very young *drnl-2* gynoecia failed to develop and became amorphous organs, which indicates that the lack of *BOL* function in these developing primordia rendered them unable to counterbalance the excess of cytokinins. The fact that the younger gynoecia are more responsive to cytokinins could be explained through BOL regulation of *AHP6*, and possibly also of *CYTOKININ OXIDASE 7* (*CKX7*), an enzyme that inactivates cytokinins. The latter

has been also proposed to be a direct target of BOL (Ikeda et al., 2006). The decrease in sensitivity in gynoecia at later stages (stages 9-11 as interpreted from their position in the inflorescence stem), was deduced from the lack of proliferating tissue that grows from the medial domain of developing gynoecia, normally observed in wild type genotypes but not in *drnl-2*. This decrease in the cytokinins sensitivity of mutant gynoecia correlates with the reduction of transcriptional response to cytokinins observed in *drnl-2 TCS: GFP* gynoecia around stages 9-10. However, we ignore if this loss of transcriptional response to cytokinins occurs only in gynoecia at these stages (9-10). For this reason, it will be very informative to observe the TCS signal at earlier stages during *drnl-2* gynoecia development.

The lower sensitivity to cytokinins in more developed gynoecia suggests that *BOL* is required for the normal response of the tissue to exogenous cytokinins. It could be that this gene is required for downstream of cytokinins signalling responses. It may also modulate cytokinins homeostasis or the sensitivity of the tissue to this hormone, either by regulating the same genes that it regulates at early stages, but in an opposite direction (e. g., repressing instead of activating), or regulating other genes. For example, it has been reported that increased expression of *BOL* caused decreased expression of some type A ARRs (Ikeda et al., 2006), which negatively regulate cytokinins signalling (Hwang et al., 2002; reviewed by Schaller et al., 2015). Also, a mutant in tomato where no leaves develop has been recently reported (Capua and Eshed, 2017). The authors found that the mutation is located in the ortholog gene of *BOL* known as *LEAFLESS (LFS).* Moreover, in *lfs* mutant plants, the development of leaf primordia is not recovered through auxins micro-application nor by the expression of *LFS* under the *DR5* promoter. The authors suggest *LFS* might be also regulating cytokinins homeostasis, because genes that participate in the cytokinins pathway (such as type A ARRs and CKXs) were found to be altered in global expression analyses performed with the *lfs* mutant (Capua and Eshed, 2017).

Finally, another interesting observation was the asynchronous response of young *drnl-2* gynoecia, in comparison to the rest of evaluated genotypes. This response may be reflecting that the loss of *BOL* function is not only affecting the morphology of floral organs but also

affects the organ spatio-temporal order of initiation or development in the floral meristem. The exacerbated asynchrony observed in treated *drnl-2*, and not detected in *ahp6* mutants, further suggests that BOL regulates more elements in the cytokinins pathway. Therefore, it might be that Arabidopsis *BOL*, and possibly the tomato *LFS*, have functions in modulating the cytokinins pathway, or the response to it, through differential gene regulation at different stages of development. Figure 2.9 shows a model depicting the participation of BOL as a modulator of cytokinins homeostasis and response during gynoecium development, proposed to play at least two different roles as gynoecium development progresses.

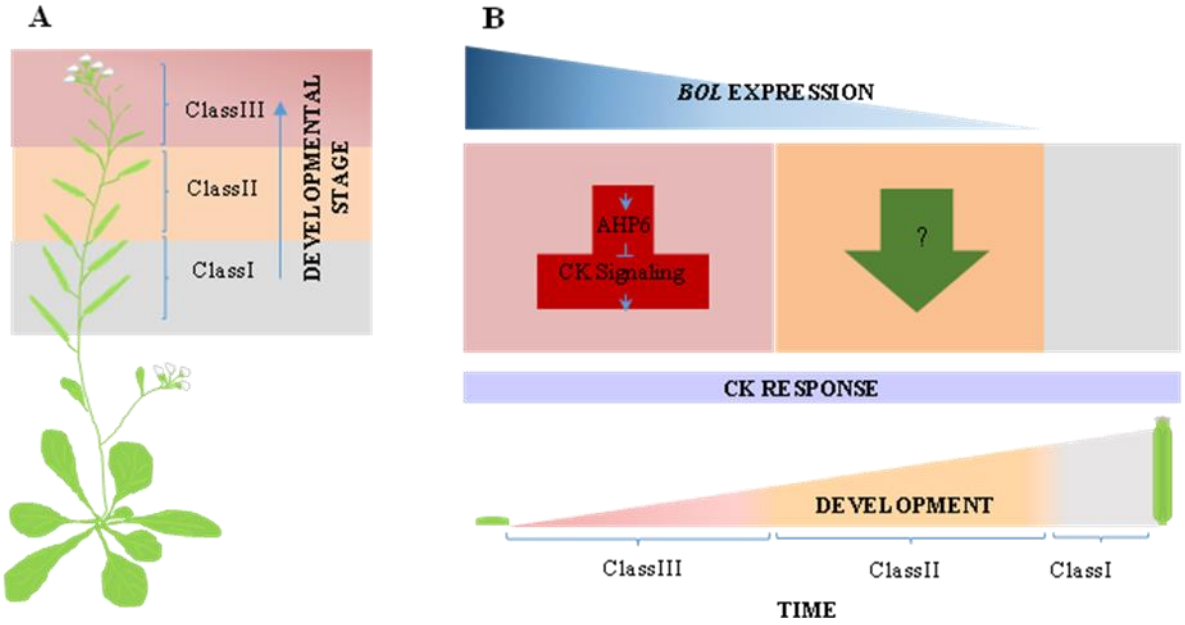

FIGURE 2.9. **Proposed model for *BOL* cytokinins response modulation during gynoecium development. A)** Three classes of phenotypes were observed along the inflorescence stem in response to cytokinins exogenous application. From bottom to top, Class I, Class II, and Class III. **B)** According to the different responses of developing *drnl-2* gynoecia to cytokinins, BOL appears to modulate cytokinins homeostasis (or to participate in the final output of cytokinins signalling) in at least two different ways during

development. At very early stages of gynoecium development, BOL represses the cytokinins response, most likely through the activation of the cytokinins signalling repressor *AHP6*, and maybe other genes, like *CKX7*. At intermediate stages of development, however, the effects of cytokinins application suggest that *BOL* positively modulates the response to cytokinins through another mechanism (or is required for cytokinins response output). In the developmental time line, orange represents early, green represents intermediate, and gray late gynoecium developmental stages, when *BOL* is not expressed.

II.5 CONCLUSION

Besides being expressed at the gynoecium initiation stage, *BOL* participates at further stages during gynoecium development. It differentially modulates the response of the gynoecium to cytokinins at distinct stages, having possibly a dual role during development. It would be interesting to test whether this is the case for other plant species and organs, and to further clarify the mechanisms through which this modulation is achieved. Moreover, considering that *BOL* has been previously associated to other hormones and is also modulating cytokinins homeostasis or response, *it* may orchestrate different hormonal pathways during the development of the gynoecium and, possibly, of new organs in general.

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CHAPTER III

BOL Modulates the Cytokinins Pathway through the Transcriptional Activation of *HISTIDINE PHOSPHOTRANSFER PROTEIN 6* and *ADENYLATE ISOPENTENYLTRANSFERASE 5*

III.1 INTRODUCTION

Plants, unlike other organisms, are sessile and are therefore subjected to changing environmental conditions such as rain, hail, drought, solar radiation, wind, low nutrient availability, etc. This makes them very susceptible to diseases, pests and herbivore attack. However, plants have mechanisms that allow them to dealing with these situations. One example of this is when plants resist some diseases through the induction of cell death in the tissues where the infection is located (Morel and Dangl, 1997). Plants can use this strategy because they can regenerate tissues and develop organs in a constant way; this ability also serves them to survive after damage has been caused by herbivores or some environmental factors.

This continuous organ development arises from meristems. Plants have different meristematic tissues which are located at different positions along the plant body. Among these meristems are the primary meristems (apical shoot and root meristems) and lateral or secondary meristems (procambium and vascular cambium). Thus, meristems are important to model plant architecture adapting the growth to changing environmental conditions and to renew themselves after damage (Jouannet et al., 2015).

A meristem contains clusters of cells called stem cells which are placed in the central zone. Stem cells are relatively undifferentiated cells defined by their abilities for self-renewal and give rise to one or more differentiated cell types. Thus, stems cells are programmed to form an entire new organ with its multiple tissues (Bäurle and Laux, 2003). This shows that stem cells have plastic fate regulation. This plasticity is regulated by the current position of

the cells. Cells that are in central zone do not differentiate whereas cells that are passively displaced to the peripheral zone by cell divisions are exposed to different cues or biochemical signals until the cell is incorporated into a final position (Gaillochet et al., 2015).

Some biochemical signals that modulate stem cell plasticity are phytohormones. Auxins and cytokinins were the first phytohormones for which the ability to modulate cell fate was observed in tissue culture. When meristematic tissues are cultivated *in vitro*, ratio modulation between auxins and cytokinins is decisive in fate specification to form calli, shoots or roots (Skoog and Miller, 1957). Recent studies have begun to shed light on the auxins/ cytokinins crosstalk. These studies show that auxin and cytokinins act together dynamically to control plant development (Vanstraelen and Benková, 2012; El-Showk et al., 2013). Their interactions can be agonistic or antagonistic depending on the plant developmental process to confer distinct cell fates to stem cells or precursor cells in close proximity (Bishopp et al., 2011, Schaller et al., 20015). With the level of information constantly generated, the complexity of development and cell fate modulation is evident. This indicates there is still much to know about development and cell fate regulation.

The BOL transcription factor is expressed at very early stages of aerial organ development. It has even been proposed that it is expressed in organ founder cells (Chandler et al., 2011b). Its over-expression induces the development of ectopic organs in tobacco and green calli formation in Arabidopsis roots, which subsequently can form leaves, inflorescences and flowers when detached from the root (Marsch-Martinez et al., 2006, Ikeda et al., 2006). Apparently its function is closely related to phytohormonal pathways, because a global gene expression analysis using young leaves from a BOL dominant mutant (*bol-D*) showed changes in genes related to cytokinins among other hormones such as auxins, ethylene, jasmonates and gibberellins (Marsch-Martinez et al., 2006). Ikeda et al. (2006) through microarray analyses using *35S::DRNL-ER* root explants, identified that *CKX7* and *AHP6* (genes involved with cytokinins pathway) are up regulated by BOL. On the other hand, Eklund et al., (2011) proposed that BOL indirectly regulates auxins biosynthesis, through *STILISH1* (*STY1)* transcriptional activation that in turn results in

YUCCA4 activation (Eklund et al., 2010). Coincidences between auxins maxima and *DRNL* expression have been observed (Chandler et al, 2011b; Chandler and Werr, 2014), and the relationship between BOL and auxins has been explored (Eklund et al., 2011, Chandler et al., 2007; Chandler et al., 2011a; Chandler et al., 2011b ;Chandler and Werr, 2014, Capua and Eshed, 2017). However, there is no evidence of direct relationship between BOL and auxins, and there is scattered data about its relation to other phytohormone pathways.

Because BOL is a transcription factor, we consider that one way to know more about BOL's function is to identify BOL transcriptional targets. Because there is evidence linking BOL with phytohormones, it would be interesting to know if among these targets, there are genes related to auxins and cytokinins, since these hormones play an important role in cell fate modulation for new organ development. Because the relationship between BOL and cytokinins is less clear, the main focus of this chapter is to explore its possible regulation of elements of the cytokinins pathway.

III.2 MATERIALS AND METHODS

III.2.1 Plant materials and growth conditions

The lines used in this study were wild type (WT) Columbia (Col); reporter lines *BOL::GUS* (Marsch-Martinez et al., 2006), *IPT5::GUS* (Raffaele Dello Ioio), *AHP6*::*GFP* (Mähönen et al., 2006a), *TCS::GFP* (Müller and Sheen, 2008) and the inducible *BOL* line *DRNL-ER* (Ikeda et al., 2006; Eklund et al., 2011).

All plants were grown *in vitro*. The seeds were disinfected with ethanol (70%) and sodium hypochlorite (20%), remaining 5 min in each solution. Subsequently, they were washed three times with distilled water. Then, they were sown in 0.5× MS medium (PhytoTechnology Laboratories TM), 1.4% plant agar (PhytoTechnology Laboratories TM) and 1% sucrose. They were placed at 4°C for 48 h, and finally transferred to a growth chamber for germination and growth, under long day conditions (16 h light and 8 h dark) at 22 ° C.

III.2.2 Gene expression analysis

For qRT-PCR analysis, RNA from a transcriptomic experiment with the inducible line was used. This RNA was extracted from aerial tissue from 9 DAG *DRNL-ER* seedlings and for this experiment we also used aerial tissue from Col-0 seedlings. The treatments and RNA extraction are described with more detail in chapter IV, and the general qRT-PCR procedure is described in chapter II. For the evaluation of *AHP6* expression, the same conditions as described for chapter II were used. The primers used to amplify *AHP6* were FW 5'-TAACGTCTGCGTTGCCTTT-3' and RV 5'CCTCCAGTCCTCTCAAGCAC3' (Reyes- Olalde et al., 2017). For the evaluation of *IPT5* expression were FW 5'-CGATGACGAAAGAAGGGAAG-3´ and RV 5'-CTCCAAGACAGCGACCAATC-3'. These were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). *ACT2* was used as a housekeeping gene. *ACT2* primers: FW 5´- AATCACAGCACTTGCACC-3' RV 5'-ATTCCTGGACCTGCCTC-3' RV (Palmeros-Suárez et al., 2015).

III.2.3 Evaluation of marker lines expression in response to BOL

To determine at what time the regulation of *AHP6* and *IPT5* expression in response to BOL in specific tissues could be observed, *DRNL-ER IPT5::GUS* seedlings sampled 6 days after germination (DAG) were grown in MS 0.5× medium and then transplanted to MS 0.5× medium with 10 μM β-estradiol and as a control treatment MS 0.5× medium with DMSO (the solvent used to dissolve β-estradiol). Treated seedlings were removed from the treatment plates at 2, 4, 6, 8, 24 and 48 h, and processed for β-Glucuronidase staining, as mentioned above . *IPT5* expression was observed under a Stemi 2000-C stereoscope coupled with Axiocam ERc 5s camera (Carl Zeiss) and a DM750 microscope coupled with ICC50 HD camera (Leica).

Once having determined the optimal time at which changes in *IPT5* expression are clearly appreciated, which was 48 h, the rest of marker plant lines were analyzed at this time.

For the *AHP6* expression analysis in response to BOL, *DRNL-ER AHP6::GFP* seedlings were observed using a confocal microscope (as described in chapter II).

For TCS signal visualization, seedlings were observed in a confocal microscope LSM 880 NLO (Carl Zeiss). GFP was excited with a 488-nm laser. *DRNL-ER TCS::GFP* seedlings were collected and fixed with paraformaldehyde 4%, then cleared with ClearSee (Kurihara et al., 2015).

III.2.4 Promoter analysis

An *in silico* analysis of the *AHP6* and *IPT5* promoters was made with the Plant Promoter Analysis Navigator (PlantPAN**;** http://PlantPAN2.itps.ncku.edu.tw**)** in order to identify putative GCC box-like regulatory element sequences. For the analysis, a sequence of 2000 bp upstream of the transcription start site and 500 pb downstream of the transcription start site was used.

III.3 RESULTS

III.3.1 Auxins and cytokinins pathway genes as possible targets of BOL

As part of the strategy towards understanding the function of BOL, we proceeded to identify genes that could be regulated by BOL. Based on background that links BOL with phytohormonal pathways, mainly auxins and cytokinins, we decided to first focus on genes related to these two phytohormones. For this, we took advantage of data generated from a previous transcriptomic analysis (described in chapter IV). This transcriptome was performed using aerial tissues of *DRNL-ER* seedlings. From this experiment we obtained a list of genes that are up- and down-regulated after the induction of BOL activity for 30 min and 8 hours. An analysis comprising broader gene categories found in the transcriptomic analysis is discussed in chapter IV, but in the present chapter we focused in the auxins and cytokinins pathways and performed a search for genes related to these phytohormones. In number, there were more genes related to auxins and most of them appeared to be down-regulated (Figure 3.1 and Table 3.1). Fewer genes were up regulated and their expression changes were moderate (Table 3.1). We also found different genes related to cytokinins, which, though less in number, interestingly presented more pronounced changes in expression than the auxins-related genes (i. e., the fold change of this set of cytokinins-

related genes was higher than the one observed in auxins-related genes) (Table 3.2). Here, the term "fold change" or "fold" refers to the Logarithm (base 2) of the fold change.

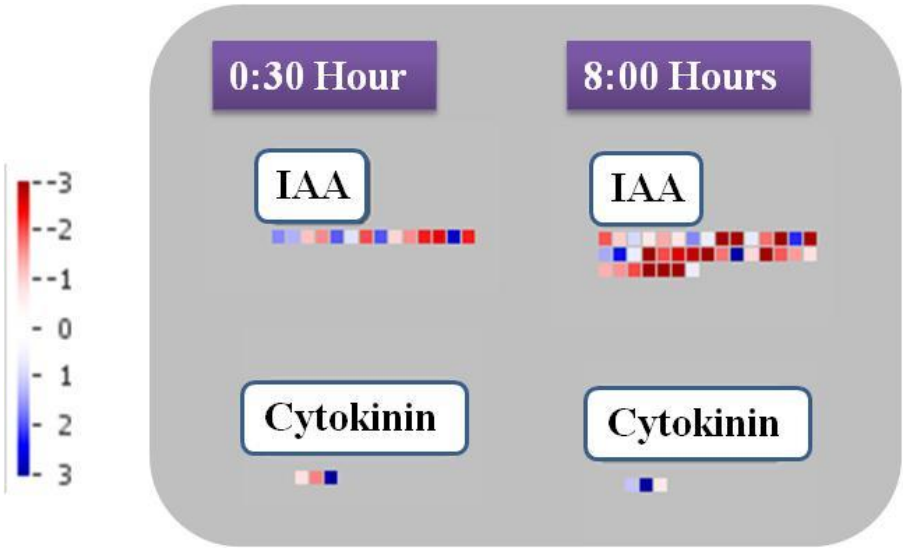

Figure 3.1 MapMan Analysis. MapMan visualization of expression changes of genes related to the auxins and cytokinins pathways at 0:30 and 8:00 h after BOL induction.

The identified genes participate in different steps of the auxins and cytokinins pathways, including synthesis, conjugation, transport, signalling and response (Figures 3.2 and 3.3). For the auxins biosynthesis pathway (Table 3.1), we found *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1)* and *YUCCA5 (YUC5)*. *TAA1* was down regulated at 30 min and 8 h, while *YUC5* was up regulated after 8 h of BOL induction. Other up-regulated genes were related to auxin transport, such as *PIN-FORMED 1* (*PIN1)*, *PIN-LIKE* (*PILS3* and *PILS5*) and *PILS5*. *AINTEGUMENTA-LIKE 6* (*AIL6)*, another AP2 / ERF trascription factor was also up-regulated. This gene has been reported to be induced by *AUXIN RESPONSE FACTOR 5/ MONOPTEROS* (*ARF5/MP*) (Yamaguchi et al., 2013). Interestingly, among early auxins response genes, 17 members of ***SMALL AUXIN UP REGULATED RNA*** (SAUR) gene family showed expression changes. *SAUR 34* and *SAUR 38* were up-regulated, while the rest were down-regulated.

On the other hand, several genes related to cytokinins were also identified in these data. The number of identified genes related to cytokinins was lower than the number of auxins-related genes (figure 3.1). However, the up regulated genes show a higher increase in their expression than the auxins related genes (table 3.2).

The transcriptomic data indicates that BOL also regulates *AHP6* at earlier stages of development, in vegetative aerial tissue of seedlings. As mentioned in previous chapter, AHP6 is a negative regulator of cytokinins signalling. However, among the differentially expressed genes, there were also other cytokinins signalling regulators in addition to *AHP6*, such as *KISS ME DEADLY 2* (*KMD2)* which has been also proposed to act as a negative regulator. On the other hand, we also found *AHP4* in the list with differential expression upon BOL induction. *AHP4*, unlike *AHP6*, positively regulates cytokinins signalling. The positive regulation of cytokinins signalling also seems to be favored by the repression of *ARABIDOPSIS RESPONSE REGULATORS*, *ARR4* and *ARR6* (negative regulators). Other factors involved in cytokinins signalling are CYTOKININ RESPONSE FACTORS (CRFs). *CRF1* and *CRF6* were up regulated. It should be noted that we found other genes involved in several processes of this pathway. There were genes encoding synthesis enzymes I*sopentenyl transferase* (*IPT5* e *IPT7*), a degradation enzyme (*CKX7*), possible transporters (*PUP7*, *14*, *18*), a receptor *Arabidopsis Histidine Kinase 4 / WOODEN LEG* (*AHK4/ WOL*), genes whose expression is influenced by cytokinins and some other genes that do not participate directly in the cytokinins pathway. However, when the expression of the latter genes is altered, changes in the levels of cytokinins or phenotypes that increase their severity with cytokinins application have been observed.

Table 3.1 Candidate BOL target genes related to the auxins pathway

Gene	Short description	0.30 H	LogFC	8:00 H	LogFC
TAA1	Tryptophan aminotransferase of Arabidopsis 1	✓	-1,5	✓	-1,1
YUC5	Flavin-binding monooxygenase family protein			✓	1,7
GH3.3	Auxin-responsive GH3 family protein	✓	-1,0	✓	-2,0
GH3.6 /	Auxin-responsive GH3 family protein			✓	-1,0
GH3.2	Auxin-responsive GH3 family protein	✓	-1,2	✓	-1,8
GH3.5	Auxin-responsive GH3 family protein			✓	-1,6
UGT74E2	Uridine diphosphate glycosyltransferase 74E2			✓	2,5
MES17	Methyl esterase 17			✓	-2,0
AUX1	Transmembrane amino acid transporter protein			✓	0,8
LAX2	Like AUXIN RESISTANT 2	✓	-1,0	✓	-1,9
LAX3	Like AUX1 3			✓	-1,4
PIN1	Auxin efflux carrier family protein	✓	1,7	✓	1,7
D6PK	D6 protein kinase	✓	-0,6		
PIN3	Auxin efflux carrier family protein			✓	-0,7
PIN7	Auxin efflux carrier family protein			✓	-0,8
PID	Protein kinase superfamily protein			✓	-1,3
FKD1	Pleckstrin domain and DUF828-containing protein			✓	-1,1
PILS3	Auxin efflux carrier family protein			✓	1,2
PILS5	Auxin efflux carrier family protein			✓	0,9
ABCB19	ATP binding cassette subfamily B19	✓	-1,1		
GRH1	GRR1-like protein 1			✓	-1,2
VFB3	VIER F-box proteine 3	✓	0,8		
IAA1	Indole-3-acetic acid inducible			✓	-1,8
IAA2	Indole-3-acetic acid inducible 2			✓	0,9
IAA3	AUX/IAA transcriptional regulator family protein	✓	-0,8	✓	-1,6
IAA4	AUX/IAA transcriptional regulator family protein			✓	-0,9
IAA14	Indole-3-acetic acid inducible 14			✓	-1,4
IAA16	Indoleacetic acid-induced protein 16	✓	-0,7		
ARF6	Auxin response factor 6	✓	-0,7		
AIL6	AINTEGUMENTA-like 6	✓	1,8	✓	0,9
SAUR4	SAUR-like auxin-responsive protein family			✓	-2,6
SAUR16	SAUR-like auxin-responsive protein family			✓	-2,8
SAUR21	SAUR-like auxin-responsive protein family			✓	-5,8
SAUR23	SAUR-like auxin-responsive protein family			✓	-3,0
SAUR24	SAUR-like auxin-responsive protein family			✓	-4,2
SAUR31	SAUR-like auxin-responsive protein family			✓	-1,3
SAUR34	SAUR-like auxin-responsive protein family	✓	2,0	✓	2,2
SAUR38	SAUR-like auxin-responsive protein family	✓	2,0		
SAUR46	SAUR-like auxin-responsive protein family	✓	-2,3		
SAUR62	SAUR-like auxin-responsive protein family	✓	-2,0	✓	-2,0
SAUR63	SAUR-like auxin-responsive protein family	✓	-2,3	✓	-4,9
SAUR64	SAUR-like auxin-responsive protein family	✓	-2,5	✓	-5,2
SAUR65	SAUR-like auxin-responsive protein family			✓	-6,3
SAUR66	SAUR-like auxin-responsive protein family			✓	-1,5
SAUR67	SAUR-like auxin-responsive protein family			✓	-3,4
SAUR76	SAUR-like auxin-responsive protein family			✓	-5,9
SAUR78	SAUR-like auxin-responsive protein family	✓	-1,6		
PAR1	Phy rapidly regulated 1	✓	-2,6	✓	-1,6
PAR2	Phy rapidly regulated 2			✓	-1,2
STY2	A member of SHI memeber			✓	1.2
LRP1	Lateral root primordium (LRP) protein-related	✓	0,9	✓	1,0
AT5G35735	Auxin-responsive family protein			✓	2,4
AT3G25290	Auxin-responsive family protein			✓	-2,0
AT4G12980	Auxin-responsive family protein	✓	1,3	✓	0,7
HAT2	Homeobox-leucine zipper protein 4 (HB-4)			✓	-1,4
NAC101	NAC-domain protein 101			✓	-1,8
ACS8	1-amino-cyclopropane-1-carboxylate synthase 8	✓	-1,6	✓	2,4
GH3.11	Auxin-responsive GH3 family protein	✓	0,8	✓	0,7
AT5G13370	Auxin-responsive GH3 family protein			✓	0,7
AT5G51470	Auxin-responsive GH3 family protein			✓	-1,8
SAG12	Senescence-associated gene 12	✓	4,5	✓	9,5
PAP1	Putative MYB transcription factor			✓	-1,8
NPY3	Phototropic-responsive NPH3 family protein	✓	1,4	✓	1,5
NPY5	Phototropic-responsive NPH3 family protein		-0,7	✓	-0,8
ACX4	Acyl-CoA oxidase 4			✓	1,3

Colors in the table indicate the relationship of each gene with key steps of the auxins pathway showed in figure 3.1.White indicates an

Auxins Pathway

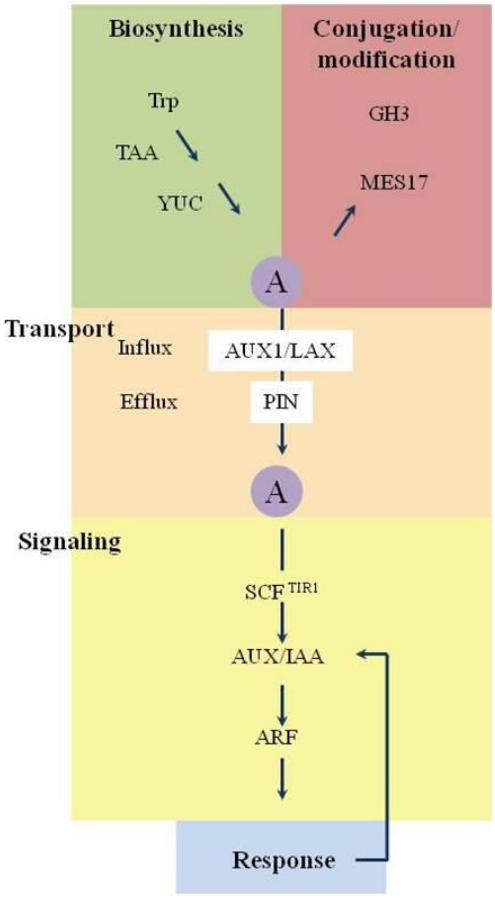

Figure 3.2 Key elements involved in auxins biosynthesis, conjugation/ modification, transport and signalling (Modified from Schaller et al., 2015)

Table 3.2 Candidate BOL target genes related to the cytokinins pathway

Gene	Short description	0:30 H	logFC	8:00 H	LogFC
IPT5	Isopentenyltransferase 5	✓	3,3		
IPT7	Isopentenyltransferase 7			✓	5,0
CKX7	Cytokinin oxidase 7	✓	1,3		
PUP7	purine permease 7			✓	1,6
PUP14	Purine permease 14			✓	1,5
PUP18	Purine permease 18			✓	2,4
WOL	Histidine kinase protein			✓	-0,7
AHP4	Histidine phosphotransfer protein 4	✓	1,6		
AHP6	Histidine phosphotransfer protein 6	✓	4,6	✓	4,5
ARR4	Response regulator 4	✓	-1,5	✓	-1,0
ARR6	Response regulator 6	✓	-2,3	✓	-1,8
KMD2	Kiss Me Deadly			✓	1,3
CRF1	Cytokinin response factor 1	✓	3,8	✓	4,2
CRF6	Cytokinin response factor 6			✓	1,4
UGT76C2	UDP-glucosyl transferase 76C2			✓	1,2
SOB5	Suppressor of phytochrome b 5			✓	1,4
TNY	AP2-EREBP. Integrase-type DNA binding superfamily protein	✓	-1,5		
GIS3	C2H2 and C2HC zinc fingers superfamily protein			✓	-3,8
GA2OX2	Gibberellin 2-oxidase			✓	1,1
ST4C	Sulfotransferase 4C			✓	-3,3
MYBD	Myb-like transcription factor family protein	✓	-1,0		
SAG12	Senescence-associated gene 12	✓	4,5	✓	9,5
ACC1	Acetyl-CoA carboxylase 1	✓	0.8		
POM1	Chitinase family protein			✓	-1,0
VND6	Vascular Related NAC-Domain 6			✓	-1,8

Colors in the table indicate the relationship of each gene with key steps of the cytokinins pathway showed in figure 3.1.White indicates an unclear relationship with the cytokinin pathway.

Cytokinins

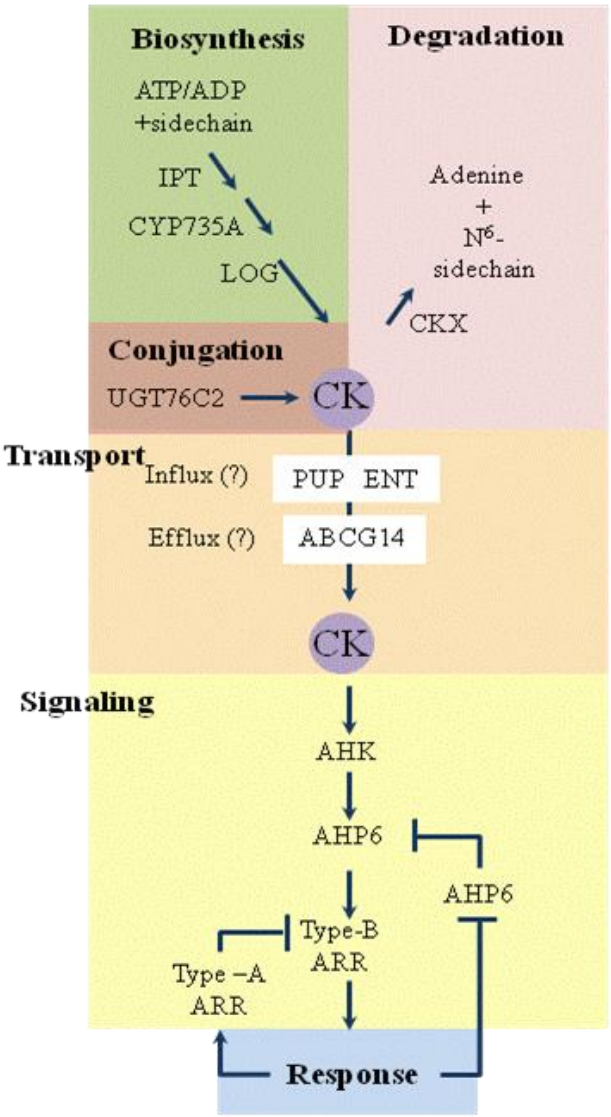

Figure 3.3 Key elements involved in cytokinins biosynthesis, degradation, transport and signaling (Modified from Schaller et al., 2015.

III.3.2 *AHP6* and *IPT5* are transcriptionally activated by BOL

Due to the identification of several genes regulated by BOL and related to cytokinins, we proceeded to explore some of these genes in greater depth. We focused on those that had a Log2 fold change higher than 30 minutes after induction, since there is a greater chance of them being BOL direct targets. As in the previous chapter we showed that BOL can promote a negative regulation of cytokinins signalling through *AHP6*, but on the other hand we found indications of a positive effect in the pathway. *IPT5* was chosen as the target gene to begin the exploration of this positive effect. We were also interested in understanding the biological relevance of this dual regulation of cytokinins by BOL, so we continued working with *AHP6* in parallel with *IPT5*.

As a first step, we proceeded to validate the upregulation of these two genes in response to BOL through qRT-PCR. In this analysis, wild type seedlings were included to corroborate that the changes in their expression are due to the induction of BOL transcription factor and not to the treatments (CHX and β-estradiol), since the transcriptome comparison was only carried out with the inducible line (not induced and induced BOL activity, respectively), due to the cost of the experiment.

The qRT-PCR results showed that BOL promotes an increase in *AHP6* and *IPT5* expression. This upregulation is very strong for *AHP6* and moderate in *IPT5*. Further information provided by this experiment was that the inducible line (without induction) shows a higher basal level of expression of these 2 genes than the wild type seedlings. This suggests that there is BOL transcription factor activity leakage in the inducible line. This is interesting because this presumed leakage appears to be sufficient to promote an increase in the expression of *AHP6* and *IPT5* (Figure 3.4), but not to cause the characteristic phenotypes produced when induced.

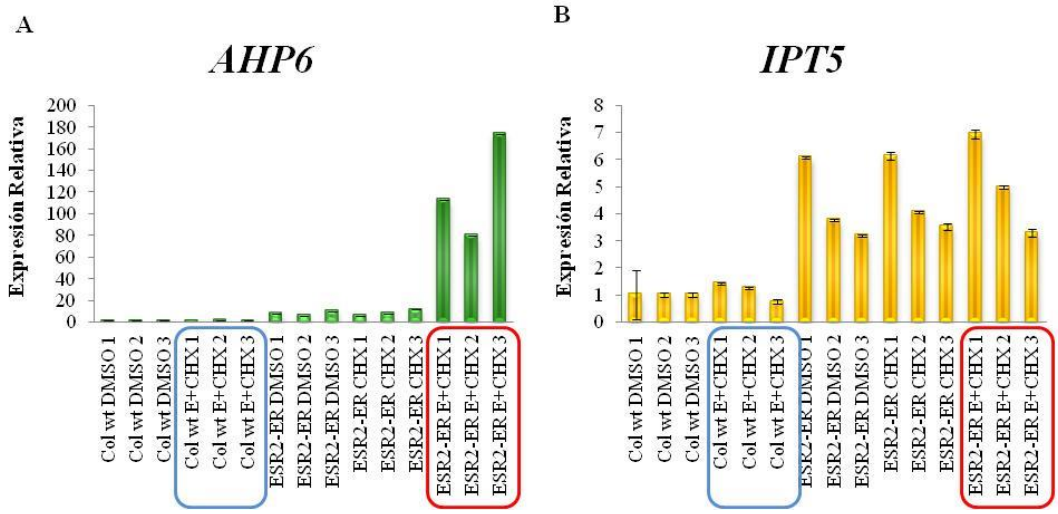

Figure 3.4 Regulation of *AHP6* and *IPT5* expression by BOL in seedling aerial tissue. **A)** *AHP6* and **B)** *IPT5* relative expression. The blue box highlights wt seedlings treated with β-estradiol, and the red box highlights *DRNL-ER* seedlings treated with β-estradiol. DMSO, solvent; E, β-estradiol; CHX, cycloheximide.

III.3.3 *AHP6* and *IPT5* are simultaneosly expressed with *BOL* in some tissues.

Once we confirmed *AHP6* and *IPT5* transcriptional regulation (direct or indirect) by BOL in Arabidopsis seedlings, the next step was to identify in which tissues this regulation could be occurring. As a first approximation, we proceeded to explore the expression patterns of *AHP6* and *IPT5* and compare them with *BOL* expression and localization of the BOL protein.

It has been reported that *BOL* is expressed in the leaf primordia of Arabidopsis seedlings (Marsch-Martínez et al., 2006; Nag, et al., 20079). However *BOL::GUS* seedlings histological sections were made to better characterize *BOL* expression. In addition, the location of the BOL protein was also analyzed with the line *BOL::BOL:GFP*. We confirmed BOL expression in the meristem peripheral zone, at the region where a new

primordium will emerge (Figure 3.5 B). The BOL protein is also located in the leaf primordia (Figure 3.5 A). As the primordium begins to differentiate, *BOL* expression is restricted to the vasculature. Interestingly, *BOL* expression was also found in stipules (Figure 3.5 B, C). Because there are no reports that explore the expression of *AHP6* and *IPT5* in vegetative aerial tissue of seedlings, the expression of these genes was sought in those tissues using the reporter lines *AHP6::GFP* and *IPT5::GUS*,. From this exploration we were able to identify *AHP6* and *IPT5* expression in some regions of aerial vegetative tissue that coincided with *BOL* expression.

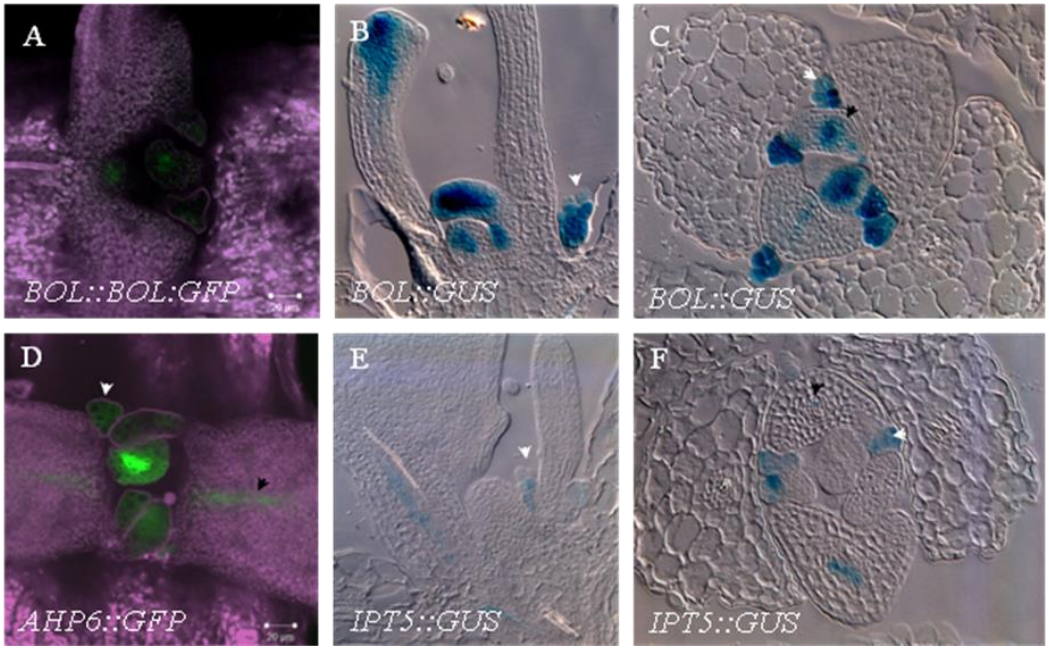

Figure 3.5 Comparison of *BOL* and *AHP6* and *IPT5* expression patterns in seedling aerial tissues. A) BOL localization in leaf primordia. **B)** and **C)** *BOL* expression. **D)** *AHP6* expression. **E)** and **F)** *IPT5* expression. **B** and **E** are longitudinal sections of seedlings, **C** and **F** are transversal sections. White arrowheads highlight stipules and black arrowheads highlight expression in vascular tissue.

Again, a high coincidence was found with *BOL* and *AHP6* expression, similalrly to what was found in the gynoecium. *AHP6* is expressed in a similar way to *BOL* in leaf primordia, vasculature and stipules; however the expression in vasculature is weaker, to such an extent

that in some plants it cannot be observed (Figure 3.5 D). In the case of *IPT5*, its expression is very weak in these tissues in comparison to *BOL* expression. However, high coincidence in expression can be observed, mainly in young tissues (leaf vasculature at a very early stage of development) and stipules (Figure 3.5 E, F). While in more developed tissues, some differences can be appreciated, since there is *IPT5* expression in the vasculature of leaves at later stages of development, and cotyledons (Figure 3.6 B), while the expression of *BOL* remains very discrete in the leaf apex and cotyledons (Figure 3.6 A).

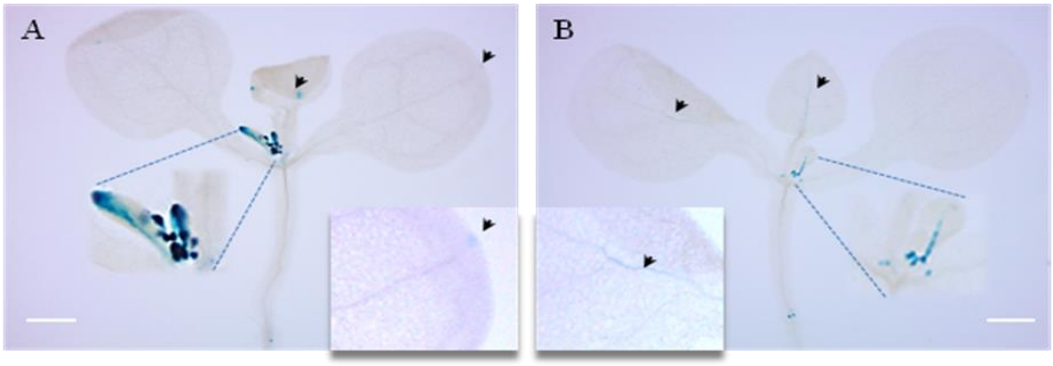

Figure 3.6 Comparison of *BOL* and *IPT5* expression in Arabidopsis seedlings. **A)** *BOL* expression. **B)** *IPT5* expression. Coincidences in expression are observed in some tissues, and also differences at later stages of leaf development (highighted by black arrowheads). Bars = 500µm.

These results are very interesting since they allowed us to identify expression patterns that had not yet been described for *AHP6* and *IPT5*. In addition, we found common expression in tissues that have been very little studied, such as stipules. Having found common tissues where *BOL*, *AHP6* and *IPT5* are expressed (e. g., vasculature and stipules) further supports that the idea that BOL could regulate the expression of these genes.

III.3.4 Increased BOL activity promotes *AHP6* and *IPT5* expression changes in vascular tissue.

Once we found that there were tissues in which there was coincidence between *BOL*, *AHP6*

and *IPT5* expression, we proceeded to evaluate whether *BOL* overactivation affected the expression of these two genes in such tissues. To perform this objective, it was decided to analyze the expression of *AHP6* and *IPT5* in the *drnl-2* mutant and in the inducible line *BOL DRNL-ER*. However, the cross between *drnl-2* and *AHP6::GFP* was not possible to obtain, so it was not included in the analysis. The *IPT5* expression was analyzed in the *drnl-2* mutant background. In this analysis we observed a decrease of *IPT5* expression in the vasculature of the cotyledons (in 100% of the seedlings) and of the hypocotyl (48% of the seedlings) (Figure 3.7).

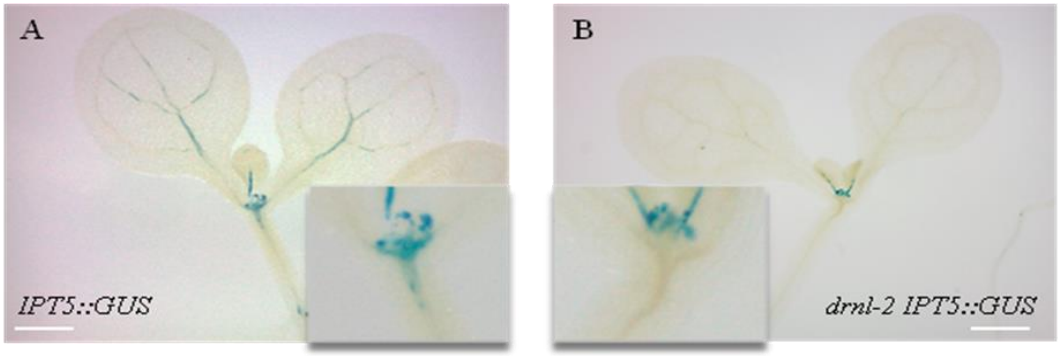

Figure 3.7 *IPT5* expression in the *drnl-2* background. A) *IPT5* expression in a wild type background. **B)** *IPT5* expression in the *drnl-2* background. Magnifications show changes in expression at the hypocotyl vasculature. Bars = 0.5mm.

On the other hand, we also proceeded to evaluate whether the activation of *AHP6* and *IPT5* expression upon BOL over-expression, was generalized or localized to specific tissues. Once the crosses of the *AHP6* and *IPT5* marker lines with the *DRNL-ER* line were obtained, tests were carried out to determine the times at which *AHP6* and *IPT5* expression changes could be visualized *in planta*. These tests were carried out in the *DRNL-ER IPT5::GUS* line. Different times of induction were tested: 2, 4, 6, 8, 24 and 48 h. Overnight staining of these plants did not detect evident changes at the first hours after induction (2 to 8). Only 24 h after induction, changes in the *IPT5* expression pattern could be observed. However at 48 h, these changes in expression were even more evident. So 48

h were taken as reference to also analyze *AHP6* expression and for the rest of experiments (Figure 3.8).

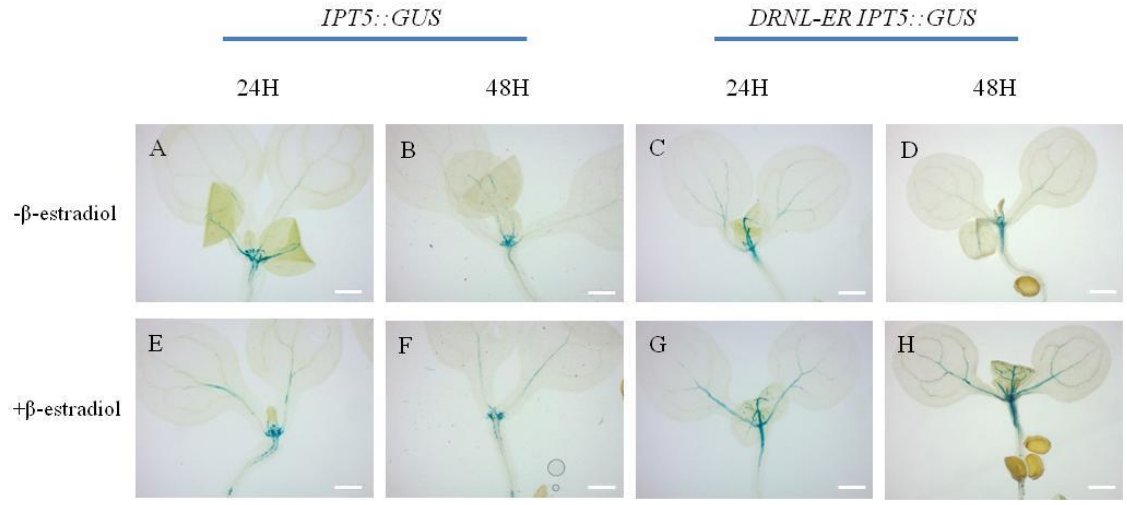

Figure 3.8 *IPT5* expression in response to *BOL* induction at different times. From **A)** to **D),** seedlings treated with mock solution and from **E)** to **H),** seedlings treated with inductive solution. **A)**, **B)**, **E)**, **F)** IPT5::GUS control plants and **C)**, **D)**, **G)**, **H)** *DRNL-ER IPT5::GUS* seedlings. Bars = 0.5mm.

These results, corroborated that BOL promote *AHP6* and *IPT5* upregulation, and that this activation occurs in specific tissues. It is interesting to note that although *BOL-ER* transcription is regulated by a *35S* promoter, the increase in *AHP6* and *IPT5* expression is restricted to the vasculature. In non-induced *DRNL-ER IPT5::GUS*, the *IPT5* expression is slightly higher in some regions of cotyledons, leaf and hypocotyl vasculature, compared to wild type plants (Figure 3.9 A). But when BOL activity is induced, the GUS staining expands completely throughout the entire vasculature of these organs. (Figure 3.9 E) For *AHP6*, we saw an expression increase in the vasculature of the petiole of the cotyledons (Figure 3.9 G).

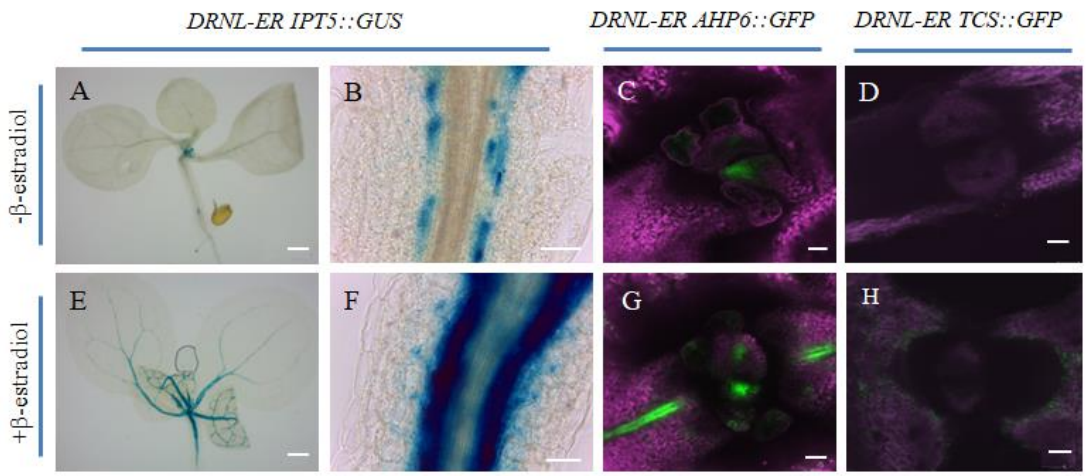

Figure 3.9 BOL regulates *IPT5* and *AHP6* expression in vascular tissues, and alters TCS signal. A), **E)** *DRNL-ER IPT5::GUS* seedlings. **B)**, **F)** *DRNL-ER IPT5::GUS* hypocotyl magnifications. **C)**, **G)** *DRNL-ER AHP6::GFP* seedlings. **D)**, **H)** *DRNL-ER TCS::GFP* seedlings. Seedlings were observed at 48 h after BOL induction. Bars = 0.5 mm in **A)**, **B)**, **E)** and **F);** 20 µm in **C)**, **D)**, **G)** and **H).**

Because *AHP6* and *IPT5* transcripts can be detected in the early transcriptome after BOL is activated in the presence of the translation inhibitor cycloheximide, this could suggest that they are direct targets of this transcription factor. However it would be necessary to corroborate if BOL is able to recognize a particular sequence in the promoter region of these genes. For this, an analysis of the *AHP6* and *IPT5* promoter regions was carried out with the Plant Promoter Analysis Navigator (PlantPAN; http://PlantPAN2.itps.ncku.edu.tw) , searching for the GCC box-like regulatory element. For *AHP6*, two regions with similarity to the GCC sequences were identified: one located in the positive chain at position -293, with a similarity of 83%, and another in the negative chain, at -200 bases and with a similarity of 100%. For *IPT5*, a sequence was identified in position -289 with a similarity of 83% (Figure 3.10). Therefore, these genes are likely direct targets of BOL. However, further experiments are needed to confirm this.

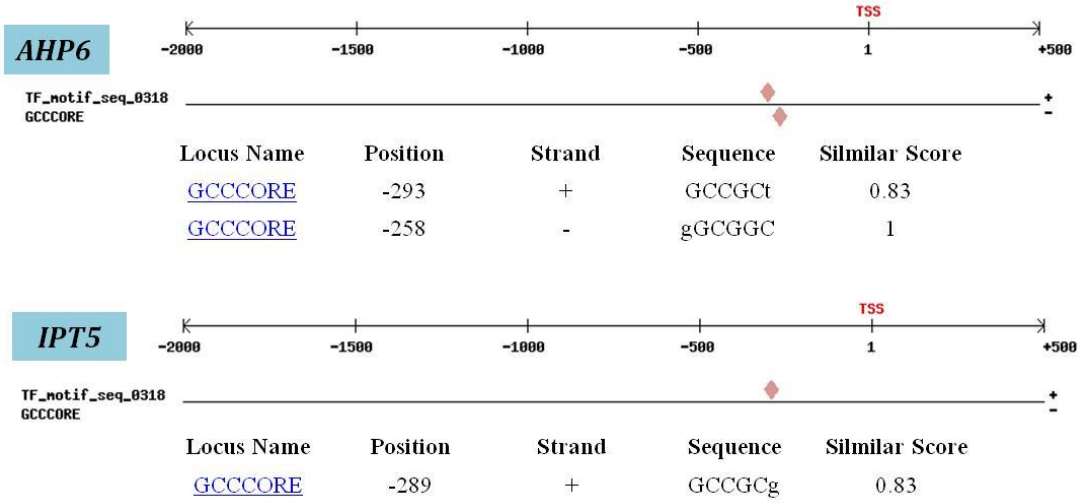

Figure 3.18. *AHP6* and *IPT5* as possible direct BOL targets, GCCbox-like regulatory elements in *AHP6* and *IPT5* promoters. TSS: Transcription Start Site. Pink diamonds represent GCC box.

III.3.5 *AHP6* and *IPT5* and their possible role during callus development

In addition to the effect of BOL on *AHP6* and *IPT5* expression, its effect on the response to cytokinins was also evaluated. For this, the inducible line was crossed to the the *TCS::GFP* cytokinins reporter. With this reporter line we could not detect any signal in the vegetative aerial tissue of seedlings. However, when the activity of BOL was induced, fluorescence could be detected in some regions, although not specifically localized to any tissue or organ.

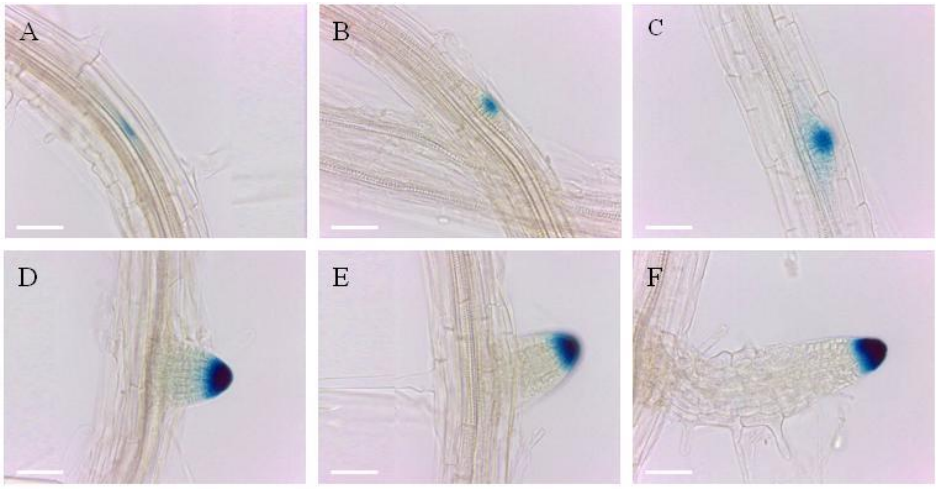

Figure 3.11 *IPT5* expression during lateral root development. **A), C)** Initial stages of lateral root development. **D), F)** Later stages of lateral root development. **A)** *IPT5* expression in adjacent cells to the xylem. **B)** Initial cell divisions. **C), F)** *IPT5* expression maintenance at the root apex. Bars = 0.05mm.

In addition to *AHP6* and *IPT5* expression in vegetative aerial tissue identified in this work, we also analyzed their expression in previously reported tissues. It has been reported that *AHP6* and *IPT5* are expressed in roots. *IPT5* is expressed in the region where a lateral root will emerge and its expression is maintained at the apex as the root elongates (Figure 3.11). *AHP6* shows expression in the root protoxylem (Figure 3.12 C). We found that the induction of BOL activity was also able to alter the expression of these genes in the root. The *AHP6* expression domain in the protoxylem was expanded; in addition, ectopic expression was observed in the epidermal cell layer (Figure 3.12 F). For *IPT5*, interestingly, not only an increase in its expression domain was observed, but it also showed ectopic expression in tissues where it is not commonly observed, such as the main root apex (Figure 3.12 E).

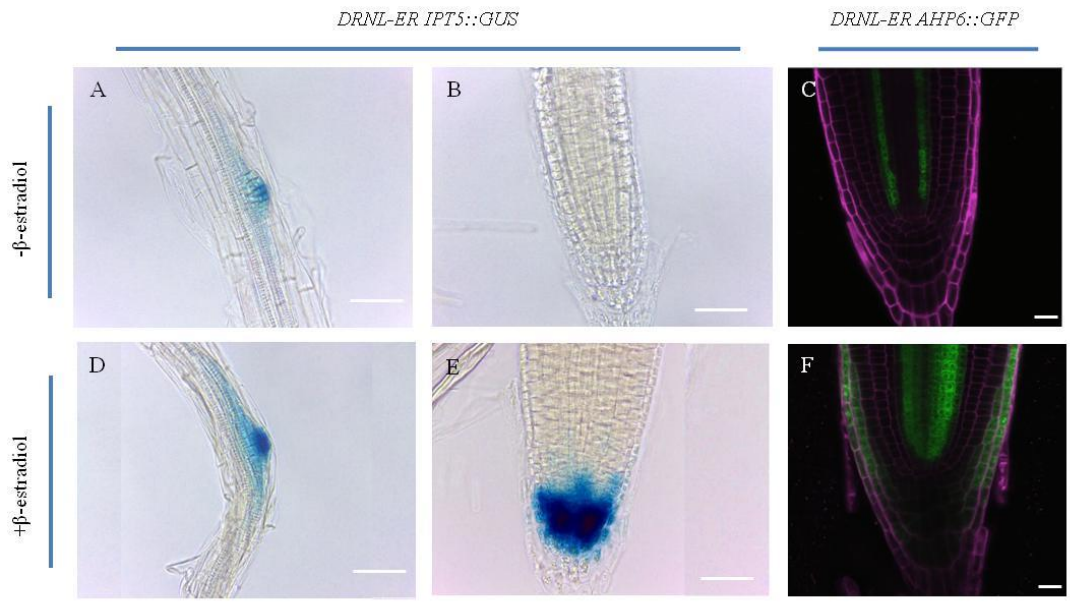

Figure 3.12 Changes in *IPT5* and *AHP6* expression in root promoted by BOL activity. A) and **D)** *IPT5* expression during lateral root development. **B)** and **E)** *IPT5* expression in the apex of the main root. **C)** and **F)** *AHP6* expression in the protoxylem of the main root. Visualizations were done 48 h after induction. Bars = 0.05 mm in **A)**, **B)**, **D)** and **E)**; 20 μm in **C)** and **F)**.

BOL over-expression, besides promoting changes in *AHP6* and *IPT5* expression, also promotes evident morphological alterations in the roots, apparently related with an increase in cell division (Figure 3.12 E, F). One of these effects is the promotion of callus development in the root. Based on this evidence we propose the hypothesis that BOL is closely related to the cytokinins pathway. Based on this, we thought that the root could be very useful to study the regulation of *AHP6* and *IPT5* by BOL, and explore the expression of these genes during the BOL-induced callus development process.

Based on this premise, the development of calli promoted by BOL was characterized in one of the marker plant lines. This characterization was carried out with the *DRNL-ER IPT5::GUS* line. We tried to visualize the callus formation process and at the same time the *IPT5* expression changes throughout this process. As a first approximation of this analysis we proceeded to observe induced seedlings during 8 days. These seedlings already had

evident morphological alterations in aerial tissue and in root. The induced seedlings showed a darker green color in the aerial part compared to the non-induced plants (Figure 3.13 A and B). In the roots, the developmental changes were very evident. Lateral roots of induced seedlings were more developed than lateral roots in not induced seedlings. In addition, the main root apex became thickened and coiled, and the tissue acquired a green color. Interestingly, *IPT5* expression was expanded through the entire region that was curved from the root. Based on these observations, we decided to make a more detailed observation of the process at earlier stages.

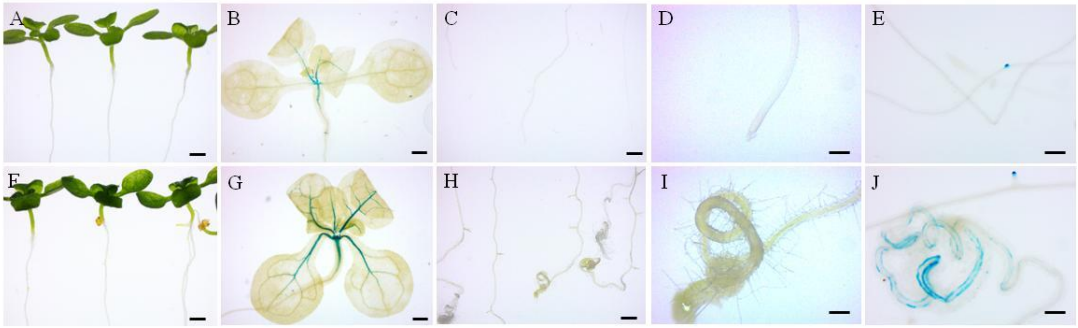

Figure 3.13. *IPT5* expression in aerial and root tissue 8 days after the induction of BOL activity. A), E) uninduced *DRNL-ER IPT5::GUS* seedlings. **F), J)** are induced seedlings. More drastic changes are observed in the tissue near the root apex, the same region where *IPT5* expression is increased. Bars: 1mm in **A)**, **F)**, **C)** and **H);** 0.5 mm in **B)** and **G)**, and 0.2 mm in **D)**, **I)**, **E)** and **J)**.

We proceeded to observe roots of *DRNL-ER IPT5::GUS* seedlings from 2, until 8 days of induction. As previously mentioned, the non-induced seedlings did not present *IPT5* expression at the main root apex, but induced seedlings did. Figure 3.14 B) and C) show that 2 days after induction, expression at the root apex can be observed, as well as a slight increase in the width of the root that increases according to the induction time. At 4 days after the induction, *IPT5* expression at the root apex is maintained and a slight expression in the distal part of the root apex begins to be expressed (Figure 3.14 C and H, arrows). At 6 days after the induction, this expression extends along the whole root as two continuous lines, which might correspond to expression along the vasculature. At 8 days after the

induction, the root architecture is completely altered, and an evident increase in cell number is observed. *IPT5* expression at the root apex begins to decrease, while the expression in the rest of tissues is maintained.

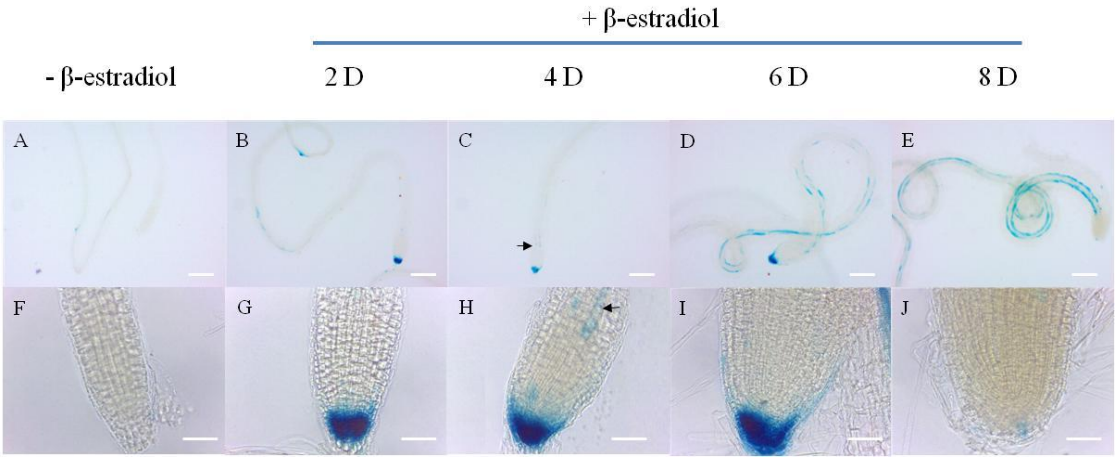

Figure 3.14 Sequential changes in *IPT5* expression in the main root 2 (B) to 8 days (D) after BOL induction. Arrows in **C)** and **H)** highlight the first visualization of ectopic *IPT5* expression in the root vasculature. Uninduced roots **A)** and **F)** and those induced for 2 days, belong to 8 DAG seedlings. Bars: 0.2 mm in **A)- E)** and 0.05 mm in **F) – J).**

However, in spite of the drastic changes in the root apex, this tissue does not continue to proliferate and form calli. For this reason, other regions of the root were explored. We observed alterations in the distribution of the lateral roots. It was even possible to observe, at longer times after induction, the emergence of lateral roots very close to existing ones (Figure 3.15 E), a phenomenon that rarely occurs in the growth conditions employed. At a later stage, the tissue adjacent to the base of the lateral roots widened (Figure 3.15 F) and gradually grew to develop a callus (Figure 3.15 H). When the callus had developed, delocalized *IPT5* expression could still be observed (Figure 3.15 H).

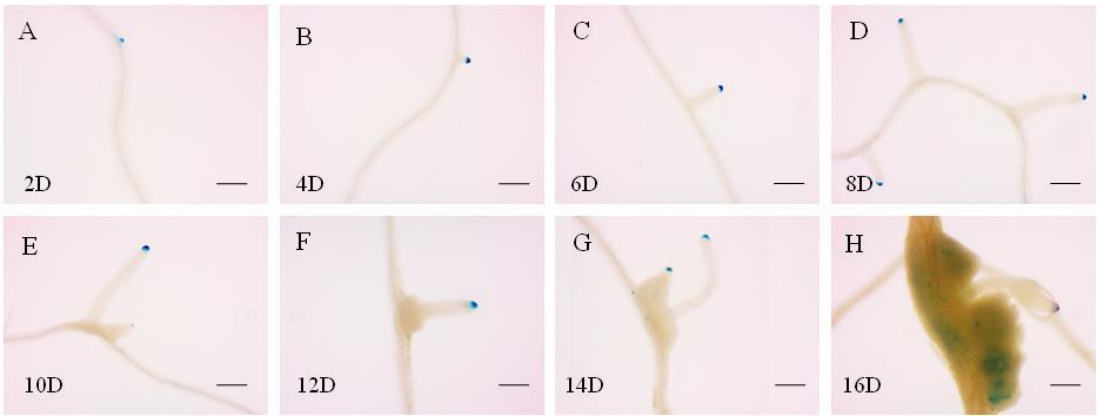

Figure 3.15 Sequential process BOL-induced callus development from lateral roots. Development of **A), D)** uninduced, and **E), H)** induced *DRNL-ER IPT5::GUS* lateral roots at different times after BOL activity induction. Bars: 0.2 mm

In summary, although cell proliferation was observed at the main root apex, it did not continue to form a callus, but rather calli developed from tissue adjacent to lateral roots (Figure 3.16).

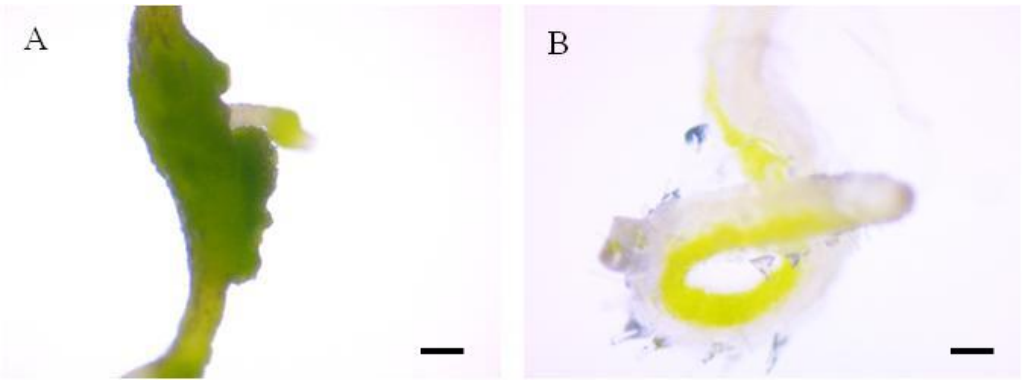

Figure 3.16 Comparison between induced DRNL-ER lateral and main roots. DRNL-ER **A)** lateral, and **B)** main roots 16 days after BOL induction. Bars: 0.2 mm. Calli develop from the tissue near where the lateral roots emerge and these do not develop from the tissue of the main root apex .

In addition to the expression analyses of *AHP6* and *IPT5* expression in response to BOL in roots, the expression of TCS, a the marker of cytokinins response was also analyzed. As

mentioned earlier, the induction of BOL activity promotes faster development of the lateral roots. This can be clearly seen in figure 3.17, where 12 DAG induced and non-induced seedlings show great difference in the length of their lateral roots. The difference in root development makes it difficult to compare the TCS signal between same age induced and non-induced seedlings. However, despite the differences between the length or developmental stage, it was possible to notice some differences in TCS signal. Induced roots generally appear to show less fluorescence than non-induced roots, although we cannot discard that this is due to differences in development. Based on less developed lateral roots observed in induced seedlings (Figure 3.17 G, J), there seems to be no detectable TCS signal at early developmental stages. At later root developmental stages, the TCS signal could be observed located at a region that could possibly be the quiescent center (Figure 3.17 I), something that was not observed in non-induced roots. Another effect of BOL induction, in addition to the faster elongation of lateral roots, was that the differentiation zone appeared to be closer in distance to the root apex (Figure 3.17 K), which could suggest an alteration in meristematic activity. On the other hand, TCS signal is commonly visualized in the columella of the main root of non-induced seedlings (Figure 3.17 E, F), but not in induced seedlings (Figure 3.17 L).

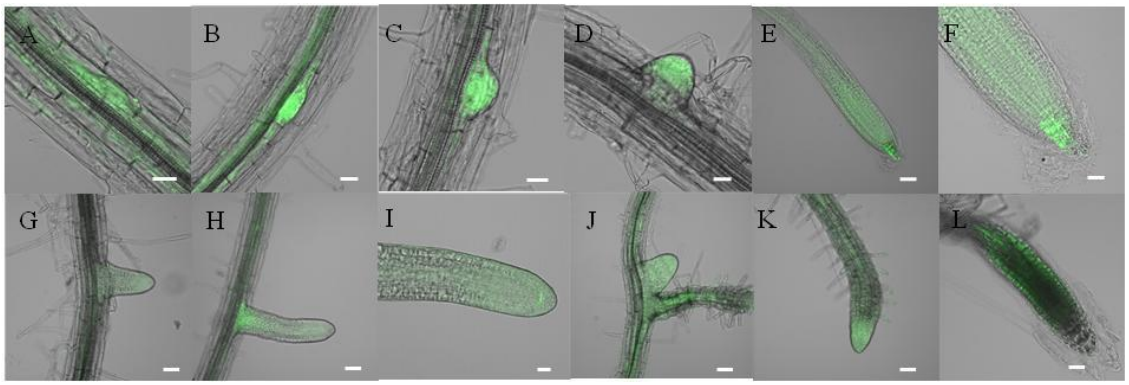

Figure 3.17. Cytokinins response in roots 6 days after BOL activity induction. **A), D)** Uninduced *DRNL-ER TCS::GFP* lateral roots. **E)** Uninduced *DRNL-ER TCS::GFP* main root, **F)** Magnification of E). **G), K)** Induced *DRNL-ER TCS::GFP* lateral roots. **L)** Induced main root. Bars: 20 μm in A), B), C), D), F) and I). 50 μm in E), G), H), K) and L).

At longer periods after induction (21 days), it was possible to appreciate the drastic morphologic changes in roots. Roots were covered with calli, and these calli were larger at the region near the hypocotyl (Figure 3.18 A), while calli located in the rest of the root were smaller (Figure 3.18 B). It also was evident that that lateral roots, which during the initial developmental stages elongated rapidly, after a certain point did not continue to elongate. Their growth became restricted while, on the contrary, the calli that formed at the base of the root proliferated. The calli that developed were green and compact, but sometimes fragments of callus tissue detached from their periphery (Figure 3.18 D). Cells present in this detached tissue did not present the typical root cell shape, but, interestingly, they presented a spherical shape (Figure 3.18 E, F).

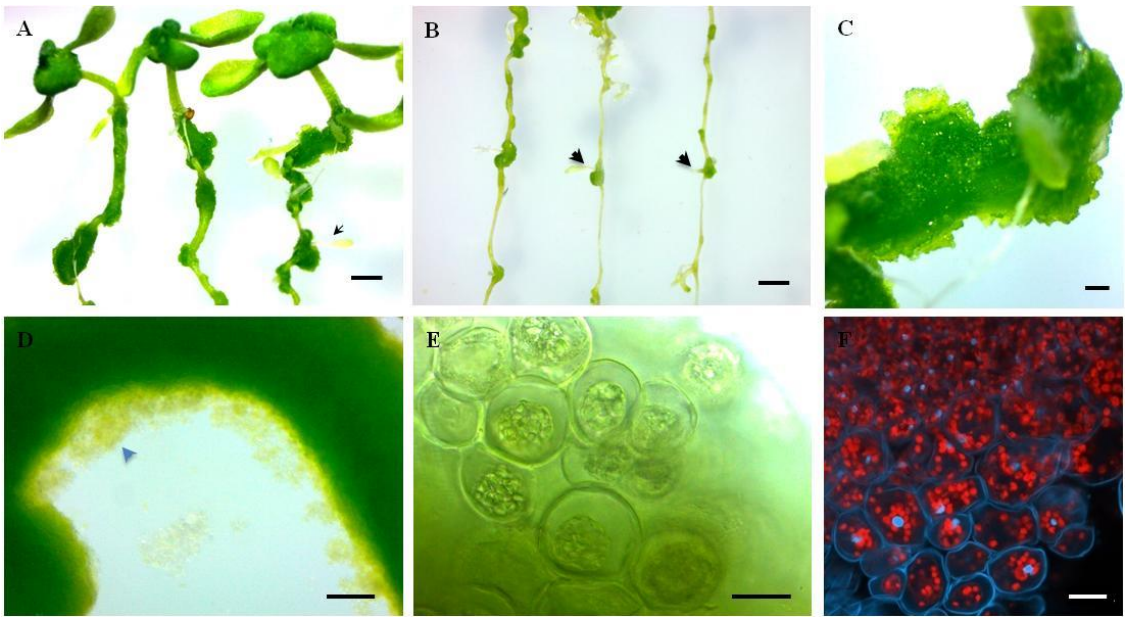

Figure 3.17. **Changes in cell identity promoted by BOL. A)** Seedlings, 21 days after BOL induction**. B)** Calli developing from the tissue near the lateral roots. **C)** Callus magnification from a seedling showed in **A). D)** Callus showing detaching tissue. **E)** Cells from the detatched tissue observed by optical microscopy. **F)** Cells from the detatched tissue observed by confocal microscopy after staining with FM4-64 and DAPI. Black arrowheads show lateral roots and blue arrowhead shows detaching tissue from the callus. Bars: 1 mm in **A)** and **B)**, 0.2 mm in **C)** and **D)**, 0.02 mm in **E)** and **F).**

III.4 DISCUSSION

The function of BOL has been mainly associated with the auxins pathway. The main reported molecular link between BOL and auxins is the SHORT INTERNODES/ STYLISH (SHI/STY) family member STY1 (Eklund et al., 2011). STY1 affects IAA biosynthesis rates and IAA levels. STY1 also acts as a transcriptional activator of genes encoding auxins biosynthesis enzymes, such as *YUC4* and *YUC8* (Eklund et al., 2010). *STY1* has been proposed as a putative BOL target. Eklund et al. (2011), based on microarray data (Marsch-Martínez et al., 2006; Ikeda et al., 2006), explored the relationship and showed that BOL induction activates the transcription of *STY1* and other *SHI*/ *STY* family members such as *LRP1*. They also demonstrate that the activation of *STY1* is dependent on a functional GCC box (a box putatively recognized by BOL).

In this work we found that there are several genes related to auxins that change their expression after β-estradiol-mediated nuclear import of the constitutively expressed BOL protein. In this list of differentially expressed genes, *STY1* was not present. However, we found other *SHI* family members, such as *STY2*, and just like Eklund et al. (2011) we found that *LRP1* was a gene regulated by BOL. *LRP1* was slightly up regulated (0.9 fold) at 30 min and *STY2* was up regulated (1.2 fold) at 8 h. On the other hand, we did not find up-regulated genes that were directly related to auxins biosynthesis at 30 min (*TAA1* was present in the list, but was found to be down regulated). However, after 8 h of BOL induction, *YUC5* was up regulated (1.7 fold). The fact that *STY1* was not found among differentially expressed genes is possibly due to the tissue used. Only the aerial tissue of seedlings were employed for the transcriptome analysis, while in the work of Eklund et al. (2011) complete seedlings were used. Another possible reason why *STY1* differential expression was not detected in our work is because the increase in *STY1* expression in response to BOL is lower than the increase in *LRP1* (Eklund et al., 2011).

IPT5 expression has been reported in lateral root primordia, columella root caps, and fruit abscission zone (Miyawaki et al., 2004). Seeking to identify in which tissues BOL regulates *IPT5* expression, we unexpectedly found that BOL regulates the expression of this gene in cotyledons, young leaves and hypocotyl vasculature. This result is interesting since cytokinins have emerged as key regulators for vasculature development. Cytokinins are important for procambium maintenance and protoxylem differentiation (Aloni, 1982; Mähönen et al., 2000; Matsumoto-Kitano et al., 2008; Hejátko et al., 2009).

With the results obtained so far we cannot determine specifically in which vascular tissue BOL regulates to *IPT5*. However, it is known that the quadruple mutant *ipt1,3,5,7* doesn´t develop cambium and shows reduced radial thickness of the root and stem (Matsumoto-Kitano et al., 2008). Therefore, it is possible that BOL regulates *IPT5* expression in the procambium or cambium cells. The procambium is a pool of stem cells and from this stem cells the exylem and phloem develop (Miyashima et al., 2013). It was observed that BOL not only regulates the *IPT5* expression in aerial tissue of seedlings, but it also regulates *IPT5* expression in roots. It has been reported that *IPT5* is expressed in pericycle cells (a layer of root tissue surrounding the vasculature) presumably giving rise to a lateral root primordium (Miyawaki et al., 2004). It is interesting to note that BOL regulates *IPT5* expression in these tissues that possibly contain cells that are in the process of differentiating for developing vascular tissues or a new lateral root. It may resemble the localization of BOL in the peripheral zone of the Shoot Apical or Floral Meristems, just in the cells that are going to initiate the development of a new organ.

It is interesting that in addition to *IPT5* other genes involved with several steps of the cytokinins pathway are regulated by BOL. Some of these genes seem to act as positive regulators of this pathway and others as negative regulators. However, it has been reported that the alteration of the expression of other genes related to the cytokinins pathway, besides *IPT5*, also promotes alterations in vasculature development. The ectopic expression of *CKX* genes promotes the exclusive development of protoxylem in the root and abnormal development of shoot vascular tissue (Mähönen et al., 2006b; Matsumoto-Kitano et al., 2008; Nieminen et al., 2008; Hejátko et al., 2009). Loss-of-function mutants of other genes

involved in cytokinins perception also show alterations in vasculature development. The *wol* mutant (an allele of the cytokinins receptor *HK4/CRE1)* has a reduced number of procambial cells and the vascular cylinder of the primary root has only protoxylem cells (Mähönen et al., 2000; Mähönen et al., 2006b). In the triple mutant *ahk2, 3, 4* the same phenotypes were observed (Yokoyama et al., 2007).

Intrigued by the expression change of several genes related to cytokinins in response to BOL and by the possibility that these genes act as positive or negative regulators of this pathway; in addition to *IPT5* expression analyses, the expression of a negative regulator of cytokinins signalling, *AHP6*, was also analyzed. *AHP6* negatively regulates cytokinins signalling. Surprisingly, we found that BOL also regulates the *AHP6* expression in vascular tissue of petioles and root. *AHP6* expression has been associated with protoxylem cells in roots (Mähönen et al., 2006a). On the other hand, it has been reported that the mutation of *AHP6* partially restores the *wol* mutant phenotype. Compared to *wol*, the double *wol ahp6* mutant displays an increased number of vascular cell files with intervening procambial and phloem cell files in roots (Mähönen et al., 2006a).

It is very interesting that BOL promotes the expression of *IPT5* and *AHP6* in similar tissues; though this double regulation could seem contradictory: Why does BOL promote cytokinins biosynthesis through *IPT5* and at the same time activates a negative regulator of cytokinins signalling? However, there is an example of how the balance between positive and negative cytokinins regulators is required for the correct development of proxylem vessels and maintenance of procambial cell identity. *AHP6* is also involved in this developmental process as described in the next lines. The vasculature near the root tip consists of procambium, protoxylem, metaxylem and phloem (Osugui and Sakakibara, 2015). Cytokinins are important to inhibit procambium to protoxylem differentiation. Active cytokinins are produced in the protoxylem by the LONELY GUY 4 (LOG4) cytokinins activating enzyme (De Rybel et al., 2014; Ohashi-Ito et al., 2014). However, in this region cytokinins are not sensed because *AHP6* is expressed in a bisymmetric pattern in the protoxylem cells near to pericycle cells (Mähönen et al., 2006a, Bisopp et al., 2011). Conversely it has been hypothesized that cytokinins difusses and functions in the adjacent

procambium (De Rybel et al., 2014; Ohashi-Ito et al., 2014). This little network illustrates well the importance of cytokinins local activation and repression for the development of two different tissue types. It is possible that BOL also participates in this developmental process or in a similar process in which its function is to regulate different genes involved with cytokinins pathway to maintain the cytokinin balance for the proper differentiation of specific cell types in the vascular tissue.

The fact that BOL over-expression promotes callus formation in the region where the lateral roots emerge supports the idea that BOL is exerting a function on meristematic cells located between vascular tissue. It has been well stablished that callus development from root and shoot explants starts with divisions of pericycle cells (pluripotent cells) associated with xylem poles of the vasculature (Atta et al., 2008; Che et al., 2007). Recently, it has been demonstrated that the ectopic activation of a lateral root initiation program from cells equivalent to root pericycle cells is the common mechanism of callus formation from aerial organs such as petals and cotyledons (Sugimoto et al., 2010). Therefore, BOL ectopic activation could be initiating this program in cells equivalent to pericycle through *IPT5* and *AHP6* over-expression, resulting in callus formation. However, this is an hypothesis and further experiments corroborating that *IPT5* and *AHP6* are necessary for callus formation, will provide support for it.

III. 5 CONCLUSION

The results obtained showed that BOL possibly has a more direct regulatory relationship with cytokinins biosynthesis than with auxins biosynthesis. In addition, BOL is able to regulate the expression of several elements that participate in different steps of the cytokinins pathway (synthesis, signalling, transport, degradation, conjugation, etc.). A more detailed analysis of *IPT5* and *AHP6* regulation revealed that BOL activates *IPT5* and *AHP6* transcription in roots and aerial organs vasculature. Given the antecedents of *IPT5*, *AHP6* and other genes related with cytokinins pathway (*CKXs*, *AHKs*) for the correct determination of the vasculature in the root, we could suggest that BOL could be acting as a master modulator to maintain the balance between positive and negative cytokinins

elements to regulate the identity of pluripotent cells for the proper patterning of vasculature. Therefore, when BOL ectopic expression is promoted, the balance of cytokinins breaks down and the pluripotent cells do not acquire an identity and begin to proliferate uncontrollably to form calli. Some of these genes related to cytokinins that we identified as candidate targets of BOL could be key to calli development, such as *IPT5* and *AHP6*. The insights obtained during this work, open a new aspect of BOL regulation that was not previously considered, and will be further explored in the future.

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CHAPTER IV

BOL as an integrator developmental of and environmental signals to control cell differentiation and proliferation

IV.1 INTRODUCTION

Plants as sessile organisms are subject to various environmental conditions that vary widely from season to season or even within a single day. For this reason, plants are highly responsive to their environment, and their physiology and morphology are constantly fine-tuned to suit prevailing requirements. The biotic and abiotic factors present in the environment are those that shape the architecture of plants. These dictate whether a plant will lose organs or generate new organs or tissues (reviewed in De Smet et al., 2009).

In the case of aerial organs, most of them are generated post-embrionically from the shoot apical meristem (SAM), which also generates axillary meristems that can give rise to new stems and organs. The shoot apical meristem is divided in different zones: the central zone, an organizing center, the rib zone and the periphery (Wolters and Jürgens, 2009; Gaillochet et al., 2015). Aerial organs, such as leaves, form at the periphery of the shoot apical meristem, a region where daughter cells that originate from the divisions at the central zone are displaced (Golz and Hudson, 2002, Bar and Ori, 2014). At the periphery, high auxin at a specific region has been associated with new organ formation. A number of cells, termed founder cells, is recruited to give rise to the new organ (Chandler et al., 2011). After initiation of the organ, the different axis are determined by the action of antagonistic genes that provide dorsal or ventral (or adaxial/abaxial) characteristics to the developing organ. Moreover, there is also antagonistic regulation between some of the genes that maintain undifferentiated cells in the meristem, and the genes that promote the development of an organ. Many transcription factors, microRNAs and hormones participate in the regulation meristematic activity and the regulation of initial organ development (reviewed in Tsukaya, 2013; Bar and Ori, 2014; Azizi et al., 2015).

During the development of the organ, processes like cell division and cell expansion have to be also tightly controlled in order to obtain a functional organ of a defined shape and size (reviewed in Kalve et al., 2014). This control is exerted, in the case of cell division, by positive and negative regulators that participate in the different steps of the cell cycle, and cell expansion, the latter due, in a large degree, by cell wall remodeling enzymes (Inagaki and Umeda, 2011; Majda and Robert, 2018).

On the other hand, the development of a plant is modulated in response to the environment. Plants sense their natural environment through receptors. External stimuli activate the receptor molecules and initiate complex downstream signalling networks to respond to environmental conditions and activate developmental cues in an integrated way (reviewed in De Smet et al., 2009 Osakabe et al., 2013). These external signals activate intracellular signalling pathways that control molecular processes and reprogram gene expression. Signalling molecules involved in the cell–cell communication include small organic molecules, small peptides, ions, and physical stimuli, among which are cell wall fragments, glycosylated proteins and phytohormones (Osakabe et al., 2013; Nissen et al., 2016).

Receptor-like kinases have been implicated in the perception of extra cellular signals. This receptors regulate a wide range of processes such as symbiosis (Parniske, 2008), disease resistance (Afzal et al., 2008), self-incompatibility (Takayama and Isogai, 2005), brassinosteroid signalling (Belkhadir et al., 2006), cell growth regulation (Hématy and Hofte 2008), formation of the shoot stem cell niche (Clark, 2001; Stahl and Simon, 2005), among others. The best-known plant receptor-like kinase cascade during plant development control is the CLV pathway that controls the size of the central stem cell pool in the shoot apical meristem. Another RLKs involved in the control of development are BAM1-3, also involved in stem cell maintenance. RPK1 and TOAD2, are required for proper morphogenesis and differentiation of cells along the radial axis and in the basal pole of the embryo (Nodine et al., 2007). ACR4 regulates formative cell divisions in the main root tip meristem and during lateral root initiation (De Smet et al., 2008). Finally ,and PXY/ TDR, that has been identified as an important receptor-like kinase that controls oriented procambial cell divisions (Fisher and Turner 2007; Hirakawa et al., 2008).

Plant development consists in the generation of new tissues and organs that relies on well-orchestrated cell divisions coupled with the correct acquisition of cell identities. During the acquisition of cell identity, cells adopt a specific cell fate according to their developmental context. Cell fate determination is mainly dependent on positional information and mediated by cell-to-cell communication and perception of morphogens or short or long-range signalling molecules (reviewed in Xu and Zhang, 2015). The BOLITA / DORNROSCHEN-LIKE / ENHANCER OF SHOOT REGENERATION 2 (BOL/ DRNL/ ESR2 transcription factor is expressed in organ founder cells. However, its function on these cells is not known (Marsch-Martínez et al., 2006; Ikeda et al, 2007; Chandler et al., 2007). For this reason, in the present work, a global analysis of differential gene expression was performed after β-estradiol-mediated nuclear import of the constitutively expressed BOL protein. From this analysis we found that BOL is able to regulate genes involved in many processes that seem to affect the morphological, biochemical and physiological properties of the cells. BOL affects genes involved in the response to several types of stress and phytohormones, receptor-like protein kinases and genes involved with key processes of development such as cell division and DNA replication. This suggests that BOL may be responsible for integrating the perception of environmental and developmental signals to control cell proliferation and differentiation.

IV.2 MATERIALS AND METHODS

IV.2.1 Growth conditions

DRNL-ER seeds were disinfected as mentioned in chapter III. They were sown in the same medium (MS) as previously described, supplemented with 1% plant agar. Growth conditions were the same as in chapter III.

IV.2.2 Induction experiment

Nine days After Germination (DAG)) *DRNL-ER* seedlings were sprayed with four different solutions: a) 10 μM estradiol and 30 μM cycloheximide (CHX), b) 10 μM β-estradiol, c) 30 μM cycloheximide, and d) DMSO (solvent) with distilled water. The a) and b) solutions were the inductor solutions, while solutions c) and d) were used as mock controls. Cycloheximide was used to prevent new protein synthesis and β-estradiol was applied to promote nuclear transport of the chimeric transcription factor.

IV.2.3 Total RNA extraction

For RNA extraction only the aerial tissue of approximately 30 DRNL-ER seedlings was collected. In order to identify genes directly regulated by DRNL, the samples treated with a), c) and d) solutions were collected after 30 min. To identify late or indirectly regulated targets of DRNL, the samples treated with b) and d) solutions were collected after 8 h. RNA was extracted using the Quick-RNATM MiniPrep kit (Zymo Research, Irvine, CA, USA). The samples were treated with DNase I, included in the kit.

IV.2.4 RNA-Seq

RNA sequencing was performed by the LANGEBIO genomic services facility. Fifteen libraries (three for each treatment) were prepared using the TruSeq RNA Sample prep v2 kit (Illumina; city, state, country). Sequencing was performed on the HiSeq 2000 (update 2500 v1) instrument using paired-end. The samples were run on two lines, generating 10 to 15 million reads of 100 bp for each library.

IV.2.5 Bioinformatic analysis

For adapter and quality trimming, Trimmomatic version 0.36 in paired end mode was used. Alignment and quantification were done with kallisto (https://pachterlab.github.io/kallisto/) version 0.43.1. The reads were aligned onto the reference Arabidopsis genome assembly (The Arabidopsis Information resource 11; released on February 2017). Differential

expression analysis was done using transcripts counts data and edgeR, with data pre-processing using tximport (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4712774/ andhttps://bioconductor.org/packages/devel/bioc/vignettes/tximport/inst/doc/tximport.html) .Genes with false discovery rates of 10^{-4} were considered differentially expressed in the rest of analysis. GO enrichment was done with Biological Networks Gene Ontology tools (BiNGO) (https://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) and was visualized with cytoscape v3.2.1 (http://www.cytoscape.org).

IV.3 RESULTS

IV.3.1 RNA sequencing

RNA sequencing libraries were constructed from three biological replicates of aerial tissue of 30 seedlings *DRNL-ER* sampled 9 DAG (Figure 4.1), sprayed with β-estradiol and cycloheximide, which inhibits translation (solution a) or mock solutions (c and d) collected after 30 min of treatment application. By inhibiting translation, we expected to strongly diminish the regulation of secondary targets (targets of transcription factors that are regulated directly by BOL), and therefore increase the chances of identifying BOL (candidate) direct targets. To identify BOL late or indirectly regulated genes, samples treated with β-estradiol (solution b) or solvent only (solution d) were collected 8 h after treatment.

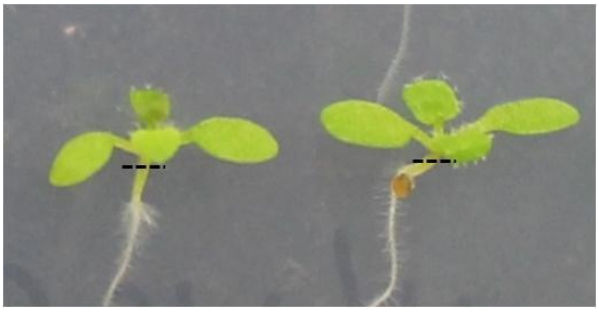

Figure 4.1 Tissue used for differential expression analyses of genes in response to BOL activity activation. Hypocotils and roots were removed (the cutting is illustrated by the black dashed lines), and only the aerial tissues of *DRNL-ER* 9 DAG seedlings of were used for RNA extraction.

The final reads obtained had a high quality (Q30 95.6%), and were subsequently used, after processing and mapping, for differential expression analyses comparing induced versus non-induced samples. Figure 4.2 shows a graph depicting the change in transcript number (as transcripts per million) of some of the genes that presented an evident increase in expression upon BOL induction. Among them there are genes of unknown function, and other genes that encode proteins or enzymes involved in different processes. There are other ERF transcription factors, such as, *RELATED TO APETALA2.9 (RAP2.9)*, *ETHYLENE RESPONSE FACTOR 9 (ERF9)* and *CYTOKININ RESPONSE FACTOR 1 (CRF1)*. CRF1 is a transcription factor that responds to cytokinins; and among genes related to cytokinins *AHP6* is also present. On the other hand, there some genes related to jasmonates, like *CYTOCHROME P450 94B3 (CYP94B3)*, *JASMONATE RESPONSIVE GENE 21 (JRG21)* and *JASMONATE ZIM DOMAIN 8 (JAZ8)*.

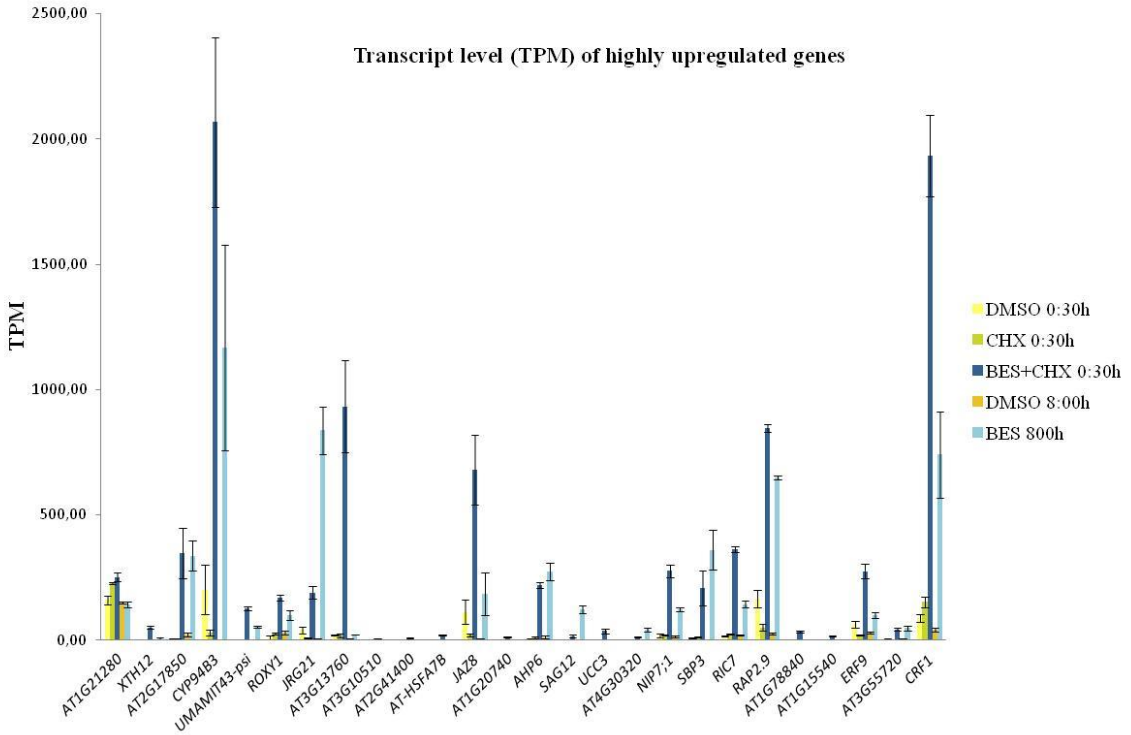

Figure 4.2 Expression levels in "Transcripts Per Million" (TPM) of some highly up-regulated genes in response to BOL activity induction with β-estradiol compared with

mock treatments. These genes are involved in different processes, some of them are related to hormones, such as cytokinins (*AHP6* and *CRF1*) and jasmonates (*CYP94B3*, *JRG21* and *JAZ8*).

Using a false discovery rate of less than 10^{-4} to define statistically significant differential expression, we identified a total of 922 genes with differential expression, at 30 min after BOL induction and 2145 genes after 8 h of BOL induction. From these genes, 480 showed differential expression exclusively at 30 min and 1703 only after 8 h of induction, while 442 showed differential expression in both sampling times after BOL induction (Figure 4.3). At 30 min after BOL induction, 480 genes were up-regulated and 442 genes were down regulated, while after 8 h of BOL induction we identified 1123 up- and 1022 down-regulated genes (Figure 4.4).

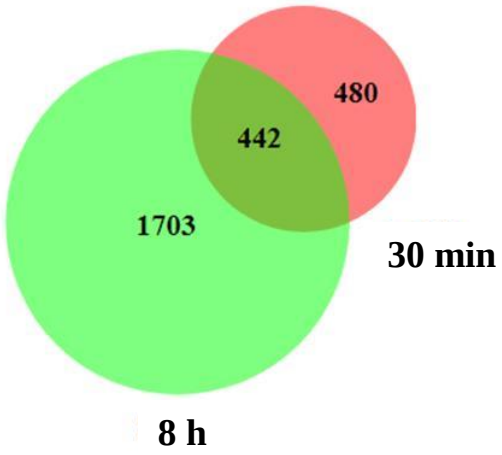

Figure 4.3 Venn-diagram depicting the overlap in the number of differentially expressed genes at 30 min (with translation inhibitor) and at 8 h after BOL induction.

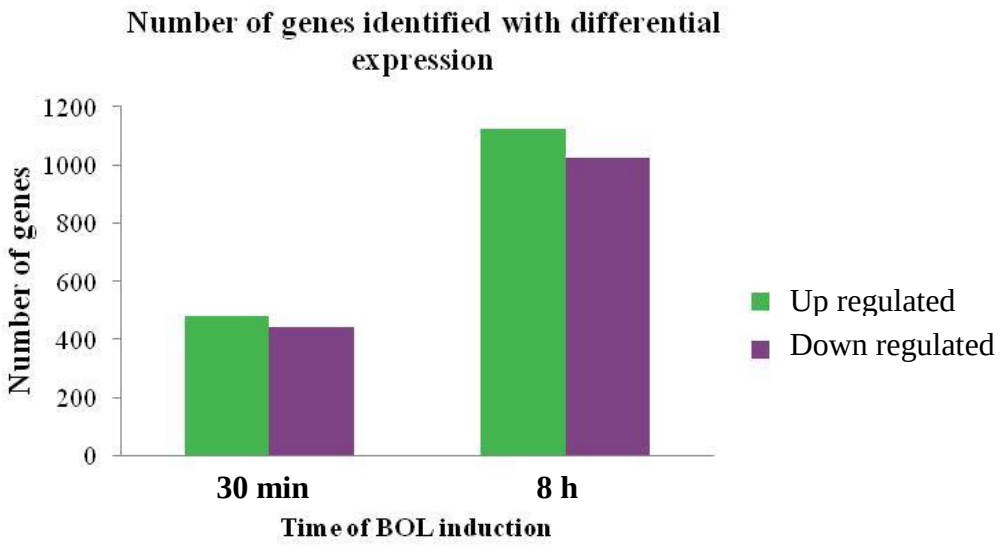

Figure 4.4 Histogram showing the number of genes up- and down-regulated 30 min and 8 h after BOL induction.

Because *BOL* is expressed in the peripheral zone of the SAM and in leaf primordia, we proceeded to search among genes having differential expression, those involved in the control of meristematic activity and leaf development. Decades of research have identified key regulators of SAM function and early organ development (reviewed in Tsukaya, 2013). Figure 4.5 provides an overview of known regulators of SAM activity and organ development, presenting their expression domains during leaf development (based on Tsukaya, 2013). Frequently, these regulators have antagonistic functions and present cross-regulation. For example, the negative feedback between the CLAVATAs (peptides and receptors) and WUSCHEL (WUS) is necessary to maintain the balance between stem cell number and proliferation rate (Mayer et al., 1998; Schoof et al., 2000), with a parallel action of SHOOTMERISTEMLESS (STM) (Gallois et al., 2002). *STM* negatively regulates genes that participate in organ development, while the other genes, in turn, also negatively regulate *STM*. Table 4.1 presents a list of genes that have known roles in the regulation of SAM activity or different stages and regions of organ development, which were found to be differentially expressed upon BOL induction. The finding genes known to participate at different stages of leaf development was interesting. As would be expected, these differential expression results show down regulation of some genes involved in the

maintenance of SAM, as *STM* and *APETALA2 (AP2)*. *AP2* functions in stem cell maintenance by modifying the *WUS-CLV3* feed-back loop (Würschum et al., 2006). However, *BEL1*, whose protein interacts with STM and KNAT1 (among other KNATs) is up regulated (Bellaoui et al., 2001). On the other hand, examples of key genes related with organ boundary formation (such as *CUP SHAPED COTYLEDON 1* (*CUC1)* and *WUSCHEL-RELATED HOMEOBOX 2 (WOX 2))* and genes related with dorso-ventrality determination (*REVOLUTA (REV)* and *KANADI (KAN*)) are up regulated (Table 4.1 and Figure 4.5). These results are consistent with the idea that BOL acts to determine the cells that will initiate the formation of a new leaf primordium, and coincides with its expresion during early stages of its development, suggesting that it can also play a role in these stages.

Table 4.1 Genes known to participate in the control of meristemactic activity and leaf development, found to be differentially expressed upon BOL induction.

Gene	Short description	30 min	logFC	8 h	LogFC
AP2	AP2/EREBP Transcription Factor	✓	-1.15		
STM	Class I Knnoted like homeodomine protein requiered for SAM formation.			✓	-1.30
BEL1	Homeodomain protein. Forms heterodimers with STM and KNAT1	✓	2.35	✓	1.61
CUC1	NAC transcription factor. Involved in SAM formation			✓	2.93
NTM1	Membrane-bound NAC transcription factor. NTM1 regulates cell division.			✓	322
NAC3	Membrane-bound NAC transcription factor			✓	2.12
BOP1	Positive regulation of AS2 in leaves	✓	-1.76		
REV	Homeobox-leucine zipper family protein. Regulates meristem initiation at lateral positions	✓	1.01		
KAN	Regulates organ polarity	✓	1.79		
PRS3	Phosphoribosyl Pyrophosphate Syntase 3	✓	1.08		
WOX2	WUSCHEL related homeobox 2	✓	2.47	✓	1.76
WOX1	WUSCHEL related homeobox 2			✓	-1.59
WOX5	WUSCHEL related homeobox 2			✓	-5.75
HAM3	GRAS family Transcription Factor. Promote cell differentiation at the periphery of shoot meristems.	✓	1.35	✓	1.82
LNG1	Regulates leaf morphology by promoting cell expansion.	✓	-2.16	✓	-2.42
LNG2	Regulates leaf morphology by promoting cell expansion.	✓	-1.16		
GRF4	Growth Regulator Factor 4. Involved in leaf development			✓	-1.00
GRF5	Growth Regulator Factor 5. Involved in leaf development.	✓	-1.47	✓	-3-39
ALE2	ABNORMAL LEAF SHAPE Protein Kinase	✓	0.79		
TCP3	TEOSINTE BRANCHED 1, cycloidea and PCF transcription factor 3	✓	-0.70	✓	-0.60
PLL4	Protein phosphatase 2C like gene, similar to POL. Involved in leaf development	✓	-0.90		

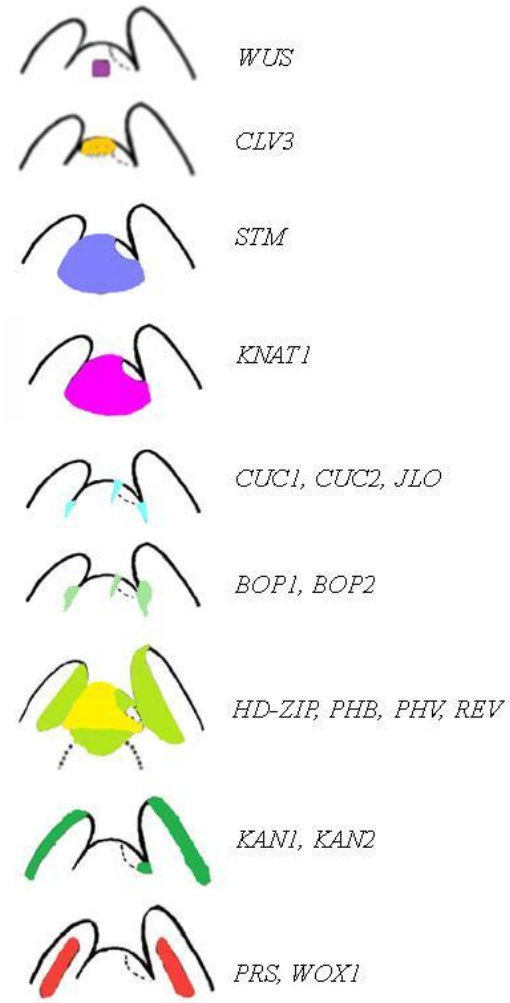

Figure 4.5 Representation of the expression pattern of key genes involved in meristematic activity and early leaf development. Sections of a Shoot Apical Meristem with two young leaf primordia and one predicted area of a leaf primordium are shown (modified fromTsukaya, 2013).

IV.3.2 Functional categorization of differentially expressed genes

To obtain a global view of gene expression changes promoted by the induction of BOL in the aerial tissue of seedlings, we proceeded to perform a Gene Ontology (GO) enrichment analysis using the lists of differentially expressed genes. From the list of genes that changed their expression 30 min after the induction of BOL, we obtained 114 enriched GO terms

(appendix 1) and 166 from genes of 8 hours (appendix 2). Some of the most significantly enriched GO terms obtained 30 min after BOL activity induction (Figure 4.6 and 4.7) were: Response to Hormone (GO: 0009725), Regulation of Transcription (GO: 0006355), Nitrogen Compound Metabolic Process (GO: 0044271), Transmembrane Receptor Protein Tyrosine Kinase Signalling Pathway (GO: 0007169), Protein Phosphorylation (GO: 0006468), and Plant Organ Development (GO: 0099402), among others. At 8 h (Figure 4.8 and 4.9), the most highly enriched categories were: Response to Hormone (GO: 0009725), Response to stress (GO: 0033554), DNA replication (GO: 0044786), Response to Water Deprivation (GO: 0009414), Cell Communication (GO: 0007154), Response to Lipid (GO: 0033993), Developmental Process (GO: 0032502). Some of these gene ontology categories were expected (such as response to hormone, organ development, regulation of transcription and DNA replication) and provide support to the findings of this study. Other categories are new in the context of this study and will be interesting for further exploration in future studies.

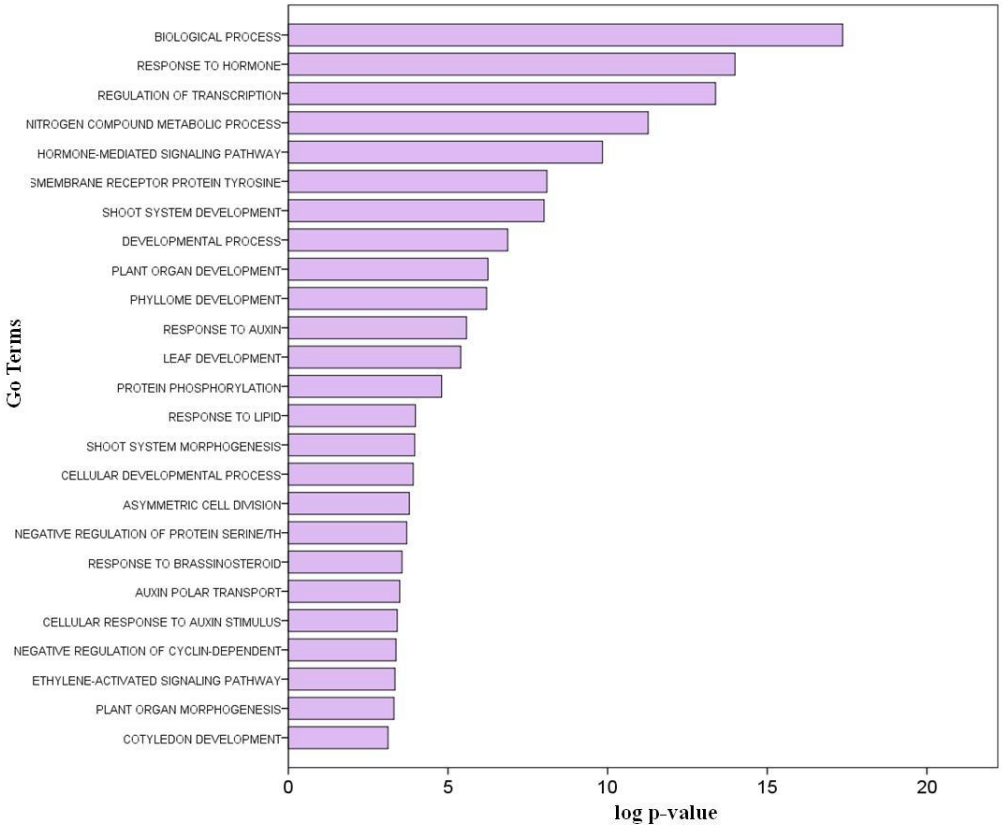

Figure 4.6 Subset of significantly enriched GO terms among differentially expressed genes 30 min after BOL induction.

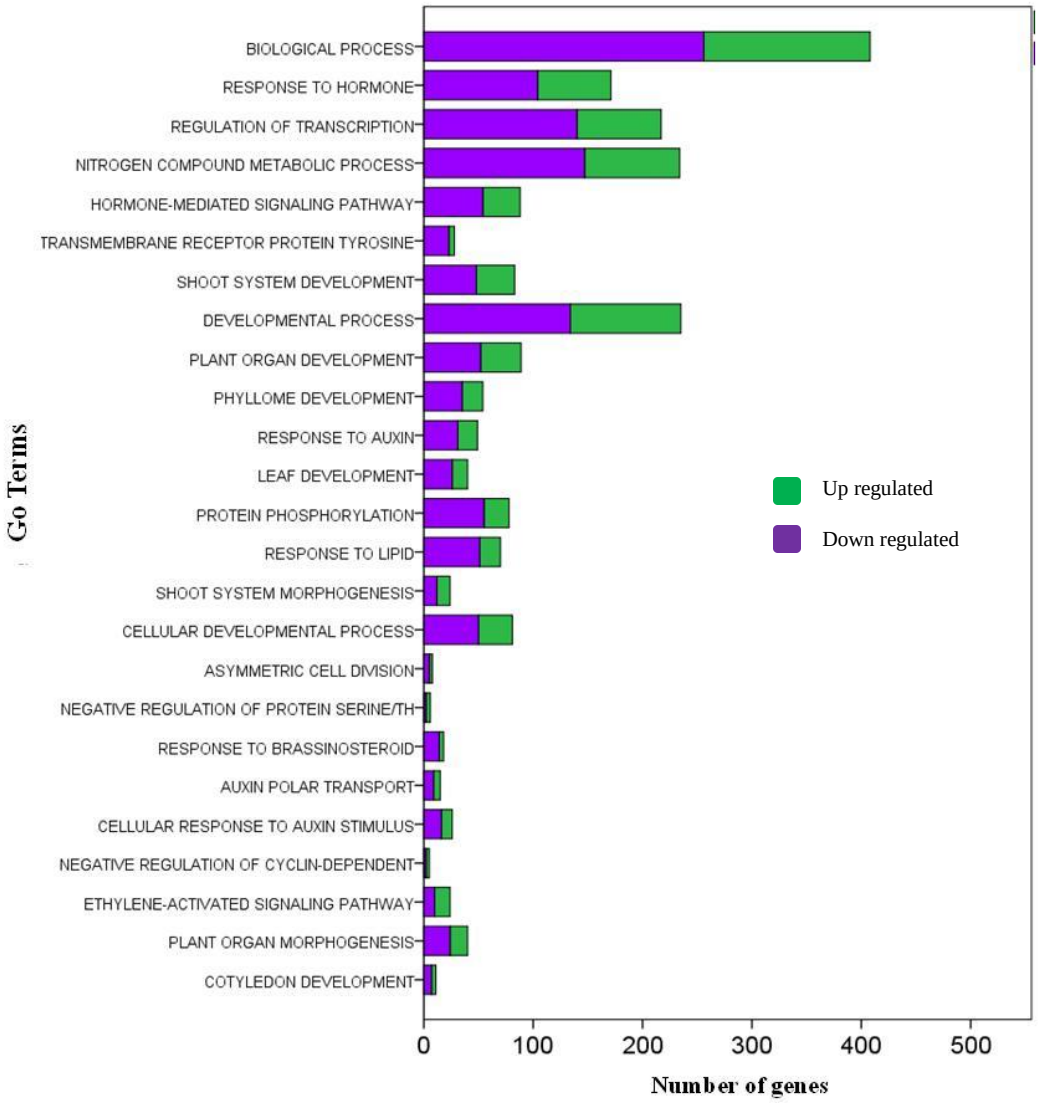

Figure 4.7 Number of genes of each significantly enriched GO category, found to be up- or down-regulated 30 min after BOL induction.

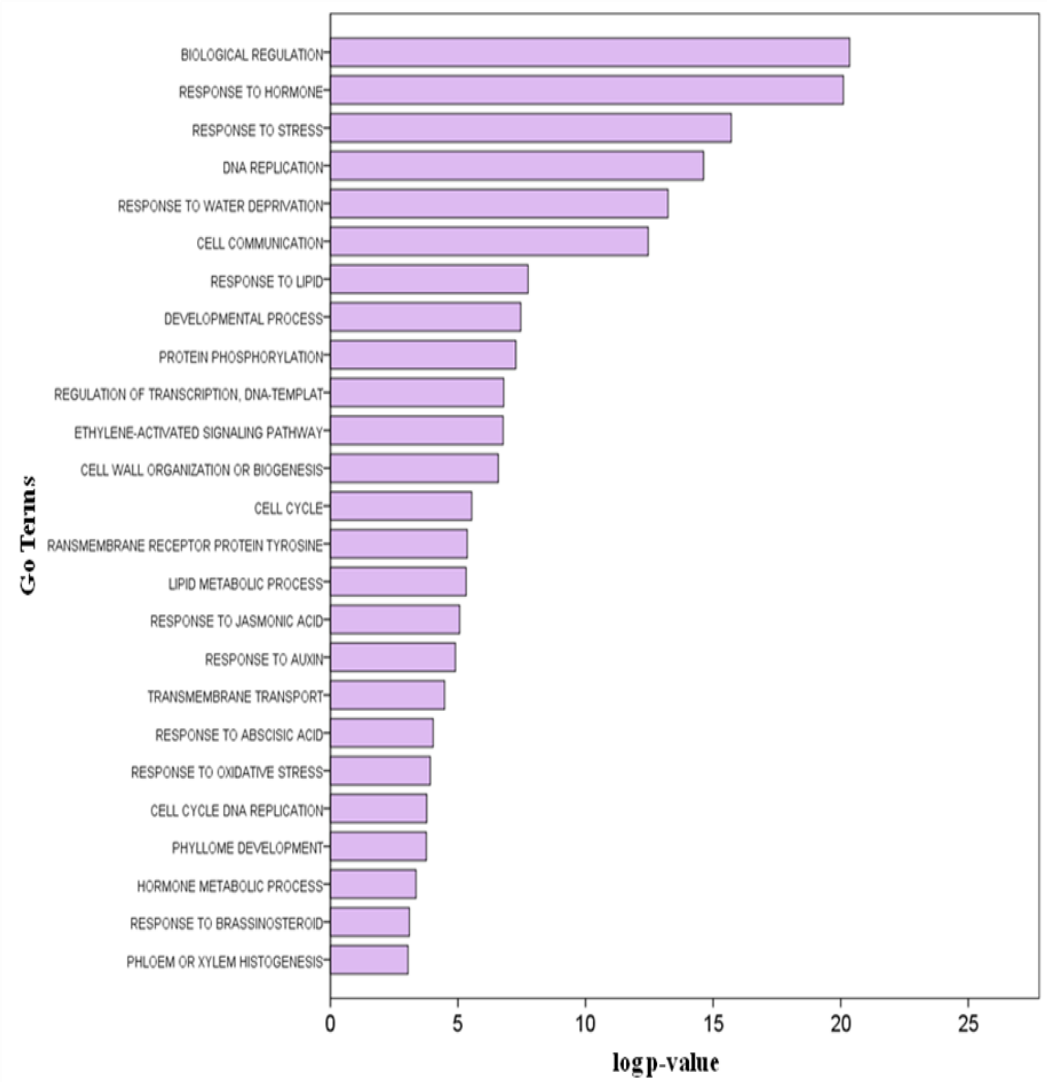

Figure 4.8 Subset of significantly enriched GO terms among differentially expressed genes 8 hours after BOL induction.

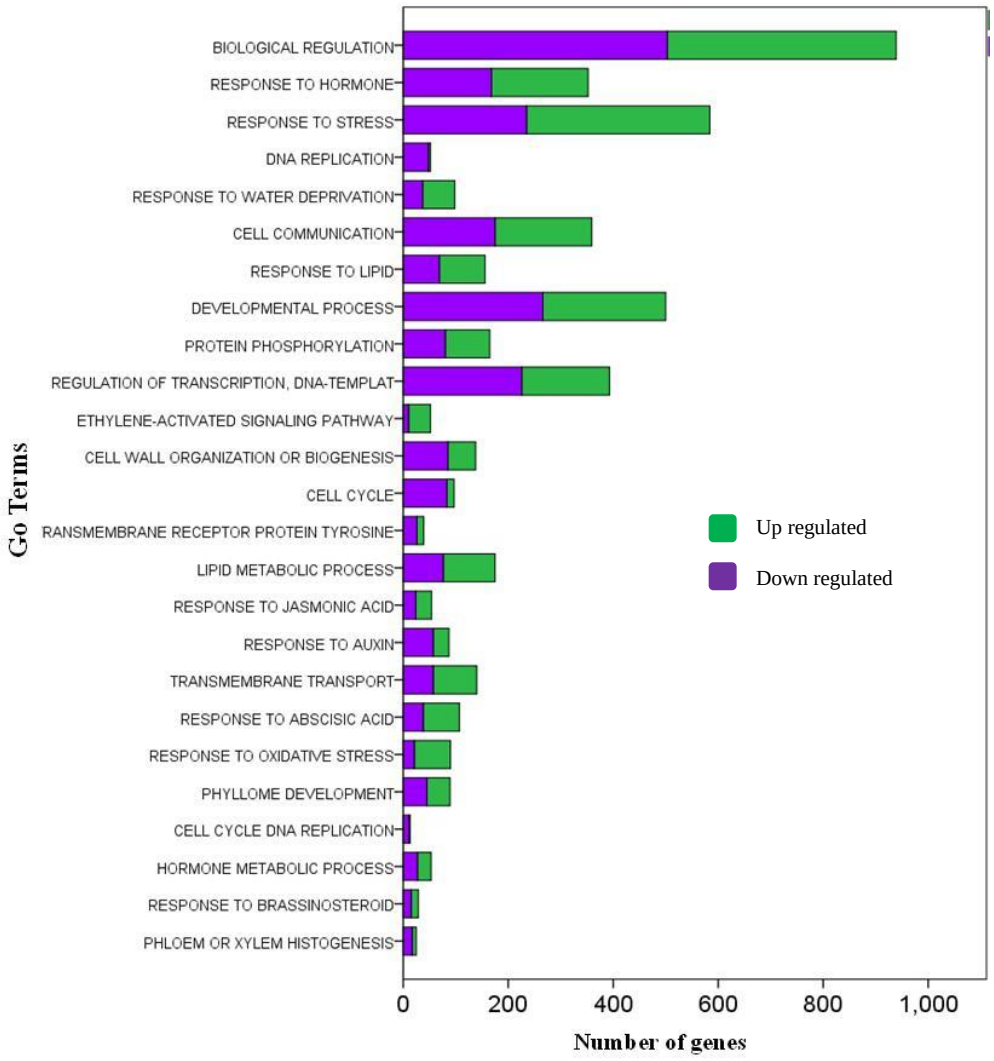

Figure 4.9 Number of genes of each significantly enriched GO category, found to be up or downregulated 8 hours after BOL induction.

In general, it can be observed that in most of the GO categories of 30 min. after BOL induction, the number of down-regulated genes is larger than those that were up-regulated (Figure 4.7), while in the categories found 8 h after induction, the number of up-regulated genes increased (Figure 4.9). Moreover, there are enriched categories at 30 minutes of BOL induction that are not shared with those from 8 hours. There are 34 enriched GO categories that are exclusively found 30 min after induction (appendix 3). Among them are: Leaf Development (GO: 0048366), Negative Regulation of Protein Kinase Activity (GO: 0006469), RNA Metabolic Process (GO: 0016070, and Auxins Transport (GO: 0060918).

Some of the 86 categories that are found at 8 h and that do not appear 30 min after BOL induction are (appendix 4): DNA Replication (GO: 0006260), Lipid Metabolic Process (GO: 0006629), Response to Stress (GO: 0006950), Cell Cycle (GO: 0007049), Auxin-activated Signalling Pathway (GO: 0009734), Negative Regulation of Transcription, DNA-templated (GO:) and DNA Conformation Change (GO: 0071103). Most of these categories contain a greater number of down-regulated genes with the exception of Negative Regulation of Protein Kinase Activity, Lipid Metabolic Process and Response to Stress (Figures 4.10 and 4.11).

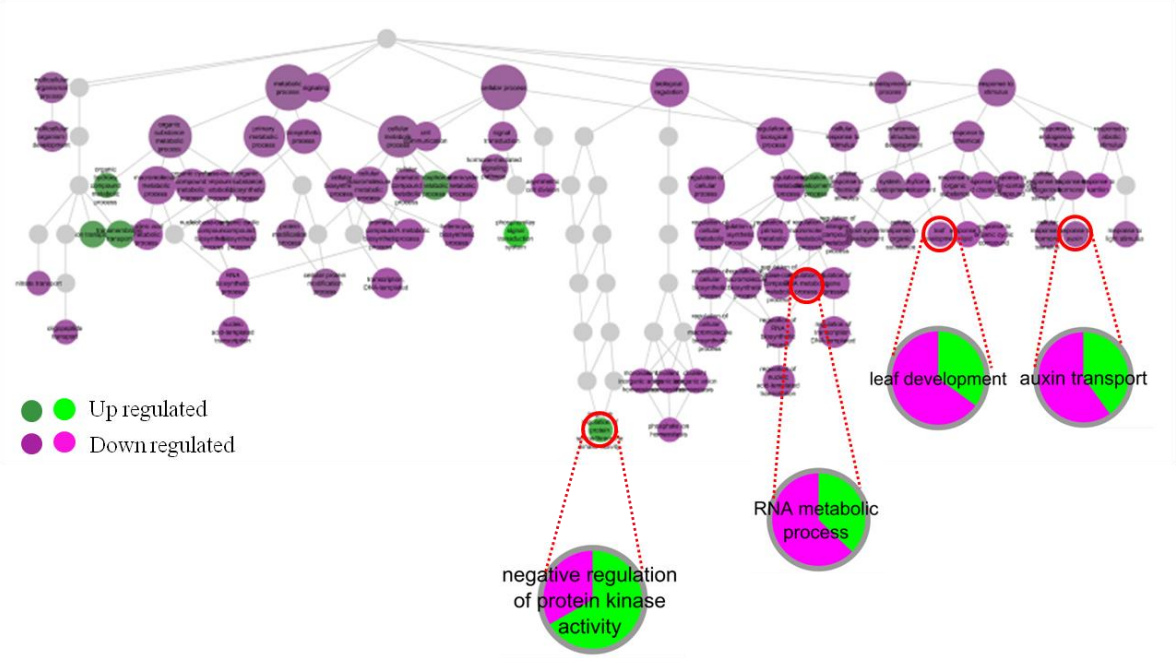

Figure 4.10 Network based on enriched gene ontology (GO) categories of genes with differential expression after 30 min of BOL induction. The diameter of the small circles in the network is proportional to the number of genes in each GO category and the color shows the median fold change among the genes in each category. The larger circles with lighter colors show the proportion between the number of up- and down-regulated genes of some categories that are not shared with those obtained after 8 h of BOL induction.

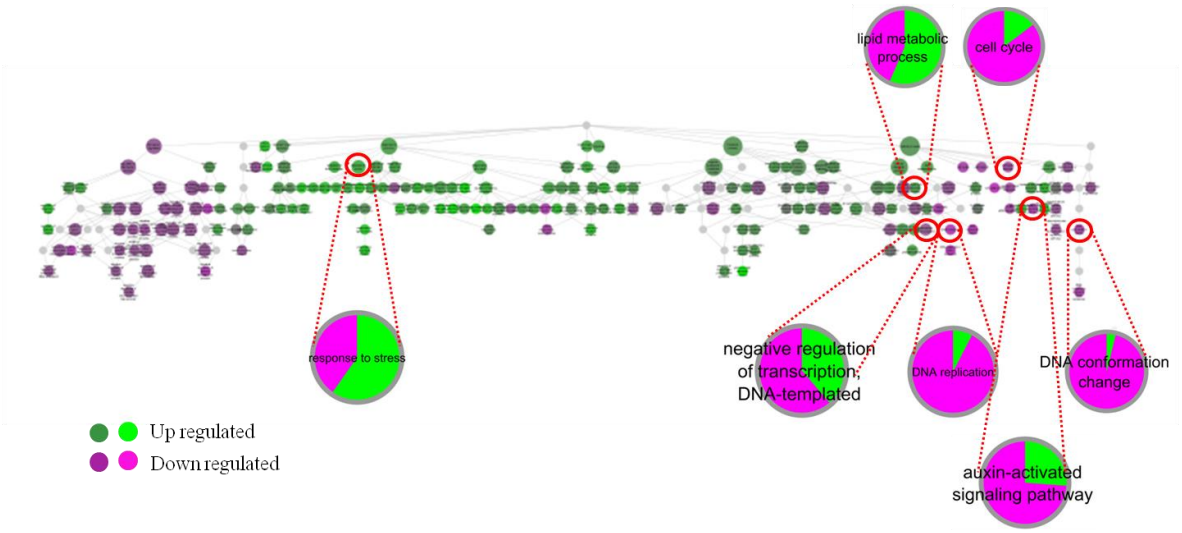

Figure 4.11 Network based on enriched gene ontology (GO) categories of genes with differential expression after 8 h of BOL induction. The diameter of the smaller circles in the network is proportional to the number of genes in each GO category and the color shows the median fold change among the genes in each category. The larger circles with lighter colors show the proportion between de number of up- and down-regulated genes of some categories that are not shared with those obtained 30 min after BOL induction.

IV.3.3 BOL as a master regulator of the expression of transcription factor genes

Of 217 total genes present in Regulation of Transcription GO category (one of the most significantly enriched GO category), 77 genes were up regulated and 140 were down regulated (Figure 4.7). The interesting fact about this, is that most of the genes present in this category encode transcription factors or putative DNA binding proteins. This means that BOL regulates the expression of several transcription factors, which makes sense given the changes in the expression of genes involved in several processes that are reflected in the number of categories represented after the 8 h of BOL induction and in the drastic morphological alterations that BOL over-expression promotes. After finding this, we used the TF2 Network tool in order to explore whether any of the transcription factors found to be up regulated 30 min after BOL induction could be regulating some of the differentially expressed genes at 8 h (an example of the output of this tool is shown in Figure 4.12). TF2

Network predicts possible regulators of a set of genes (Kulkarni et al., 2017). Among the possible regulators that this tool predicted for genes with differential expression at 8 h, we found BR-ENHANCED EXPRESSION 1 (BEE1), OBF BINDING PROTEIN 4 (OBP4), PHYTOCROME INTERACTOR FACTOR 3 (PIF3/ PAP3), MYC3, Integrase-type DNA-binding protein (TINY2), ETYLENE RESPONSE FACTOR 8 (ERF8), RAP2.9, TEOSINTE BRANCHED 3 (TCP3), HOMEOBOX PROTEIN 5 (ATHB5), ATHB6, ATHB16, NAC DOMAIN CONTAINING PROTEIN 41 (NAC041), CRF1, HOMEOBOX-LEUCINE ZIPPER PROTEIN 3 (HAT3) and ERF9. The genes that encode these transcription factors are also differentially expressed 30 min after BOL induction. From those, only PIF3/ *PAP3*, *RAP2.9*, *ERF8*, *ERF9* and *CRF1* are up regulated. PIF3/ *PAP3* encodes a transcription factor that interacts with the photoreceptors PHYA and PHYB, whereas *RAP2.9*, *ERF8*, *ERF9* and *CRF1* are AP2/ ERF transcription factors. The genes that encode AP2/ ERF transcription factors are among those that are most highly up regulated (*RAP2.9*, 3.9 fold; *ERF8*, 3.0 fold; *ERF9*, 3.9 fold and *CRF1*, 3.8 fold). "Fold" refers to the logarithm (base 2) of the fold change. So these early BOL up-regulated BOL genes detected at 30 min could be considered as possible positive regulators of some genes that change expression after 8 h, such as, *ANAC002*, *MYB DOMAIN PROTEIN 51* (*MYB51)*, *ERF11*, *SALT TOLERANCE ZINC FINGER* (*STZ)*, *RAP21*, *ANAC019*, *WRKY DNA-binding protein 6* (*ATWRKY6)*, *CRF6*, among others. These genes are also related to the Regulation of Transcription, Response to Hormones and Phosphorelay Transduction System categories. Figure 4.12 shows an example of the possible targets of ERF9 and CRF1, highlighting the genes that are related to the phosphorelay transduction system. CRF proteins rapidly accumulate in the nucleus in response to cytokinins, and this re-localization depends on the histidine kinases and the downstream histidine-containing phosphotransfer proteins (Rashotte et al., 2006). On the other hand, some CRF proteins (CRF1-CRF6) have a putative MAPK phosphorylation site motif (Cutcliffe et al., 2011). For this reason, it is very interesting to find that some possible targets of CRF1 are related with the phosphorelay transduction system and it points to a new research direction

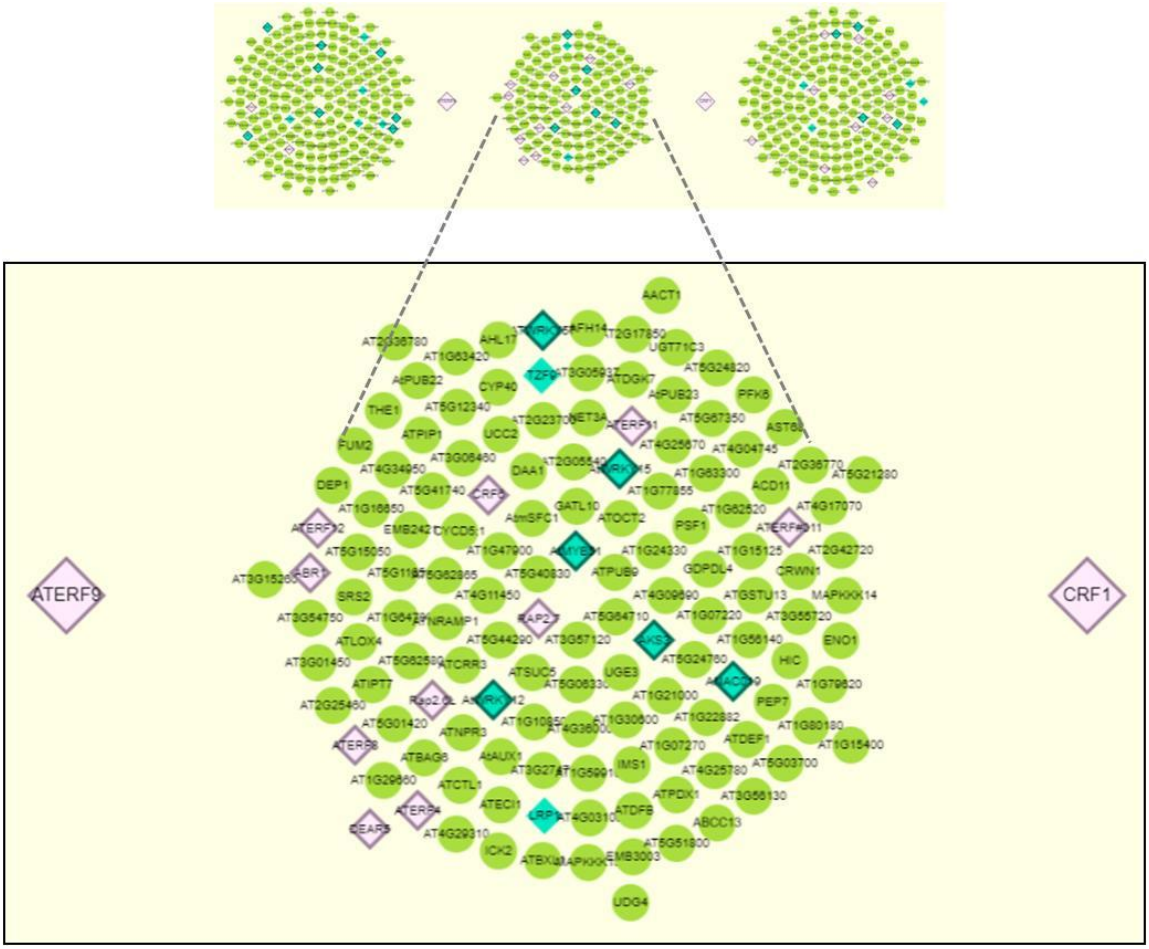

Figure 4.12 ERF9 and CRF1 as possible regulators downstream of BOL. ERF9 and CRF1were predicted to be, by the TF2 network tool, as possible regulators of genes that show differential expression 8 h after of BOL induction. Circles depict genes, and diamonds indicate genes that code for transcription factors. Purple diamonds point to genes related to the phosphorelay transduction system. The large group of genes in between ERF9 and CRF1 are their common candidate target genes, found among the list of genes that showed differences in expression 8 h after BOL induction.

IV.3.4 BOL regulates genes that participate in the control of the cell cycle

An interesting GO category present 30 min but not 8 h after BOL induction is Negative Regulation of Protein Kinase activity (GO: 0033673). Within this category are *KRP1* (-1.4 fold), *KRP4* (1.1 fold), *KRP6* (1.4 fold), *KRP7* (3.0 fold), *CYCD1;1* (-0.8 fold) and *CYD5;1*

(1.2 fold). Kip-related protein (KRP) genes encode CDK (cyclin-dependent kinase) inhibitors (CKI) (Lui et al., 2000; Nakai et al., 2006). It has been proposed that KRP proteins are negative regulators of cell division and in some circumstances promote endoreduplication. CYCLIN D1; 1 (CYCD1; 1) and CYCLIN D5;5 (CYCD5; 5) have also been associated with endoreduplication (Sterken et al., 2011).

When using the NetworkAnalyst tool (Xia et al., 2015) to predict and visualize protein-protein interactions from genes with differential expression at 30 min, it was found that the proteins encoded by the genes present in the category "Negative Regulation of Protein Kinase activity" show interactions with proteins such as Retinoblastoma-Related (RBR1), DNA Cytosine Methyltransferase (DMT1) and Cell Division cycle Protein 27 homolog A (CDC27A) (as presented in Figure 4.13). Interestingly, the genes that encode these proteins also showed differential expression 30 min after BOL induction, and are up-regulated at this time (1.4 fold, 1.8 fold, and 1.7 fold respectively).

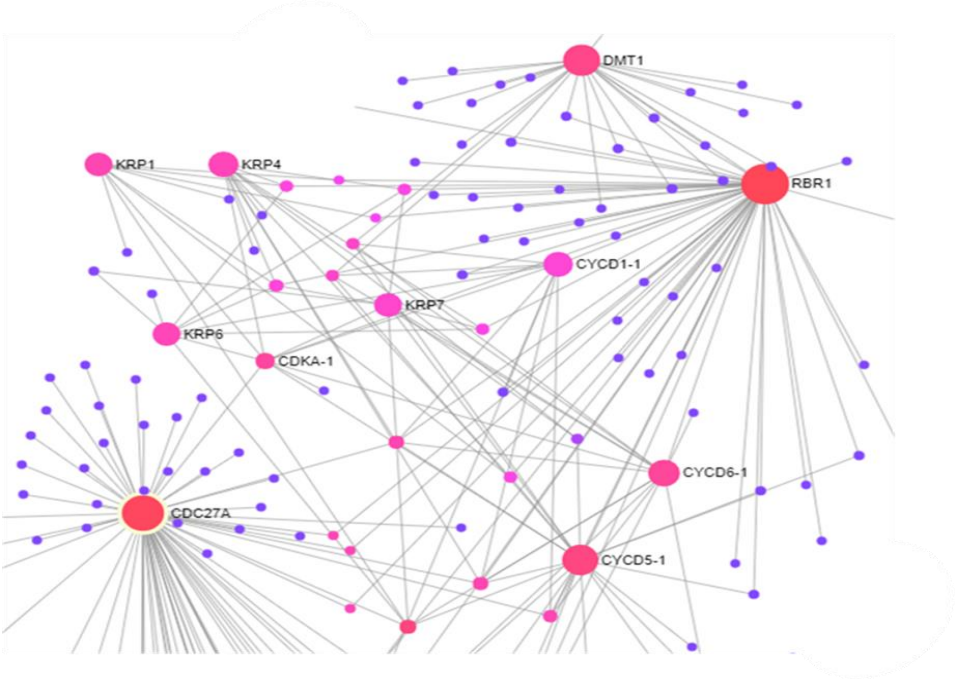

Figure 4.13 Prediction of protein-protein interactions of negative regulators of protein kinase activity (KRPs) found among differentially expressed genes 30 min after BOL induction. Prediction and visualization of protein-protein interactions revealed the presence

of other cell-cycle related genes in addition to KRPs. The size of the circles represent the number of interactions.

METHYLTRANSFERASE 1 (DMT1) and *RETINOBLASTOMA RELATED 1* (*RBR1)* are grouped within the category Asymmetric Cell Division (GO: 0008356). This category is also only present in the 30 min after induction data. Other genes such as *BLUEJAY (BLJ)* (-0.8 fold), *INDETERMINATE DOMAIN 4 (IDD4)* (-1.1 fold), *JACKDAW (JKD)* (-1.2 fold), RNA LIGASE (*RNL)* (1.4 fold), *SHORT ROOT* (*SHR)* (-1.2 fold) also fall into this category. There is evidence that indicates that RBR1 together with MULTICOPYSUPPRESSOR OF IRA1 (MSI1) repress the expression of *DMT1* (Jullien et al., 2008). SHR forms a network in combination with JKD, BLJ to regulate tissue patterning through asymmetric cell division in root (Welch et al., 2007).

After 8 h of BOL induction, the cell cycle category (GO: 0007049) is enriched. Within this category there are 97 genes of which 14 are up- and 83 are down-regulated. Up-regulated genes show a slight increase in expression, between these genes are *KRP6* (1.3 fold), *KRP4* (1.3 fold); *SOMATIC EMBRYOGENESIS RECEPTOR –LIKE KINASE 2 SERK2* (0.5 fold), *CYCD5;1* (0.82 fold), *RBR* (0.699 fold), *FORMIN HOMOLOGY 14* (*AFH14)* (1.28 fold), and *PATELLIN 2* (*PATL2*) (1.7 fold). However, since some of these genes show higher expression at 30 min, this could be interpreted as that they are being down-regulated rather than being up-regulated, such as, *CYCD5;1* and *RBR*. Among the down regulated genes are: *CYCA1;1* (-0.98 fold), *CYCA2;3*, (-0.8 fold), *CYCA2;4* (-1.21 fold), *CYCA3;1* (-1.77 fold) and *CYCD6;1* (-1.79 fold). On the other hand, most of the genes related to microtubule organization are down regulated (29 of 34), which could suggest that BOL is repressing cell division at this time point.

IV.3.5 BOL represses DNA replication

Because some KRPs (*KRP4*, *6* and *7*) are up regulated 30 min after BOL induction, and 8 h after BOL induction 83 cell cycle related genes are down regulated, among them several *CYCA*, it could be inferred that BOL has a negative effect in the progression of the cell

cycle. On the other hand, 30 min after BOL induction, genes related to endoreduplication were up regulated. For this reason, we explored the list of differentially expressed genes to obtain hints about whether BOL promotes DNA replication. DNA replication starts when the six-subunit ORIGIN RECOGNITION COMPLEX (ORC1-6) binds to origin DNA. DNA-bound ORC recruits the CELL DIVISION CONTROL 6 (CDC6) and CYCLIN-DEPENDENT PROTEIN KINASE 1 (CDT1) proteins. These proteins facilitate the loading of multiple copies of the putative replicative helicase complex (MCM2-7) onto the replication origin (Shultz et al., 2009).

In order to better understand the relationship of BOL with DNA replication we proceeded to review the genes that are present in this category. Indeed, 52 genes were present in this category, of which 4 were up- and 48-down regulated. Among the down regulated genes are: *ORC1A* (-1.41 fold), *ORC2* (-1.45 fold), *ORC3* (-1.41 fold), *ORC6* (-1.30 fold), *CDC6* (-2.04 fold), and *CDT1A* (-1.40 fold). In addition to these genes also 7 mini-chromosome maintenance (*MCM*) genes are also down regulated: *MCM2* (-1.10 fold), *MCM3* (-1.09 fold), *MCM4* (-1.15 fold), *MCM5* (-1.08 fold), *MCM6* (-1.44 fold), *MCM7* (-1.31 fold) and *MCM10* (-1.66 fold). So, in the conditions and at the times at which we evaluated the transcriptional effect of BOL, it seems that BOL promotes repression of cell division as well as inhibiting DNA replication, at least at the transcriptional regulatory level.

IV.3.6 BOL promotes changes in cell signal transduction

Since the GO categories we had analyzed so far presented a greater number of down-regulated genes, we decided to focus on searching among the most enriched categories 8 h after BOL induction, that had a higher number of up-regulated genes. We were particularly interested in the Response to Stress category (GO: 0006950) with a total of 584 genes, of which 349 are up-regulated and 235 are down-regulated. Within this category, there are genes involved in responses to cold, water deprivation, wounding, bacterial infection, nematodes, and excessive salt, among others. Among the up regulated genes is PROTEIN PHOSPHATASE 2C (*AP2C1*) (2.31 fold). AP2C1 is a Ser/ Thr phosphatase that regulates

stress hormone levels (i. e., jasmonate and ethylene), defense responses, and MAPK activity (Schweighofer et al., 2007). It is interesting that this category has a close connection with hormones and the phosphorelay signal transduction system. Response to Hormones (GO: 0009725) was another category with a higher number of up-regulated genes (184 genes up- and 168 down-regulated); among these, there are genes related to Response to Auxins, Jasmonic Acid, Abscisic Acid, Brassinosteroids and Salicylic Acid.

Within the genes related to the phosphorelay signal transduction system there are some of the ARABIDOPSIS RECEPTOR-LIKE KINASES (RLK) type (appendix 5). RLKs are serine-threonine protein kinases; their extracellular ligand-binding domain perceives signals and is commonly used to classify RLKs into distinct subgroups (Shiu and Bleecker, 2003). RLKs play important roles in defense response, but also in plant growth and development (As illustrated in Figure 4.14). We found some typical or well-known defense response RLKs in our data, such as: *PEPR1* (2.06 fold), which amplifies the innate immunity response to pathogen attacks and *RLK7* (1.12 fold), which participates in abiotic tress responses (Reviewed in Pitorre et al., 2010; Osakabe et al., 2013). Some RLKs involved in development and indicated in Figure 4.14 are: *ERECTA* (-0.82 fold), and *ERL1* (0.76 fold), genes related with stomatal development; *PSKR1* (1.14 fold), related with cell proliferation; *THE1* (1.65 fold), a receptor kinase regulated by Brassinosteroids and required for cell elongation, and *WAK1* (-1.83 fold) also involved in cell elongation; *SERK2* (0.55 fold), which participates in anther development and *PXY* (-1.41), involved in vascular development (reviewed in Osakabe et al., 2013).

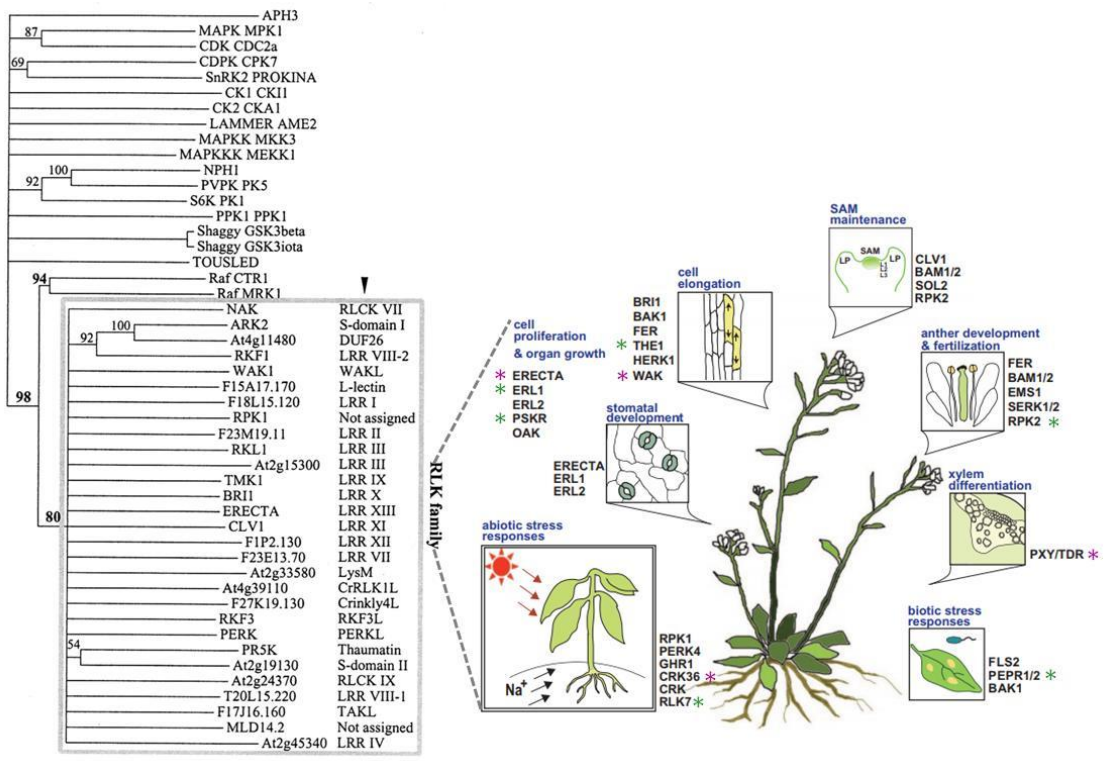

Figure 4.14 Overview of plant receptor-like kinases (RLKs) and their functions during stress response and development. The RLKs are a large gene family that form a monophyletic group distinct from all of the other protein kinases found in the *Arabidopsis* genome. Representative and well-known RLKs are shown. Green asterisks indicate those RLKs that are up-regulated and purple asterisks those that are down-regulated after BOL induction (Modified from Shiu and Bleecker 2001, and Osakabe et al., 2013).

Several of the genes related to RLKs that show differential expression in our data have not been functionally characterized. Most of the RLK-type genes belong to the subgroup of Cysteine-rich RLKs (CRKs). At 30 min of BOL induction we found *CKR12* (-3.59 fold), *CKR28* (1.17 fold), *CKR36* (-2.12 fold) and *CKR42* (-1.22 fold). At 8 h of BOL induction *CRK10* (2.88 fold), *CRK19* (1.02 fold), *CRK21* (-2.09 fold), *CRK22* (-1.13 fold), *CRK28* (1.63 fold), *CRK29* (1.86 fold) and *CRK30* (-1.62 fold) are present. It has been reported that *CRKs* are differentially regulated by reactive oxygen species (ROS). *CRK10*, *CRK19*, *CRK28*, *CRK29* and *CRK36* are up regulated in response to O_3, while *CRK22* and *CRK30*

are down regulated. On the other hand *CRK12*, *CRK22* and *CRK42* do not change their expression in response to O_3 (Wrzaczek et al., 2010).

ROS have long been known to play critical roles in processes in response to oxidative stress. However, ROS have also emerged as important regulators of plant development. It is interesting because among the genes induced 30 min after BOL induction we found *ROXY1* with a fold change of 5.77, and 8 h after BOL induction *ROXY2* (1.59 fold). ROXY1 and ROXY2 are glutaredoxins (GRXs). Glutaredoxins play a crucial role in the response to oxidative stress. However ROXY1 and ROXY2 have been also shown to have a function during Arabidopsis flower development (Li et al., 2009).

IV.3.7 BOL regulates genes involved in cell wall organization

Because with the previous analyses we observed that BOL promotes drastic molecular and biochemical changes in cells, we wanted to know if there were also indications of possible changes in processes that can affect cell morphology. We identified that there are changes in the expression of genes that are grouped within the category of Cell Wall Organization and Biogenesis (GO: 0071554) (appendix 6). Within this category, there are 138 genes of which 53 are up- and 85 are down-regulated. There are differentially expressed genes that belong to this category both 30 min and 8 h after BOL induction. Among these genes there are some that code for XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ HIDROLASES such as: *XTH12* (8.02 fold), *XTH18* (4.56 fold), *XTH19* (4.05 fold), *XTH23* (2.27 fold), *XTH22* (2.04 fold), *XTH27* (1.00), *XTH8* (-1.07), *XTH6* (1.07) and *XTH33* (-1.36); the expansin genes: *EXLA1* (2.06 fold), *EXPB3* (1.58 fold), *EXPA9* (1.03), *EXPA13* (0.77 fold), *EXPLB1* (-0.99), *EXPA6* (-0.66), *EXPB3* (-1.28) and *EXPA11* (-2.11); the cellulose synthase genes : *CSLC5* (-0.89 fold) and *CSLA01* (-4.54 fold) and the cellulase genes : *CEL5* (1.31 fold), *CEL2* (-3.17 fold). Therefore, cell wall remodeling processes appear to be also regulated by BOL.

In summary, the transcription factor BOL promotes expression changes in genes involved in many processes. Within these processes we could notice that there are early changes (30 min) in the expression of genes that encode various transcription factors and processes

related to transcription regulation. Also among our results we observed that several genes involved in key processes of development such as DNA replication and cell cycle are repressed, and those genes that are up-regulated encode repressor cell cycle proteins, such as KRPs. On the other hand, at later times (8 h) there is a greater number of up-regulated genes that are grouped mainly within diverse categories related to the response to biotic and abiotic stress and response to phytohormones; among these are some genes that encode receptor like kinases that are responsible for sensing stress and developmental signals. In addition to this we could also observe changes in genes related to microtubules and cell wall organization or biogenesis. Therefore BOL seems to promote a readjustment of cellular processes, possibly because these cells are undergoing a change in cellular identity.

IV.4 DISCUSSION

The differential expression analysis of genes after BOL activity induction at very short times provided a large amount of valuable information about the early regulatory activity of the transcription factor and possibly early processes of organ development. The diversity of processes that are putatively regulated by BOL is reflected in the number of categories in which such genes are grouped (114 for genes of 30 min and 166 for genes of 8 h). However, it is interesting to note that processes that are considered key for plant development show a greater number of down- than up-regulated genes. In some categories, like "Cell Cycle", a close look to the type of differentially expressed genes helped obtain a glimpse about the kind of regulation that BOL is exerting in this process. Within this category genes involved in the progression of the cell cycle are down-regulated, while those genes that encode proteins that have been identified as negative regulators of cell division, such as KRPs (Nakai et al., 2006; Lui et al., 2000) are up-regulated. This result could be interpreted as that, at early stages of action, BOL promotes cell cycle repression. The repression of the cell cycle by BOL draws attention because it contrasts with the phenotypic effect that characterizes the over-expression of BOL, which is cell proliferation in the roots and calli development (Marsch-Martínez et al., 2006; Ikeda et al., 2006). However, it must be considered that in this work, aerial tissue of seedlings (excluding the hypocotyls) was used and in this tissue an evident cellular proliferation is not observed as in roots. On the other hand, this early repression may be linked to the specification of founder cells at the periphery of the meristem, before evident proliferation occurs.

Another process that is important for plant development is endoreduplication. Endoreduplication is the replication of DNA without cellular division. An increase in ploidy level by endoreduplication is often correlated with cell elongation and differentiation (Caro et al., 2008). Because BOL seems to be negatively regulating the cell division we proceeded to review genes related to DNA replication. Again we found that key genes for the start of DNA replication were down regulated by BOL at these early stages. There is

evidence of a relative increase in DNA content when BOL is over-expressed in tobacco plants (March-Martínez et al., 2006) and when it is expressed in epidermal cells of Arabidopsis (Seeliger et al., 2016). It is interesting that the results obtained in this work differ from those reporting BOL phenotypic analyses. It must be considered that those phenotypic analyzes had been made with plants that show morphological alterations caused by BOL over-expression, while this work was performed at a very short time after BOL induction. The samples were collected 30 min and 8 h after the induction of BOL activity, obtaining the genes that change their expression before visible morphological changes in the seedlings occur. Therefore, it provides insights about the early regulatory activity of BOL, and complements previous differential expression data obtained from 4 week old gain-of-function mutants, where early, late and indirect effects cannot be distinguished (Marsch-Martínez et al, 2006). Again, the data obtained here may suggest that the transcription factor has different effects in time, and coincides with the conclusion obtained by studying its role in gynoecium development, which indicated that BOL affects cytokinins response in a different way during the development of this organ (Durán-Medina et al., 2017).

In addition to the above results, we also find that BOL is able to regulate the expression of genes that encode many other transcription factors, proteins involved in cell signalling and cell wall organization. The regulation of all these processes implies biochemical, physiological and morphological changes in the cells. So this could mean that BOL is reorganizing the entire cellular machinery in order to prepare the cell reprogramming or cell identity change evidenced much later in the phenotype. For this reason, BOL may initially repress for a time-lapse some processes such as DNA replication and cell division.

An important element during cell identity change is gene expression reprogramming. This process involves chromatin remodeling as well as posttranscriptional modifications. In our results we identified differential expression in genes encoding DNA methyltransferases such as *METHYLTRANSFERASE 1* (*MET1*) (1.92 fold) and

CHROMOMETHYLASE 3 (*CMT3*) (-1.04 fold), involved in transposon and gene silencing (Bartee et al., 2001; Zemach et al., 2013); RBR1, that has been reported to repress *MET1* (Jullien et al., 2008); and methylcytosine-binding proteins, *VIM1* (-0.82 fold) and *VIM3* (-0.99 fold) that regulate DNA methylation and maintain the chromatin structure (Woo et al., 2007; Kraft et al 2008). There is also down-regulation of genes that encode histones of the H2A family, such as: *HTA2*, *HTA5*, *HTA6*, *HTA7*, *HTA8*, *HTA10*, *HTA11*, *HTA13*; type H2B: *HTB1*, *HTB2*, *HTB9*, *HTB11*; and several type H3 histones. This supports the possibility that BOL activity results in genome reprogramming, which could also explain the very drastic effects observed in the phenotype of induced plants days after BOL induction (appendix 7) .

It is very interesting that several genes involved in stress responses show differential expression after inducing BOL activity. However, we call "stress" to changing external conditions to which plants are constantly exposed . Once the plants sense these signals, a series of processes that will modulate the plant development to adapt to these conditions are triggered. Therefore, when there is a change in cellular identity, there must also be an adjustment of the cellular mechanisms to perceive certain stimuli that allow them to fulfill the function they will play according to the cellular identity they acquire. This could be a possible explanation of why when we induce BOL activity we see changes in the expression of genes involved with cell signalling, such as, MAP kinases, membrane receptors (RLKs) and genes related to phytohormones and stress response. It is increasingly evident that there is a close relationship between the response to signals that come from the environment, and factors that regulate developmental processes (reviewed in Munné-Bosch and Müller, 2013). An example of this is RBR1 that has been identified as a molecular link between cell cycle and environmental responses such as circadian signalling and biotic stress (Harashima et al., 2016). On the other hand factors that have been studied as related to stress responses recently have also been found to play a role in regulating plant development. This is the case of glutaredoxins, which have been associated with redox-regulated processes involved in stress responses. However, the differentially expressed glutaredoxin gene *ROXY1* is expressed in the primordia of some floral organs, similarly to

BOL; in addition, it has been reported that nuclear interactions of ROXY1 with TGA transcription factors are required for normal petal initiation (Xing et al, 2008, Li et al, 2009). Possibly, ROXY1 performs a similar function for the primordia leaf development.

This shows that many processes are intimately linked, and that even though some genes have been classified as performing a function or process such as the response to stress, they are part of an interconnected network that allows the plant to develop correctly in the environment in which it is located.

Finally, from all these data we could speculate that possibly, for a short period of time, BOL pauses the progression of the cell cycle and DNA replication to trigger adjustments in cell signalling and gene expression reprogramming. However, we should take these results carefully, since we obtained genes with differential expression from a mixture of tissues including the shoot apical meristem, cotyledons, leaves at different stages of development, and petioles. Another possible scenario is that the negative regulation of the cell cycle occurs in most of the differentiated cells present in the samples, whereas in meristematic cells, which are very few in comparison, something different happens with the regulation of the cell cycle. Therefore these interpretations should be experimentally corroborated. It is necessary to analyze cell cycle markers and DNA replication exploring their progression at different tissues and times of BOL induction to determine if the cell cycle repression is related to the timing of BOL action or to specific cell types. Moreover, during these analyses, it would also be very interesting to analyze the relationship of BOL with histones and chromatin remodelling.

IV.5 CONCLUSION

In conclusion, genes that participate in very different cellular activities were differentially expressed 30 min and 8 h after BOL induction. Some of these genes and GO categories were expected, but also categories that would not have been predicted *a priori*, were enriched. These results open the door for new future research directions.

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Chapter V
Concluding remarks and perspectives

Different publications have reported valuable information about the expression pattern of BOL, and the phenotypic defects and effects promoted by the alteration of its expression (Marsch-Martínez et al., 2006; Ikeda et al., 2006; Chandler et al, 2007; Nag et al., 2007; Chandler et al., 2011a; Chandler et al., 2011b; Seeliger et al., 2016). However, even though different molecular approaches have been employed to elucidate them, the molecular processes that it regulates are not yet clear. Therefore, we decided to perform an exploration of the processes regulated by the BOL transcription factor. Information about its relationship with the auxins pathway was available, but its nature is elusive and there was evidence indicating a broader role for the transcription factor (Eklund et al., 2011, Chandler et al., 2011, Capua and Eshed, 2017). The initial exploration aimed to identify if at least part of the BOL function was related to cytokinins. This exploration provided interesting results, since we confirmed that the BOL function has a close relationship with cytokinins, provided new information about the BOL function and we also obtained some unexpected results. In addition to that, this exploration generated a lot of information that goes beyond the initial objective and suggests that BOL may regulate many other processes besides those related to cytokinins.

V.1 Regulation of the cytokinins pathway by BOL

We corroborated that one of the functions of BOL is related to the cytokinins pathway when we found that BOL was able to regulate *AHP6* (a negative regulator of cytokinins

signalling) expression during the early stages of gynoecium development, besides observing that the loss of *BOL* function affects the gynoecium response to cytokinins treatments. However, it was clear that the cytokinins-related function of BOL during gynoecium development can not only be explained through the regulation of *AHP6*. This gave rise to an hypothesis proposing that there were other genes of the cytokinins pathway that were regulated by BOL, possibly also involved in the correct development of the gynoecium.

On the other hand, through the differential expression analysis in vegetative tissue, we found that, indeed, BOL is able to regulate the expression of other cytokinins-related genes in addition to *AHP6*. Among these, there are genes involved in different steps of the cytokinins pathway, such as biosynthesis, degradation, conjugation, transport, signalling and response. Interestingly, some of these genes act as negative regulators and others as positive regulators of the cytokinins pathway. However, interestingly and in a way unexpectedly, we found that *AHP6* and *IPT5* (a gene encoding a cytokinins biosynthetic enzyme) show coincidence in the tissue in which they are regulated by BOL, presenting a highly specific expression in the vasculature upon activation by BOL.

Based on the *AHP6* and *IPT5* expression patterns reported in the root, we hypothesize that their antagonistic function may modulate the cytokinins pathway to promote the correct differentiation of vascular tissues, not only in the root, but also in the aerial organs. Another hypothesis that emerged from this work was that BOL over-expression causes an imbalance between these positive and negative elements of cytokinins pathway, resulting in uncontrolled proliferation of the vascular meristematic tissue, thus causing calli development.

V.2 Regulation of other cellular processes by BOL

The global analysis of differential expression revealed that in addition to regulating the cytokinins pathway, BOL regulates genes that participate in other phytohormonal pathways, including the auxins pathway. In the same way as with cytokinins, BOL regulates genes involved in different steps of the auxins pathway. However, at the earliest time of BOL induction, we did not find a relationship between BOL and auxins biosynthesis as expected (based on reports indicating that BOL promotes auxins biosynthesis, Eklund et al., 2011). Nevertheless, we observed changes in the expression of genes involved with auxins transport. Interestingly, many SAUR-like genes changed their expression in response to BOL; however, there is very little information about the function of these genes. Few genes of SAUR family have been characterized and some of them have been related to cell expansion (Spartz et al., 2012). It would be interesting to further study the function of these genes and their relationship with BOL.

At the later time of the BOL induction, in relation to auxins, we mainly found genes related to the response. In general, in this data, the genes related to the response to phytohormones such as abscisic acid, jasmonic acid, brassinosteroids and ethylene prevail; in addition to genes related to the response to other types of molecules and signals related to biotic and abiotic stresses.

We could also observe that BOL promotes expression changes in genes involved in cell signalling, mainly with the phosphorelay signal transduction system, among which are genes that encode MAP kinases and receptor-like protein kinases, which regulate several processes related to the response to stress and development (Reviewed in Osakabe et al., 2013; Xu and Zhang, 2015). Besides, BOL also seems to alter the expression of genes related to cell wall biogenesis, remodeling and organization. From these results, we hypothesize that BOL regulates these elements to adjust the cell identity and thus control the development of new organs.

Also with these results, obtained at early times (30 min and 8 h) of BOL induction, we unexpectedly found that BOL seems to trigger the repression of the cell cycle and DNA replication. The explanation that emerges from this is that, at the very first stages of the

development of an organ, BOL possibly stops these processes to reprogram the gene expression of these cells and thus shape their identity. However, further experiments are necessary to corroborate these observations as they contrast drastically with the phenotypes observed at very late times of the BOL induction, suggesting that the early and late effects of BOL may be different.

V.3 New information provided with this study

With this work we found important elements that contribute to the understanding of BOL function that had not been previously reported, or even expected. We found that BOL is important for the development of the gynoecium. BOL is expressed in the gynoecium founder cells, but expression continues beyond the earliest stages. At later stages it becomes restricted to the prospective valve regions and this expression is maintained, and gradually diminished during the gynoecium development until stage 11, when it disappears. This information provides new elements to understand BOL function, since until now it had been considered that BOL was expressed only in the flower organ founder cells. We also found that BOL activity is important for the synchronic development of the internal tissues of the gynoecium. In addition, we concluded that the BOL function during the gynoecium development it is partly carried out through the regulation of *AHP6*. An interesting observation also made during this work, was that BOL appears to have effects or functions that are dependent of the developmental stage of the organ. This, observation will surely help to better interpret the global expression data, and understand the biological role of BOL.

The result of the work described in chapter III that explored the regulation of cytokinins-related genes, is very interesting since the BOL function had not been previously associated with the development of the vasculature. In addition to the new information that was provided on the BOL function, information not reported for *AHP6* and *IPT5* was also obtained. So far, the *AHP6* and *IPT5* expression pattern was very well described in the root (Mähönen et al., 2006; Miyawaki et al., 2004), and there was information about *AHP6*

expression in the inflorescence and floral meristem (Besnard et al., 2014), but not in the vasculature of vegetative tissues.

Despite having previous differential expression data obtained using microarrays, in this work we found candidate target genes that had not been identified, possibly due to the different experimental strategy followed. For example, there was no information linking BOL with cytokinins biosynthesis. The cytokinins biosynthetic IPTs had not been identified as possible targets of BOL. Similarly, the BOL function had not been related to the cell cycle regulators KRPs and RLKs. So it was worth to perform the transcriptome analysis, since it provided valuable new information about the possible additional BOL functions.

PERSPECTIVES

The results of the present work provided an abundance of information, but they bring with them new questions to solve and hypotheses to validate. However, these questions and hypotheses are more specific and directed to very particular processes.

The near-future perspectives of this work are mainly aimed at identifying the direct targets of this transcription factor through techniques that allow us to identify the physical binding of the BOL protein to the regulatory regions of particular genes. Among these techniques we could perform Yeast One-hybrid (Y1H) and Chromatin Immunoprecipitation (ChiP) analyses. The standardization of these techniques in the laboratory has started (by members of the group). For the Y1H assays, fragments of *AHP6*, *IPT5* and *CRF1* candidate regulatory regions have already been cloned. In the future it would be interesting to include other genes related to other processes such as auxins and cell cycle regulators.

In addition, it would be important to corroborate the biological function and relevance of the regulation of *AHP6* and *IPT5* by BOL in the vasculature (and other cytokinins related genes). First of all we would like to determine at a finer level the identity of the cells in which BOL regulates these genes (whether at the pericycle, procambium, protoxylem, etc). We are also interested in determining if these genes are important for calli development,

either by mutating these genes in the inducible line of BOL or by making crosses of the inducible line with mutants of *AHP6* and *IPT5*. For the analysis of this process it would be interesting to include *IPT7*, *CRF1* and *CRF6*, since there are reports indicating that they are also expressed in the vasculature. To complement this work we would like to analyze if there is an increase in the production of cytokinins when BOL is induced and integrate this information with that available in the literature and that here generated in this study, including information about its relation with the auxins pathway.

It would also be very interesting to clarify the possible temporal regulation of DNA replication and the cell cycle by BOL, reducing these processes at the beginning, and possibly activating them later again.

Moreover, the data will be used to initiate new research directions exploring the relationship and relevance of BOL direct or indirect regulation of genes and pathways that have not been frequently linked to organ development. In this way, a better understanding of new organ development may be obtained.

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