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HD-ZIP III transcription factors regulate *ACL5* and participate with this gene in a regulatory loop controlling vascular development in *Arabidopsis thaliana* (L.) Heynh

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RIASSUNTO

I numerosi dati genetici accumulati indicano che i 5 membri della famiglia HD-ZIP III della pianta modello *Arabidopsis thaliana* sono coinvolti nella regolazione dell'attività dei meristemi, nella formazione della polarità degli organi laterali e dei fasci vascolari, e nel differenziamento del sistema vascolare con una complessa combinazione di funzioni ridondanti, sinergiche e contrastanti. Questi processi di sviluppo rappresentano delle innovazioni fondamentali acquisite durante l'evoluzione delle piante terrestri ed è stato suggerito che i geni della famiglia HD-ZIP III, che presentano una notevole conservazione filogenetica, siano stati tra i primi componenti chiave dei circuiti regolativi che hanno permesso l'evoluzione di un'ampia varietà di specie vegetali adattate alla vita fuori dall'ambiente acquatico. L'attività dei fattori di trascrizione HD-ZIP III è strettamente controllata a diversi livelli attraverso una molteplicità di meccanismi che comprendono la omo- ed etero-dimerizzazione, l'interazione proteina-proteina e la regolazione da parte dei miRNA e di piccole molecole come l'ormone auxina e un ligando lipidico la cui natura non è ancora nota. Per far luce sul meccanismo d'azione dei fattori di trascrizione HD-ZIP III, ci si é proposti di ricercare i geni direttamente regolati da queste proteine. Utilizzando un approccio bioinformatico sono stati identificati nel genoma di *Arabidopsis* 390 geni contenenti il sito di legame delle proteine HD-Zip III (BS-III). Tra questi, è stata notata la presenza del gene *ACAULIS5 (ACL5*), codificante per la termospermina sintetasi, che presenta una significativa sovrapposizione funzionale e di espressione con i geni della famiglia HD-ZIP III. Esperimenti di legame *in vitro* e analisi di espressione hanno fornito una prima conferma sperimentale all'ipotesi che *ACL5* sia un autentico gene target della famiglia HD-ZIP III. L'oggetto di questa tesi è stato quindi la verifica *in vivo* della regolazione di *ACL5* da parte della famiglia HD-ZIP III e lo studio della relazione tra questi geni nella regolazione dello sviluppo del sistema vascolare in *Arabidopsis*.

Per verificare *in vivo* la rilevanza funzionale del sito di legame della famiglia HD-ZIP III nel promotore di *ACL5* sono state generate piante transgeniche di *Arabidopsis* esprimenti la sequenza codificante di *ACL5* fusa alla sequenza codificante il reporter GUS sotto il controllo del promotore di *ACL5* nella versione selvatica o mutata nell'elemento BS-III in modo da abolire il legame delle proteine HD-ZIP III. L'analisi istochimica del profilo d'espressione del reporter GUS ha mostrato che l'espressione di *ACL5* è preceduta da quella di *ATHB8*, un membro della famiglia *HD-ZIP III* la cui espressione è necessaria per definire l'identità pre-procambiale delle cellule, e che la mutazione dell'elemento BS-III abolisce l'espressione di *ACL5* negli stadi più precoci del differenziamento del procambio nei primordi fogliari,

nell'apice radicale e nei primordi delle radici laterali. Questo risultato rafforza l'ipotesi che *ACL5* sia un target autentico regolato direttamente dalle proteine HD-ZIP III attraverso il legame all'elemento BS-III.

Per verificare ulteriormente il ruolo funzionale dell'elemento BS-III *in vivo*, i costrutti *ACL5::ACL5:GUS* e *ACL5mut::ACL5:GUS* sono stati introdotti anche nel mutante *acl5-1*. L'analisi fenotipica di queste piante transgeniche ha mostrato che la mutazione del promotore non impedisce la complementazione di tutte le alterazioni fenotipiche del mutante *acl5-1*. In modo inatteso, l'analisi dettagliata della nervatura delle foglie di queste piante ha permesso di estendere la caratterizzazione funzionale di *ACL5* suggerendo che il differenziamento dello xilema è correlato al livello di ACL5. Infatti, sebbene sia stato dimostrato che *ACL5* è necessario per permettere il corretto sviluppo vascolare impedendo la morte prematura delle cellule dei vasi, i dati presentati qui indicano chiaramente che alti livelli di ACL5 possono ritardare o inibire completamente il differenziamento delle cellule procambiali in elementi xilematici maturi.

Durante questo studio, inoltre, sono stati descritti dei nuovi fenotipi del mutante *acl5-1,* come l'iponastia delle foglie, l'alterazione dello sviluppo radicale, l'accumulo di antocianine e l'anticipo della fioritura, che hanno permesso di estendere la caratterizzazione funzionale di *ACL5* suggerendo un ruolo per questo gene nel controllo della proliferazione cellulare e nelle risposte da stress oltre che nel controllo del differenziamento vascolare.

Infine un'attenta caratterizzazione fenotipica di mutanti doppi e multipli ottenuti dall'incrocio di *acl5-1* con mutanti dei geni *HD-ZIP III* ha mostrato che, sebbene *acl5-1* sia epistatico su *rev-5* rispetto all'allungamento del fusto, alla dimensione della rosetta e alla fertilità, alcune difetti del mutante *acl5-1* sono compensati dalla perdita di funzione dei geni *HD-ZIP III*, indicando che questi partecipano anche ad alcuni degli eventi regolativi a valle dell'azione di *ACL5.* In particolare, i geni *HD-ZIP III* sono necessari nel mutante *acl5-1* per permettere la formazione di nervature extranumerarie e per sostenere il circuito di regolazione a feedback negativo che regola l'espressione di *ACL5*.

Infine, poiché è noto che *ACL5* influenza l'espressione dei geni *HD-ZIP III,* è stato proposto un modello di regolazione a feedback secondo il quale la progressione del differenziamento delle cellule procambiali é regolata dall'auxina e dai fattori di trascrizione HD-ZIP III attraverso l'induzione di un reostato enzimatico, costituito dalla proteina ACL5 e dal suo prodotto termospermina, che a sua volta modula i livelli di espressione dei geni *HD-ZIP III*.

SUMMARY

Comprehensive genetic studies in the model plant *Arabidopsis thaliana* have deduced that the five-member *HD-ZIP III* gene family regulates, through a complex pattern of overlapping, distinct, and antagonistic roles, meristem initiation, vascular and leaf development, and organ polarity. These developmental processes represent key innovations in land plant evolution, and it has been suggested that HD-ZIP III family genes, which present extensive phylogenetic conservation, were an early component of the land plant developmental tool-kit. The activity of HD-ZIP III family transcription factors is tightly controlled at several different levels by many different mechanisms including miRNA, homo- and hetero-dimerisation, protein-protein interaction and by the action of small molecules such as the hormone auxin and an as yet unidentified lipid ligand.

To gain more information on the regulatory circuits in which these transcription factors are involved, one of the major goals is the identification of the target genes directly regulated by HD-ZIP III proteins. A database mining of the *Arabidopsis* genome searching for genes containing the consensus binding sequence (BS-III) recognised by HD-ZIP III proteins *in vitro* yielded a list of 390 putative target genes. Among these, the presence of *ACAULIS5* (*ACL5*), encoding a thermospermine synthase, and showing an expression pattern and functional overlap with *HD-ZIP III* genes seemed particularly meaningful. *In vitro* binding experiments and expression analysis have given support to the hypothesis that *ACL5* could be a genuine primary target gene of *HD-ZIP III* genes. Thus, the aim of this thesis has been further testing of *ACL5* regulation by HD-ZIP III family *in vivo* and analysis of the functional relationship between these genes in the control of vascular system differentiation in *Arabidopsis*.

In order to confirm *in vivo* the functional relevance of the HD-ZIP III binding site present in the *ACL5* promoter, transgenic *Arabidopsis* plants expressing the *ACL5* coding sequence fused to the sequence encoding the GUS reporter under the control of the *ACL5* promoter sequence, either wild-type or mutated in the BS-III element to abolish HD-ZIP III proteins binding, have been generated and characterized. Histochemical analysis of the GUS expression pattern has revealed that the HD-ZIP III family member *ATHB8*, necessary to define the pre-procambial cells identity, is expressed earlier than *ACL5* and that mutations in the BS-III element abolish *ACL5* expression in the very early phases of vascular development in leaf primordia, primary root meristem and lateral roots primordia. These results strongly reinforce the hypothesis that *ACL5* is a genuine target directly regulated by HD-ZIP III proteins through binding to the BS-III element.

To further test *in vivo* the functional relevance of the BS-III element, *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* were introduced also in *acl5-1*. A careful examination of these transgenic plants clearly demonstrated that BS-III element mutation does not impair complementation of all the phenotypic alterations of *acl5-1* plants. Intriguingly, a detailed analysis of leaf veins pattern in these plants have extended our current knowledge of *ACL5* role suggesting that xylem differentiation is linked to ACL5 dosage. In fact, although *ACL5* has been demonstrated to be necessary to ensure proper vascular differentiation by preventing premature cell death of xylem elements, the data presented here clearly indicate that high ACL5 levels delay or even totally inhibit the differentiation of procambial cells into mature tracheary elements. Additional clues for the functional characterisation of *ACL5* were also provided by the observation during this study of previously unnoticed phenotypes of *acl5-1* mainly linked to cell proliferation and stress response, such as leaf hyponasty, alteration of root development, anthocyanins accumulation and early flowering.

Finally, a careful analysis of the phenotype of double, triple and quadruples mutant combinations of *acl5-1* with HD-ZIP III mutants pointed out that, although *acl5-1* is epistatic to *rev-5* respect to stem elongation, rosette size and fertility, HD-ZIP III proteins also take part to developmental regulatory events downstream of *ACL5* as some of the developmental defects of the *acl5-1* mutant are compensated for by loss of HD-ZIP III function. In particular, *HD-ZIP III* genes are required to sustain the formation of extranumerary veins and the *ACL5* autoregulatory negative feedback loop in the *acl5-1* mutant. As *HD-ZIP III* genes expression has been reported to be affected by *ACL5*, a working model is proposed in which progression of procambial cells differentiation is regulated by auxin and HD-ZIP III transcription factor through induction of an enzymatic rheostat, formed by ACL5 and thermospermine, acting back on the expression level of *HD-ZIP III* genes.

INTRODUCTION

Overview of plant development

The form of a multicellular plant organism ultimately depends on the planes of cell division and cell expansion. In fact, as they are encased into a rigid cell wall, plant cells cannot move and their position within an organ is fixed since their origin. Plants are characterised by two main axes that are laid down early during embryogenesis and maintained throughout plant life. These are the apical-basal axis, from the shoot apex to the root, corresponding to the direction of growth and the central-peripheral axis, with a radial symmetry, corresponding to the distribution of the different tissues. A peculiar aspect of plant development is that their growth is indefinite. In fact, organogenesis is not restricted to the embryonic phase of development but continues during the whole plant life due to the presence of groups of undifferentiated, pluripotent cells called meristems that retain the ability to proliferate. New organs are then formed by the differentiation, according to a genetically defined developmental program, of the cells produced by the meristems. A fundamental characteristic of meristems is the ability to coordinate cell proliferation and differentiation in order to promote organ formation while maintaining a stem cell pool. Genetic and molecular analysis in the model plant *Arabidopsis thaliana* have shown that this coordination is ensured by the activity of several transcription factors families and the synthesis and distribution of a continuously increasing number of hormone and hormone-like small mobile signalling molecules (reviewed in Carraro et al., 2006; Wolters and Jurgens, 2009). In addition, being sessile organisms, plants have evolved the ability to integrate the endogenous developmental program with environmental signals to adapt to changes in the environment and survive in stress conditions. Recently accumulated evidences have shown that translation of environmental cues into a growth response also depends on modulation of hormonal and transcriptional responses (reviewed in Wolters and Jurgens, 2009). In addition, extensive functional redundancy among gene family members as well as feedback regulation and crosstalk within and between different signalling pathways has been reported during both developmentally and environmentally regulated growth. Thus, both the plasticity of plant development and its responsiveness to a multitude of environmental situations requires the orchestration of very complex regulatory mechanisms.

The plant vascular system and the control of its differentiation

In plants, embryonic and post-embryonic development are tightly linked to the patterning of the internal vascular systems (for a complete and up-to-date review on the vascular system and the genetic and hormonal factors controlling its development see Jung et al., 2008). The vascular system connects the leaves and all the other aerial parts of the plant with the root and allows the long distance transport of water, mineral salts and organic compounds between organs (Fig. 1). The evolution of a conducting system that provides also mechanical support to the plant body was a necessary adaptation to the life in a terrestrial habitat. The vascular apparatus of higher plants is composed of two types of conducting tissues: xylem, which transport water and mineral salts from the roots to the shoot, and phloem, which transports photoassymilates in the opposite direction. Both xylem and phloem are complex tissues composed of conducting cells, respectively tracheary (vessels) elements and sieve tubes elements, supporting parenchyma cells and reinforcing fiber cells. The conducting elements are arranged in strands of highly specialised, elongated cells, which are connected end to end through particular cell wall structures. Terminal differentiation of tracheary cells requires their complete cellular autolysis while mature sieve elements remain viable despite the extensive cellular modifications such as disappearance of the nucleus and almost all the organelles.

All types of vascular cells differentiate from two meristems, the procambium and the cambium (Fig. 1). The procambium, recognisable as continuous strands of dense, narrow, elongated cells aligned with the longitudinal axis of the organ is responsible for the formation of the vascular system in the primary body of the plant. Procambial cells are differentiated early during embryonic development and, in eudycots, are morphologically distinguishable in the central part of the embryo since globular stage. The vascular cambium is a lateral meristem that ensures continuous production of new conducting tissue during secondary growth of the plant. The origin of the ring of cambial cells, which unlike procambial cells are highly vacuolated, is duplex; in fact, they are differentiated partly by residual procambial cells within mature vascular tissue and partly from interfascicular parenchyma or pericycle cells. Although vascular tissues are usually originated from proliferating procambial and cambial cells according to a highly predictable, genetically controlled pattern, in certain physiological or experimental instances, such as lateral root formation, wounding and tissue culture, other cells can trans-differentiate to form vascular tissue demonstrating a remarkable degree of adaptability.

The positions at which procambial cells differentiate in leaves, stems and roots always foreshadow the sites of the future vascular network. Interestingly, organ-specific and species -

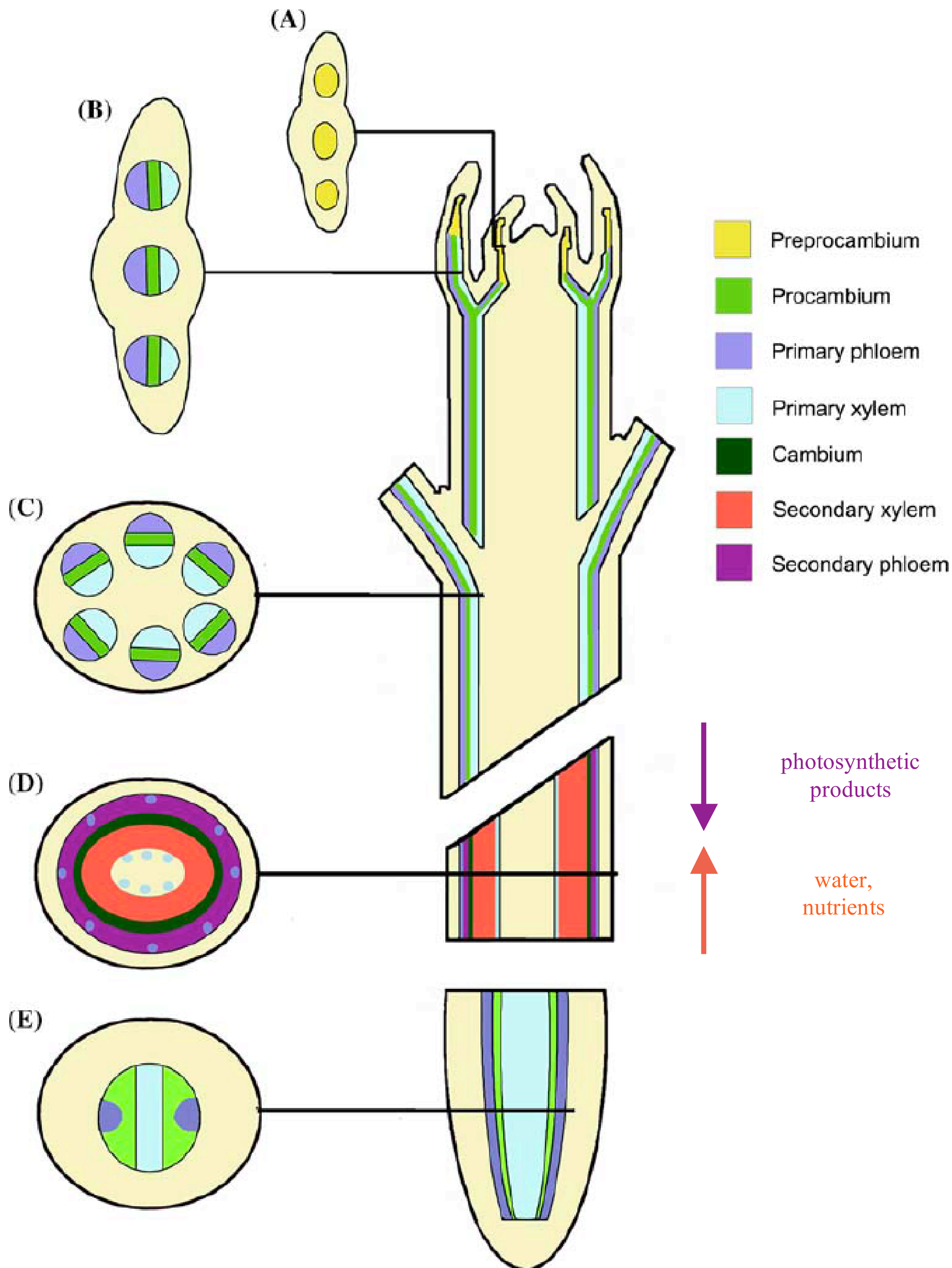


Figure 1. Schematic representation of the organization of the vascular tissues during the primary and secondary growth phases in higher plant (A) Cross section of early developing leaf. Pre-procambial cells precede the vascular development. (B) Cross section of leaf. Within the vascular strands primary xylem and phloem tissues differentiate asymmetrically from the procambium. (C) Primary structure shown in stem cross section. (D) Secondary structure shown in stem cross section. A continuous ring of cambium is differentiated and cambial cells proliferation forms secondary xylem and phloem. (E) Cross section of root tip showing vascular organization during primary development. Later during development root vascular tissues proceed also into the secondary development phase (not shown in figure but similar to D). The direction of flow in the conducting cells is also indicated. (from Dettmer et al., 2009.)

specific cues seem to genetically regulate vascular system development, as remarkably different vascular patterns are found in leaves, stem and roots of dicot and monocot plants. However, how the vascular system is patterned during the development of lateral organs such as leaves, flowers and lateral roots is a fundamental but still largely unanswered question. For example, leaf venation pattern is always consistent with leaf morphology, and abnormal venation pattern or vascular bundles have been detected in lots of leaf morphology mutant but it is not clear yet whether the abnormality in leaf lamina resulted from or in the change of vein pattern. As vascular cells need to be precisely connected in order to carry out their functions, an important point is how continuity with the existing vascular system of stem and root is ensured during the recruitment of meristematic initials and differentiation of procambial strands in developing lateral organs. Patterning of the different cell types within the vascular system is also organ-specific, species-specific and essential for the continuity between the main axes and lateral organs. Remarkably, the arrangement of xylem and phloem within the vascular bundles indicate a close association between the dorso-ventral patterning of lateral organs and the establishment of central versus peripheral identities within the stem (Jung et al., 2008). In particular, in stem and leaves of *Arabidopsis* and most other plants, xylem is localised on the dorsal (adaxial, away from the axis) / central side, while phloem is on the ventral (abaxial, toward the axis) / peripheral side and procambium is located between (Fig. 1). As it will be discussed in more detail later in this chapter, two classes of antagonistic transcription factors have been shown to play a fundamental role for the establishment of this pattern.

As already indicated by the pioneering work of Sachs, recent genetic and molecular studies have clearly shown that the hormone auxin is the major signal involved in the control of several aspects of plant vascular development (reviewed in Jung et al., 2008 and Dettmer et al., 2009). Auxin is necessary and sufficient to trigger vascular differentiation under both normal and experimental conditions, and controls both the initiation of xylem differentiation and the final size of the xylem elements. Moreover, auxin constitutes a positional signal and controls cambial growth rate by regulating the number of dividing cells. The physiological source of auxin for procambial differentiation in the stem is supposed to be the leaf primordium but in experimental condition it can be replaced by exogenous application of auxin or by the auxin released from a severed vascular bundle. One of the main peculiarities of auxin is that, of all the known plant hormones, it is the only one that is directionally transported. Detailed studies have shown that polar auxin transport occurs in a cell-to-cell manner and relies on cell type-specific expression and asymmetric subcellular localisation of the membrane-localized protein carriers for auxin influx and efflux (reviewed in Vanneste and Friml, 2009). Interestingly, available data indicate

that the *PIN* family of auxin efflux transporters is regulated by auxin itself at multiple levels including transcription, turnover, and plasma membrane localization of PIN proteins (reviewed in Vanneste and Friml, 2009). Thus, it seems that feedback regulation between auxin signalling and transport constitutes a self-organizing auxin mediated polarization and patterning mechanism that links individual cell polarity with tissue and organ polarity (reviewed in Vanneste and Friml, 2009). These recent molecular data provides experimental support to the auxin-flow canalization hypothesis proposed by Sachs (1981). This model suggests that auxin induced differentiation leads in some cells to the amplification of auxin transport capacities by positive feedback loop and to establishment of a directional intercellular transport mechanism. This, in turn, promotes auxin transport, leading to the canalization of auxin flow along a narrow column of cells. This continuous polar transport of auxin through cells ultimately results in the differentiation of strands of procambial cells and, subsequently, vascular strands. Indeed, a number of genetic and pharmacological inhibition studies have shown that polar auxin transport from source to sink is required for continuous vascular pattern formation and establishment of procambial strands (Mattsson et al., 2003; Scarpella et al., 2006; Vanneste and Friml, 2009).

Other signals controlling vascular development have only recently started to emerge through biochemical, genetic, and genomic approaches in several model plants (reviewed in Dettmer et al., 2009 and Lehesranta et al., 2009). These signals include classical hormones (brassinosteroids, and cytokinins, in particular), and other small regulatory molecules (such as the proteoglycan-like xylogen and the TFID/CLE41 dodecapeptide) that act non-cell-autonomously and ensure the intercellular communication necessary for coordinated differentiation within the vascular tissue and between the vascular meristem and shoot and root meristems as well as developing organs. Several genes encoding for components of the signal transduction pathways and for transcription factors involved in xylem or phloem identity specification have also been described. Although there is currently little knowledge on what regulates the expression of these genes and on how they interact and regulate each other, the control of vascular development seems to be a highly integrated process with extensive cross-talk between cell types and tissues.

The HD-Zip transcription factors

Homeobox (HB) genes encoding for transcription factors characterised by a 60 amino acids DNA binding domain called homeodomain (HD) are present in all eukariots. A peculiar class of

transcription factors characterised by the presence of a leucine-zipper dimerisation domain immediately at the C-terminal of the homeodomain, hence the name HD-Zip, is present only in plants (Ruberti et al., 1991). These factors interact with DNA differently from classical HD proteins and form a distinct class of regulatory proteins. In fact, *in vitro* binding experiments have shown that HD-Zip proteins bind DNA as dimers and recognize pseudopalindromic sequences (Sessa et al., 1993).

Forty eight HD-Zip genes are present in the genome of the model plant *Arabidopsis thaliana* and they represent nearly 50% of all the HD encoding genes in these organism (Mukherjee et al., 2009). HD-Zip proteins have been grouped in four families, named HD-ZIP I-IV, on the basis of structural homologies in the HD-Zip domain that predict the formation of heterodimeric complexes only between members of each family (Sessa et al., 1994). A greater similarity has been noted between members of the HD-ZIP I and II families (characterised by short genes with low sequence homology outside the HD and the HD-Zip domain in a central position) and members of the HD-ZIP III and IV families (long genes with high homology along the entire coding sequence and the HD-Zip domain at the N-terminal of the protein). Noteworthy, genetic and molecular studies have shown that this classification based on structural features reflects also a functional distinction as each family take part in different developmental processes. In particular, members of the HD-ZIP I and II families, like *ATHB1* and *ATHB2*, are involved in the regulation of plant development in response to environmental stimuli while members of the HD-ZIP III and IV, like *ATHB8* and *ATHB10/GLABRA2,* are necessary for the endogenous developmental programs controlling tissue patterning and cell fate determination (reviewed in Ariel et al., 2007).

The HD-ZIP III family

In *Arabidopsis*, the HD-ZIP III family is composed of 5 genes located on four different chromosomes: *ATHB8, ATHB9/PHAVOLUTA (PHV), ATHB14/ PHABULOSA (PHB)/, ATHB15/CORONA (CNA)*, and *REVOLUTA (REV)/INTERFASCICULAR FIBERLESS1 (IFL1)* (Baima et al., 1995; Talbert et al., 1995; Sessa et al., 1998; Zhong and Ye, 1999; Ratcliffe et al., 2000; McConnell et al., 2001; Green et al., 2005). The homology between these genes is very high (61-85%), extended to the entire coding sequence, and the position of the introns (16-17) is also highly conserved. Phylogenetic analysis indicates that *PHB* and *PHV* are most closely related to each another, sharing 85% amino acid identity (Fig. 2A). Likewise, *ATHB8* and *CNA*

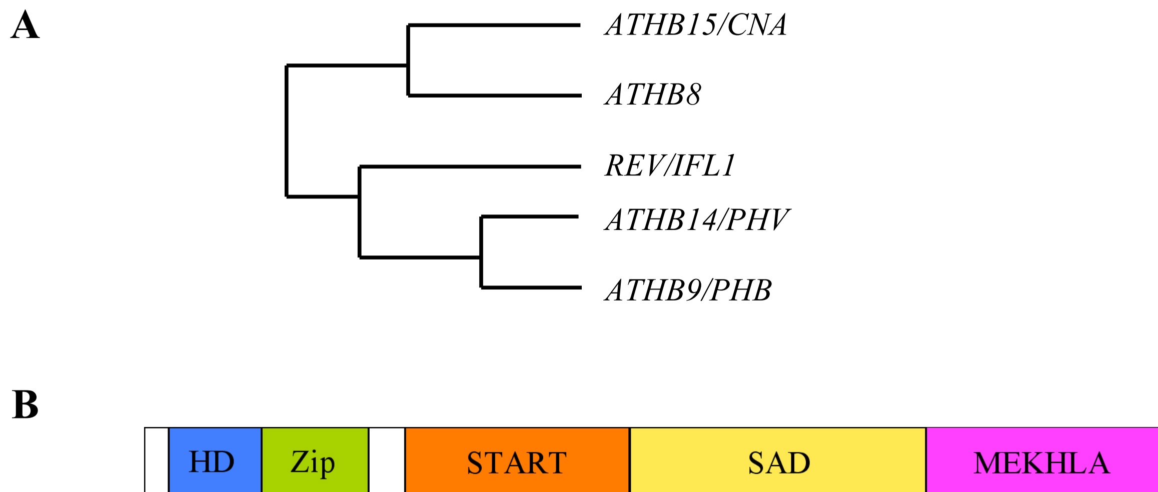


Figure 2. (A) Dendrogram showing the relationship between the five *Arabidopsis HD–ZIP III* genes. (B) Schematic representation of the functional domains of *Arabidopsis HD–ZIP III* proteins.

form a relatively closely related pair, sharing 75% amino acid identity. *REV*, that has not been recently duplicated, is more related to the *PHB* and *PHV* pair but shares between 60% and 66% amino acid identity with all other members of the group.

In vitro DNA binding experiments have shown that the HD-ZIP III domain recognises an 11 bp pseudopalindromic sequence (GTAAT(G/C)ATTAC) and binds to it as a dimer (Sessa et al., 1998).

In addition to the DNA binding domain, the proteins of this gene family, that are composed of 833-852 amino acids, are characterised by the presence of other three conserved regions. These are a 200 residues domain called START (STeroidogenic Acute Regulatory protein-related lipid Transfer) present downstream of the HD-ZIP domain, an adjacent conserved region of 290 residues called SAD (START-adjacent domain) and a 150 residues domain, at the C-terminal of the protein, called MEKHLA (after a goddess of lightning, water and rain) (Fig. 2B). Interestingly, each of these regions can occur separately in other proteins, confirming the notion that each region represents a distinct functional unit. The START domain, that is present also in the closely related HD-ZIP IV family, is found, both in animals and in plants, in proteins involved in lipid transport and in signal transduction and has an hydrophobic pocket that is able to bind several type of lipids such as steroids, phospholipids and carotenoids (Ponting and Aravind, 1999; Tsujishita and Hurley, 2000; Schrick et al., 2004). The SAD region is always found associated to the START domain and is also conserved in HD-ZIP III and IV proteins but

its function is unknown (Mukherjee and Burglin, 2006). The MEKHLA domain has been identified by a bioinformatic analysis only in HD-ZIP III proteins in plants and shares significant similarity with the PAS domain, found in many proteins throughout all kingdoms of life and considered an intracellular sensor of light, oxygen and redox potential (Mukherjee and Burglin, 2006). Recently it has been demonstrated that the MEHKLA domain is necessary and sufficient to mediate the dimerisation of HD-ZIP III proteins with two members of the AP2/ERF transcription factor family, *DORNROSCHEN* (*DRN*) and *DORNROSCHEN-LIKE* (*DRNL*), involved in embryo development in *Arabidopsis* (Chandler et al*.,* 2007). Finally, the redox potential of the cell seems to affect HD-ZIP III DNA binding affinity and specifity also through the effect on two conserved cysteine residues in the homeodomain (Comelli and Gonzalez, 2007).

Genetic and functional analysis of the HD-ZIP III family

Loss-of function mutants

The isolation and characterization of several mutants corresponding to HD-ZIP III family members and the study of multiple mutants have shown that these genes have partially overlapping but also distinct and in some cases antagonistic function during *Arabidopsis* development (Prigge et al., 2005). *REV*/*IFL1* is the only HD-ZIP III family member that has not been recently duplicated and whose loss-of-function (recessive) mutation causes phenotypic alterations. Unlikely, the role on plant growth of the strictly related gene pairs, *PHB-PHV* and *ATHB8-CNA,* is masked by their functional redundancy and has been characterised only through gain-of-function (dominant) alleles or overexpression (Byrne, 2006 and references therein). Mutations in the *REV/IFL1* gene affect the proliferation of the apical, axillary and vascular meristems and impair interfascicular fibers differentiation, vascular development and auxin polar transport (Talbert et al., 1995; Zhong et al., 1997; Zhong and Ye, 1999; Otsuga et al., 2001). *REV* mutants exhibit a diverse range of phenotypes and are characterised by elongated and downward curled bracts (hence the name), expanded asymmetric leaves, longer and weaker stems lacking the interfascicular fibers and part of the xylematic cells in the vascular bundles, reduced number of lateral inflorescences due to a failure in initiation of secondary meristems (empty axils), and irregular formation of flower organs leading to partial sterility (Talbert et al., 1995) (Fig. 3A-H, R). Loss-of-function mutations in *PHB* and *PHV* genes, that either as single mutants or together in a double mutant, have no evident phenotypic consequences, enhance *rev*

mutant phenotype, indicating that the function of these two genes is redundant and overlaps with that of *REV* both during embryogenesis and post-embryonic growth (Prigge et al., 2005) (Fig. 3I-P). Although *PHB* and *PHV* act synergistically with *REV* in the regulation of organ polarity, where they are necessary to specify adaxial (dorsal) identity, they have a role distinct from *REV* but coincident with *CNA* in the regulation of apical meristem size. In fact, the shoot apical meristem (SAM) phenotype of the *phb phv cna* triple mutant is not enhanced by the addition of the *rev* mutation (Prigge et al., 2005). Conversely, loss of *ATHB8* and *CNA*, that also have no gross phenotypic effect alone or in combination, antagonise loss of *REV* gene function in the inflorescence as demonstrated by the partial suppression of the axillary and floral meristem defects of the *rev* single mutant in the *rev cna athb8* triple mutant (Prigge et al,2005)(Fig.3M-P).

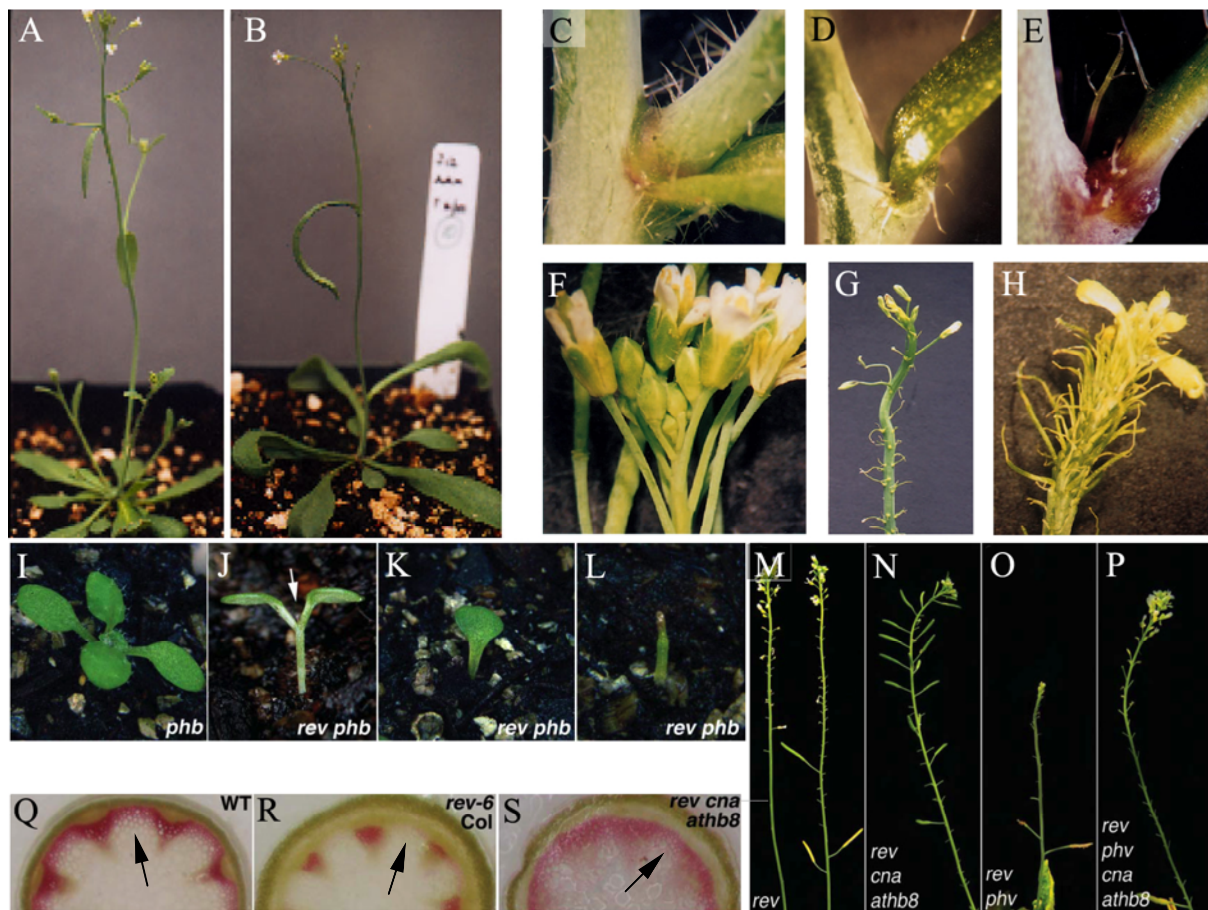
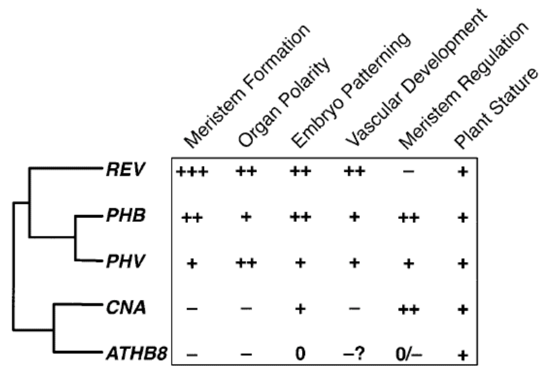


Figure 3. Phenotype of wild-type (A, C, F) and *rev-1* (B, D, E, G, H) plants. (C-E) A detail of cauline leaf axil. Note that in (E) the axil is empty and in (F) a filamentous structure is formed in place of a lateral inflorescence. (F-H) Inflorescence. Filamentous structures are formed in place of flowers in rev-1 (G, H). (from Talbert et al., 1995). (I) Wild-type seedling. (J-L) Phenotypes of *rev-6 phb-13* seedlings. Most *rev phb* double mutants produce two cotyledons (J), but others produce a single cotyledon (K) or a radially symmetric organ (L). Arrow in (J) indicates the radial symmetric structure emerging from apical region. (M-P) Inflorescences of (M) *rev-6*, (N) *rev-6 cna-2 athb8-11*. (O) *rev-6 phv-11*, (P) *rev-6 phv-11 cna-2 athb8-11* plants. (Q-S) Cross sections of inflorescence stems from 1 cm above the rosettes stained with phloroglucinol (red) to detect lignified tissues. (Q) Wild type (*er-2*). (R) *rev-6* (Col). (S) *rev-6 cna-2 athb8-11*. Arrow in (R) indicate the interfascicular region. (from Prigge et al., 2005).

An antagonistic function of *REV*, *CNA* and *ATHB8* has been observed also in the differentiation of the vascular system. In fact, the vascular defects of the *rev* mutant are partially suppressed in the *rev cna athb8* triple mutant (Prigge et al., 2005) (Fig. 3Q-S). Finally, the phenotype of quadruple *phb phv cna athb8* and quintuple rev/+ *phb phv cna athb8* mutants indicates that each of the HD-ZIP III family members redundantly regulates plant stature (Prigge et al., 2005). Very recently, a detailed expression analysis of early markers of leaf vein differentiation has uncovered a subtle phenotypic alteration in the *athb8-11* loss-of-function mutant and has confirmed the specific involvement of *ATHB8* in vascular development (Donner et al., 2009). In fact, this analysis has pointed out that *ATHB8* is required to stabilize pre-procambial cell identity specification against auxin transport perturbations, to restrict pre-procambial cell state acquisition to narrow zones and to synchronize procambial cell identity assignment within and between veins (Donner et al., 2009). Interestingly, loss of ATBH8 function has only a transient effect and mature leaves of null mutants display no obvious alterations in vein patterns unless auxin flow is genetically or pharmacologically perturbed (Baima et al., 2001; Prigge et al., 2005; Donner et al., 2009). In conclusion, analysis of loss-of-function alleles of HD-ZIP III family members has revealed roles for these genes in embryo patterning, meristem initiation, organ polarity, meristem regulation, and vascular development, with different subsets of genes being involved in each process. A schematic summary of the functional relations of the *HD-ZIP III* genes in the different plant developmental processes is shown in Fig. 4A (Prigge et al., 2005).

Although the differential expression patterns can account for the functional differences and genetic interactions of *HD-ZIP III* genes, these are likely due also to differences in protein function. In fact, cross-complementation of the *rev* mutant with the cDNA corresponding to each of the five *HD-ZIP III* genes under the control of the *REV* promoter has shown that, despite the high homology, the protein-coding sequences have evolved distinct functions and are not fully interchangeable (Fig. 4B). Therefore, the complex interactions shown by *HD-ZIP III* genes could be due to the production of homo and heterodimers, with different activities depending on the protein(s) involved in the dimers as has been suggested by Prigge and Clark (2006). Ultimately, the phenotype of specific mutants would result from the relative level of each HD-ZIP III product and the types of dimers formed in the different tissues.

A



B

Figure 4. *HD-ZIP III* genes play overlapping, antagonistic, and distinct roles in development and HD-ZIP III proteins are not fully interchangeable. (A) Diagram showing the functional relationships of *Arabidopsis HD-ZIP III* genes. The (+) symbols indicate the relative strength of defects seen in single and multiply mutant lines lacking the genes. The (-) symbol indicates that the gene antagonises the roles of other genes with respect to this function. A (0) indicates that no role was observed. Meristem Formation, development of lateral shoot meristems and floral meristems; Organ Polarity, specification of adaxial polarity during leaf development; Embryo Patterning, patterning the apical end of the embryo; Vascular Development, formation of lignified cells in inflorescence stems; Meristem Regulation, control of shoot apical meristem and floral meristem size; Plant Stature, role in growth to normal size. (B) The ability of each *HD-ZIP III* gene to complement the *rev* inflorescence phenotype was assessed by expression of the corresponding cDNA under the control of the *REV* promoter (P_{REV}) in the *rev-6* mutant background. The genotype or the transgene introduced is indicated below each image. *er-2*, wild-type. *rev-6* C1, untransformed mutant. (from Prigge et al., 2005).

Gain-of-function mutants

In contrast with recessive mutations that, apart for the *REV* gene, lack discernable phenotypes, dominant mutations in HD-ZIP III genes profoundly affect plant development (Byrne, 2006 and references therein). Dominant mutations in *PHB* and *PHV* (*phb-1d* and *phv-1d*) have revealed a role for these genes in the establishment of organ polarity (McConnell et al., 2001). Leaf morphology is strictly connected to its highly specialised function as photosynthetic organ. In *Arabidopsis*, as in most eudicots, leaves present a dorsoventral polarity with the dorsal (adaxial) side facing the light source specialised for the light capture and the ventral (abaxial) side opposite to it specialised for the gas exchange. Interestingly, polar identity within the leaf is established very early during leaf primordium development and is strictly correlated to its position respect to the shoot apical meristem (SAM), with the side towards the meristem becoming the adaxial part of the lateral organ and the side distal from the meristem becoming

the abaxial part. As a result, leaf polarity reflects the central/peripheral differentiation of the main plant body axis. This correspondence is maintained also in vascular bundles organisation. In fact, the xylem is adaxial in the leaf and central in the stem while the phloem is abaxial in the leaf and peripheral in the stem (Fig. 5). Classic surgical experiments have shown that primordium polarisation depends on the interpretation of a morphogenetic gradient emanating from the SAM (reviewed in Golz, 2006). In the absence of the shoot–derived signal, the leaf fails to differentiate adaxial and abaxial surfaces and develop into a radialised structure that do not produce blade. In radialised leaves, the arrangement of the vascular tissue is also affected, and amphivasal or amphicribral vascular bundles, characterised by xylem surrounding the phloem and *viceversa*, are formed in place of the normal collateral vascular strands. Thus, it has been proposed that the juxtaposition of adaxial and abaxial domains is required for lamina outgrowth (Waites and Hudson, 1995) (Fig. 5). The dominant *phb-1d* and *phv-1d* mutants form adaxialised trumpet-like or rod-like radial leaves surrounded by ectopic axillary meristems (Fig. 6A-D) (McConnell and Barton, 1998; McConnell et al., 2001). Instead of the normal collateral arrangement, the vascular strands within severely affected leaves also show a radial arrangement of the conducting elements with the xylem surrounding the phloem. A similar arrangement of the vascular strands is observed also in stems of the dominant mutant alleles of *REV*, *rev-10d* and *amphivasal vascular bundles1* (*avb1*), although these mutations do not noticeably affect dorsoventral patterning of leaves (see Fig. 8D) (Zhong et al., 1999; Emery et al., 2003; Zhong and Ye, 2004). Therefore, *HD-ZIP III* genes are determinants of adaxial/central identity.

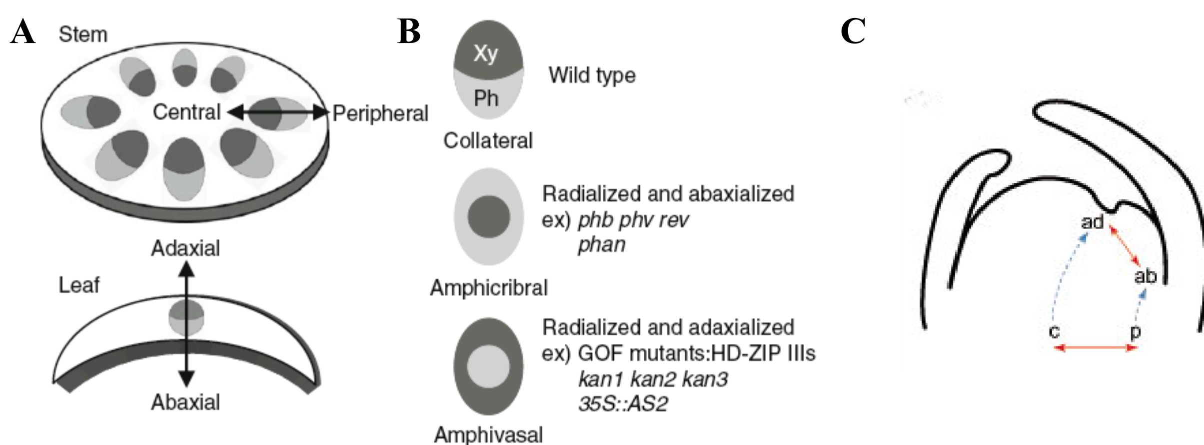


Figure 5. (A) Radial pattern of vascular tissues and adaxial-abaxial identities in stem and leaf of *Arabidopsis* plants (transverse sections). (B) Organization of vascular tissues in wild-type *Arabidopsis* plants and in mutants affecting organ polarity. (from Jung et al., 2008). (C) Correspondence between the central (c) - peripheral (p) polarity of the main axis and the adaxial (ad) - abaxial (ab) polarity of lateral organs formed by the apical meristem.

A number of genetic studies have clearly shown that, at molecular level, the antagonistic interaction of *HD-ZIP III* genes with *KANADI* (*KAN*) genes encoding another transcription factors family necessary to define the abaxial/peripheral fate, plays a central role in organ polarisation (reviewed by Floyd and Bowman, 2007; Jung et al., 2008). Noteworthy, *KAN* and *HD-ZIP III* gene families are expressed in two complementary, mutually exclusive domains in leaf, root and embryo and several observations indicate that *HD-ZIP III* genes regulate the expression domains of *KAN* genes and these, in turn, regulate the expression domain of HD-ZIP III genes (reviewed by Floyd and Bowman, 2007; Jung et al., 2008). Interestingly, the analysis of embryos in which most members of both gene families are mutated has suggested that the antagonistic functions of HD-ZIP III and KANADI transcription factors are linked to the control of auxin flow and perception (Izhaki and Bowman, 2007). In particular, it has been proposed that *KAN* genes could pattern tissues within the plant by regulating auxin flow while *HD-ZIP III* genes may modulate the response of cells to auxin maxima, promoting meristem fate and preventing leaf initiation in response to auxin in the central part of the embryo and the SAM. Interestingly, the defined spatial and temporal expression patterns of members of these gene families, in turn, may be influenced by auxin, suggesting a complex interplay between these hormonal and genetic factors.

Unlike the *KAN* genes, *HD-ZIP III* genes are expressed also in the apical meristem and, in addition to their role in adaxial/central polarity establishment, clearly play a role in meristem function and stem cell proliferation. In fact, gain of HD-ZIP III function determine the development of ectopic axillary meristems (McConnell and Barton, 1998) and promote vascular meristem proliferation (Baima et al., 2001) while in *rev-1* loss-of-function allele axillary meristems are missing (Talbert et al., 1995; Otsuga et al., 2001) and in *rev phb* double mutant even the embryonic apical meristem fails to develop (Prigge et al. 2005). In particular, the genetic analysis of the dominant negative *cna-1* mutant, causing a subtle enlargement of the shoot apical meristem that is dramatically enhanced in a *clavata1* (*clv1*) background, have shown that *CNA* is required to maintain the delicate balance between stem cell specification and differentiation at the shoot meristem and functions in parallel to the *CLV* loci to promote organ formation (Green et al., 2005) (Fig. 6E, F).

The gain-of-function *incurvata4-1* (*icu4-1*) allele of *CNA* displays incurvated rosette leaves (with upwards curled lamina) and increased production of vascular tissues in the stem, supporting a role for *CNA* in promoting adaxialisation and vascular development (Ochando et al., 2006, 2008) (Fig. 6G-J). Interestingly, although the *icu4-1* allele of *CNA* carries the same point mutation of several semidominant alleles of *PHB* and *PHV* that affects the microRNA

complementary site (see page 17), and increases the transcript level of the corresponding genes, it does not induce the formation of radially symmetric leaves and does not affect *per se* the normal collateral polarity of the vascular bundles (Ochando et al., 2006, 2008).

Despite the absence of characterised gain-of-function alleles of *ATHB8*, an effect on plant development similar to that of the dominant *icu4-1* allele of *CNA* has been demonstrated for the overexpression of *ATHB8* in *35S::ATHB8* transgenic plants (Baima et al., 2001) (Fig. 6K-N). Besides the up-curled and hyponastic leaves, *35S::ATHB8* transgenic plants exhibit a short inflorescence stem, and a detailed histological analysis of this organ at various developmental stages has shown that *ATHB8* overexpression promotes vascular meristems differentiation increasing and accelerating procambial and cambial cells activity resulting in xylem overproduction (Baima et al., 2001).

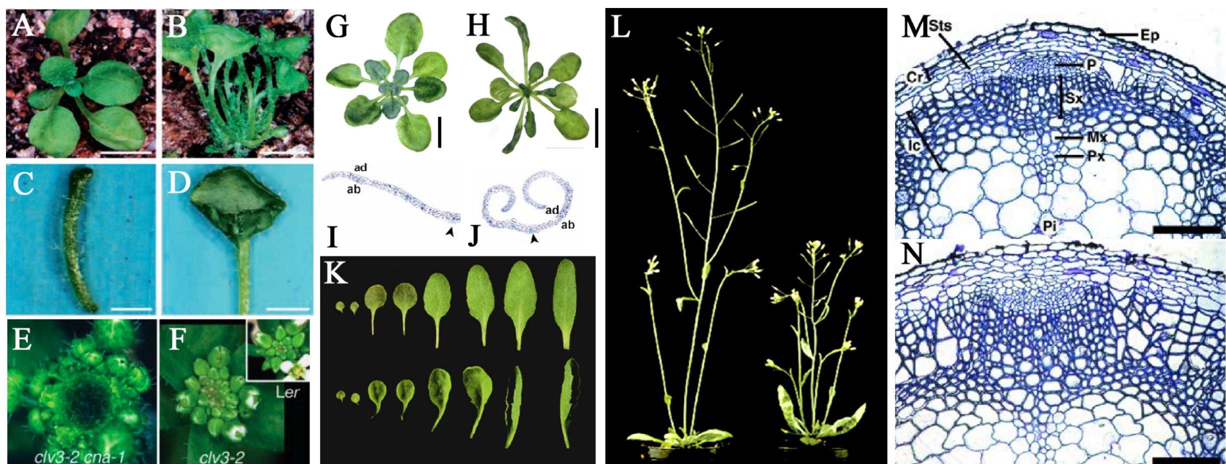


Figure 6. Phenotype of wild-type (A) and *phb-1* (B-D) plants. Radialised rod-like (C) and trumpet-like (D) leaves are formed by *phb-1* (from McConnel and Barton, 1998). Phenotype of the apical inflorescence meristem of *clv3-2 cna-1* (E) and *cna-1* (F) mutants. The wild-type (L*er*) is shown in the inset (from Green et al., 2005). Phenotype of wild-type (G) and *icu4-1* (H) plants. Scale bar: 5 mm. Transverse sections of wild-type (I) and *icu4-1* (J) leaves. Arrowheads indicate the position of the midvein. Ad, adaxial. Ab, abaxial. (from Serrano-Cartagena et al., 2000) (K) Comparison of wild-type (upper) and *35S:ATHB8* (lower) leaves. (L) Phenotype of wild-type (left) and *35S:ATHB8* (right) plants. Transverse sections of the inflorescence stem of wild-type (M) and *35S:ATHB8* (N) plants in secondary structure showing the vascular anatomy (from Baima et al., 2001). Cr, cortex. Ep, epidermis. Ic, interfascicular cells. Mx, metaxylem. P, phloem. Pi, pith. Px, protoxylem. Sx, secondary xylem. Sts, starch sheet. Scale bar: 100 μm.

Regulation of the HD-ZIP III family

Considering their involvement in many fundamental aspects of plant development, it is perhaps not surprising that an activity-buffering system has evolved for regulating seed-plant *HD-ZIP III* genes function. In fact, many experimental data clearly indicate that HD-ZIP III transcription factors are subjected to multiple levels of regulation to ensure a fine spatial, temporal and quantitative tuning of their expression and activity. Evidences for the mechanisms acting at the different levels are presented in the next sections.

Transcriptional regulation

HD-ZIP III genes are expressed since the earliest stages during both embryogenesis and cell differentiation in the adult plant. In agreement with their differential contributions to plant development, the expression patterns of *HD-ZIP III* genes are similar but not perfectly overlapping and differ in timing, spatial extension and relative intensity (Fig. 7) (Baima et al., 1995; McConnel et al., 2001; Otsuga et al., 2001; Prigge et al., 2005). In particular, *ATHB8*, unlike all the other members of the family, that are expressed in the apical meristem (both in the vegetative and the reproductive phase), in the adaxial domain of lateral organs and in developing vascular strands, is expressed exclusively in the latter tissue and is considered one of the earliest markers of vascular differentiation (Baima et al., 1995; Dengler and Kang, 2001; Scarpella et al., 2004; Donner et al., 2009).

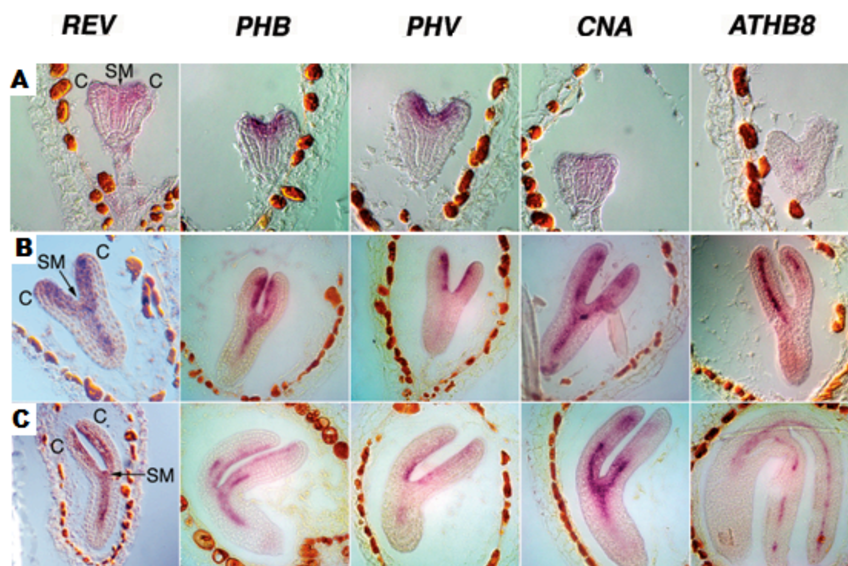


Figure 7. The expression pattern of each *HD-ZIP III* gene in wild-type embryos was determined by *in situ* hybridization. A) Heart stage, B) torpedo stage, C) bent cotyledon stage. The gene hybridized is indicated at the top of each column of panels. Gene expression is indicated by the indigo staining while the dark orange-brown tissue is the seed coat. SM, shoot meristem; C, cotyledon. (from Prigge et al., 2005)

In addition, *ATHB8* is the only gene of the family whose expression is positively regulated by auxin, the main hormonal factor controlling vascular differentiation (Baima et al., 1995). Very recently, a deletion analysis of the *ATHB8* promoter have shown that a 13 bp sequence corresponding to an auxin response element (ARE) is essential for both the vascular specific expression pattern and the auxin inducibility of the gene (Donner et al., 2009). Moreover, it has been demonstrated that the auxin response factor (ARF) MONOPTEROS (MP/ARF5), necessary for the establishment of the apical-basal polarity and for the differentiation of the vascular system, directly binds to this sequence and positively regulates *ATHB8* expression (Donner et al., 2009). Cellular studies with *Zinnia elegans* xylogenic cultures and molecular and genetic studies in *Arabidopsis* have indicated that *HD-ZIP III* genes expression is enhanced by brassinosteroids, another class of hormones that promote xylem formation. In turn, *HD-ZIP III* genes positively regulates brassinosteroid signalling (reviewed by Jung et al., 2008 and Dettmer et al,. 2009).

miRNA mediated post transcriptional regulation

HD-ZIP III genes expression is regulated at post-transcriptional level by microRNA (miRNA). These are small non-coding RNA, 21-25 nucleotides long, transcribed from genes (*MIR* genes) different from their targets, that regulate at post-transcriptional level the expression of genes which present sequence complementarities with them (Voinnet, 2009). In plants, the interaction with the miRNA determines the cleavage of the target mRNA and its degradation but, recently, miRNAs have been shown to be involved in translational repression also in plants (Voinnet., 2009). The perfect complementarity of plant miRNAs to their target mRNAs has allowed the bioinformatic prediction of miRNA-regulated genes in *Arabidopsis* (Zhang et al., 2006). Interestingly, many of the putative miRNA target genes identified encode for members of transcription factors families controlling several aspects of plant development. Among these there are the *HD-ZIP III* family genes. In fact, miR165, perfectly complementary to *PHB*, *PHV*, *REV* and *ATHB8*, and miR166, perfectly complementary to *CNA*, encoded by two and seven *MIR* genes, respectively, have been among the first plant miRNA isolated and experimentally validated (Rhoades et al., 2002; Reinhart et al., 2002). Intriguingly, most dominant mutations in *HD-ZIP III* genes map to a short region at the junction between the fourth and the fifth exons (Fig. 8A). The discovery that this region is complementary to miR165/166 has suggested that the effect of these mutations is due to an alteration in the post-transcriptional regulation of *HD-ZIP III* genes expression and not to a change in the START domain as originally proposed

(McConnell et al. 2001). This hypothesis has been confirmed by *in vitro* experiments showing that miR165/166 induce the degradation of wild type *PHB* and *PHV* transcripts, while the mutant *phv-1d* transcript is resistant to miRNA mediated cleavage (Tang et al., 2003). Moreover, transgenic plants expressing a *REV* or *PHB* miRNA resistant variant (*REV-δmiRNA* and *PHB-δmiRNA*) in which two nucleotides in the miRNA complementary region have been changed without affecting the encoded amino acids, show the same phenotype of the corresponding dominant mutations (Emery et al., 2003; Mallory et al., 2004) (Fig. 8B-E). Finally, the study of miR165/166 overexpression (Zhou et al., 2007; Jung and Park, 2007) and the identification of two activation tagging mutants, *meristem enlarged1* (*men1*) and *jabba-1D* (*jab-1D*) (Kim et al., 2005; Williams et al., 2005), overexpressing two of the nine *MIR* genes encoding these miRNAs, all characterised by a phenotype opposite to that of *phb-1d* and *phv-1d* as well as by a reduction of *HD-ZIP III* family transcripts levels, support the hypothesis that miRNA negatively regulate *HD-ZIP III* expression and that the effect is dependent on the relative amounts of miRNA and target mRNA (Engstrom et al, 2004; Zhou et al., 2007; Jung and Park, 2007).

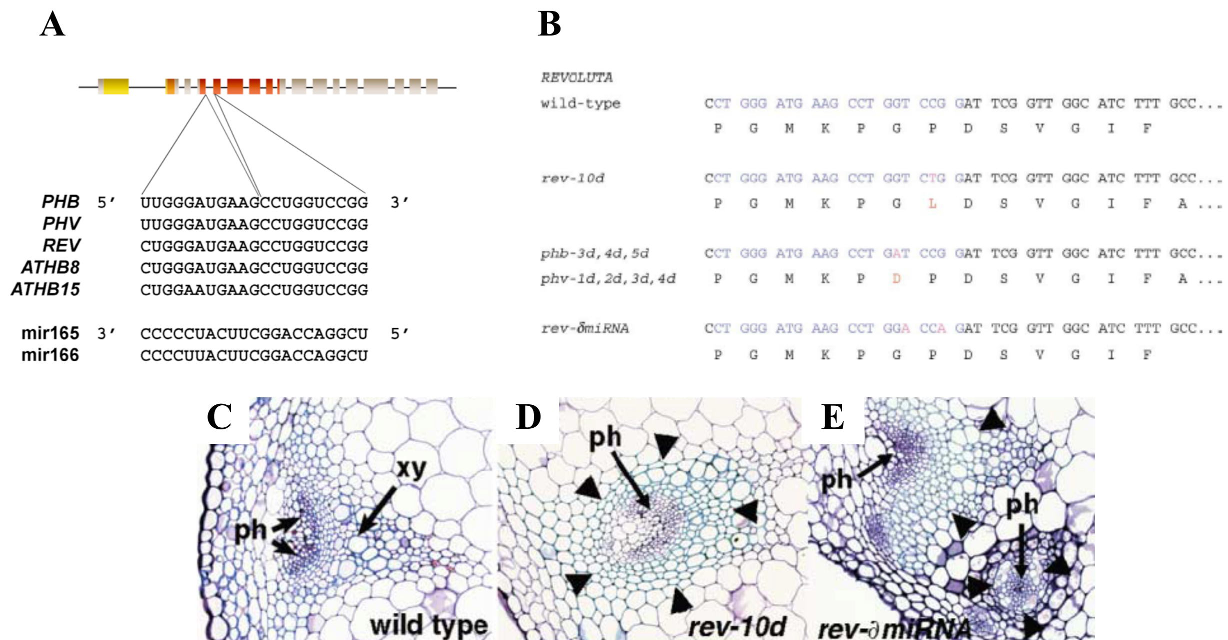


Figure 8. (A) The sequence of the miRNA binding site in the five *HD-ZIP III* genes and of miR165 and miR166 is indicated below the schematic representation of an HD-ZIP III gene. Exons are represented as boxes and introns as lines. Yellow, HD domain. Orange, Zip domain. Red, START domain. (from Byrne, 2006) (B) Nucleotide and amino acid sequences spanning the miR165/166 binding site in *REV* alleles. Blue nucleotides denote those complementary to miR165, pink nucleotides represent those altered in *rev* (or *phb*/*phv*) mutant alleles, red amino acids represent those altered in *phb*, *phv*, and *rev* mutant alleles. (from Emery et al., 2003) (C-E) Phenotypic alterations of vascular patterning in *rev-10d* and *rev-δmiRNA* plants (C) In wild-type stem vascular bundles have xylem (xy) located centrally and phloem (ph) strands located peripherally. (D) In *rev-10d* stems, vascular bundles are often radialized and amphivasal, with xylem tissue (arrowheads) surrounding phloem tissue (ph). (E) The stem vascular bundles of *rev-δmiRNA* plants resemble those of *rev-10d* plants. (from Emery et al., 2003).

Besides the quantitative effect, miR165/166 also affect the spatial regulation of *HD-ZIP III* genes expression. In fact, *in situ* hybridisation experiments have shown that *HD-ZIP III* genes and miR165/166 precursors are expressed in complementary domains in leaf primordia, and that *PHB* is ectopically expressed in the abassial domain when its transcript cannot be cleaved as in dominant *phb* mutants or in mutants impaired in miRNA function (Kidner and Martienssen, 2004; Juarez et al. 2004). In addition, a comparison of GFP localization in transgenic plants expressing the GFP protein under the control of the *HD-ZIP III* promoters or as a translational fusion with HD-ZIP III proteins under the control of their own promoters have shown that, in root, HD-ZIP III proteins are expressed in a domain that is smaller than the one in which the promoter is active (Lee et al., 2006). These observations suggest that *HD-ZIP III* transcripts are specifically wiped out in certain cell types by the action of miR165/166.

Taken together these data support the hypothesis that the phenotype of the dominant mutants is not due to constitutive activation of the encoded protein but is caused by ectopic and increased expression of the corresponding genes owing to impairment of mRNA degradation.

The miR165/166 complementary sequence has been implicated also in the transcriptional regulation of *PHB* and *PHV* genes (Bao et al., 2004). In fact, it has been reported that, in differentiated cells, these loci are heavily methylated downstream of the miRNA complementary region and that this methylation is reduced in *phb-1d* and *phv-1d* dominant mutants. This observation has suggested that miR165/166 could induce, by an unknown mechanism linked to transcription, chromatin methylation and transcriptional gene silencing of these genes.

Translational regulation

Two recent studies suggest that *HD-ZIP III* genes could be regulated also at translational level (Pinon et al*.,* 2008; Yao et al., 2008). In fact, mutations in the three *PIGGYBACK1* (*PGY1*)*, PGY2* and *PGY3* genes and in *ASYMMETRIC LEAVES 1/2 ENHANCER5* (*AE5)* and *AE6* genes, coding for the ribosomal proteins L10aB, L9C, L5A, L28A and L5A respectively (*PGY3* and *AE6* are alleles), and isolated as *enhancers* of *asymmetric leaves1* and *2* (*as1* e *as2*), cause defects in the establishment of polarity in the leaf lamina and vascular tissue, a developmental process controlled by *HD-ZIP III* genes. In particular, genetic analysis has shown that *PGY1* promotes leaf adaxial fate (or repress abaxial fate), independently of *AS1,* partecipating in the same regulatory circuit as *HD-ZIP III* genes and their antagonist *KANADI* genes. Mutations in *HD-ZIP III* genes enhance the phenotype of *ae5-1* and *as1 pgy1* double mutants by inducing the formation of radial abaxialised leaves while *pgy1* mutation enhances the *rev* phenotype in a manner similar to the combination of *rev* with *phv* and *phb* (Pinon et al., 2008) (Fig. 9).

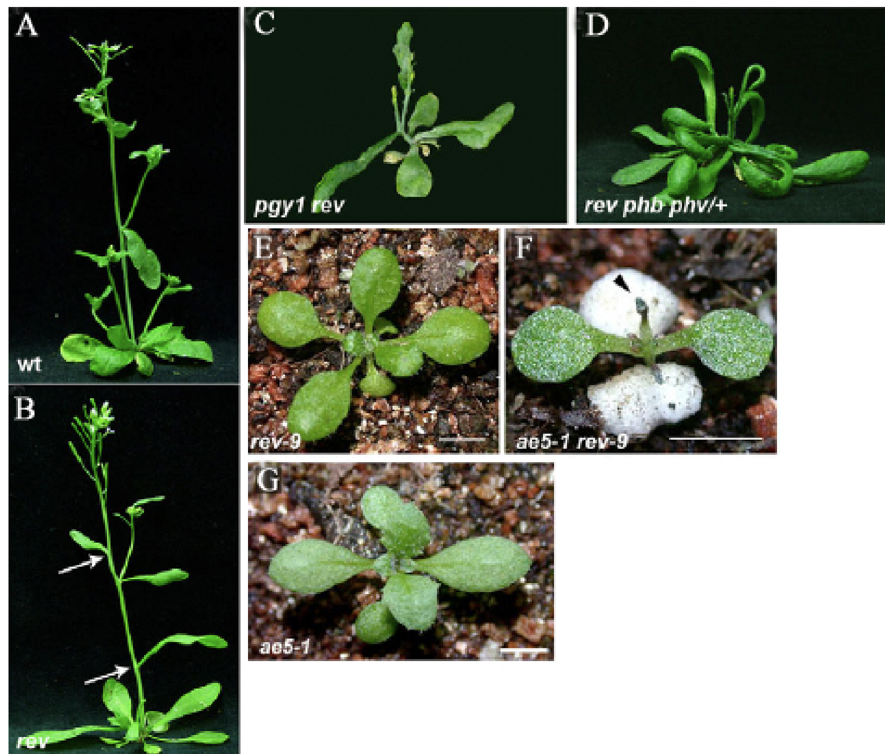


Figure 9. (A) Wild-type flowering plant. (B) *rev* mutants lack axillary meristems at the base of some cauline leaves (arrows). (C) *pgy1 rev* plant with reduced stature compared with wild type and *rev*. Inflorescence forms few or no flowers. (D) *rev phb phv*/+ plant with reduced stature and inflorescence with few flowers. (from Pinon et al., 2008). (E) *rev-9* seedling. (F) Severe *ae5-1 rev-9* seedling with only expanded cotyledons. An arrowhead indicates a needle-like leaf. (G) *ae5-1* seedling. (from Yao et al., 2008).

Although genetic data indicate that *HD-ZIP III* genes could be downstream targets of *PGY1*, *REV*, *PHB* and *PHV* transcript levels are not significantly altered in the *as1 pgy1* double mutant and this suggests that *PGY1* could regulate *HD-ZIP III* genes at translational level (Pinon et al., 2008). Although it cannot be ruled out that the proteins encoded by the *PGY* genes have also a ribosome-independent function as reported for other ribosomal proteins, a more likely hypothesis is that they contribute to the formation of specific ribosomes controlling key events in plant development (Byrne, 2009). It has been suggested that the sensitivity of some transcripts to ribosome heterogeneity could be due to intrinsic characteristics of the mRNAs such as the presence of small upstream ORFs in the 5' leader sequence or targeting by microRNAs (Byrne, 2009). Intriguingly, *HD-ZIP III* genes transcripts possess both these two characterisctics.

Post-translational regulation

Another mechanism regulating *HD-ZIP III* genes at post-translational level has been uncovered by the identification of a family of four *LITTLE ZIPPER* genes (*ZPR1-4*) encoding small proteins characterised by the presence of a leucine-zipper motif similar to that of HD-ZIP III proteins. *ZPR* genes have been identified as direct targets of *REV*. In fact, microarray analysis have shown that their expression increases, also in the presence of cycloheximide, after dexametasone induction in transgenic *35S::GR:REVd* plants expressing an inducible version of *REV* mutagenised in the region complementary to the miR165/166 (Wenkel et al., 2007). In agreement with the microarray result, the *ZPR3* transcript level is reduced in the triple HD-ZIP III loss-of-function mutant *phb phv rev* while it is increased in the *phb-1d* and *phv-1d* mutant resistant to the miRNA mediated degradation (Wenkel et al., 2007). *ZPR* genes have been identified also by the functional characterisation of a dominat mutant expressing high levels of *ZPR3* (*zpr3-1d;* Kim et al., 2008). Plants overexpressing *ZPR3* are characterised by leaf polarity defects opposite to those observed in mutants with an increased activity of *HD-ZIP III* genes (Wenkel et al*.,* 2007; Kim et al., 2008) (Fig. 10). In addition, *ZPR3* overexpression in the *zpr3-1d* mutant partially suppresses the phenotypic alterations determined by HD-ZIP III overexpression in the *phb-1d* dominant mutant and in *35S::REV* transgenic plants (Kim et al. 2008). Loss of *ZPR2* and *ZPR3* function have only subtle effects on plant growth while a more severe phenotype is observed in the double *zpr2 zpr3* mutant suggesting a functional redundancy within this gene family (Wenkel et al*.,* 2007; Kim et al., 2008).

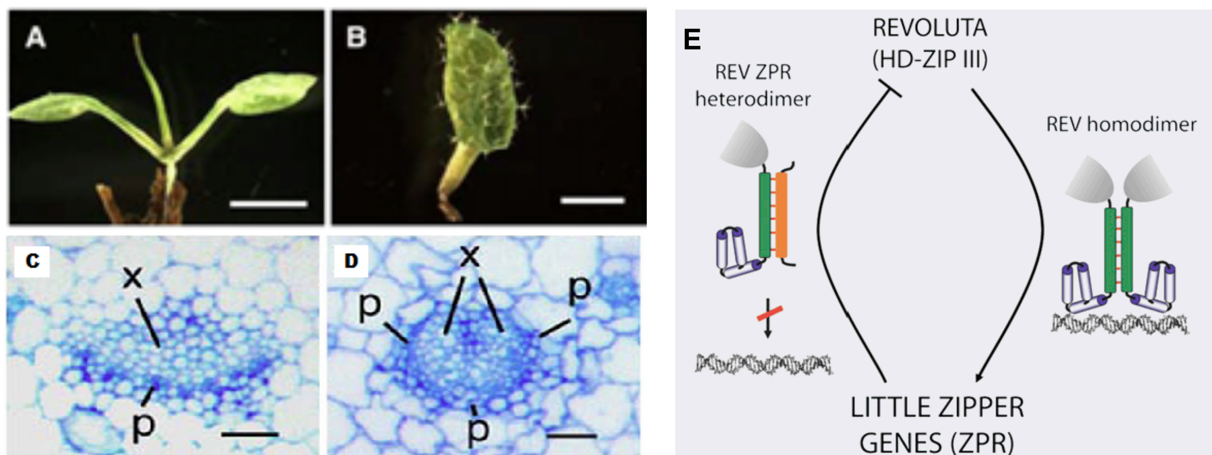


Figure 10. (A) ZPR3-overexpressing plant showing meristem termination phenotype. (B) Abaxialized trumpet-shaped leaf from ZPR3-overexpressing plant. (C) Section through wild-type petiole. Note that xylem is located adaxially, above the phloem. (D) Section through petiole from ZPR3-overexpressing plant. Xylem is nearly surrounded by phloem. x, xylem; p, phloem (E) Model for a negative feedback loop between HD-ZIP III proteins and ZPR proteins. Active HD-ZIP III dimers induce the transcription of ZPR genes. The ZPR proteins dimerise with the HD-ZIP III proteins, preventing them from forming homodimers and thus inactivating them. (from Wenkel et al., 2007).

Protein-protein interaction analysis *in vitro* and in yeast have shown that HD-ZIP III proteins can heterodimerise with ZPR proteins through the leucine zipper domain but that this interaction abolish their DNA binding abillity (Wenkel et al., 2007; Kim et al., 2008). Based on structural features of ZPR proteins and functional relationship with *HD-ZIP III* genes, it has been proposed that these two families participate in a negative feedback regulatory circuit (Fig. 10). In this model, the HD-ZIP III transcription factors activate the transcription of *ZPR* genes whose products, in turn, inhibit HD-ZIP III activity by forming non-functional heterodimers (Wenkel et al., 2007).

Post-translational regulation of HD-ZIP III activity could be also mediated by the START domain. In analogy with steroid hormones nuclear receptors in animals, it has been proposed that HD-ZIP III activity could be regulated through binding of a hydrophobic ligand to the START domain. Alternatively, the interaction of the START domain with membrane phospholipids could inhibit HD-ZIP III protein activity by sequestering them into the cytoplasm (Ponting and Aravind, 1999; Tsujishita and Hurley, 2000). A third hypothesis suggests that binding of the ligand to the START domain could induce a conformational change affecting the affinity for the dimerisation partner, therefore favouring the formation of HD-ZIP III/HD-ZIP III dimers respect to the formation of HD-ZIP III/ZPR heterodimers (Wenkel et al., 2007). Intriguingly, the peculiar modular structure of HD-ZIP III proteins raises the possibility that their transcriptional activity could be regulated at post-translational level by both START and MEKHLA in order to ensure development of lateral organs in coordination with SAM activity and only if appropriate resources are available. In fact, START and MEHKLA domains, that are sensitive to the concentrations of lipid metabolites and to the redox potential, could act either as receptors for the polarising signal(s) emanating from the SAM and/or as sensors of the nutritional and energy status of the cell (McConnell et al., 2001; Mukherjee and Burglin, 2006).

Evolution of the HD-ZIP III family in land plants

The HD-Zip III family is the only transcription factors family for which sequences have been compared from representatives of all major land plant lineages (Aso et al.1999; Sakakibara et al. 2001; Floyd et al., 2006; Prigge and Clark 2006; Floyd and Bowman, 2007). Recent molecular phylogenetic analyses along with comparative analysis of ultrastructure and biochemistry indicate that land plants (embryophytes) compose a monophyletic group that evolved from a freshwater charophycean green algal ancestor (Graham et al. 2000; Karol et al. 2001).

Therefore, it is interesting that an HD-Zip III family homologue was also identified in *Chara corallina*, a member of the algal sister group, indicating that this gene family originated before the origin of land plants (Floyd et al., 2006). The phylogenetic analysis of HD-Zip III inferred amino acid sequences performed by Floyd et al. (2006) suggests that a single gene was inherited from an algal ancestor before the divergence of *Chara* and land plants but after the separation from the Coleochaetales approximately 450 million years ago (Fig. 11A). A first duplication event probably took place in the genome of the euphyllophyte ancestor but one of the two genes seems to have been lost before Angiosperm radiation (Floyd et al. 2006). On the other hand, a subsequent duplication before the Angiosperms – Gymnosperms separation gave rise to the ''*REV* clade'' (including the *REV*, *PHB* and *PHV Arabidopsis* genes) and the ''*CNA* clade'' (including *CNA* and *ATHB8 Arabidopsis* genes). A further duplication event occurred early within the Angiosperm lineage before the divergence of monocots and eudicots, separating the *REV* and *PHB/PHV* ancestor clades. The separation of the *CNA* and *ATHB8* clades occurred later only within the eudicot lineage while the duplication of the *PHB/PHV* ancestral gene took place independently in monocot and eudicot lineages (Floyd et al., 2006).

Deduced amino acid sequences of all land plant and *Chara HD–Zip III* genes are highly conserved and easy to align along their entire length, apart for the region between the HD–Zip domain and the START domain that displays a great variation in its sequence accounting for most protein length differences (Floyd et al., 2006). The coding sequences range in length from 2457 nt to 2733 nt, encoding amino acid sequences of 818–910 aa. The average pairwise identity of all identified HD–Zip III protein sequences is 59.6% with the low being 41% (between the *Chara* sequence and three different vascular plant sequences) and the highest identity being 91% (between two sequences of *Ginkgo* and *Taxus*). Introns and splice sites are largely conserved indicating that genomic structure of the coding region has also largely been conserved, at least in land plants (Floyd et al., 2006).

The phylogenetic tree of HD–Zip III genes is largely consistent with the evolution of the major developmental innovations in land plant body-plan such as apical meristems, vascular tissue, and leaves suggesting a parallel evolution and diversification of *HD-ZIP III* genes and land plants (Fig. 11A) (Floyd et al., 2006; Floyd and Bowman, 2007). It is interesting in this respect that the distribution of functions among *Arabidopsis HD–ZIP III* genes reflects their phylogenetic relationship. In fact, genes from both *REV* and *CNA* clades are involved in the control of the more ancient developmental processes such as vascular patterning and differentiation and apical meristem function while only genes of the *REV* clade are involved in the control of a more recent innovation, such as leaf development and polarity, suggesting that

Figure 11. (A) Phylogeny and functional evolution of *HD-ZIP III* genes. Cladogram (right) depicts relationships of HD-ZIP III genes in land plants inferred from Bayesian analysis of amino acid sequences. Gray boxes indicate the three seed plant clades. The yellow box indicate the algal sequence. The small cladogram (upper left) depicts functional evolution of *HD-ZIP III* genes mapped onto a simple land plant phylogeny. Known functions/expression patterns are labelled next to lineages in dark grey boxes. The darker branches indicate the lineages for which functional and expression data are available. The putative ancestral function and a new function acquired in the common ancestor of land plants are indicated with question marks. Patterning of vascular tissues was a novel function acquired by a single gene in the vascular plant ancestor, and leaf polarity was new function acquired by one of three genes in the seed plant ancestor. (from Floyd and Bowman, 2007) (B) Conservation of the nucleotides composing the miRNA165/166 binding site (in red) in land plant *HD-ZIP III* genes. X, conserved position. -, variable position. ., third base of a codon. The cleaved nucleotides are underlined. *Tg, Taxus globosa. Cr, Ceratopteris richardii. Sk, Selaginella kraussiana. Pp, Physcomitrella patens. Mp, Marchantia polymorpha. Pc, Phaeoceros dorffii.* (from Floyd and Bowman, 2004)

this is a new function acquired after the separation of the two clades (Emery et al., 2003; Prigge et al., 2005). Based on the observation that most of the known functions of *HD-Zip III* genes in flowering plants are clearly associated with key innovations in land plant evolution, it seems likely that expansion and diversification of *HD-ZIP III* gene family through gene duplications, neofunctionalization, subfunctionalization and modification of expression patterns played an important role in the evolution of increasingly complex land plant architecture. In particular, it has been suggested that these genes are part of an ancient developmental patterning tool kit that diversified and acquired new functions that allowed the modification of land plant development and the origin of new tissues and organs (Floyd and Bowman, 2007). Nevertheless, what was the function of the ancestral *HD-ZIP III* gene is still an open question. Given the simple anatomical structure of the most ancient relatives of land plants, possible functions for the ancestral *HD-ZIP III* gene could have been the control of apical growth or meristematic growth and patterning of three-dimensional tissues (Floyd et al., 2006). In particular, considering the topological correspondence in *Arabidopsis* between the central/peripheral polarity in stem tissues and vascular strands and the adaxial/abaxial polarity in lateral organ as well as the involvement of HD-ZIP III genes in the establishment of both, it is also possible that an ancestral *HD-Zip III* gene function was the regulation of tissue polarity (Prigge et al., 2005). Alternatively, the primary role of the ancestral *HD-ZIP III* gene could have been in other, more basic, signalling pathways, such as auxin signalling. In fact, even if *HD-ZIP III* genes are not directly involved in auxin signalling, some of them have been implicated in auxin response and polar transport in *Arabidopsis* (Baima et al., 2001; Zhong and Ye, 2001; Mattsson et al., 2003; Izhaki and Bowman, 2007; Donner et al., 2009). Since the use of auxin to regulate growth appears to predate the evolution of land plants (Cooke et al., 2002) and many of the effect of HD-ZIP III on plant architecture are also controlled by auxin, it is tempting to speculate that these two regulatory pathways were linked very early in plant evolution and were recruited together to help in patterning the new tissues during evolution (Prigge et al., 2005).

It is well known that changes in gene expression regulation can significantly contribute to functional diversification of gene paralogs and lead to morphological novelty. As described previously, among the many regulatory mechanisms controlling HD-ZIP III activity in *Arabidopsis*, a major role is played by miRNAs. As miRNA themselves are encoded by multiple loci and can be transcriptionally regulated in a complex manner, they add further plasticity for the evolution of regulatory and functional diversity. Interestingly, comparison of the aligned nucleotide sequences of streptophyte *HD–ZIP III* genes indicates that, the miR165/166 binding site is conserved in all land plant sequences but not in *Chara*, suggesting that microRNA

regulation of *HD-Zip III* mRNAs is associated with the origin of the embryophytes (Floyd and Bowman, 2004; Floyd et al. 2006) (Fig. 11B). Given that a key innovation of land plants respect to their algal relatives was the development of truly three-dimensional tissues (Graham et al., 2000) and that miR165/166 in *Arabidopsis* are required for fine spatial and temporal regulation of *HD-ZIP III* expression patterns, it is possible that the ability to precisely regulate the expression of the ancestral *HD-Zip III* gene in a three dimensional, multicellular tissue allowed the transition from filamentous apical growth to three-dimensional growth and patterning (Floyd and Bowmann, 2007).

ACAULIS5, a putative target of HD-ZIP III factors involved in vascular tissue differentiation

In an attempt to identify target genes directly regulated by HD-ZIP III transcription factors, we performed a database mining of the *Arabidopsis thaliana* genome sequences searching the 11 bp pseudo-palindromic consensus binding sequence of HD-ZIP III proteins GYAAT(G/C)ATTRC (BS-III) (Sessa et al., 1998) and the genes associated to it (Forte et al., manuscript in preparation). Among the 390 genes identified, we noticed the presence of the *ACAULIS5* gene (*ACL5*) that encodes an enzyme of the polyamine biosynthetic pathway, the thermospermine synthase (Knott et al., 2007; Kakehi et al., 2008). Polyamines, the most common and abundant being putrescine, spermidine and spermine, are ubiquitous low-molecular-mass polycations that can interact with proteins, nucleic acids and phospholipids and involved in a wide range of cellular processes, including chromatin condensation, maintenance of DNA structure, RNA processing, regulation of translation, modulation of enzyme activities and stabilisation of membranes (Kusano et al., 2008). In plants, polyamines, that can be transported in the vascular system, frequently exert effects resembling those of plant hormones and appear to function in stimulation of cell division, fruit ripening and stress signalling (Kusano et al., 2008).
ACL5 is structurally and functionally unrelated to the genes *SPMS*, encoding spermine synthase, and *SPDS1* and *2*, encoding spermidine synthase and it has been suggested that the ACL5 protein and its product, thermospermine, have a specific regulatory role different from the major polyamines (Panicot et al., 2002; Imai et al., 2004; Kakehi et al., 2008; Rambla et al., 2009). Conversely, *ACL5* seems to be functionally linked to *HD-ZIP III* genes and, in particular, to *ATHB8*. In fact, *ACL5* expression is specific of provascular cells (Birnbaum et al., 2003; Clay and Nelson, 2005) and differentiating xylem vessel elements (Muñiz et al., 2008) (Fig. 12I, J).

Moreover, similarly to *ATHB8* overespressing plants, the *acl5-1* mutant is characterised by a reduced plant stature and an abnormal development of the stem vascular tissue (Hanzawa et al., 1997) (Fig. 12A, B). In addition, after the transition to the reproductive phase, the *acl5-1* mutant produces smaller leaves resulting in dwarf plants (Hanzawa et al., 1997). A dramatic effect on vascular tissue differentiation has been reported also for the other *ACL5* mutant allele, *thickvein* (*tkv*) (Clay and Nelson, 2005).

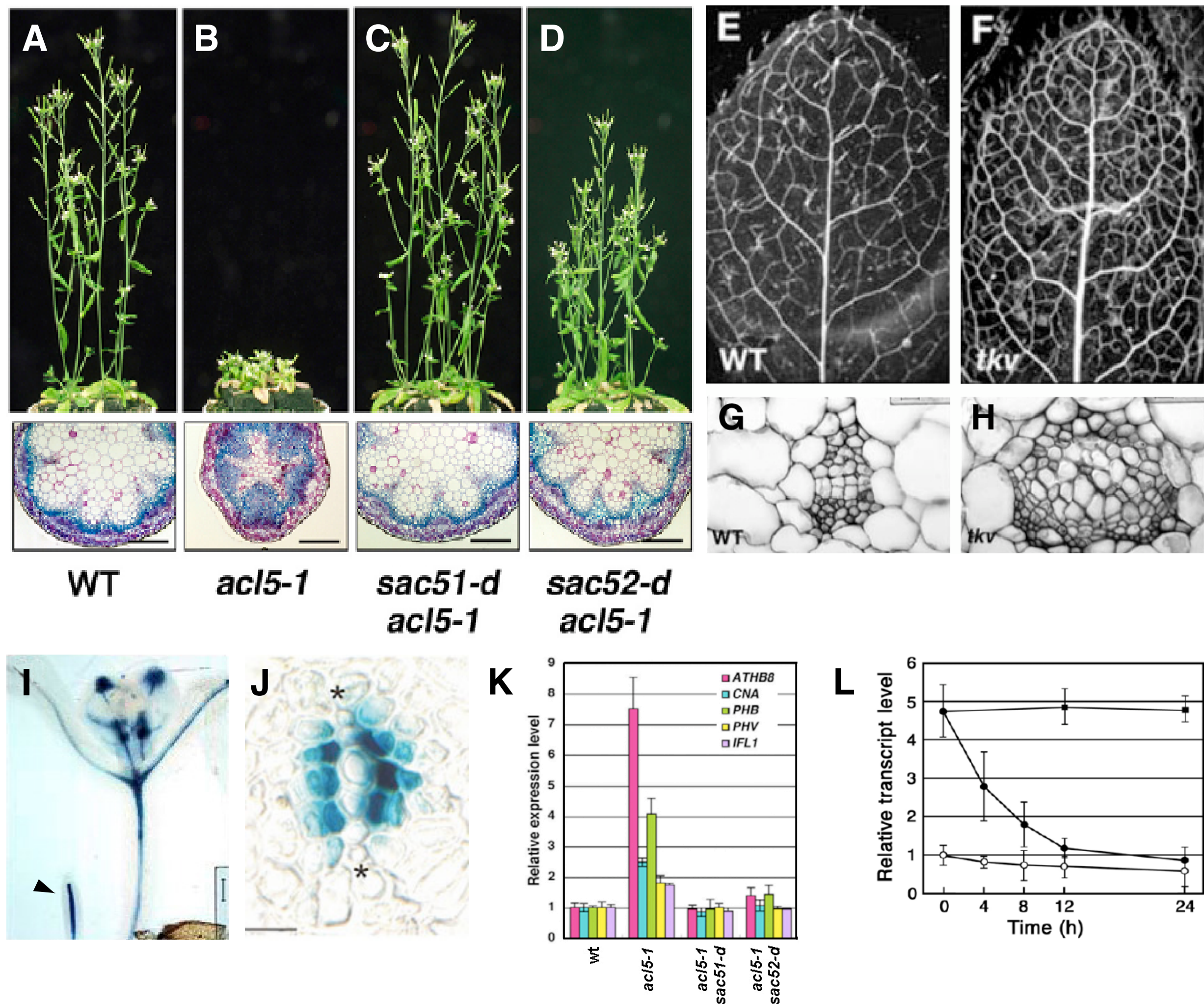


Figure 12. (A-D) Phenotype of 6-week-old plants (upper panel) and anatomy of the first inflorescence stem internode (lower panel, transverse sections) of wild-type (WT), *acl5* and *sac acl5* plants. (E-H) Increased vein thickness and vascularisation in (E, F) cleared adult leaves viewed under dark-field illumination and (G, H) transverse sections through the midvein of fully expanded juvenile leaves of *tkv* (F, H) compared to wild type (E, G) plants. (I, J) Histochemical localisation of *ACL5* promoter-driven β-glucuronidase (GUS) reporter activity in (I) 1-week-old whole seedling and (J) a transverse section of the hypocotyl of a 4-day-old seedling. The arrowhead indicate the root apex and asterisks indicate the protoxylem poles. (K) Quantitative RT-PCR analysis of *HD-ZIP III* genes expression in seedlings of 12-day-old wild-type (WT), *acl5* and *sac acl5* plants. (L) Real-time PCR quantification of *ACL5* transcript levels in 7-day-old wild-type seedlings incubated with 0.1 mM thermospermine (open circles), and *acl5-1* seedlings incubated with 0.1 mM thermospermine (closed circles) or spermine (closed squares) for indicated hours. (K, L) Relative values were normalized to the expression of *ACTIN8*. The basal level of the transcript of each gene in the untreated wild-type sample was set at 1.0. Bars show mean±SD (*n*=3). Scale bars: (A-D) 200 μm, (G, H) 16 μm, (I) 100 μm, (J) 20 μm. (A-D, K) from Imai et al., 2006. (E-I) from Clay and Nelson, 2005. (J) from Muniz et al., 2008. (L) (from Kakehi et al., 2008).

This mutant displays an increase in the number and thickness of leaf veins as well as enlarged stem vascular bundles with a greater number of procambial, xylem and phloem cells (Fig. 12E-H). This phenotype is in good correlation with the increased *ATHB8* and *PHB* expression reported in *acl5-1* (Imai et al., 2006) (Fig. 12K). Root growth and anatomy is reported to be unaffected in *acl5-1* and *tkv* but the latter mutant has been shown to be hypersensitive to exogenous cytokinin (Clay and Nelson, 2005). Recently it has been proposed that the effect of *ACL5* on vascular differentiation is due to its ability to prevent premature cell death of the tracheary elements and to control the duration of xylem cells differentiation (Muñiz et al., 2008). In fact, histochemical analysis of GUS reporter activity have shown that expression of a vascular specific cell death marker gene normally expressed only in mature xylem elements with secondary walls is anticipated in *acl5-1* respect to wild type plants and appears since the first stages of procambial cells differentiation (Muñiz et al., 2008). Moreover, transgenic plants expressing the cell lethal diphteria toxin under the control of the *ACL5* promoter largely phenocopy the xylem defects associated with loss of *ACL5* function (Muñiz et al., 2008). Finally, exogenous spermine prolonged xylem element differentiation and stimulated cell expansion and cell wall elaboration in xylogenic cell cultures of *Zinnia elegans*, suggesting that *ACL5* prevents premature death of the developing vessel elements to allow complete expansion and secondary cell wall patterning (Muñiz et al., 2008).

Interestingly, the level of the *acl5-1* transcript, encoding for an inactive enzyme, is much higher in mutant seedlings than the *ACL5* transcript level in wild type seedlings and addition of exogenous thermospermine to *acl5-1* restores normal expression levels (Hanzawa et al., 2000; Kakehi et al., 2008) (Fig. 12L). This observation indicates that *ACL5* expression is regulated by its reaction product through a negative feedback loop and suggests a requirement for strict control of the endogenous thermospermine level. In addition, like *ATHB8*, *ACL5* expression is positively regulated by auxin probably through binding of ARF transcription factors to two putative auxin response elements (ARE) present in the 5' upstream region of the gene (Hanzawa et al., 2000). Interestingly, a reduced polar auxin transport capacity has been reported for the stem of *tkv* plants (Clay and Nelson, 2005).

The identification and characterization of two extragenic dominant suppressors of *acl5-1*, the *SUPPRESSOR OF ACL5 51* and *52* genes (*SAC51* and *SAC52*) have pointed out a role for translational control in *ACL5* dependent phenotypes (Imai et al., 2006 and 2008) (Fig. 12C, D). In fact, *sac51-d* disrupts one of the five short upstream open reading frames (uORFs) present in the 5' untranslated region (5' UTR) of *SAC51*, which encodes a bHLH-type transcription factor (Imai et al., 2006). Interestingly, the sequence of the peptide encoded by this uORF is conserved

in *Arabidopsis* and rice (Imai et al., 2006; Hayden and Jorgensen, 2007) and premature termination of this uORF in *sac51-d* results in an increased abundance and translational efficiency of the mutated transcript as shown by reporter gene analysis (Imai et al., 2006). Therefore, suppression of the mutant phenotype in *sac51-d acl5-1* may be attributable to the overproduction of the SAC51 protein. *ACL5* has been shown to be required for the acceleration of *SAC51* translation although the addition of exogenous thermospermine did not have the same effect (Imai et al. 2006; Kakehi et al., 2008). In contrast, exogenous thermospermine resulted in up-regulation of *SAC51* transcript level and *SAC51*::GUS activity in both *acl5-1* and wild-type seedlings (Kakehi et al., 2008). The *sac52-d* mutation resides in a nucleotidic change in the SAC52 gene encoding the ribosomal protein L10, which is highly conserved among eukaryotes and implicated in translational regulation (Imai et al., 2008). Experimental data indicate that suppression of the *acl5-1* phenotype by *sac52-d* is attributable, at least in part, to enhanced translation of certain transcripts, including *SAC51,* that could require thermospermine, produced by ACL5, for optimal translation (Imai et al., 2008). The mode of interaction of the ribosomal protein L10A encoded by *SAC52* and thermospermine remains to be elucidated. Besides restoring normal stem elongation, combination of the *sac51-d* and *sac52-d* alleles with *acl5-1* in the double mutants down-regulates the *acl5-1* transcript level (Imai et al., 2006) (Fig. 12K). These findings suggest that the negative feedback circuit controlling *ACL5* expression does not require thermospermine but involves a transcriptional repressor (that could be SAC51 itself or a component downstream of it) whose activation or translation requires *SAC52/RPL10A*. The transcript levels of *ATHB8* and *PHB* were also restored to wild-type levels in *acl5-1 sac51-d* and *acl5-1 sac52-d* double mutants suggesting a close correlation between *HD-ZIP III* gene expression and *acl5-1* mutant phenotype (Imai et al., 2006). Noteworthy, induction of *ACL5* under the control of a heat-shock promoter in the *acl5-1* mutant results in recovery of stem growth and in transient increase in *SAC51* transcript abundance prior to reduction in the transcript levels of *HD-ZIP III* genes (Hanzawa et al., 2000; Imai et al., 2008). Down-regulation of *ATHB8* and *PHB* transcripts was also induced by application of exogenous thermospermine to *acl5-1* seedlings (Kakehi et al., 2008). Taken together, these data indicate that *SAC51* may play a key role in *ACL5*-dependent developmental processes through repression of expression of HD-ZIP III genes although it remains to be determined whether the SAC51 bHLH protein acts as a transcription activator or repressor.

AIM OF THE WORK

Although genetic and functional data clearly indicate that HD-ZIP III transcription factors are involved in key developmental processes in plants, the molecular details of their action and how their activity is integrated with that of other factors controlling the same processes are still largely unknown. Identification of primary target genes regulated by this family of transcription factors could help to elucidate their role in the basic mechanisms controlling meristem function, establishment of organ polarity and vascular differentiation. To this goal, a bioinformatic approach as been undertaken to identify genes directly regulated by HD-ZIP III proteins in the model plant *Arabidopsis thaliana.* Experimental verification of one of the putative targets identified by database mining, the *ACAULIS5* (ACL5) gene, encoding a polyamine biosynthetic enzyme, thermospermine synthase and showing a significant functional overlap with HD-ZIP III genes, have given support to the hypothesis that this gene could be a genuine primary target. Therefore, the aim of this work has been further testing of *ACL5* regulation by HD-ZIP III family *in vivo* and the study of the functional relationship between these genes in the control of vascular system differentiation in *Arabidopsis*.

RESULTS

Identification of putative targets of HD-ZIP III transcription factors

In an attempt to identify target genes directly regulated by HD-ZIP III transcription factors, we performed a database mining of the *Arabidopsis* genome sequences searching for genes containing the 11 base pairs (bp) pseudo-palindromic consensus binding sequence of HD-ZIP III proteins GYAAT(G/C)ATTRC (BS-III) (Sessa et al., 1998) using the Pattern Matching tool of TAIR (The Arabidopsis Information Resource, www.arabidopsis.org). Of the 390 genes identified 27,3% contain the BS-III sequence in the 3000 bp upstream of the translation start site, 33,0% in the transcribed region and 39,7% within the 3000 bp downstream the coding sequence (Forte et al., manuscript in preparation). Although the function of many of these genes is unknown or putative, the annotation of 305 genes suggests that they are involved in transcriptional control, hormonal response, signal transduction, protein degradation, growth, transport and metabolism.

As already mentioned in the Introduction, within the list of putative target genes we noticed the presence of *ACAULIS5* (*ACL5*), a gene with a significant expression and functional overlap with *HD-ZIP III* genes. Although regulatory sequences have been found in any position respect to the protein coding region of a gene in eukaryots, the vast majority are located within the promoter in the first 500 bp upstream the transcription start site. It is therefore noticeable that a GCAATCATTAC sequence, corresponding to the HD-ZIP III consensus DNA binding site, is present at -89 bp from the *ACL5* transcription start site. An Electrophoretic Mobility Shift Assay (EMSA) has demonstrated that the HD-Zip III domain actually binds *in vitro* to a region of the *ACL5* promoter including this sequence and that mutation of four nucleotides in the BS-III element at positions predicted to be directly involved in DNA-protein interaction abolishes this binding (Forte et al., manuscript in preparation; Sessa et al., 1998). In addition, by quantitative real-time analysis, *ACL5* transcript level was found to be induced between two- and four-fold by constitutive or transient inducible overexpression of *ATHB8* in transgenic plants (Forte et al., manuscript in preparation). Interestingly, the fold-induction is higher in primary inflorescence stems of 5-week-old *35S::ATHB8* plants than in seedlings, in agreement with the strong effect of *ATHB8* overexpression on stem vascular tissue differentiation. Conversely, *ACL5* expression has been found to be down-regulated approximately threefold in microarray analysis of RNA extracted from 1-week-old transgenic seedlings overexpressing miR165, a negative regulator of *HD-ZIP III* genes (Zhou et al., 2007).

Taken together, these data clearly indicates that HD-ZIP III proteins directly bind *in vitro* to the BS-III site in the *ACL5* promoter and positively regulate *ACL5* expression, indirectly confirming that *ACL5* is a genuine target of HD-ZIP III transcription factors.

Effect of HD-ZIP III binding site mutation on *ACL5* expression pattern

In order to confirm *in vivo* the functional relevance of the HD-ZIP III binding site present in the *ACL5* promoter, a reporter gene fusion strategy was employed. To this purpose, the region 2 kb upstream the *ACL5* translation initiation codon was amplified from genomic DNA and cloned into the pENTR^{TM}/D-TOPO^{R} vector, and four nucleotidic changes in the putative BS-III element at positions shown to be essential for DNA-protein interaction *in vitro* were introduced by site-directed mutagenesis using mutated oligonucleotides (Fig. 13). The *ACL5* promoter region harbouring either the wild type or the mutated BS-III sequence and the *ACL5* coding sequence, amplified from a seedling cDNA, were then translationally fused to the β-glucuronidase (GUS) reporter gene into the pMDC162 binary vector. After sequence verification, the two chimeric genes driving the expression of the ACL5 protein fused to the GUS reporter under the control of the *ACL5* promoter sequence wild type (*ACL5::ACL5:GUS*) or mutated in the BS-III site (*ACL5mut::ACL5:GUS*) were then introduced into *Agrobacterium tumefaciens* to transform *Arabidopsis* wild type plants of the Col-0 ecotype by infiltration.

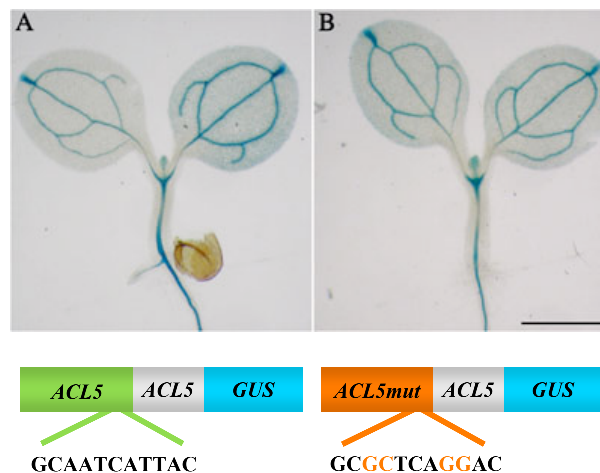


Figure 13. Histochemical β-glucuronidase (GUS) staining of (A) *ACL5::ACL5:GUS* and (B) *ACL5mut::ACL5:GUS* 1-week-old transgenic *Arabidopsis* seedlings. A scheme of the chimeric genes with the wild-type and mutated BS-III site sequence is presented below the images. Bar: 1 mm.

The progeny of 9 and 12 independent *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS*, respectively, primary transformants was tested on hygromycin and F2 families deviating from the expected 3:1 segregation ratio of the antibiotic resistance marker associated with the T-DNA were discarded in order to avoid transgenic lines with multiple insertions at different sites. Hygromycin selection of the T3 generation allowed the identification of homozygotes for 6 and 5 independent lines containing a single insertion of *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS*, respectively. A preliminary expression pattern analysis by histochemical localisation of GUS activity in these transgenic lines showed that, in 1-week-old seedlings, both wild type and mutated promoter drive specific GUS expression in procambial and differentiating xylem cells as shown by Clay and Nelson (2005) (Fig. 13). Nonetheless, a careful analysis of GUS staining in the first pair of true leaves and in root apices revealed that mutation of the BS-III site affects *ACL5* expression in the early phases of vascular cells differentiation. In order to select transgenic lines with comparable expression levels, GUS activity was quantitatively assessed by a fluorometric assay (Table I). To exclude any influence of transgene position on promoter activity, all subsequent detailed analyses were performed on two homozygote independent lines for each reporter construct (wt_c1 and wt_c2 for *ACL5::ACL5:GUS* and mut_c2 and mut_c3 for *ACL5mut::ACL5:GUS*).

	line	GUS activity (pmol MU/µg/min)
ACL5::ACL5:GUS	**wt_c1**	4,50
	wt_c2	4,28
	wt_c3	4,17
	wt_c4	3,72
	wt_c5	3,57
	wt_c6	0,94
ACL5mut::ACL5:GUS	mut_c1	5,99
	mut_c2	5,05
	mut_c3	4,39
	mut_c4	1,57
	mut_c5	0,71

Table I. Quantitative evaluation of GUS reporter activity in 1-week-old seedlings of homozygous T3 *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* transgenic lines. The experiment was repeated three times with similar results. The lines indicated in bold were selected for the subsequent analysis.

In *Arabidopsis* leaves, veins of subsequent orders are formed sequentially (Kang and Dengler, 2004; Jung et al., 2008; Sawchunk et al., 2008). Leaf vein differentiation is preceded by a pattern of continuous narrow files of elongated, non-vacuolated procambial cells, the primary meristem that differentiate all vascular cells. In turn, procambial strands are preceded by *de novo* formation of a pattern of anatomically inconspicuous, isodiametric ground meristem cells that progressively acquire pre-procambial identity through a still largely unknown mechanism involving auxin transport and distribution. The temporal sequence of vascular developmental events in *Arabidopsis* leaf primordia and definitions of the stages and terminology used in this study are schematically illustrated in Fig. 14 A-D. First, a single procambial strand extends from the stem vascular bundles into the leaf primordium to form the primary vein. Second, the secondary veins arise as continuous loops, with the first loops formed in the apical portion of the leaf and new ones added basipetally. Tertiary and higher-order veins also appear as continuous strands that span intercostal areas and are added basipetally until the complete reticulate vein pattern is formed. The expression pattern of the *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* constructs at different stages during leaf primordium development was analysed in parallel with the expression pattern of *ATHB8::GUS*. As already mentioned in the Introduction, *ATHB8* is considered one of the earliest markers of vascular differentiation and, in the leaf primordium, is expressed in polygonal, morphologically indistinguishable pre-procambial cells before they acquire the typical elongated shape of procambial and vascular cells (Baima et al., 1995; Dengler and Kang, 2001; Scarpella et al., 2004; Donner et al., 2009). Available evidence indicates that *ATHB8* expression identifies a crucial and typically irreversible stage in procambial cell fate acquisition, accurately predicting sites of vascular differentiation, that is mutually exclusive with a "pre-mesophyll" cell state presaging mesophyll fate assignment (Scarpella et al. 2004; Donner et al., 2009). In leaf primordia of 4-, 5-, 6- and 7-day-old seedlings, *ATHB8* expression initially proceeds acropetally along the midvein and then appears in secondary vein loops originating from the midvein (Fig. 14 E). For each time point, pattern comparison was made in leaf primordia of the same area to ensure that all were at the same developmental stage. *ACL5* expression closely matches that of *ATHB8* but is clearly delayed respect to it (about 1 day). In particular, the onset of *ACL5* expression is subsequent to the differentiation of morphologically distinct procambial cells while *ATHB8* expression precedes it (Fig. 14 E, inset). Noteworthy, *ACL5* expression is further delayed (about 1 day) by mutation of the BS-III site (Fig. 14 E).

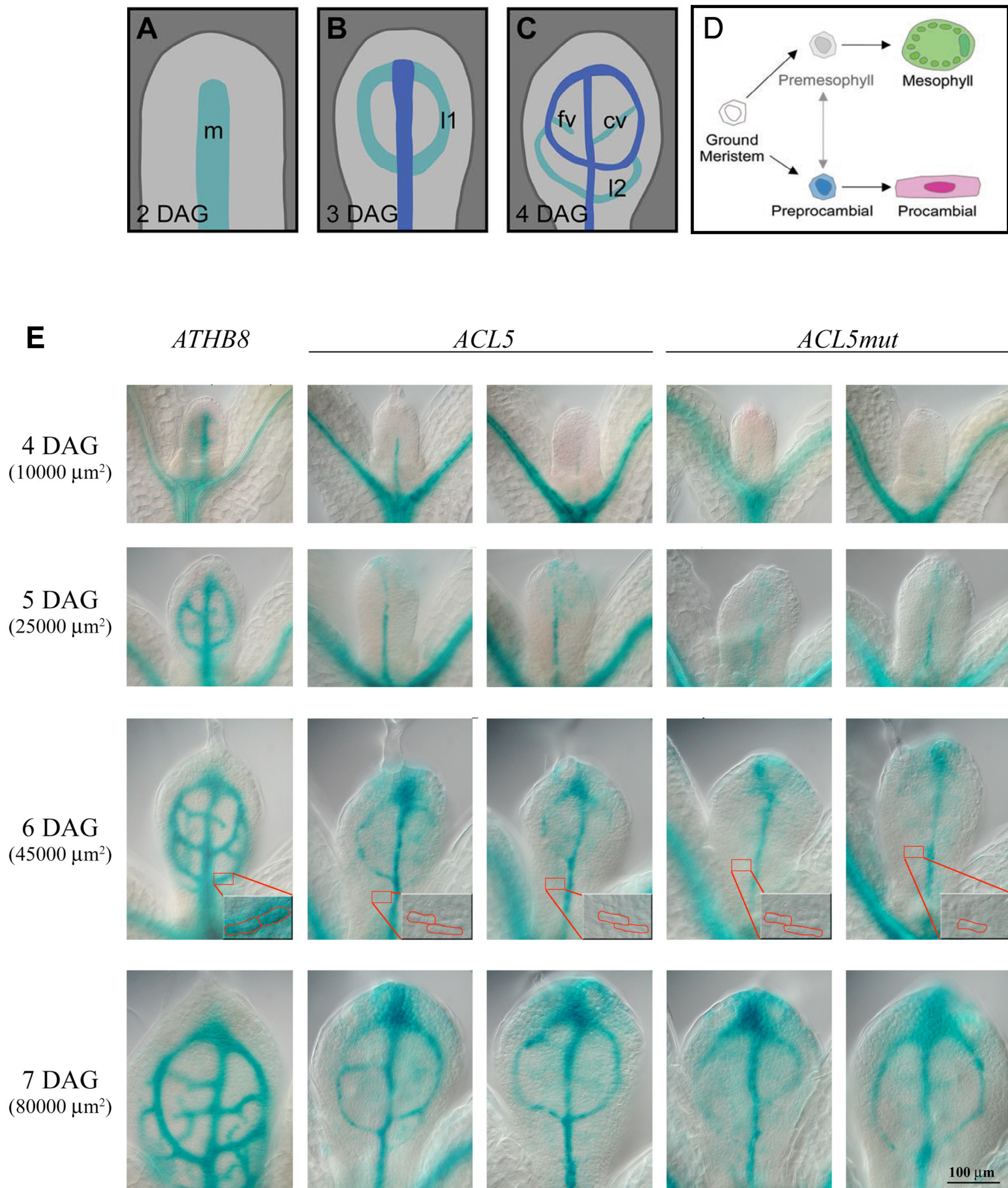


Figure 14. (A-C) Schematic representation of the spatiotemporal course of vein formation in the first leaf primordium 2-, 3- and 4-days after germination (DAG) (from Donner et al., 2009). Cyan, pre-procambial stages; blue, procambial stages m, midvein; l1, first loop; l2, second loop;fv, free-ending vein; cv, connecting vein. (D) Schematic representation of ground meristem cells differentiation in the leaf primordium during vein patterning. Pre-procambial and pre-mesophyll states are mutually exclusive (from Sawchunk et al., 2008) (E) Histochemical localization of GUS activity in leaf primordia 4, 5, 6 and 7 DAG in seedlings of *ATHB8::GUS* (*ATHB8*), *ACL5::ACL5:GUS* (*ACL5*) and *ACL5mut::ACL5:GUS* (*ACL5mut*) transgenic lines. Vein development is retarded respect to the scheme in (A-C) probably due to different growth conditions. The area of the primordia is indicated below the age. The inset shows a detail of the elongated procambial cells. Scale bar: 100 µm

Interestingly, a striking difference between the GUS expression patterns of the two chimeric constructs was observed also in the root apical meristem and lateral root primordia.
The anatomy of the *Arabidopsis* root tip is shown in Fig. 15M-O. In longitudinal sections, three zones can be distinguished directly related with cell growth stages: a distal meristematic zone characterised by small cells overlaid by the root cap, a central elongation zone where cells are elongated by 10 times compared to the original cell length, and a basipetal differentiation zone, in which elongated cells from the different tissues mature into fully differentiated cells (Fig. 15M). A striking feature of the *Arabidopsis* root is that the organisation and numbers of cells is remarkably constant. In transverse sections, three concentric single-cell layers, the epidermis, cortex and endodermis, surrounding a core of cells, the stele or vascular cylinder, can be distinguished (Fig. 15N) (Dolan et al., 1993). The central vascular cylinder is diarch with two phloem poles located on an axis perpendicular to the xylem axis and is lined on the outer edge by pericycle cells. All root tissues arise from files of cells that originate from dividing cells at the root apical meristem. The stem cells at the end points of these files are defined as the meristem initial cells. In *Arabidopsis*, the initial cells surround and contact a small group of cells that are mostly mitotically inactive, the quiescent center (QC) (Fig. 15D) (Dolan et al., 1993). The organisation of cells in lateral roots, arising from stereotyped divisions of the pericycle cells facing the xylem poles, is similar to that of the primary, embryonically derived root.
When expression is driven by the wild type *ACL5* promoter, GUS activity is detected in the meristematic region in vascular precursor cells immediately adjacent to the QC, as previously reported (Birnbaum et al., 2003; Clay and Nelson, 2005) (Fig. 15A, D). On the other hand, when the expression is driven by the mutated promoter, GUS activity disappear in the stele of the meristematic region and it is at first visible at the transition between the meristematic and the elongation zone of the root (Fig. 15G, J). Moreover, *ACL5* expression in emerging lateral root primordia is lost upon mutation of the BS-III site (compare H, I, K, L to B, C, E, F in Fig. 15) and becomes visible only at later stages in the provascular tissue in connection with the primary root stele (not shown).
In conclusion, reporter analysis suggests that, in leaf primordia, *ACL5* acts downstream of *ATHB8* and is involved in differentiation rather than in patterning of the leaf veins. Moreover, mutation of the BS-III element results in loss of expression at early procambial stages in leaf, root meristem and lateral root primordia strongly supporting that HD-ZIP III transcription factor could developmentally activate *ACL5* expression through binding to this site.

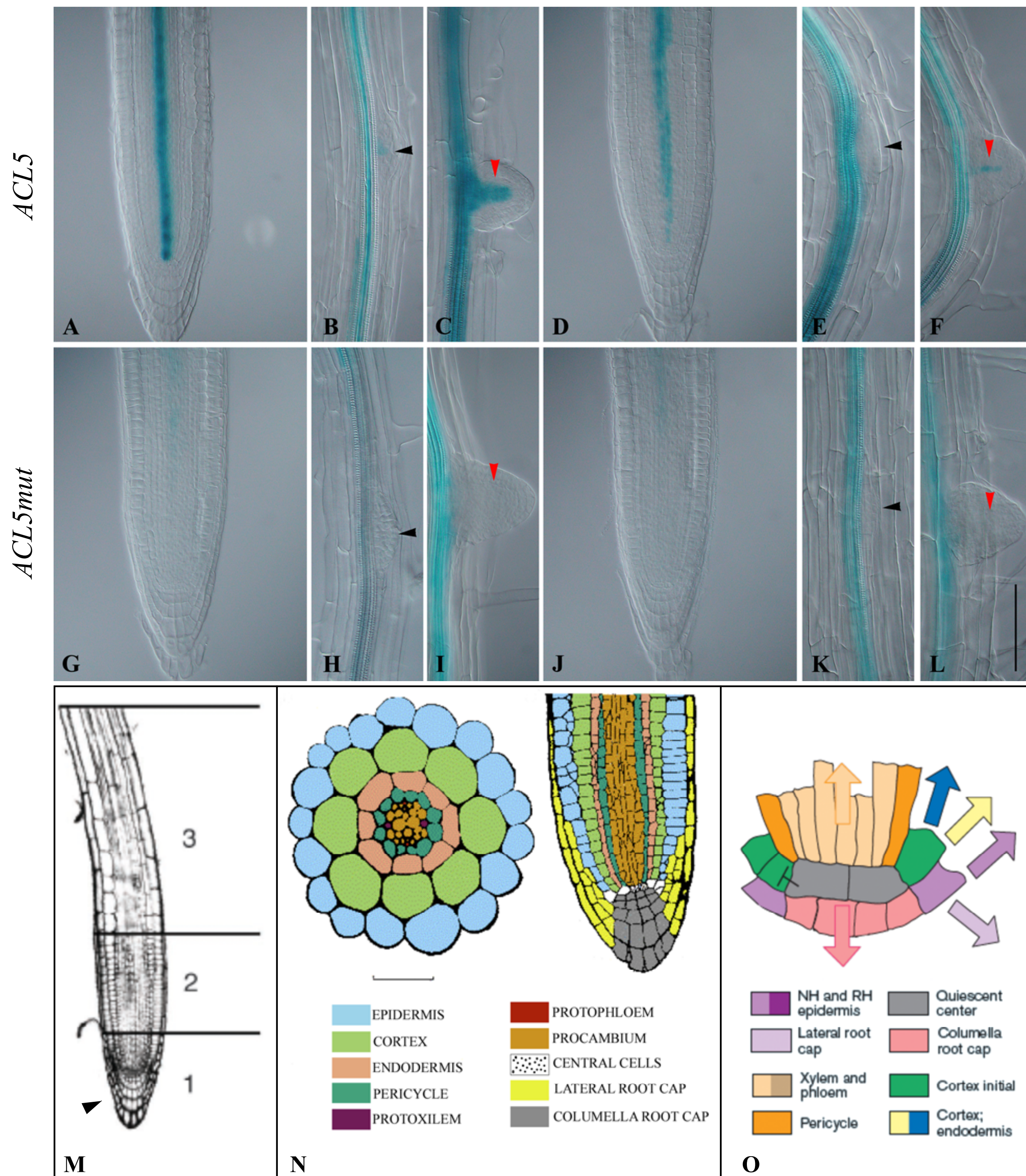


Figure 15. (A-L) Histochemical localization of GUS activity in roots of 6-day-old seedlings of (A-F) two *ACL5::ACL5:GUS* (*ACL5*) and (G-L) two *ACL5mut::ACL5:GUS* (*ACL5mut*) transgenic lines. (A, D, G, J) Primary root apex. (B, E, H, K) Lateral root primordium before emergence. (C, F, I, L) Lateral root primordium after emergence. Arrowheads indicate the position of vascular cells precursors. Scale bar: 100 μm. (M) The different zones corresponding to subsequent growth stages in the *Arabidopsis* root apex are indicated by numbers: 1, meristematic zone; 2, elongation zone; 3, differentiation zone. At the tip of the root, the root cap (arrowhead) provides mechanical protection to the meristematic tissue as the root advances through the soil. (from Birnbaum et al., 2005) (N) Pseudo-coloured transverse (left) and longitudinal (right) sections showing the organisation of tissues in the *Arabidopsis* root tip. The different cell types are indicated in the legend. Scale bar: 25 μm. (from Dolan et al. 1993) (O) Schematic representation of the stem cells (meristem initials) and their direction of cell division in the *Arabidopsis* root meristem (from Benfey and Scheres, 2000).

Extended phenotypic characterisation of the *acl5-1* mutant

The different expression patterns observed in the primary and lateral roots in plants transformed with the wild-type or the mutated *ACL5* promoter suggest that regulation of *ACL5* could be important for vascular differentiation in the root system. On the contrary, it has been reported previously that mutation of the *ACL5* gene does not affect root growth, except for an increased sensitivity to cytokinin (Hanzawa et al., 1997; Clay and Nelson, 2005). Nonetheless, in our growth conditions, a strong reduction of the root apparatus was noticed for *acl5-1* plants when grown both in soil (not shown) and on agar plates (Fig. 16A). Unexpectedly, a more detailed analysis of the growth of the root apparatus in 5-day-old *acl5-1* seedlings pointed out that, although the length of the primary root is only slightly and occasionally reduced, the number of lateral roots primordia is consistently and significantly increased (Fig. 16B).

A few other *acl5-1* phenotypes that had not been reported previously were also observed during this study. First, while the rosette of wild-type plants is flat with slightly convex leaves, when grown *in vitro*, *acl5-1* plants present erect leaves (the petiole angle increases with respect to the horizontal) with the leaf lamina curved upward and then inward from its lateral sides (Fig. 16D, E). Intriguingly, this phenotype strikingly resembles mutants and transgenic plants with increased expression of *HD-ZIP III* genes. Second, bolting of the flowering stem is anticipated in *acl5-1* by approximately 1 week and a reduced number of rosette leaves are formed (Fig. 16F, G). Third, the rosette of plants grown in soil often displays strong accumulation of purple pigments after flowering probably due to induction of anthocyanin synthesis (Fig. 16C). Finally, the *acl5-1* hypocotyl (especially in the upper part) is transformed into a callus-like structure in 5-week-old plants (Fig. 16H-M). Groups of disorganised, round or digit-like cells (like callus cells), apparently originated by an abnormal proliferation of epidermal cells are visible on the *acl5-1* hypocotyl surface (Fig. 16H, K). In transverse sections, *acl5-1* hypocotyl displays an impressive disorganised structure (Fig. 16I, L). All parenchyma cells are characterized by a hypertrofic expansion and all tissues show an abnormal proliferation that causes the destruction of normal radial symmetry of the organ. The ring of cambium is no longer recognisable, but many anomalous proliferating regions, more or less well separated and with variable division planes, are identified. Many of these proliferating regions appear as nodule-like structures with ectopic cambium-like cells in the outer part. Remarkably, abnormal division planes and proliferation of groups of cells causing a loss of radial symmetry are already visible in the vascular cylinder of hypocotyls of 2-week-old *acl5-1* plants (Fig. 16J, M.)

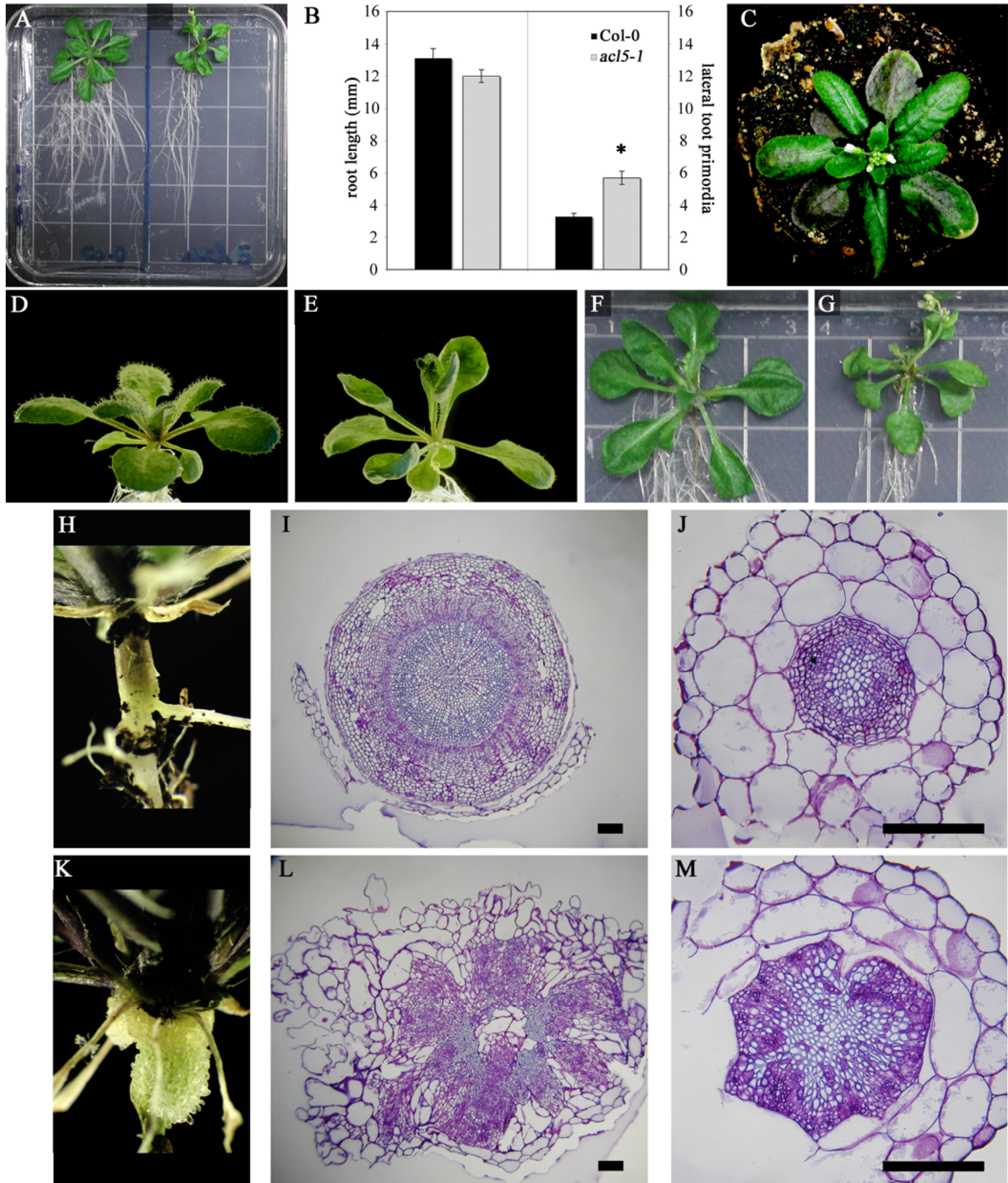
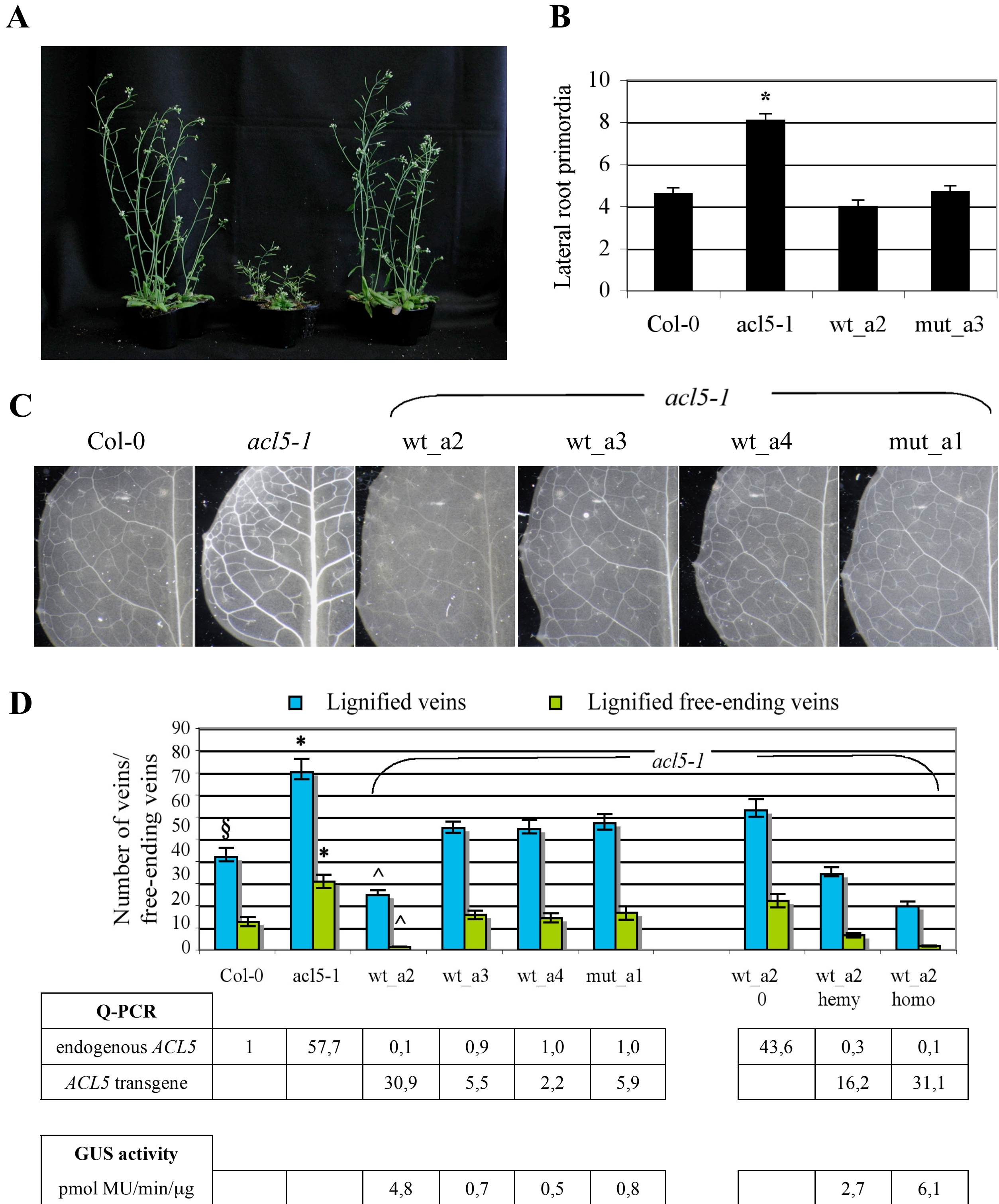


Figure 16. New phenotypes of the *acl5-1 Arabidopsis* mutant. (A) Three-week-old plants grown *in vitro* in vertical orientation. Left, wild-type; right, *acl5-1*. (B) Length and number of lateral root primordia of roots of wild-type (Col-0) and *acl5-1* 5-day-old seedlings. Values are mean ± SE (n= 20) * P<0,01. (C) Four-week-old *acl5-1* plant grown on soil. (D-G) Three-week-old wild-type (D, F) and *acl5-1* (E, G) plants grown in Phytatrays (D, E) or in vertically oriented plates (F, G). (H-M) Hypocotyls of wild-type (H-J) and *acl5-1* (K-M) plants. (H, I, K, L) Five-week-old plants grown on soil. (J, M) 2-week-old plants grown *in vitro*. Transverse sections (I, J, L, M) were stained with toluidine blue. Scale bar = 100 μm.

Taken together, these observations suggest a role for *ACL5* in leaf polarity, lateral root formation, cell proliferation and coordinated growth in the hypocotyl, anthocyanin accumulation and flowering time control in addition to its established role in vascular cells differentiation.

Complementation of *acl5-1* with *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* constructs

The differences observed in the GUS expression pattern of *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* transgenic plants suggest that mutation of the BS-III element cause the loss of a relevant regulatory region in the *ACL5* promoter. To further verify this hypothesis, the two chimeric construct expressing the *ACL5:GUS* fusion protein under the control of the wild-type or the mutated *ACL5* promoter were introduced into *acl5-1* by *Agrobacterium* mediated transformation to test their ability to complement the *ACL5* mutation. All the 12 and 16 T1 hygromycin resistant plants transferred to soil transformed with the *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* constructs, respectively, displayed normally elongated inflorescence stems (Fig. 17A). Similarly, after the selection of T3 homozygous lines containing a single T-DNA insertion, phenotypic analysis has shown that transformation with both constructs suppresses all the developmental defects of *acl5-1*, including leaf curvature and hyponasty, early flowering, hypocotyl hyper-proliferation and lateral root formation (Fig. 17B). A detailed analysis of the fifth rosette leaf have shown that expression of ACL5:GUS protein under the control of both *ACL5* wild-type and mutated promoter complements the *acl5-1* mutation respect to the number and thickness of veins formed in adult leaves (Fig. 17C, D). Surprisingly, in a few transgenic lines, the number of lignified leaf veins observed in dark field owing to the autofluorescence of the cell walls decreased even below that observed in wild-type plants (Fig. 17C, D). Quantitative evaluation of transgene expression by real-time PCR and of *ACL5:GUS* protein activity by a flurometric GUS assay of T3 homozygous lines and of a T2 segregating population clearly indicates that the effect on leaf vein number and thickness is correlated to the level of *ACL5:GUS* expression/activity attained by each transformation event and to transgene dosage (Fig. 17D). Interestingly, the milder leaf vein defect of the *acl5-1* plants segregating in the T2 generation of the wt-a2 transgenic line respect to the *acl5-1* parental plants could suggest a maternal effect of the *ACL5::ACL5:GUS* transgene expression. Although the values of the relative expression level of endogenous and transgenic *ACL5* transcripts cannot be directly compared, as they were measured with different real-time PCR assays, reduction of lignified leaf vein formation is clearly linked to *ACL5* overexpression (Fig. 17D).

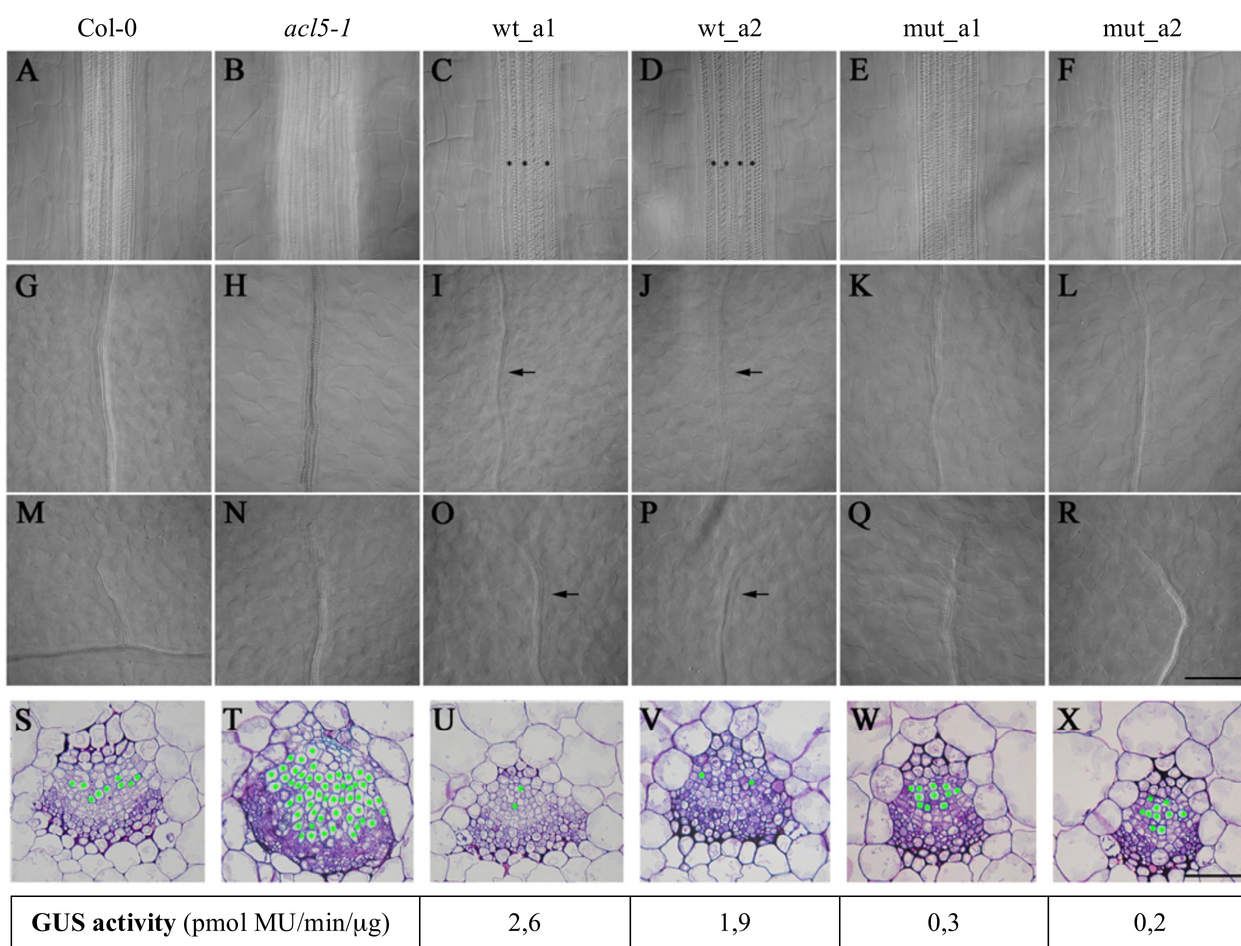


Q-PCR	Col-0	acl5-1	wt_a2	wt_a3	wt_a4	mut_a1	wt_a2 0	wt_a2 hemy	wt_a2 homo
endogenous *ACL5*	1	57,7	0,1	0,9	1,0	1,0	43,6	0,3	0,1
ACL5 transgene			30,9	5,5	2,2	5,9		16,2	31,1

GUS activity	Col-0	acl5-1	wt_a2	wt_a3	wt_a4	mut_a1	wt_a2 0	wt_a2 hemy	wt_a2 homo
pmol MU/min/µg			4,8	0,7	0,5	0,8		2,7	6,1

Figure 17. (A) Phenotype of *acl5-1* mutant (center) complemented with *ACL5::ACL5:GUS* (left) and *ACL5mut::ACL5:GUS* (right) constructs. (B) Analysis of lateral roots formation in 6-day-old wild-type (Col-0), *acl5-1* and *acl5-1* seedlings complemented with *ACL5::ACL5:GUS* (wt_a2,) and *ACL5mut::ACL5:GUS* (mut_a3) constructs. *P<0,01 *acl5-1 vs*. all other lines. (C) Venation pattern of the fifth leaf of 3-week-old wild-type (Col-0), *acl5-1* and *acl5-1* plants complemented with *ACL5::ACL5:GUS* (wt_a2, wt_a3, wt_a4) and *ACL5mut::ACL5:GUS* (mut_a1) constructs. Dark-field images of cleared leaves. (D) Number of lignified veins and free-ending veins in 16 mm^2 of the fifth leaf of 3-week-old plants. Values are mean ± SE (n=7). § P< 0,01 in wt *vs*. all other lines; * P< 0,01 in *acl5-1 vs*. all other lines; ^ P< 0,01 in wt_a2 *vs*. all other lines. Below the graph is reported the quantitative analysis of ACL5:GUS expression/activity in the transgenic lines and the level of the endogenous *ACL5* transcript.

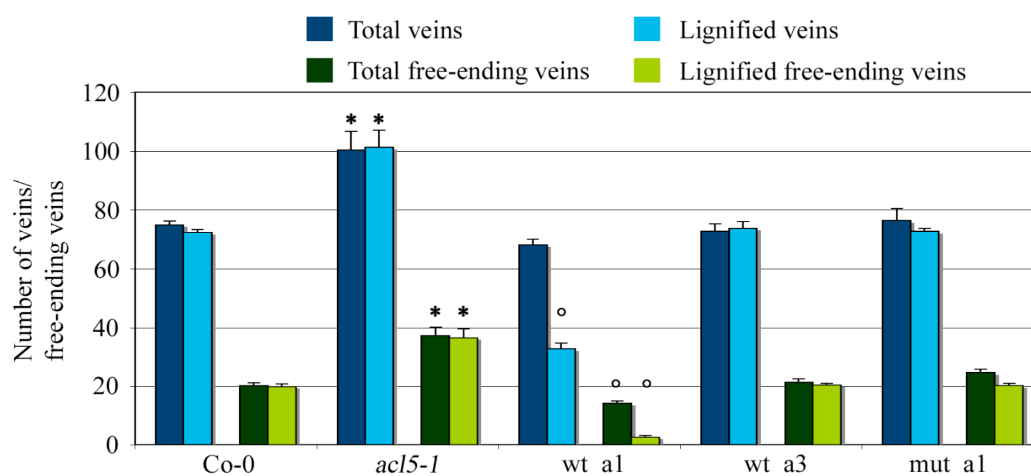
In this respect, it is important to point out that, as ACL5:GUS production is driven by the *ACL5* promoter, high transgene transcript levels result from increased expression in the same cell types in which it is normally expressed (entopic overexpression) and not by ectopic expression, as confirmed by the unchanged histochemical localization of GUS reporter activity (data not shown). Noteworthy, due to the negative autoregulatory feedback loop, the dramatic increase of the mutated *acl5-1* transcript observed in the *acl5-1* mutant is also recovered by the activity of the chimeric ACL5:GUS protein while its entopic overexpression further reduces *acl5-1* transcript by approximately ten fold respect to the wild-type *ACL5* transcript level (Fig. 17D). A careful inspection of leaf veins anatomy in whole mounts and in transverse sections has subsequently revealed that lignification of xylem cells in the midvein and in higher order veins is largely impaired in transgenic lines with high levels of ACL5 activity (Fig. 18).



GUS activity (pmol MU/min/µg)	2,6	1,9	0,3	0,2

Figure 18. Morphology and anatomy of midvein (A-F, S-X), third order vein (G-L) and free-ending vein (M-R) from the fifth leaf of 3-week-old plants. (A-R) Dark-field images of cleared leaves. (S-X) Transverse sections from the basal portion of the leaf stained with toluidine blue. (A, G, M, S) Wild-type. (B, H, N, T) *acl5-1*. (C, I, O, U) *ACL5::ACL5:GUS* transgenic line wt_a1 and (D, J, P, V) wt_a2. (E, K, Q, W) *ACL5mut::ACL5:GUS* transgenic line mut_a1 and (F, L, R, X) line mut_a2. Asterisks and arrows indicate not lignified vascular elements in the midvein and in the leaf lamina. Lignified elements in (S-X) have been coloured in green. Scale bar: 100 µm

As in the previous analysis vein counting had been based on visualisation in dark field of the autofluorescent lignified vascular elements, the number of veins formed in transgenic lines with high *ACL5* expression/activity could have been underestimated due to their reduced lignification. For this reason, vein quantification was repeated comparing the numbers of morphologically distinguishable veins and free-ending veins observed using Differential Interference Contrast (DIC) optics and autofluorescent veins and free-ending veins observed in dark-field (Fig. 19). This study has revealed that introduction of wild-type *ACL5* sequence into *acl5-1* determines the formation of a number of procambial strands similar to that of wild-type plants independently from the ACL5:GUS expression/activity level reached in the different transgenic lines. Conversely, xylem elements differentiation seems to be strictly linked to ACL5:GUS protein level, being accelerated in the loss-of-function mutant (Muñiz et al. 2008) and impaired in transgenic lines with elevated levels of ACL5:GUS protein (Fig. 19).



Q-PCR	Co-0	acl5-1	wt_a1	wt_a3	mut_a1
endogenous ACL5	1,0	59,0	0,1	0,5	0,4
ACL5 transgene			20,20	3,7	4,7

GUS activity	Co-0	acl5-1	wt_a1	wt_a3	mut_a1
pmol MU/min/µg			2,6	0,4	0,3

Figure 19. Comparison of the number of lignified *vs* the total number of veins and free-ending veins in 16 mm^2 of the fifth leaf of 3-week-old plants. Values are mean ± SE (n=15). * P<0,01 in *acl5-1 vs*. all other lines; ° P<0,01 in wt_a1 *vs*. all other lines. Below the graph is reported the quantitative analysis of ACL5:GUS expression/activity in the transgenic lines and the level of the endogenous *ACL5* transcript.

Genetic interaction between *HD-ZIP III* genes and *ACL5*

Although the data presented in the previous sections indicate that *ACL5* is a downstream target positively regulated by HD-ZIP III transcription factors, it has been previously reported that *HD-ZIP III* genes expression is negatively regulated by *ACL5*. In fact, as already mentioned in the Introduction, *ATHB8* and *PHB* transcripts levels are increased in *acl5-1* and this increase is rapidly reversed upon heat-shock induction of ACL5 activity in *HS::ACL5* transgenic plants or by addition of its enzymatic product, thermospermine (Imai et al., 2006; Imai et al., 2008; see Fig. 12). In order to investigate in more detail the genetic interaction between *ACL5* and *HD-ZIP III* genes, *acl5-1* was crossed to *rev-5* and *athb8-1 cna-3 rev-5*. To discriminate the different genotypes segregating in the F2 generation, genomic DNA extracted from a cotyledon of individual seedlings was screened by PCR. A combination of gene specific and insertion event specific primers pairs was used to detect individuals homozygous for *athb8-1* and *cna-3* (insertional mutants) while sequence specific probes were used for allelic discrimination and genotyping of *acl5-1* and *rev-5* (single nucleotide mutants). Both *acl5-1 athb8-1 cna-3* triple and *acl5-1 athb8-1 cna-3 rev-5* quadruple mutants were identified among the F2 progeny of the *acl5-1* x *athb8-1 cna-3 rev-5* cross. Like *acl5-1* and *rev-5* single mutants, all mutant combinations are similar to control plants until the transition from vegetative to reproductive growth phase. At this stage the rosette of *acl5-1* appears smaller than that of wild-type plants and exhibits round leaves with short petioles while that of *rev-5* and *athb8-1 cna-3 rev-5* presents leaves with long petioles and rolled-under margins. The rosette of mutant combinations is similar to that of *acl5-1* but, in addition, shows rolled-under leaf margin when the *rev-5* allele is present (Fig 20A). As already shown by Prigge et al. (2005), loss of *ATHB8* and *CNA* functions in the *athb8-1 cna-3* double mutant has no visible phenotypic consequences but reduces the severity of loss of *REV* function in the *athb8-1 cna-3 rev-5* triple mutant due to the partial antagonism between these genes (Fig. 20A, B). After bolting, *rev-5* is characterised by an overgrowth of leaves, that become abnormally large and distorted, and stem, that later becomes longer than that of wild-type plants due to prolonged activity of the inflorescence meristem, empty axils, curled down cauline leaves and semisterility (Fig. 20B; see also Fig. 3. Talbert et al., 1995; Zhong et al., 1997). Conversely, *acl5-1* is characterised by a small rosette and a short inflorescence stem due to reduced cell elongation and precocious proliferative arrest of the apical inflorescence meristem (Fig. 20B; see also Fig. 12. Hanzawa et al., 1997).

A

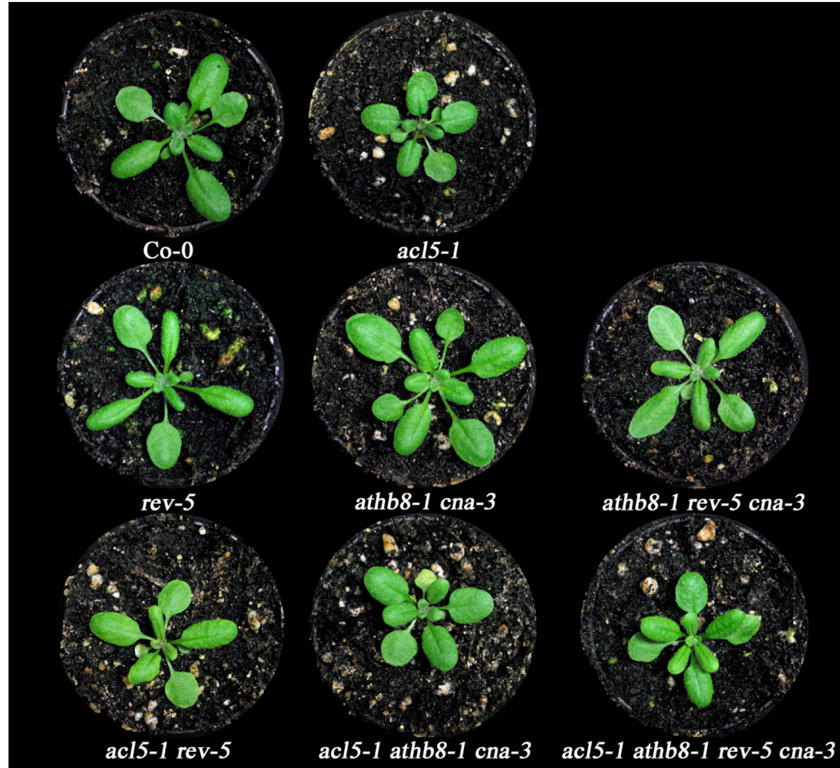


Figure 20. Phenotype of *acl5-1* and *hd-zip III* family single, double and triple mutants and their multiple mutant combinations. (A) 20-day-old plants grown in soil. (B) 38-day-old plants grown in soil. (C) 21-day-old plants grown *in vitro*.

All *acl5-1 rev-5* double, *acl5-1 athb8-1 cna-3* triple, and *acl5-1 athb8-1 cna-3 rev-5* quadruple mutants are characterised by a small rosette and a short inflorescence stem similar to *acl5-1* but displays slightly ruffled and distorted leaves when the *rev-5* allele is present (Fig. 20B). Noteworthy, the semisterility defect of *rev-5* is recovered by the presence of the *acl5-1* allele suggesting that it could be due to thermospermine synthesis deregulation (Fig. 20B). Thus, *acl5-1* appears epistatic to *rev-5* respect to stem elongation, rosette size and fertility but not to leaf growth defects. However, other phenotypes of combinations of *acl5-1* and *hd-zip III* mutants suggest that HD-ZIP III activity is required for the developmental events downstream of *ACL5*. At macroscopic level, combination of *acl5-1* with *rev-5* or *athb8-1 cna-3 rev-5* reverts the upward curling of leaves displayed by *acl5-1* mutant plants grown *in vitro*, giving support to the hypothesis that this phenotype is due to overexpression of *HD-ZIP III* genes (Fig. 20C). In contrast, loss of HD-ZIP III function does not revert the newly described effects of *ACL5* mutation on anthocyanin accumulation and flowering time while hypocotyl hyperproliferation is partially or completely restored in *acl5-1 rev-5* and *acl5-1 athb8-1 cna-3 rev-5,* respectively (data not shown). At microscopic level, a detailed analysis of the fifth leaf revealed that the absence of *REV*, *ATHB8* and *CNA* functions, does not affect *per se* leaf vein patterning but counteracts the increase in veins number caused by *acl5-1* in the *acl5-1 athb8-1 cna-3 rev-5* quadruple mutant (Fig. 21A, B). Intriguingly, mutations in these *HD-ZIP III* genes do not contrast the effect of *acl5-1* on vein thickness (Fig. 21A). Analysis of the root of 5-day-old seedlings has pointed out an antagonistic role of *ACL5* and *HD-ZIP III* genes in this organ. In fact, although none of the mutants significantly affects root length, the number of lateral root primordia is increased in *acl5-1* and decreased in *athb8-1 cna-3* and, even more, in *athb8-1 cna-3 rev-5* respect to wild-type indicating that *ACL5* is necessary to limit and *HD-ZIP III* genes to promote lateral root formation (Fig. 21C; Hawker and Bowman, 2004). Combination of *acl5-1* with *hd-zip III* mutants shows an additive effect, determining the formation of a number of lateral root primordia similar to the wild-type in *acl5-1 athb8-1 cna-3* triple mutant or only slightly reduced in *acl5-1 athb8-1 cna-3 rev-5* quadruple mutant (Fig. 21C). At molecular level, expression analysis by quantitative real-time PCR of RNA extracted from root apices of 1-week-old seedlings has shown that, loss of *REV*, *CNA* and *ATHB8* function does not affect the abundance of *ACL5* transcript in single, double or triple *hd-zip III* mutants while loss of ACL5 activity enhances the level of its own transcript approximately by four-fold (Fig. 22A). Nonetheless, combination of *athb8-1 cna-3* and *athb8-1 cna-3 rev-5* with *acl5-1* partially or completely suppresses the up-regulation of the mutated *ACL5* transcript displayed by the *acl5-1* single mutant (Fig. 22A).

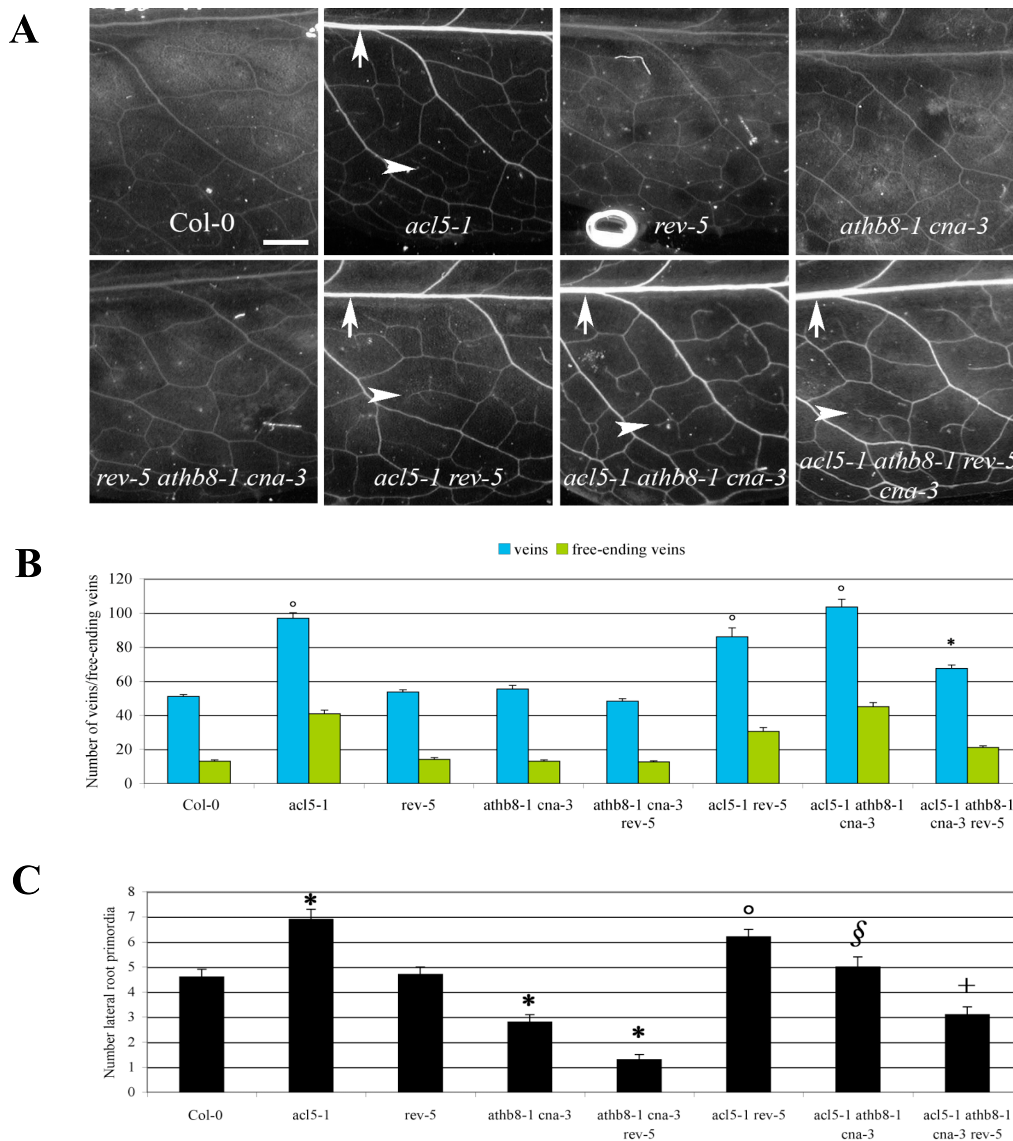


Figure 21. Effect of *acl5-1*, *hd-zip III* family single, double and triple mutants and their combinations on (A, B) leaf vascular development and (C) lateral root formation. (A) Dark-field images of cleared leaves. Arrow, midvein. Arrowhead, free-ending vein. Scale bar: 1 mm. (B) Number of lignified veins and free-ending veins in 16 mm^2 of the fifth leaf of 3-week-old plants. °P<0,01 line *vs*. Col-0; *P<0,01 line *vs*. Col-0, and acl5-1 (C) Number of lateral roots primordia in 5-day-old seedlings. Values are mean ± SE (n=20). *P<0,01 line *vs*. Col-0, °/§/+ P<0,01 line *vs*. the parental lines.

In order to assess whether the different expression levels observed could be due to expansion or reduction in the mutants of the cell populations normally expressing this gene, the root apex of wild-type, *acl5-1* and *acl5-1 athb8-1 cna-3 rev-5* has been analysed in histological sections. This analysis has demonstrated that the anatomy of the differentiation zone at 0.5 cm from the root apex of *acl5-1* and *acl5-1 athb8-1 cna-3 rev-5* is identical to that of wild-type seedlings (Fig. 22B). Therefore, all the changes in expression levels observed directly reflect differential regulation of *ACL5*.

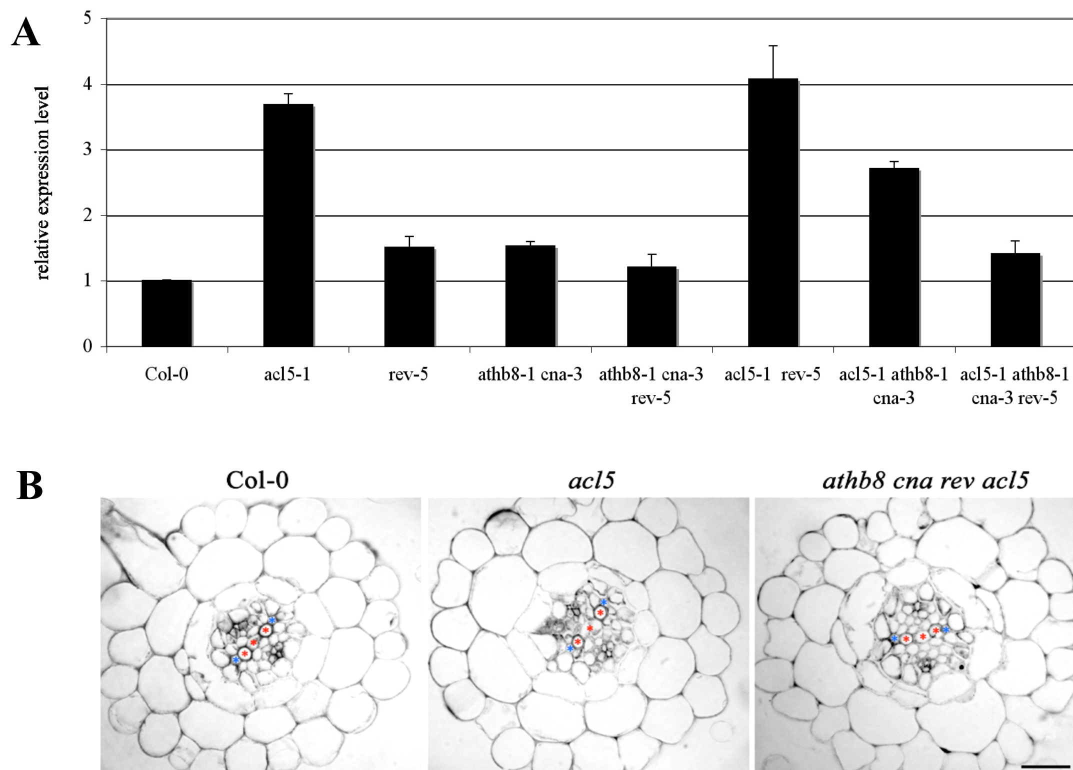


Figure 22. (A) Relative expression levels of *ACL5* wild type and mutant transcripts in the root apex (5 mm) of 1-week-old seedlings of the indicated genotype were measured with quantitative RT-PCR using the *ACTIN2* gene as reference. Values represent averages of relative transcript levels ± SD of three technical replicates normalised to the expression level of each gene in the wild-type set to 1.0. (B) Transverse sections of the root apex of wild-type, *acl5-1* and *acl5-1 athb8-1 cna-3 rev-5* 1-week-old seedlings stained with toluidine blue. Scale bar: 20 μm.

Finally, to extend the characterisation of the functional interaction between *ACL5* and the *HD-ZIP III* family, *acl5-1* was also crossed to *35S::ATHB8* transgenic plants. Seeds of the F2 generation were germinated on kanamycin to select seedlings with at least one copy of the transgene, and genomic DNA was extracted from a cotyledon of individual antibiotic resistant seedlings. After PCR with allele specific probes, *acl5-1* homozygous plants were identified and transferred to soil. Kanamycin resistance segregation analysis of the resulting F3 generation allowed the recovery of double homozygous *35S::ATHB8 acl5-1* plants. A preliminary phenotypic observation indicates a sinergistic interaction between *ATHB8* overexpression and absence of *ACL5*. In fact, *35S::ATHB8 acl5-1* plants have a very severe phenotype with small rosettes, short inflorescence stems, severely hyponastic and up-curled leaves, fewer secondary inflorescences and reduced fertility (Fig. 23A). In agreement with the induction of *ACL5* expression by constitutive or inducible overexpression of *ATHB8* in transgenic plants (Forte et al., manuscript in preparation) and with the result of the molecular analysis of multiple mutants reported above, *ATHB8* overexpression in root tips of *35S::ATHB8 acl5-1* seedlings enhances

the accumulation of the *acl5-1* unproductive transcript (Fig. 23B).

In conclusion, this genetic and molecular study demonstrates that *ACL5* and *HD-ZIP III* genes may act upstream, downstream and independently of each other in plant development and indicates that HD-ZIP III transcription factors are involved in the negative autoregulatory feedback mechanism controlling *ACL5* expression.

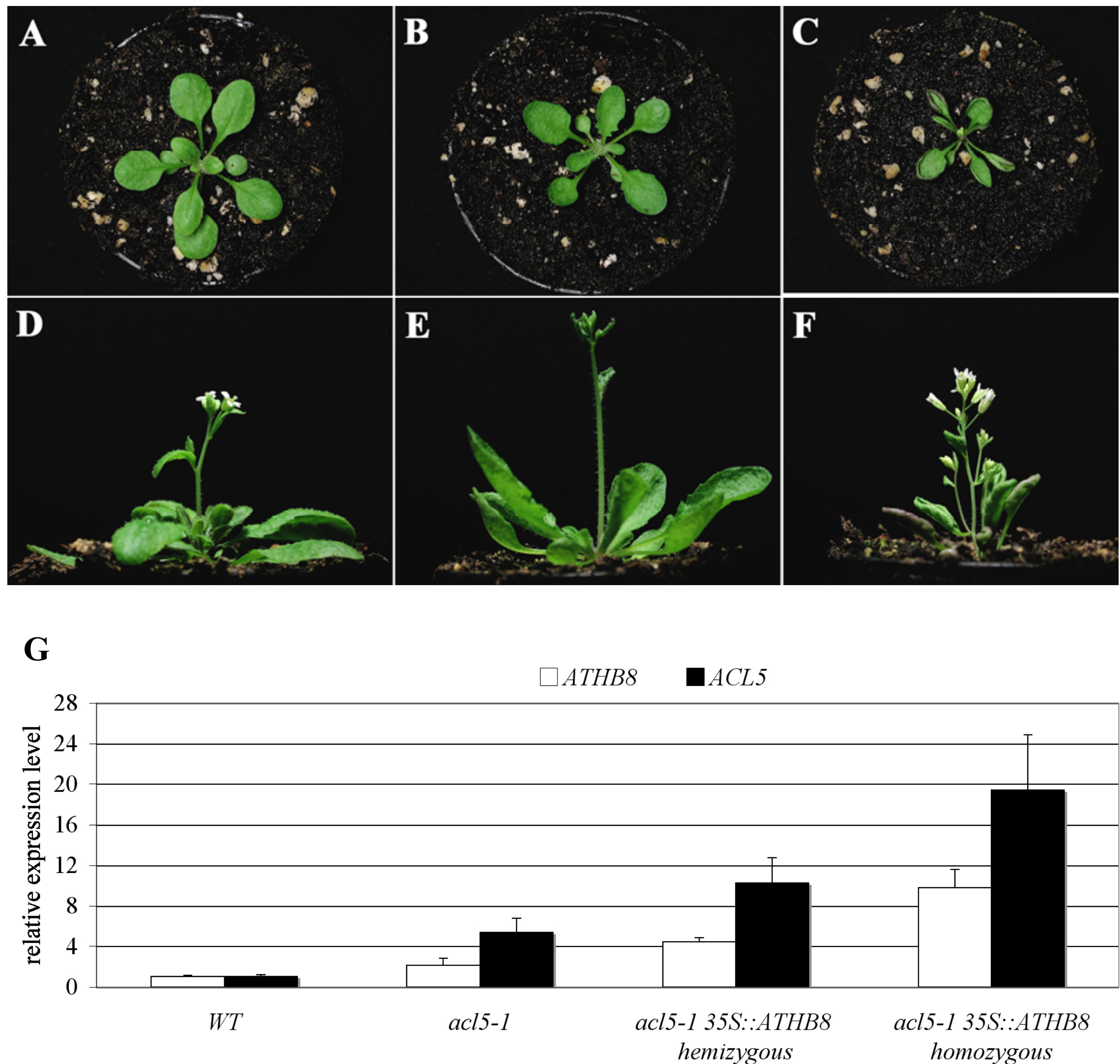


Figure 23. Phenotype of 3-week-old (A-C) and 4-week-old (D-F) *acl5-1* (A, D), *35S::ATHB8* (B, E) and *acl5-1 35S::ATHB8* (C, F) plants. (B) Relative expression levels of *ACL5* wild type and mutant transcripts and *ATHB8* endogenous and transgene transcripts in the root apex (5 mm) of 1-week-old seedlings of the indicated genotype were measured with quantitative RT-PCR using the *ACTIN2* gene as reference. Values represent averages of relative transcript levels ± SD of three technical replicates normalised to the expression level of each gene in the wild-type set to 1.0.

DISCUSSION

The HD-ZIP III binding site is necessary for *ACL5* expression in the early phases of vascular cell differentiation but its function is masked in *acl5-1* complementation

This study, in combination with the data available in the literature, have revealed the existence of a complex functional relationship between the HD-ZIP III family of transcription factors and one of their putative target genes, *ACAULIS5* (*ACL5*), in *Arabidopsis thaliana*.
ACL5 is one of the 390 putative target genes identified by a bioinformatic screening that is associated to the 11 bp pseudo-palindromic sequence (BS-III) bound by HD-ZIP III proteins *in vitro* (Forte et al., manuscript in preparation; Sessa et al., 1998). *ACL5,* that encodes a polyamine biosynthetic enzyme, thermospermine synthase, presents a striking expression and functional overlap with HD-ZIP III genes (Hanzawa et al., 1997, 2000; Clay and Nelson, 2005; Knott et al., 2007; Kakehi et al., 2008; Muñiz et al., 2008). Moreover, the BS-III site is located in the *ACL5* promoter 89 bp upstream the translation initiation codon, in an ideal position to influence its transcription. *In vitro* binding assays and expression analyses have given experimental support to the hypothesis that *ACL5* could be a genuine primary target gene of HD-ZIP III genes (Forte et al., manuscript in preparation).
To test whether the BS-III element plays a functional role on *ACL5* gene regulation *in vivo*, the BS-III sequence in the *ACL5* promoter was mutated so as to abolish binding of HD-ZIP III transcription factors and the effects on *ACL5* expression profile were studied in transgenic *Arabidopsis* plants using a reporter gene fusion strategy. The comparison of ACL5:GUS fusion protein expression patterns driven by the wild-type and mutated *ACL5* promoter clearly demonstrates that the BS-III sequence plays a fundamental role for the expression of *ACL5* in vascular meristem cells in leaf and root. In leaf primordia, *ACL5* expression is at first detected in morphologically distinguishable procambial cells and is preceded by *ATHB8* expression in pre-procambial cells. Detailed analysis of leaf primordia at different developmental stages has shown that mutagenesis of the BS-III sequence in the *ACL5* promoter delays the appearance of GUS reporter expression indicating that it negatively impacts *ACL5* expression in the early phases of procambial cells differentiation. In the root, *ACL5* expression initiates in the meristematic region of the root apex in vascular precursor cells immediately above the quiescent center, while in the lateral roots primordia it appears in vascular precursor cells when the new root meristem and the connection to the vascular system of the primary root have been re-established. Also in the primary and lateral roots, mutagenesis of the BS-III site impairs *ACL5* expression in the first stages of differentiation of the meristematic cells of the vascular tissue

and *ACL5* expression is activated only in the elongation zone at the end of the phase of active proliferation.

These observations confirm the functional relevance of the BS-III element *in vivo* and, together with other experimental evidences (Forte et al., manuscript in preparation), indicate that *ACL5* is a genuine direct target of this transcription factors family. However, chromatin immunoprecipitation experiments (ChIP) are in progress to definitively demonstrate direct binding of HD-ZIP III proteins to the *ACL5* promoter *in vivo*.

The altered GUS expression pattern of *ACL5mut::ACL5:GUS* transgenic plants suggests that functional disruption of the BS-III element could reduce the ability of the ACL5:GUS fusion protein to complement the *acl5-1* mutation when expressed under the control of the mutated *ACL5* promoter. However, a careful macroscopic and microscopic examination of the phenotype of *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* transgenic plants clearly demonstrated that this is not true. In fact, all the phenotypic alterations of *acl5-1* plants could be rescued by the ACL5:GUS fusion protein expressed under the control of both the wild-type and mutated promoter. Thus, the regulatory potential of the BS-III element appears to be concealed in the *acl5-1* mutant complemented with *ACL5mut::ACL5:GUS*. The fact that *ACL5:GUS* expression under the control of the *ACL5* promoter mutated in the BS-III element is equally able to complement the *acl5-1* mutation could be explained by at least two different mechanisms. One possibility is that functional compensation could rectify defects due to *ACL5* misexpression consequential to BS-III element mutagenesis. BS-III element mutagenesis delays but does not completely abolish *ACL5* expression in differentiating vascular cells, indicating a possible redundancy of regulatory elements recruiting the same or other types of transcription factors. Intriguingly, several other non canonical (degenerated) potential binding sites for HD-ZIP III proteins have been identified in the *ACL5* regulatory region (A. Peñalosa, personal communication) and these could substitute for the mutated BS-III element although with a lower affinity. Thus, at least in normal conditions, vascular differentiation can proceed in *acl5-1* plants complemented with the *ACL5mut::ACL5:GUS* construct even if with a possible delay respect to plants complemented with the *ACL5::ACL5:GUS* construct. Amelioration of early vascular defects during organ development is not unprecedented and, in particular, it has been recently described also for the *athb8-11* mutant (Donner et al., 2009). In fact, the defects observed at early stages of vein formation in *athb8* leaves are subsequently corrected and eventually resolve into a normal leaf vascular pattern (Baima et al., 2001; Prigge et al., 2005; Donner et al., 2009). It has been hypothesised that *ATHB8* could have an ephemeral role confined to early stages of vascular strand formation without consequences on later stages or/and could be functionally

compensated by other members of the HD-ZIP III family (Donner et al., 2009). In a similar fashion to *ATHB8*, *ACL5:GUS* expression under the control of the *ACL5* promoter mutated in the BS-III element could determine vascular defects in the early phases of leaf primordia development that are without consequences on later stages or that have been unnoticed owing to the subsequent recovery of normal vein patterning. Moreover, the fact that *ACL5* is a target of HD-ZIP III transcription factors raises the possibility that recovery of irregular vein formation in leaves of *acl5-1* plants complemented with *ACL5mut::ACL5:GUS* could be due to the same rescue mechanism operating in *athb8* leaves. Alternatively, as *ACL5* function is non-cell-autonomous (Hanzawa et al., 2000) and polyamines can be transported over distances through xylem and phloem (Hanzawa et al., 2000), the presence of ACL5 activity in some cells could be sufficient to overcome its absence in other cell type caused by the altered expression of *ACL5mut::ACL5:GUS* in *acl5-1* plants. The study of the dynamics of vein differentiation in *acl5-1* plants complemented with *ACL5mut::ACL5:GUS* by expression analysis of early vascular specific markers will be necessary to discriminate between these hypotheses and to elucidate the mechanism that conceal the regulatory potential of the BS-III element.

ACL5 affects the progression of xylem cells differentiation in a dose-dependent way

This study has also provided additional clues for the functional characterisation of *ACL5*. Recently, it has been proposed that *ACL5* controls the progression of procambial cells differentiation and ensures proper xylem specification and cell wall deposition by preventing premature cell death of tracheary elements (Knott et al., 2007; Muñiz et al., 2008). Intriguingly, the data presented here have further extended this hypothesis by indicating that xylem differentiation is linked to ACL5 dosage. In fact, a detailed analysis of leaf vein pattern in the *acl5-1* mutant complemented with the *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS* has pointed out that although ACL5 is necessary and sufficient to restore the number of procambial strand formed, at high levels it causes a reduction in vein lignification. Thus, enhanced expression of *ACL5* demonstrates that increased levels of ACL5 delay or even totally inhibit the progression of procambial cells differentiation into tracheary elements. In this respect, it is important to point out that, high transgene transcript levels result from increased expression in the same cell types in which it is normally expressed (entopic overexpression) as ACL5:GUS production is driven by the *ACL5* promoter. Whether transcript levels higher than in the wild-type obtained upon complementation of *acl5-1* are due to an increased stability of the

ACL5:GUS transcript, to the loss of a negative regulatory element in the chimeric gene or to the insertion of the transgene near an enhancer is currently unknown. Noteworthy, due to the negative autoregulatory feedback loop, entopic overexpression not only abolishes the *acl5-1* transcript up-regulation but also reduces the level of the endogenous *ACL5* transcript by approximately ten fold with respect to the wild-type level indicating an attempt of the plant to buffer excessive ACL5 production.

ACL5 plays additional roles in plant development

In addition to its dosage-dependent regulation of vascular system development, previously unnoticed phenotypes of *acl5-1* mutant were described in this thesis suggesting that *ACL5* has pleiotropic effects and affects several other plant developmental processes mainly linked to cell proliferation and stress response.

Root development is under the control of hormonal, metabolic, and environmental cues that can act on genetically controlled developmental programmes and thus affect the plasticity of root architecture. In addition to the five 'classical' plant hormones, also polyamines have been involved in the regulation of the different aspects of root development such as primary root growth and lateral and adventitious root formation in several plant species (Couee et al., 2004). Although it has been previously reported that mutation of *ACL5* has no effect on root growth apart for enhanced sensitivity to cytokinins (Hanzawa et al., 1997; Clay and Nelson, 2005), formation of a significantly higher number of lateral root primordia (LRP) was observed in the *acl5-1* mutant seedlings during this study. Conversely, primary root length is not significantly and consistently affected in *acl5-1*. Similarly, despite the precocious and high expression of *ACL5* in the root vascular cylinder (Birnbaum et al., 2003; Clay and Nelson, 2005; this study), patterning and differentiation of the vascular tissue in the primary root apex is unchanged in *acl5-1*. These observations separate the effect of *acl5* mutation on lateral root formation from primary root growth and differentiation. This is not totally unexpected as there is considerable evidence that the developmental stages of lateral root formation (initiation, LRP formation, LRP emergence, and apical meristem activation) are independently regulated (Malamy, 2005). By contrast, a marked reduction in the development of the root apparatus was noticed in adult plants. Further experiments will be needed to assess if the discrepancy between the number of lateral root primordia formed in young seedlings and the low complexity of the root system of adult plants is due to a premature arrest of primordia elongation or to a different rate of new

primordia formation in different phases of plant growth. In the latter case, long-distance communication between shoot and root could coordinate growth of the root apparatus with reduced aerial growth after the transition to reproductive phase. Alternatively, as polyamine metabolism is responsive to stress conditions (see below) and polyamines may play a role in environmentally-induced plasticity of root development (Couee et al., 2004), root architecture of adult *acl5-1* plants could indicate a differential plant response to external stimuli. Nonetheless, defective development of the root apparatus rather seems to be the cause than the consequence of an altered stress response as reduction of the root apparatus is observed also when plants are grown *in vitro* under optimal conditions (see below).

As lateral root formation is dependent on auxin-mediated reactivation of the cell cycle in pericycle cells adjacent to xylem poles, and polyamines have been associated with cell proliferation in animal and plant cells being required for cell cycle progression (Bouchereau et al., 1999; Couee et al., 2004) it is intriguing that mutation of *ACL5* also affects proliferation in the hypocotyl. In flowering plants, both hyperproliferation and hypertrophy are strikingly evident in all cell layers but the most remarkable features of *acl5-1* hypocotyls are the loss of coordination between adjacent cells and tissues, and the presence of abnormal, variable division planes resulting in the formation of nodule-like structures and in complete destruction of the normal radial symmetry of the organ. Abnormal proliferation and loss of symmetry are already visible in hypocotyls of 2-week-old *acl5-1* plants, but restricted to the vascular cylinder. Asymmetry of the stele and of the whole hypocotyl as well as increased xylem proliferation in hypocotyls has also been described by Muñiz et al. (2008) in seven-day-old seedlings and thirteen-day-old seedlings, respectively. However, in contrast to this study, no further secondary growth was observed in 35-day-old *acl5* mutant plants, resulting in hypocotyls significantly thinner than those of the wild type (Muñiz et al., 2008). A possible explanation for this discrepancy is that different *acl5* alleles in different backgrounds possibly harbouring genetic modifiers were used in the two studies. Taking into account that abnormal proliferation and loss of symmetry first appear in the stele, it is tempting to speculate that these alterations could be a consequence of defective vascular differentiation. However, a direct effect of *ACL5* and thermospermine on cell proliferation and differentiation or on hormone signalling pathways independent from its role on vascular development cannot be excluded at present, and further histological characterisation with the aid of molecular markers will be necessary to better understand the origin of the cambium-like cells and of uncoordinated growth. Remarkably, while a positive correlation is generally found between polyamine levels and cell proliferation, these observations indicate that thermospermine acts negatively on cell-cycle progression.

Interestingly, a negative effect on cell division apparently due to a decrease in spermidine levels has also been observed for spermine in the unicellular green alga *Chlamydomonas reinhardtii* indicating that cell-cycle regulation could depend on fine tuning of the relative levels of the different polyamines.

Abnormal differential proliferation and growth of petiole, that bents upward, and of adaxial and abaxial leaf surfaces, that curves the leaf lamina upward and then inward from its lateral sides, likely is also at the origin of the hyponastic and curled leaves phenotype when *acl5-1* mutant is grown *in vitro*. Intriguingly, the up-curled character of *acl5-1* rosette leaves strikingly resembles the phenotype of mutant and transgenic plants overexpressing HD-ZIP III genes (Baima et al., 2001; McConnell et al., 2001; Ochando et al., 2006, 2008; see also Fig. 6) or missing genes that antagonise their function (Wenkel et al., 2007; Kim et al., 2008).

Another prominent phenotype of *acl5-1* rosette is the appearance, after bolting of the flowering stem, of purple pigments, which is most probably due to an accumulation of anthocyanins. Synthesis of anthocyanins is stimulated by abiotic and biotic stresses and is regulated by a complex interaction between internal and external stimuli such as temperature, light, sugar, water deficit, inorganic macronutrients limitation and several plant hormones. (Loreti et al., 2008). In higher plants, synthesis of polyamines is also stimulated by abiotic and biotic stresses such as salinity, drought, osmotic stress, mineral nutrient deficiencies, heat and chilling, hypoxia and is affected in response to exogenous hormones (namely auxins, cytokinins and gibberellins) (Bouchereau et al., 1999). Hence, this pigmentation phenotype could indicate an enhanced (water) stress response of *acl5-1* possibly due to abnormal root and vascular development. It is interesting in this respect that purple pigment accumulation is not observed when *acl5-1* is exposed to high humidity such as when it is grown *in vitro.* Intriguingly, purple pigmentation and induction of genes involved in the anthocyanin biosynthetic pathway has been observed also in transgenic plants overexpressing a negative regulator of *HD-ZIP III* genes, the complementary miR165, that also down-regulates *ACL5* expression (Zhou et al., 2007).

Finally, the effect of *acl5-1* on bolting and flowering time is not totally surprising as available experimental evidences indicate that polyamines affect not only vegetative growth but also reproductive development, modulating flowering and fruit maturation in various plant species including *Arabidopsis* (Huang et al., 2004; Applewhite and Galston, 2000). In constrast to the phenotype observed in this study, the same *acl5-1* allele but in a different *Arabidopsis* background (Lansberg *erecta*, L*er*) has been reported to form the same number of leaf and to flower at the same time as wild-type plants (Hanzawa et al., 1997). Thus, further physiological and genetic interaction studies with flowering time mutants will be needed to discriminate if

acl5-1 accelerates the transition from the vegetative to the reproductive phase of growth or if it simply accelerates bolting of the flowering stem, and to elucidate the molecular basis of the effect of *acl5* mutation on this processes. Flowering time is controlled by both endogenous factors, such as an autonomous genetic program and gibberellins, and by interaction with environmental stimuli, such as light quality and period as well as low temperatures (Yant et al., 2009). In addition, early flowering is also often induced in response to stress conditions. This raises the possibility that anticipated flowering could be another consequence of reduced growth and enhanced stress response of *acl5-1* plants. Interestingly, a recent study searching for quantitative trait loci (QTL) controlling secondary growth in *Arabidopsis* hypocotyls has also highlighted a correlation between induction of flowering and induction of xylem expansion through the stimulation of the late stage of cambial activity (Sibout et al., 2008).

ACL5 and HD-ZIP III transcription factors act upstream, downstream and independently of each other in plant development

Together, the molecular and functional data obtained by means of different *in vitro* and *in vivo* approaches strongly indicate that *ACL5* is a genuine downstream target of HD-ZIP III transcription factors. However, it has also been shown that *ACL5* negatively regulates *ATHB8* and *PHB* transcripts levels suggesting that *HD-ZIP III* genes are downstream of *ACL5* (Imai et al. 2006, Kakehi et al. 2008). Thus, a comprehensive genetic analysis of double and multiple combinations of *acl5* and *hd-zip III* mutants was undertaken to extend our knowledge on the functional relationship between *ACL5* and the HD-ZIP III gene family.

Detailed phenotypic analysis of flowering plants have shown that while *rev-5* have larger leaves and longer stems than wild-type and is semi-sterile, the progeny of the cross of *acl5-1* to *rev-5* and to *athb8-1 cna-3 rev-5* has small rosettes and short inflorescence stems and is fully fertile like *acl5-1*. Interestingly, the effect of *rev-5* on leaf curvature, causing downward curling of the leaf margin, and distortion is maintained in all mutant combinations. These observations indicate that *acl5-1* is epistatic to *rev-5* with respect to stem elongation, rosette size and fertility, and are consistent with *ACL5* acting downstream of *HD-ZIP III* genes. However, other phenotypes of *acl5-1 rev-5* and *acl5-1 athb8-1 cna-3 rev-5* have revealed that HD-ZIP III proteins also take part to the developmental regulatory events downstream of *ACL5*. First, as already suggested above owing to the phenotypic similarity with *HD-ZIP III* overexpressing mutants and transgenic plants, HD-ZIP III activity is necessary for the curled phenotype

observed *in vitro* upon mutation of *ACL5*. In fact, this phenotype is reverted in *acl5-1 rev-5* and *acl5-1 athb8-1 cna-3 rev-5*. Second, combination with three *hd-zip III* mutants ameliorates the leaf venation phenotype of *acl5-1*. Available evidences indicates that, in *Arabidopsis*, vein pattern arises through recruitment of ground meristem cells into new pre-procambial strands until this process becomes terminated by the differentiation of mesophyll (Scarpella et al., 2004). It has been recently shown that *ATHB8* plays a fundamental role in leaf vein patterning being required to restrict and stabilise the identity of the pre-procambial cells in leaf primordium (Donner et al., 2009). Moreover, it has been suggested that other HD-ZIP III family members could act redundantly and compensate *ATHB8* function as no obvious changes are seen in leaves of *athb8* mutant plants (Donner et al., 2009). Therefore, it is not surprising that the presence of functional *HD-ZIP III* genes is essential to sustain the formation of an increased number of veins in *acl5-1* enabling the recruitment of ground meristem cells into pre-procambial strand. Accordingly, *ATHB8* and *PHB* are found to be upregulated in *acl5-1* (Imai et al., 2006). Intriguingly, loss of *ATHB8, CNA* and *REV* activity decreases the number of veins induced by *acl5-1* but not their thickness. Although it has been suggested that the increase in vascular cell numbers found in *acl5* mutant leaf veins could also be due to an increase in outside cell recruitment (Clay and Nelson, 2005), the observation that HD-ZIP III genes are not necessary for the vein thickness phenotype would argue against this hypothesis, and would rather suggest that it is due to an *HD-ZIP III* independent increase of cell divisions within the procambium. Third, HD-ZIP III activity is necessary for the hypocotyl hyperprolipheration phenotype observed upon mutation of *ACL5* as demonstrated by partial and complete restoration of this phenotype observed in *acl5-1 rev-5* and *acl5-1 athb8-1 cna-3 rev-5*, respectively. Fourth, *HD-ZIP III* genes are necessary to maintain the negative-feedback loop controlling *ACL5* expression. In fact, although the level of *ACL5* transcript is unchanged by loss of *REV*, *CNA* and *ATHB8* function *per se*, combination of *athb8-1 cna-3* and *athb8-1 cna-3 rev-5* with *acl5-1* partially or completely counteracts the up-regulation of the mutated *acl5* transcript that is observed in *acl5-1*. In addition, a much higher level of *acl5* unproductive transcript accumulation is obtained in *35S::ATHB-8 acl5-1* plants. Noteworthy, the effect of *hd-zip III* mutants and *ATHB8* overexpression on *acl5* transcript abundance in root apices directly reflect differential regulation of *ACL5,* as histological analysis has shown that the anatomy of *acl5-1* and *acl5-1 athb8-1 cna-3 rev-5* root tips is identical to that of wild-type seedlings, as previously described for *35S::ATHB-8* (Baima et al., 2001) and *tkv* (Clay and Nelson, 2004).

In addition to the genetic interactions described above, other data indicates that *HD-ZIP III* genes and *ACL5* can also have additive effects. In agreement with the phenotypic similarities

displayed by *acl5-1* and *35S::ATHB8* plants, that are both characterised by a reduced stem elongation, hyponastic and up-curled leaves and increased xylem differentiation (Baima et al., 2001; Hanzawa et al., 1997, 2000; Clay and Nelson, 2004), a preliminary observation have shown that *35S::ATHB-8 acl5-1* plants exhibit a markedly more severe phenotype. This synergistic interaction between *ATHB8* overexpression and absence of *ACL5* suggests that *ACL5* and *ATHB8* have antagonistic roles. Similarly, an antagonistic role of *HD-ZIP III* genes and *ACL5* has been observed also in the formation of lateral roots. In fact, in comparison with the wild-type, the number of lateral root primordia is moderately and severely decreased in *athb8-1 cna-3* and *athb8-1 cna-3 rev-5,* respectively, and is increased in *acl5-1*. Also in this case the effect of the genetic interaction of *ACL5* and *HD-ZIP III* genes is additive, since a number of lateral root primordia similar to the wild-type or only slightly reduced is formed in the *acl5-1 athb8-1 cna-3* triple and *acl5-1 athb8-1 cna-3 rev-5* quadruple mutants, respectively.
Finally, to further complicate matters, *ACL5* seems to act independently of *HD-ZIP III* genes on some aspects of plant growth. In fact, two of the new phenotypes described for *acl5-1,* namely anthocyanin accumulation and flowering time anticipation, remain unchanged when *acl5-1* is combined with *hd-zip III* mutants. Interestingly, these two phenotypes seem to be more linked to an altered adaptative response to environmental conditions than to developmental regulation.

A working model for the regulatory loop mediated by *HD-ZIP III* genes and *ACL5* for the control of vascular development in *Arabidopsis*

Taken together, the data presented in this work demonstrate a complex functional interaction between *HD-ZIP III* genes and *ACL5*. Regulatory complexity is emerging as a recurrent theme in plant developmental biology, and molecular and functional data accumulated in *Arabidopsis* in recent years clearly indicate that genetic interactions between plant growth regulators can best be described by feedback loops than by linear pathways. The acknowledgement that both auxin and transcription factors may be both upstream and downstream in patterning events is a striking example of this concept (Izhaki and Bowman, 2007).
By integrating the data obtained here with available evidences from the literature, the functional relationship between *HD-ZIP III* genes and *ACL5* in the control of vascular development can be described as a negative feedback regulatory loop as shown in the working model proposed in Figure 24. Although it is not currently possible to assess the peculiar contribution of each individual *HD-ZIP III* family member in this circuit nor to exclude any of them, *ATHB8* is

indicated in the model as it is the gene more specifically involved in vascular differentiation and for which most of the functional relations indicated has been actually demonstrated.

The hormone auxin initiates and controls patterning and differentiation of the vascular system by activating *ATHB8* transcription through the *MONOPTEROS (MP) / AUXIN RESPONSIVE FACTOR 5 (ARF5)* transcription factor. Another not yet identified ARF family member could mediate the auxin dependent increase of *ACL5* transcription (Hanzawa et al., 2000; Donner et al., 2009). In turn, ATHB8 activity is required to restrict and stabilise the identity of the meristematic pre-procambial cells in organs primordia and to promote vascular differentiation (Baima et al., 2001; Donner et al., 2009). ACL5 activity is also necessary for proper progression of this process and to avoid premature cell death during the differentiation of procambial cells into xylem elements but high ACL5 levels delay or even totally inhibit vessels maturation (Muñiz et al., 2008; this work). *ATHB8* expression precedes that of *ACL5* during the establishment of the vascular meristem (being visible in morphologically undistinguishable pre-procambial cells the former, and in already elongated procambial cells the latter) and ATHB8 protein binding to the BS-III element in the promoter positively regulates *ACL5* expression (this work). In turn, ACL5 negatively regulates *ATHB8* and *PHB* expression by a mechanism likely involving the positive effect of its enzymatic product, the polyamine thermospermine, on the expression of *SAC51*, a bHLH transcription factor whose dominant gain-of-function mutation suppresses the *acl5-1* phenotype (Imai et al., 2006; Kakhei et al., 2008). In this way, *ATHB8* and other *HD-ZIP III* genes become an essential integral part of the feedback loop, sustained by its product thermospermine, that negatively regulates *ACL5* expression (Hanzawa et al., 2000; Kakehi et al., 2008; this work). In addition, ACL5 and thermospermine provide a further mechanism ensuring fine-tuning of HD-ZIP III activity, in a similar fashion to the other known targets of the HD-ZIP III family, the LITTLE ZIPPER (ZPR) proteins (Wenkel et al., 2007; Kim et al., 2008).

In summary, this model suggests that progression of procambial cells differentiation is regulated by auxin through an enzymatic rheostat, formed by ACL5 and thermospermine, acting on the expression levels of *HD-ZIP III* genes.

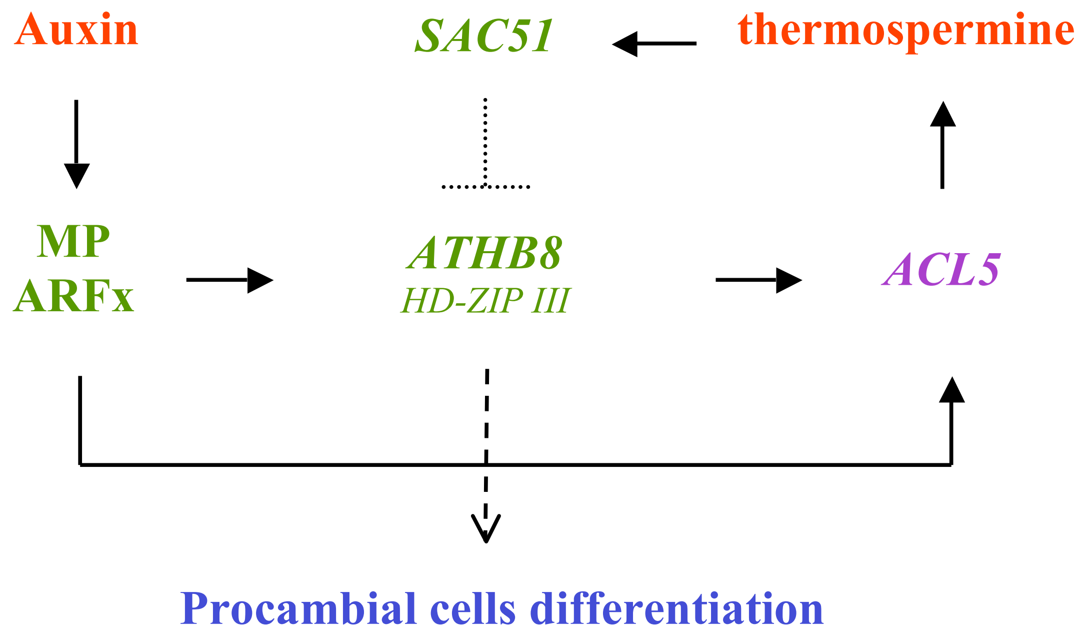


Figure 24 A working model showing the regulatory loop mediated by *HD-ZIP III* genes and *ACL5* for the control of procambial cells and tracheary elements differentiation. Red, signalling molecules. Green, transcription factors. Lilac, enzyme.

MATERIALS AND METHODS

Plant materials and Growth conditions

All the *Arabidopsis thaliana* mutants and transgenic lines were in the Columbia (Col-0) background except when differently specified. The *acl5-1* allele (Imai et al, 2006) and *rev-5* allele (Talbert et al., 1995) were kindly donated by Taku Takahashi and Luca Comai, respectively. The *35S::ATHB8* transgenic plants, in Wassilewskija (WS) background, and the *athb8-1* allele were described previously (Baima et al. 2001). The *athb15-1/cna-3* allele was isolated from the BASTA T-DNA activation tagging mutant collection (Sussman et al., 2000) of the *Arabidopsis* Knockout Facility (AKF, University of Wisconsin, Madison; http://www.biotech.wisc.edu/Arabidopsis/default.htm) by a PCR screen according to Krysan et al. (1999) and is in Wassilewskija (WS) background. The *athb8-1 cna-3* double and *athb8-1 cna-3 rev-5* triple mutants have been generated previously in our laboratory. The *athb8-1 cna-3* double mutant has a mixed Col-0/WS background while the *athb8-1 cna-3 rev-5* triple mutant was backcrossed three times to *athb8-1 rev-5* to eliminate WS background modifiers.
Arabidopsis plants were grown in growth chambers at 21 °C, with a 16 h light /8 h dark cycle, and a light intensity of 140 μmol sec^{-1} either in soil or on synthetic MS medium (1x Murashige and Skoog salts (pH 5.8), 1% sucrose, 0.8% agar) in plates or Phytatrays (ICN) closed with 3MM Micropore tape. To synchronise germination, seeds were germinated after being cold treated for 3 to 4 d at 4 °C in the dark (stratification). For *in vitro* growth, seeds were surface sterilized for 10 min in 30% (v/v) commercial bleach and 0.02% Triton X-100, rinsed three times with sterile distilled water, dried in a laminar flow hood and placed on synthetic medium before stratification.

Plasmid constructions and Plant transformation

The 1983 bp of the *ACL5* genomic sequence upstream of the ATG was amplified from *Arabidopsis* Col-0 DNA with P_ACL5_F and P_ACL5_R (see Table 2) using Accuprime Pfx polymerase (Invitrogen) and cloned into the pENTR^{TM}/D-TOPO^{R} vector (Invitrogen). The wild-type promoter sequence was then used as a template for a two-step mutagenesis protocol to introduce four point mutations in the putative HD-ZIP III binding site. PCR amplification was first performed using P_ACL5_F/mutACL5_R and mutACL5_F/P_ACL5_R primer pairs (see Table 2). The PCR products were subjected to a second round of amplification with P_ACL5_F and P_ACL5_R, and cloned into pENTR^{TM}/D-TOPO^{R} vector. After checking the sequence, both

the wild type and mutated promoter were cloned by LR clonase (Invitrogen) recombination into pMDC162 GATEWAY^{TM} binary vector (Curtis and Grossniklaus, 2003) that contains the sequence encoding the β-glucuronidase (GUS) reporter, resulting in *ACL5::GUS* and *ACL5mut::GUS*. RNA from 8-d-old Col-0 seedlings was retro-transcribed with Superscript III (Invitrogen) and amplified with ACL5_*Asc*I_F and ACL5_*Asc*I_R primers and Accuprime Pfx polymerase to obtain the 1017 bp ACL5 coding sequence without the stop codon. The amplified fragment was digested with *Asc*I and cloned into *Asc*I digested *ACL5::GUS* and *ACL5mut::GUS* resulting in *ACL5::ACL5:GUS* and *ACL5mut::ACL5:GUS*. The two constructs were checked by sequencing to confirm the orientation and the absence of errors in the *ACL5* coding sequence, introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 and used to transform *Arabidopsis* by floral dip method (Clough and Bent, 1998). Both Col-0, for reporter gene analysis, and *acl5-1*, for complementation analysis, were used for *in planta* transformation. Transformants were selected on agar medium containing 20 μg ml^{-1} of hygromycin. Independent transgenic lines that segregated 3:1 for antibiotic resistance marker in the T2 generation were selected for further analysis. Plants homozygous for the transgene were identified in the T3 generation as those showing 100 % antibiotic resistant progeny.

GUS reporter activity analysis

Localisation of GUS activity in plant tissues was analysed by histochemical staining as described by Scarpella et al. (2004). Seedlings were fixed in 90% acetone at –20 °C for 1 hour, washed briefly with 100 mM phosphate buffer (pH 7,7) and incubated at 37 °C in the dark in freshly prepared buffer containing 100 mM sodium phosphate (pH 7,7), 10 mM sodium EDTA, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-gluguronic acid (INALCO) and 0,5 (roots) or 2,5 mM (leaf primordia) ferrocyanide/ferricyanide mix. For analysis of leaf primordia, seedlings were pre-treated with vacuum at room temperature for 15 min prior to incubation at 37°C to facilitate the uptake of the X-gluc substrate. After staining for 2 (roots) or 3 (leaf primordia) hours the plants were transferred to an ethanol:acetic acid (3:1, v/v) mix for 1-16 h at room temperature to terminate the reaction and fix the tissues. Samples were stored at 4 °C in 70% ethanol, and cleared in chloral hydrate:glycerol:water mix (8:2:1, w/v/v; Weigel and Glazebrook, 2002) before observation with an Axioskop2 Plus microscope (Zeiss) equipped with Differential

Interfase Contrast (DIC) optics. Micrographs were taken with a Coolpix 990 (Nikon) digital camera.
Quantification of GUS activity in plant tissues was determined by fluorometric assay as described previously (Jefferson et al., 1987). Crude protein extracts were obtained by resuspending grinded frozen plant material (from a pool of at least 8 individuals) in a buffer containing 50 mM sodium phosphate (pH 7,6), 10 mM sodium EDTA, 0,1 % Triton X-100, 0,1% SDS and 10 mM DTT. An aliquot of the extract was incubated at 37 °C in the dark for 1 h in the presence of 4-methyl-umbelliferyl-β-D-glucoronide (MUG) at the final concentration of 1 mM. After stopping the reaction with 0.2 M Na_2CO_3, fluorescence of the MU produced was measured using an Infinite M200 spectrofluorophotometer (TECAN) setting λ_{ex}= 365 nm and λ_{em}= 455 nm. For each extract, the fluorescence of a reaction stopped immediately after addition of the MUG substrate was also measured to subtract aspecific fluorescence background. For normalisation, protein content was determined using the Bradford assay (BioRad). Triplicate assays for each extracts were averaged.

Gene expression analysis

For gene expression analysis in transgenic lines, all the aerial part of 3-week-old plants grown in Phytatrays was collected and immediately frozen in liquid N_2 for RNA extraction. For gene expression analysis in multiple mutants, about 500 seeds were surface sterilised, stratified, and sawn in a row on a nylon mesh (Nytex 44, SEFAR) laid on square plates containing MS medium and grown in vertical orientation. After 1 week, only the root tips (approximately 5 mm) of the seedlings were collected and immediately frozen in liquid N_2 for RNA extraction.
Total RNA was extracted with the RNAeasy Plant Mini Kit (QIAGEN) according to manufacturer's specifications with the addition of the on-column DNAse I digestion step, and 2 μg were used for cDNA synthesis with random examers and Superscript III first strand synthesis kit (Invitrogen). Real-time PCR was performed with the ABI Prism 7900 HT Sequence Detection System and SYBR-Green PCR Master Mix (Applied Biosystem) as described in the manufacturer's protocol. Amplification reactions were performed in triplicate and were prepared in 20 μl using 1 μl of five-fold diluted cDNA, and 300 nM of forward (F) and reverse (R) primers. PCR primers were designed using the Primer Express Software installed into the system and sequences of primers used in this study are reported in Table 2. To discriminate

between the expression of the endogenous *ACL5* gene and the expression of the *ACL5:GUS* transgene, primers pairs were designed, respectively, on the 3'UTR region (ACL5_3'_F and ACL5_3'_R) and on a short vector sequence inserted at the junction between the *ACL5* promoter and coding region (ACL5_F and ACL5_tg_R). Relative transcript levels were calculated with the $2^{-\Delta\Delta Ct}$ method using *ACTIN2* (A3g18780) as internal control for normalisation of the variations in cDNA amounts used. The ΔΔCt is calculated using the formula: $\Delta\Delta Ct = (Ct_{Target\ sample} - Ct_{End\ sample}) - (Ct_{Target\ Cal} - Ct_{End\ Cal})$ where Ct = threshold cycle, Target = gene under analysis, End = endogenous control gene (*ACTIN2*), Sample = mutant or transgenic plant, Cal = calibrator (wild-type).

Phenotypic analysis and Microscopy

Analysis of phenotypic and anatomical characters was performed on at least 5 specimens, randomly chosen within homogeneous populations of *Arabidopsis* plants. Macroscopic images were captured using a Coolpix 990 (Nikon) digital camera directly or mounted on a Wild MZ8 dissecting microscope (Leica).

For venation pattern analysis, the fifth leaf of 3-week-old plants was detached and cleared as described by Weigel and Glazebrook (2002). Cleared samples were observed with an Axioskop2 Plus microscope (Zeiss) either in dark-field or using a Differential Interfase Contrast (DIC) optics and images taken with a Coolpix 990 (Nikon) digital camera were used to count the number of veins and free-ending veins in a defined area in the central part of the leaf.

For root growth analysis, seedlings grown in square plates incubated in vertical position for 5 days were clarified according to Weigel and Glazebrook (2002) and mounted on microscope slides. Images taken under a Wild MZ8 (Leica) dissecting microscope were used to measure the root length with the NIH Image analysis Software (Research Service Branch (RSB) of the National Institute of Mental Health (NIMH), USA, http://rsb.info.nih.gov/ij/). The number of lateral root primordia, including those at very early stages of development and not yet emerged from the primary root, was counted upon observation under an Axioskop2 Plus (Zeiss, Germany) with DIC optics.

T-test statistical analysis has been performed with the Quickcalcs Ondine Calculators for Scientists (GraphPad Software, Inc. http://graphpad.com/quickcalcs/) software.

For histological analysis, hypocotyls were fixed overnight in 1% glutaraldehyde - 4%

formaldehyde in 50 mM sodium phosphate buffer pH 7.2. After washing 30 minutes in the same buffer, the samples were dehydrated through a graded series of ethanol and embedded in Technovit 7100 (Kulzer, Hereaus) as indicated by the manufacturer. Sections were made on a Microm HM 325 microtome at 5 μm thickness and stained with 0.1% toluidine blue.

Generation of multiple mutants

The *acl5-1 athb8-1 cna-3* triple and *acl5-1 athb8-1 cna-3 rev-5* quadruple mutant were both obtained from the cross of *acl5-1* x *athb8-1 cna-3 rev-5*. The *acl5-1 rev-5* and *acl5-1 35S::ATHB8* double mutants were obtained from the cross of *acl5-1* x *rev-5* and *acl5-1* x *35S::ATHB8* transgenic plants. Multiple mutant lines were identified in segregating F2 or F3 populations by PCR-based genotyping and, when appropriate, antibiotic selection (BASTA for *cna-3,* kanamycin for *35S::ATHB8*). Genomic DNA was extracted from a single cotyledon of one-week-old seedlings according to Mannerlof and Tenning (1997). All primers used for genotyping are listed in Table 2. The genotype of insertional alleles (*athb8-1* and *cna-3*) was determined using combinations of two gene-specifics and one insertion-specific primers (73*Eco*/73REV8 and 73*Eco*/En205 primer pairs and 15FOR/15V4 and 15FOR/JL202 primer pairs for wild-type and mutant alleles of *ATHB8* and *CNA/ATHB15*) in standard PCR reactions. Allelic discrimination of the point mutation alleles (*acl5-1* and *rev-5*) was performed by end-point detection on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) of products amplified with gene specific primers in the presence of VIC and FAM labelled wild-type and mutant alleles specific MGB probes using the TaqMan Universal PCR master mix (Applied Biosystems) according to manufacturer's protocol.

Application	Name	Sequence
Plasmid construction	P_ACL5_F	5'- CACCTTGTAGCGTGTGTTTTGTCTG-3'
	P_ACL5_R	5'- CCATATCCAAGTTGAGGAGAAG-3'
	mutACL5_F	5'- CGCGCTCAGGACTCATGCTAGCATTTTTC-3'
	mutACL5_R	5'- AGTCCTGAGCGCGACAGATTACAACTCTAC-3'
	ACL5_AscI_F	5'- AAAAAGGCGCGCCTGGGTGAAGCCGTAGA -3'
	ACL5_AscI_R	5'- AAAAAGGCGCGCCATATGCCGGTACGCCA -3'
Gene expression	ACTIN2_F	5'- GACCAGCTCTTCCATCGAGAA -3'
	ACTIN2_R	5'- CAAACGAGGGCTGGAACAAG -3'
	ACL5_3'_F	5'- CCGGCATATTTAAAGACGAACC -3'
	ACL5_3'_R	5'- GAGAAAAGAAACCGGCCTTT-3'
	ACL5_F	5'- CGTCCCATCACCAAACATTAAGTAGC -3'
	ACL5_tg_R	5'-TTCACCCAGGCGCGCCCA -3'
	ATHB8_F	5'- -3'
	ATHB8_R	5'- -3'
Genotyping	73*Eco*	5'- GTGAGAATTCACAGCAAGAG -3'
	73REV8	5'- TTGTAGAGTCTCTCCAGA-3'
	En205	5'- AGAAGCACGACGGCTGTAGAATAGGA-3'
	15FOR	5'- GTGCTTAGGATTTGAGATGGCTTAGATTT -3'
	15V4	5'- GCATCTGAACCCACTCAACAGCGGT-3'
	JL202	5'- CATTTTATAATAACGCTGCGGACATCTAC-3'
	ACL5_SDS_F	5'- TGTGTTTATAATGGGAGGAGGTGAA-3'
	ACL5_SDS_R	5'- AACTTTCTCGATCGTCGTGTGTT -3'
	ACL5_MGB	5'-VIC-TGCAAGAgAAATAC -3'
	acl5-1_MGB	5'-FAM-TGCAAGAaAAATAC -3'
	REV_SDS_F	5'- AGATGAATGAATAAAGTTGTGACATTTGT -3'
	REV_SDS_R	5'- TGTATCTCAGGGTCCAGAAATCG -3'
	REV_MGB	5'-VIC AGACGTATGcACCAAC -3'
	rev-5_MGB	5'- FAM-CGTATGtACCAACGAC -3'

Table 2. Sequence of primers and MGB probes used for plasmid construction, gene expression analysis and genotyping.

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