Regulation of stem cell fate and organ development in *Arabidopsis thaliana*

Inaugural–Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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aus Mailand, Italien

2005

Gedruckt mit der Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Heinrich-Heine Universität Düsseldorf.

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Tag der mündlichen Prüfung: . .2005

“Una vita senza ricerca non è degna di essere vissuta”

(Socrate)

… I quoted this sentence on the first page of my diploma thesis, written in Milano four years ago. Oddly, I still believe in it …

Acknowledgments

This research work was started in the Institute of Developmental Biology at the University of Cologne and finished in the Institute for Genetics of the Heinrich-Heine University in Düsseldorf under the supervision of Prof. Dr. Rüdiger Simon. I would like to thank Rüdiger for the opportunity he gave me to do this PhD. I am particularly grateful to him for his excellent supervision, practical help and every kind of support I received during this project.

Special thanks to all the members of the labs, both in Cologne and in Düsseldorf, in particular to Yvonne Stahl for the final correction of my thesis, and to everybody else in both the departments for making this a really good experience.

Thanks to Anastassia, my wife, for giving me great support in the last three years.

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1 Introduction

1.1 Organ production in plants: a meristem's duty

Small groups of undifferentiated and totipotent cells are located in plant shoot and root apices. Plants require this type of cells to promote postembryonic development, i.e. the production of all the adult structures like leaves, roots, internodes, flowers and lateral shoots. In the root apex, the root meristem (RM) generates the primary and the secondary roots; at the shoot tip, the shoot apical meristem (SAM) produces all the above ground organs. The SAM is composed of an outer *tunica* and an inner *corpus*. Clonally distinct cell layers, the so-called L1 and L2, form the *tunica*; the inner L3 layer forms the *corpus* (Vaughan, 1952; Steeves, 1989). The L1 layer cells divide anticlinally: their daughter cells after division remain in the same clonal layer. In the L2 layer, first divisions are anticlinal, but in lateral organ development they are also periclinal, thus causing the sporadic invasion of *tunica* cells into the *corpus* area. In the L3 layer, cell division axes are variable. Clones originated from these three cell layers have different fates, as shown in plant chimeras (Satina et al., 1940): the L1 layer produces the cells of the epidermal layer; the L2 layer creates sub-epidermal cells and the gametes; the L3 layer gives rise to the vasculature and ground tissue (Satina et al., 1941; Stewart and Dermen, 1970; Dermen, 1973).

The SAM can be further subdivided into a central zone (CZ) in the centre of the meristem, a peripheral zone (PZ) surrounding the central zone, and a rib meristem (RM) beneath both of them. The CZ contains slowly dividing stem cells; in the PZ are the founder cells of organ primordia; cell divisions in the RM are necessary for stem elongation. The CZ and PZ of the meristem are symplastically isolated, because plasmodesmata connect the L1, L2 and L3 cell layers with each zone, but not the totipotent CZ with the differentiated PZ

(Rinne et al., 1998). Every time a stem cell of the CZ undergoes a mitotic division, it produces two daughter cells: one may remain in the CZ and maintain its undifferentiated state, whereas the other cell can be shifted to the PZ, where it becomes the founder of an organ primordium or enters a pathway towards differentiation (Medford et al., 1992).

During the vegetative stage, the Arabidopsis SAM produces only rosette leaves. After the transition to reproductive development, the SAM (which is now called inflorescence meristem, IM), will develop floral and axillary meristems (FM and AXM). Each floral meristem produces the full range of flower organs, which are usually found in dicotyledonous plants, in four concentric organ whorls: four green sepals, four white petals, six stamens producing pollen and two fused carpels, containing ovules. The FM, in contrary to the SAM, is determinate: it arrests after producing the flower organs. The AXMs produce lateral shoots.

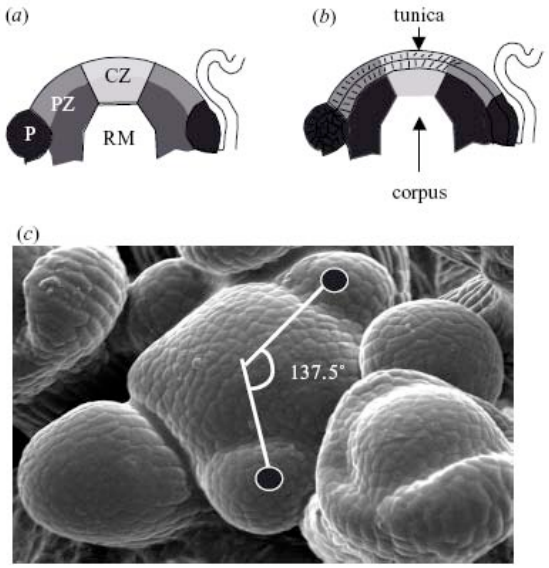

Fig. I. (a,b) Organization of the meristem in angiosperm. The shoot apical meristem can be divided into overlapping zones and layers. (a) Division into zones: The CZ at the summit of the meristem is responsible for meristem maintenance. Descendants of the CZ cells are shifted to the PZ where the primordia (P) are initiated. (b) Division into layers: The surface layer or tunica remains separated from the inner domain (corpus) because the cells divide only in anticlinal orientations. The tunica in angiosperms is again divided into sub layers (called L1 and L2). The L2 cells divide in different orientations when the primordia are initiated. (c) Phyllotaxis at the inflorescence meristem in Arabidopsis. The flower buds are initiated in a spiral fashion. The divergence angle between successive primordia is approximately 137.5°. Modified from Traas and Vernoux, 2002.

1.2 Feedback regulation between CLAVATA3 and WUSCHEL controls the stem cell population

For continuous growth and the ability to initiate new organ primordia, plants need to maintain an active stem cell population in their meristems. Stem cell identity in shoot and floral meristems is promoted by the WUSCHEL (WUS) homeodomain transcription factor. *WUS* is expressed in some cells of the L3 layer, in the so-called organizing centre (Laux et al., 1996; Mayer et al., 1998). *wus* mutant plants have an arrested SAM: the stem cells in the CZ, already established during embryo development, are sufficient to produce only a few leaves before they are lost by differentiation. This indicates that *WUS* is necessary for the maintenance of the SAM. Later in plant development, axillary meristems produce lateral shoots, inflorescences and flowers lacking organs in the two inner whorls. The mutant phenotype of *wus* flowers implies that *WUS* is required to promote stem cell maintenance also in floral meristems (Laux et al., 1996; Mayer et al., 1998). *WUS* promotes the stem cell identity in the upper CZ through an unknown pathway. *WUS* expression, and in consequence the size of the stem cell population, are under control of the *CLAVATA (CLV)* pathway (Schoof et al., 2000; Brand et al., 2000).

The genes acting in the *CLV* pathway are *CLV1*, *CLV2* and *CLV3* (Leyser and Furner, 1992; Clark et al., 1993, , 1995; Clark et al., 1997; Kayes and Clark, 1998). *CLV1* encodes a receptor-like kinase (RLK). Its extracellular domain is composed of leucin rich repeats (LRR); the intracellular domain is a protein-kinase. *CLV1* is expressed in the L3 meristem layer, partly overlapping with the WUS expression domain (Clark et al., 1997; Mayer et al., 1998). The mutant phenotype of *clv1* plants is already visible in embryos, which have larger shoot apical meristems in comparison to the wild type (Running et al., 1995). Later in development, *clv1* mutant plants are fasciated

and have vegetative, inflorescence and floral meristems up to 1000 fold larger in comparison to the wild type. Microscope analyses showed that the increased meristem size of *clv1* mutants is due to accumulation of stem cells in the central zone of meristems (Clark et al., 1993). Because of this higher number of stem cells, the organ production in the peripheral zone of the mutant meristem is altered, and flowers of *clv1* plants have an increased number of organs in all four whorls. Analyses of the *wus/clv1-4* double mutant showed that *CLV1* and *WUS* act on the same pathway (Laux et al., 1996).

The phenotypes of *clv1* and *clv3* mutants are identical. The phenotype of the *clv1/clv3* double mutant is not distinguishable from *clv1* or *clv3* single mutants. *clv1* and *clv3* mutant phenotypes and the gene expression patterns of *CLV1* and *CLV3* appear to be specific to shoot and flower meristems. Thus, the conclusion is that these two genes function in the same pathway to regulate meristem development (Clark et al., 1993, , 1995; Clark et al., 1997; Fletcher et al., 1999). *CLV3* is expressed in the L1 to L3 layers of the meristem's CZ and it encodes a secreted protein of 96 amino acids. CLV3 is supposed to interact with the extracellular domain of the CLV1 receptor (Rojo et al., 2002).

Compared to *clv1* and *clv3* mutants, all *clv2* mutant alleles display weak phenotypes in shoot and floral meristems (Kayes and Clark, 1998). *clv2* mutations also affect the development of several organ types, including elongated flower pedicels and reduced anthers and valves. Double mutant analysis showed that *clv1* and *clv3* mutants are not fully epistatic to *clv2*. The involvement of *CLV2* in the *CLAVATA* pathway is not yet fully understood. *CLV2* encodes a receptor-like protein with a leucin rich repeat extracellular domain, and with a short cytoplasmic tail without a kinase domain.

A non-functional *CLAVATA* pathway, as in the case of *clv1, clv3*, and partially *clv2* mutant plants, leads to enlargement of the stem cell population and to stem fasciation because of a failure in repressing *WUS* expression, as demonstrated in Brand et al., 2000. Brand and colleagues showed that plants

ectopically expressing *CLV3* (35S::CLV3) phenocopy *wus* mutant plants. As the ectopic CLV3 strongly downregulates *WUS* through the signal generated by the activated CLV1 receptor, *WUS* does not promote stem cell identity anymore and the meristem arrests. In line with this, *clv3* mutant plants show a larger SAM than wild type plants, because *WUS* can promote stem cell identity without the restricting signal originated from the CLV3-CLV1 interaction. Thus, CLV3, together with CLV1, controls the balance of stem cell proliferation and differentiation through the regulation of *WUS* expression. From these observations, the *WUS/CLV3* feedback loop model was established: *CLV3*, expressed in stem cells, negatively regulates through the *CLAVATA* pathway *WUS* expression, which on the other hand promotes stem cell identity and *CLV3* expression. This feedback loop compensates alterations in *CLV3* and *WUS* expressions and is necessary to maintain a stable number of stem cells in the SAM. Thus, the tuning of *CLV3* expression plays a central role in the regulation of the stem cell population size. The *CLV3* promoter deletion analysis presented in this thesis is an attempt to investigate how *CLV3* expression is regulated.

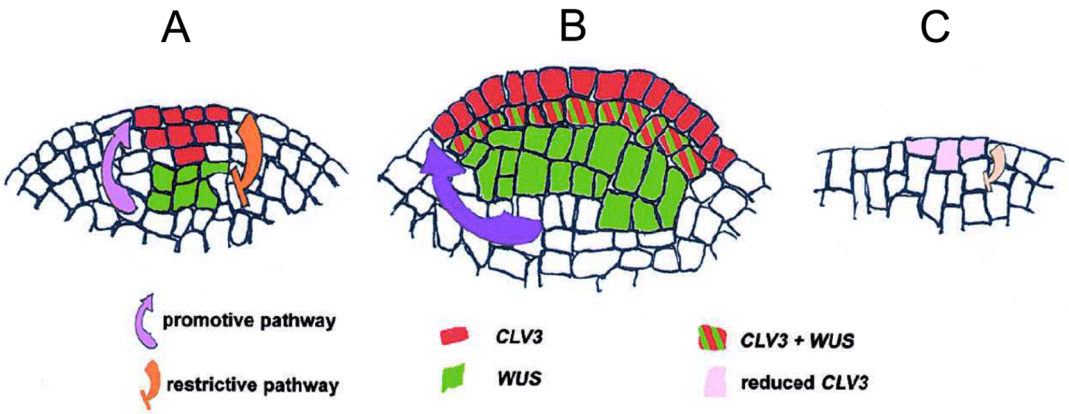

Fig. II. Meristem organization and regulation of stem cell number: *CLV3* expressing stem cells are shown in red. (A) View of a wild-type shoot meristem. *WUS* expression (shown in green) promotes stem cell fate and *CLV3* expression in overlaying cells. Expression of *CLV3* in stem cells activates a restrictive signal transduction pathway that represses *WUS* activity. (B) In *clv* mutants, the restrictive pathway fails, and stem cells accumulate. (C) *wus* mutants (or plants ectopically expressing *CLV3*) are unable to maintain stem cells and the expression of the stem cell marker *CLV3* is reduced in the central zone of the SAM. Modified from Waites and Simon, 2000.

Many components of the *CLAVATA* signalling pathway are probably not yet known. Starting point of this pathway are the cells in the central zone of the meristem, which encode and secrete CLV3 in the extracellular space (Rojo et al., 2002). CLV3 is supposed to diffuse laterally and downwards into the meristem layers and to be sequestrated by the CLV1 receptor in the L3 layer (Lenhard and Laux, 2003). The CLV3-CLV1 ligand-receptor interaction promotes the formation of a protein complex of 450 kDa and the activation of the CLV1 kinase domain probably by auto-phosphorylation (Trotochaud et al., 1999). The signal originated from the activated CLV1 may be transmitted by plant Rho GTPases and mitogen-activated protein kinases (MAPK) to the cell nucleus, where it restricts *WUS* expression. Despite the large numbers of putative RLKs encoded in plant genomes, a general model describing signal transduction has yet to be determined. Arabidopsis genome sequencing has revealed the presence of at least 610 putative RLK genes, 222 of which belong to the large LRR-RLK subfamily (Shiu and Bleecker, 2001). A kinase associated protein phosphatase (KAPP) is part of the complex formed after the CLV1-CLV3 interaction. KAPP binding to CLV1 depends on the kinase activity of CLV1 and on the phosphorylase activity of KAPP. Transgenic plants overexpressing KAPP phenocopy *clv1* mutants. KAPP may negatively regulate the CLV1 signal transduction pathway, possibly through dephosphorylation. KAPP is known to interact also with other receptor kinases and thus to regulate different signal transduction pathways (Stone et al., 1998; Trotochaud et al., 1999; Trotochaud et al., 2000).

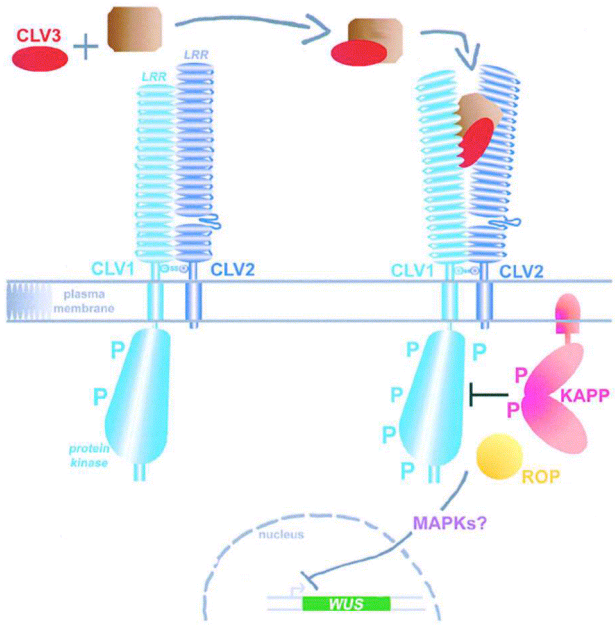

Fig. III. Scheme of the CLV signalling complex. The CLV1 LRR-RLK possibly forms a heteromeric complex with the CLV2 receptor-like protein at the plasma membrane. Binding of the CLV3 polypeptide (shown in red), possibly in association with another protein X (shown in brown), is proposed to stimulate the assembly of an active signalling complex that also contains a phosphatase (KAPP) and a Rho-like GTPase (Rop). The signal is relayed from the cytosol to the nucleus, potentially via a MAPK cascade, to limit *WUS* expression. P, phosphorylation site. Modified from Waites and Simon, 2000.

Other proteins are known to have a function in the CLV3/WUS feedback loop. *POLTERGEIST* (*POL*) encodes a protein phosphatase 2C (PP2C) with a predicted nuclear localization sequence, indicating that it could have a role in signal transduction modulation downstream of the CLV1 receptor (Yu et al., 2003). *clv/pol* double mutant plants produce less stem cells than single *clv* mutants (Yu et al., 2000). The *pol* mutant phenotype is comparable to wild type. Thus, *POL* is supposed to function as a regulator of meristem development, partially redundant with WUS. The ubiquitous expression of *POL* suggests that it may be a common regulator of many other signalling pathways.

SHEPHERD (*SHD*) encodes a HSP-90-like protein mainly localized in the endoplasmic reticulum. The SHD protein is supposed to be responsible for the correct folding of CLV3 protein or to help CLV3 to bind to the CLV1

receptor (Ishiguro et al., 2002). The *shd* mutant phenotype is pleiotropic, but the shoot and the floral meristems are comparable to *clv* mutants. The *shd/clv* double mutants are not distinguishable from *clv* single mutants, and overexpression of *CLV3* in a *shd* mutant background does not have an effect. Thus, *CLV3* function probably depends on SHD activity.

1.3 The CLE gene family

CLV3 belongs to the *CLV3/ESR* (*CLE*) gene family, which contains 26 Arabidopsis members that share a conserved C-terminal domain with CLV3, and 3 maize *EMBRYO SURROUNDING REGION (ESR)* genes, which encode for proteins secreted by cells neighbouring the embryo (Bonello et al., 2000; Cock and McCormick, 2001; Bonello et al., 2002). The majority of the predicted CLE proteins contain N-terminal signal sequences and are exported to the extracellular space (Sharma et al., 2003). CLE proteins are all transcribed in one or more tissues during development, indicating that they encode functional products.

One member of the *CLE* family, CLE40*,* encodes a potentially secreted protein distantly related to CLV3. While *CLV3* is expressed in the stem cell domain of the shoot apex, *CLE40* is expressed at low levels in all tissues. Misexpression and promoter swap experiments show that *CLE40* can substitute *CLV3* to activate the *CLV* pathway in the shoot, indicating that CLV3 and CLE40 are functionally equivalent proteins that differ mainly in their expression patterns. *cle40* loss-of-function mutants do not cause alterations in cell number in the SAM, showing that *CLE40* does not contribute to *CLV* signalling in wild-type. High-level expressions of *CLV3* or *CLE40* result in a premature loss of root meristem activity, indicating that activation of a *CLV*-like signalling pathway may restrict cell fate also in roots. The cellular organization of *cle40* root meristems is normal, but mutant roots

grow in a strongly waving pattern, suggesting a role for CLE40 in a signalling pathway that controls movement of the root tip (Hobe et al., 2003).
Another CLE protein, CLE19, has been reported to be expressed in leaves, flowers, siliques, and pollen (Sharma et al., 2003). Overexpression of CLE19 in roots was shown to restrict the size of the root meristem. This result suggests that CLE19 acts by activating an endogenous CLV-like pathway involved in root meristem maintenance (Casamitjana-Martinez et al., 2003).
Recently, in vitro applications of synthetic 14-amino acid peptides, corresponding to the conserved CLE motif of CLV3, CLE40 and CLE19, were shown to mimic their overexpression phenotype, i.e. the consumption of the root meristem. These short peptides are proposed to represent the major active domain of the corresponding CLE proteins (Fiers et al., 2005). As *clv2* mutant plants failed to respond to the peptide treatments, Fiers et al suggest that CLV2 is involved in the CLE peptide signalling in roots.

1.4 Establishment of meristem identity

Several genes control initiation and maintenance of the SAM, among them are the genes belonging to the *knotted1-like homeobox* (*KNOX*) family. *knotted1* (*kn1*), founder of the *KNOX* gene family, was isolated as a dominant mutant in maize. Leaves of the *kn1-D* mutants showed tissue over-proliferation, chaotic patterning and abnormal cellular structure of the vasculature (Smith et al., 1992). Recessive mutant alleles of *kn1* revealed a role for this gene in meristem maintenance, particularly because they affect branching and lateral organ formation (Kerstetter et al., 1997). Using the *kn1* homeobox as a heterologous probe, *KNAT1* and *KNAT2*, two *KNOTTED1-like* genes from *Arabidopsis thaliana*, were cloned (Lincoln et al., 1994). *KNAT1* and *KNAT2* promote SAM identity in Arabidopsis. Ectopic expression of *KNAT1* or *KNAT2* causes the formation of leaves that are lobed and with ectopic meristems formed in the sinuses close to the leaf veins (Chuck et al.,

1996). *KNAT1* is expressed in the SAM. Its expression decreases during the floral transition and it is then restricted to the vasculature of the stem. *KNAT1* is downregulated in emerging organ primordia by *ASYMMETRIC LEAVES1* and *ASYMMETRIC LEAVES2 (AS1, AS2),* two genes required to specify lateral organ symmetry (Semiarti et al., 2001; Lin et al., 2003). *KNAT2* seems to have an additional function in carpel development in the flower meristem, as its overexpression promotes the formation of ectopic carpels (Pautot et al., 2001). Even if the overexpression of *KNAT1* and *KNAT2* promotes formation of ectopic meristems in differentiated tissues, their loss of function mutations do not affect meristem formation. Other components of the *KNAT* family (*KNAT3, KNAT4, KNAT5 and KNAT6)* are not necessary for the maintenance of the SAM and have a function in organ primordia formation (Serikawa et al., 1997; Dean et al., 2004). Interestingly, *KNAT1*, but not *KNAT2*, was shown to have a partially redundant function with *SHOOTMERISTEMLESS* (*STM*) in maintaining meristem identity (Byrne et al., 2002).

STM also belongs to the *KNOX* gene family and encodes a protein similar in sequence and function to *knotted1*. *STM* expression is required to define the embryonic SAM (Barton and Poethig, 1993). Seedlings homozygous for the strong *stm-1* mutant allele do not produce leaf organs. *STM* expression is detected in the meristem during embryogenesis between the two emerging cotyledon primordia (Long and Barton, 1998). As soon as cotyledon primordia are initiated, *STM* downregulates the expression of the organ primordia genes *AS1* and *AS2,* thus defining the meristem niche where stem cells are contained. Therefore, STM does not initiate the SAM but is needed to maintain its identity (Byrne et al., 2000). *STM* has a partially redundant function with *KNAT1*: *as1/stm* double mutants still have a SAM during embryonal and vegetative development even if *STM* is not expressed, because *KNAT1* is ectopically expressed (Byrne et al., 2002).

Plants mutant for *AS1* or *AS2* show alterations in cotyledon, leaf and flower development. Proximal-distal and abaxial-adaxial polarity is disturbed in leaves of *as1* mutant plants: rosette leaves have petiole growth underneath

the leaf lamina, are occasionally curled upwards and shaped like lotus-leaves. In *as2* mutant plants, lobed leaves and leaflet-like structures emerging from the petioles are often visible. Interestingly, in both mutant plants, ectopic SAMs on the adaxial side of the leaves are produced. These mutant phenotypes are comparable to plants overexpressing *KNAT1* or *STM* (Ori et al., 2000; Semiarti et al., 2001). Indeed, in *as1* and *as2* mutants, *KNAT1* and *KNAT2,* but not *STM*, are ectopically expressed. In wild type, *KNAT1* and *KNAT2* are usually expressed in the SAM, overlapping with the *STM* expression domain, but not in the lateral organs (Lincoln et al., 1994; Serikawa et al., 1996). Therefore, *AS1* and *AS2* promote leaf differentiation through repression of *KNAT* genes in leaves. *AS1* encodes a putative transcription factor of the MYB family. *AS2* is *LBD6*, a member of the *LATERAL ORGAN BOUNDARY* (*LBD*) gene family (Shuai et al., 2002). Both of these genes are expressed only in organ primordia, in a domain complementary to *STM* and *KNAT1* (Byrne et al., 2000). Byrne et al. showed that *AS1* and *AS2* are downregulated by *STM* in the SAM, and that in *stm* mutant plants ectopic expression of *AS1/AS2* is detected also in the meristem. The interactions described between *AS1*, *AS2*, *STM* and *KNAT1* show that genes expressed in organ primordia interact with genes expressed in the meristem, thus to control cell fate in the shoot apex in a concerted action. Recently, AS1 and AS2 were shown to form homo- and hetero-dimers, suggesting that AS1-AS2 complexes regulate the establishment of leaf polarity (Lin et al., 2003).

DONRÖSCHEN (*DRN*) seems to be involved in both organ polarity maintenance and meristem organization. In the *drn-D* dominant mutant, *CLV3* expression is initially promoted in both the central and peripheral zone of the meristem. After the shoot meristem of *drn-D* mutants arrests the formation of lateral organs and initiates radialized leaves, *CLV3* and *WUS* are still upregulated, but their expression pattern is shifted into deeper meristem layers. *DRN* encodes an AP2/ERF transcription factor expressed in the primordia anlagen in the first two layers of the meristem's central zone.

DRN does not seem necessary to promote stem cell identity, as the *drn* loss of function mutant is aphenotypic, possibly due to functional redundancy with other *DRN*-like genes. Furthermore, *CLV3* does not seem necessary for *DRN* functionality: the *drn-D/clv3-2* double mutants are comparable to *drn-D* single mutants (Kirch et al., 2003). Even if from the result of my research DRN seems to be a direct activator of *CLV3*, a role for *DRN* in controlling *CLV3* expression is not yet clear. As the enhanced expression (or mis-expression) of *DRN* promotes accumulation of stem cells in the central zone of the meristem and alterations in organ primordia development, *DRN* could be involved in a pathway controlling cell division in the peripheral zone of the meristem (Kirch et al., 2003).

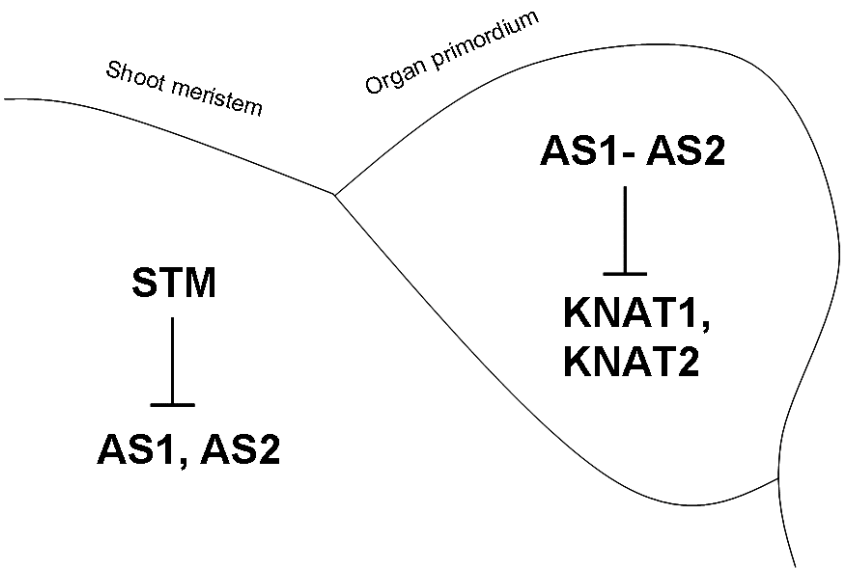

Fig. IV. A model of genetic interactions between meristem and primordia in the SAM. *STM* represses *AS1* and *AS2* in the SAM, thus maintaining meristem identity. AS1-AS2 heterodimers repress the expressions of *KNAT1* and *KNAT2* in emerging organ primordia, thus allowing organ differentiation.

1.5 CUC genes define the STM expression pattern

STM expression and meristem initiation are under control of the *CUP-SHAPED-COTYLEDON (CUC)* boundary genes (Takada and Tasaka, 2002). *CUC1*, *CUC2* and *CUC3* belong to the *NO APICAL MERISTEM (NAC)* family of transcriptional factors. They share the *NAC* DNA-binding domain, originally isolated in the *NO APICAL MERISTEM (NAM)* gene from Petunia. *CUC1* and *CUC2,* respectively, are expressed in the early and late globular stages in the embryonic meristem (Takada et al., 2001). *cuc1* or *cuc2* mutants show weak defects in the separation of cotyledons, stamens and sepals (Aida et al., 1997). Also *CUC3* is expressed from early stages of embryo development onward in the presumptive meristem. Besides the expression in embryonic boundaries, *CUC3* is additionally detected in a wide variety of boundaries, like at the base of lateral roots or around trichomes. *CUC3* generally separates two organs or an organ from its surrounding cells. Analysis of a *cuc3* null mutant indicates that *CUC3* function is partially redundant with that of CUC1 and CUC2 in the establishment of cotyledon boundaries and shoot meristems. However, in *cuc1cuc2cuc3* triple mutant plants, cotyledons are fully fused, not only partially as was observed in *cuc1/cuc2* double mutants. This, plus the broad *CUC3* expression pattern, suggests a primary role for *CUC3* in the establishment of boundaries (Vroemen et al., 2003). Interestingly, *cuc1/cuc2* double mutants lack the SAM and do not express *STM*, and overexpression of *CUC1* promotes *CUC2* and the formation of ectopic meristems where *STM* expression is detected. Therefore, these results show that *STM* expression is under control of *CUC1* and *CUC2*.

1.6 Expression domains of CUC1 and CUC2 are controlled by auxin transport

The expression patterns of *CUC1* and *CUC2*, and therefore the establishment of a functional SAM, are controlled by PIN-FORMED1 (PIN1) and PINOID (PID).

PIN1, member of the PIN protein family, facilitates the transport of the phytohormone auxin. Auxin moves apoplastically in the plant and promotes cell differentiation and elongation. PIN proteins encode for auxin efflux facilitators and help auxin to exit the cell, while the permease AUXIN INFLUX FACILITATOR1 (AUX-1) facilitates auxin to enter the cell (Swarup et al., 2001). *PIN1* is expressed mainly in the vasculature, young organ primordia and in the meristem L1 layer cells (Galweiler et al., 1998; Reinhardt et al., 2003). Mutations affecting *PIN1* highly disturb auxin transport and the whole plant structure. *pin1* mutants have rosette leaves which are often fused and inflorescences that produce organs with high delay in comparison to wild type plants. In the meristem of *pin1* mutants, PZ cells are not recruited to distinct primordia: instead, a ring expressing primordia specific genes and the boundary marker *CUC2* is observed around the meristem. The other characterized PIN proteins, PIN3, PIN4 and PIN7, are not known to have a function in the SAM. Relocalization of PIN3 in response to gravity provides a mechanism for redirecting auxin efflux to promote asymmetric growth. PIN3 is a component of the auxin transport system that regulates tropistic growth. In *pin3* loss of function plants, roots have a weak sensitivity to gravity. *pin4* mutants fail to canalize externally applied auxin and display various defects in both embryonic and seedling roots. PIN4 seems to be essential for auxin distribution and patterning during root development (Friml et al., 2002b; Fu and Harberd, 2003). PIN7 was recently shown to be required during early stages of embryo development. The asymmetric division of the zygote produces a basal cell that transports auxin and an apical cell that responds to

it. PIN7 maintains the apical-basal auxin gradient that promote the specification of the apical embryo (Friml et al., 2003). Interestingly, embryos mutant for *pin1pin3pin4pin7* are arrested in development (Blilou et al., 2005).
The localization of PIN proteins in the cell membrane establishes the direction of auxin efflux, and thus the functionality of auxin transport. Plants overexpressing *PINOID (PID)* show a basal-to-apical shift in PIN1 localization. Conversely, in plants mutant for *pid,* an apical-to-basal shift in PIN1 polar targeting is detected. In both of the cases, plant organogenesis is defective. Therefore, *PID*, which encodes a serine-threonine protein kinase, controls PIN1 localization in the cell membrane. *PID* was also shown to be auxin inducible and to have a single auxin responsive TGTCTC element in its promoter region (Ulmasov et al., 1997). Thus, *PID* controls auxin transport direction and responds to local auxin concentrations (Christensen et al., 2000).
Single mutations in *PIN1* or *PID* have moderate effects on cotyledon symmetry and on the *CUC2* expression pattern. In contrast, the *pin1/pid* double mutant completely lacks cotyledons and bilateral symmetry. In the double mutant embryo, the expression domains of *CUC1*, *CUC2* and in consequence of *STM*, expand to the meristem periphery and overlap with the cotyledon-initiation area. Without *PIN1* and *PID* expression, i.e. with disturbed auxin transport, the set up of the SAM and boundaries is defective and the onset of organ primordia formation in the peripheral zone of the meristem is not allowed. Therefore, auxin transport is required to restrict *CUC* gene expressions to the boundaries between meristem and cotyledon primordia, thus to permit organ primordia formation.

1.7 Auxin promotes organ primordia initiation

Auxin transport does not only restrict the expression of boundary genes during embryo development. It is also actively involved in the initiation of

primordia anlagen throughout the whole life of a plant. Several mutants in auxin transport show defects in organ initiation (Okada et al., 1991; Mito and Bennett, 1995; Gälweiler et al., 1998). Expression patterns of auxin responsive genes indirectly indicate a high level of auxin activity at the sites of organ primordia formation (Benkova et al., 2003). Therefore, auxin may be considered the signal required to promote lateral organ formation in the peripheral zone of the meristem. No vasculature is present in the meristems to transport auxin to the sites of organ formation. Interestingly, auxin transport could be performed by the cells of the meristem L1 layer, where AUX-1 and PIN1 are expressed, as reviewed in Friml, 2003.

Taken together, these observations support a model that describes organ initiation as a process mediated by auxin transport and accumulation in the peripheral zone of the meristem. a) Auxin is delivered from the vasculature to the periphery of the meristem without a preferred position. b) Auxin accumulates at the sites of organ primordia formation and is depleted in the vicinity, where therefore no new organ is initiated. c) In line with this, auxin accumulates at a certain distance beyond the reach of the previously formed primordia. d) Auxin promotes *PIN1* and *PID* expressions. e) Auxin accumulates actively: PIN1 creates a sharp auxin peak that leads to organ initiation. f) A new organ primordium is produced. g) The new organ primordium develops and starts to synthesize auxin, which is drained back to the vasculature because of an hypothesized auxin sink formed by PIN1 (Reinhardt et al., 2003). Thus, auxin transport could be required for both the promotion of organ primordia initiation, and for the establishment of the distance between emerging organ primordia.

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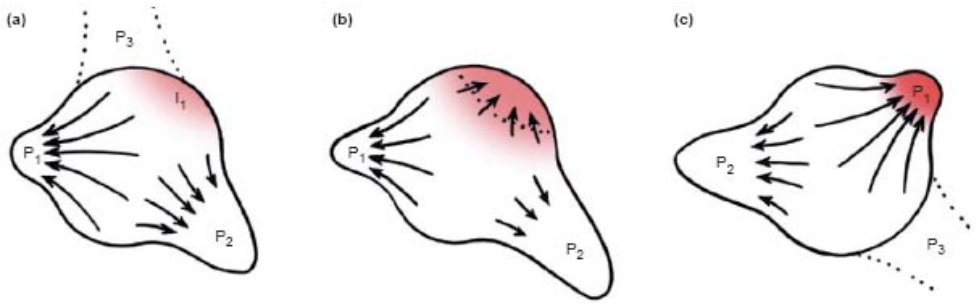


Fig.V. Progression of organ positioning and outgrowth in the shoot meristem. (a) As a result of the sink function of P1 and P2, auxin that is delivered to the meristem becomes diverted into the primordia (arrows). As a result, auxin (red) can accumulate only slowly at a defined distance from P1 and P2, which corresponds to the site of incipient organ formation (I1). (b) At a certain threshold level of auxin, PIN1 becomes induced and begins to actively accumulate auxin at I1. At the same time, the sink activity of P1 and P2 decreases, as they start to produce auxin by themselves. (c) Auxin has been focused to a sharp peak at I1, leading to the outgrowth of a new organ. Arrows represent the direction of polar auxin transport; auxin distribution is represented in red. For clarity, only auxin at I1 is depicted. Modified from (Reinhardt, 2005).

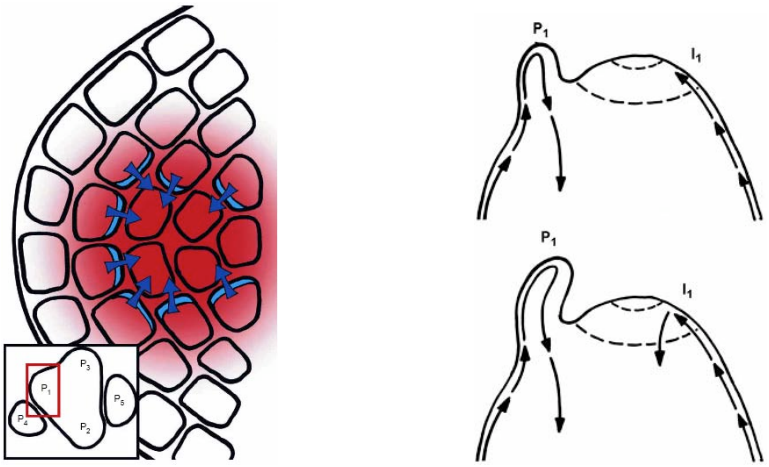


Fig.VI (left). Auxin accumulation in young primordia. PIN1 is induced in young primordia. It becomes localized to the side of the cells that points to the centre of the primordium (light blue). This results in the accumulation of auxin in the primordium and its withdrawal from the surrounding cells (blue arrows). The resulting auxin gradient (red) confers positional information to the cells allowing them to establish organ and boundary identity. Inset: Location of the P1 position in the context of the apex. Modified from (Reinhardt, 2005)

Fig. VII. (right) Schematic representation of an apex in longitudinal section through P1 and I1 at an early (top) and a later stage (bottom) of incipient primordium formation. Polar auxin flux is indicated with arrows. Top, acropetal auxin flux is diverted by P1 preventing auxin accumulation on the left flank of the meristem, while auxin can reach the right flank (I1). Bottom, accumulation of auxin at I1 promotes primordium formation, and establishment of a new auxin sink. Modified from (Reinhardt et al., 2003).

1.8 KNOX genes control hormonal activities in meristems

KNOX gene activities, required to maintain meristem identity, are mediated by the downregulation of the enzyme GA20ox1 in the meristem. GA20ox1 performs the penultimate step of the pathway that produces the phytohormone gibberellin (GA). GA promotes cell differentiation, growth and elongation. GA is normally synthesized only in lateral organs, where *KNOX* genes are not expressed and thus GA20ox1 is active. If *KNOX* genes are ectopically expressed, GA20ox1 is downregulated also in lateral organs, permitting the (partial) induction of meristem identity. Plants overexpressing *KNAT1*, for example, have ectopic meristems formed on the adaxial side of the mutant leaves. Interestingly, if ectopic GA is applied on the mutant leaves of 35S::KNAT1 plants, the mutant phenotype is partially rescued. In conclusion, in meristems, *KNOX* genes downregulate GA biosynthesis; in lateral organs, *KNOX* genes are downregulated (by AS1 and AS2), GA is synthesized, and lateral organ differentiation is permitted. GA is produced only in lateral organs and does not diffuse in the meristem of wild type plants, even if meristem and organ primordia are neighbouring areas. This restriction may be due to the activity of AtGA2ox2 and AtGA2ox4, two enzymes that inactivate GA and that are expressed at the base of the meristem, between meristem and organ primordia, thus forming a “shield” against diffusion of active GA into the meristem. Interestingly, *KNOX* upregulation promotes overexpression of AtGA2ox. Thus, *KNOX* genes are supposed not only to downregulate AtGA20ox in the meristem, but also to upregulate expression of AtGA2ox in the boundaries between meristem and lateral organs, avoiding “leaking” of GA into the meristem (Hay et al., 2002).

KNOX functions are also mediated by the phytohormone cytokinin (CK), a growth regulator that promotes cell division and meristem activity. Low level of GA and high level of CK are required for the maintenance of a functional

shoot meristem (Jasinski et al., 2005). Jasinsky et al showed that in plants overexpressing *KNOX* genes, the expression level of *isopentenyltransferase* (*IPT*), which encodes a CK biosynthetic enzyme, is upregulated. They showed that also the expression of *ARABIDOPSIS RESPONSE REGULATOR GENE5* (*ARR5*), an early induced gene under control of CK, and of GA2ox2 and Ga2ox4 are upregulated. Therefore, *KNOX* genes promote meristem identity through the upregulation of CK expression and, mediated by CK, through the suppression of GA activity in the meristem.

Little is known about the role of *KNOX* genes in controlling polar auxin transport (PAT) in plants. In maize, *SEMAPHORE1* (*SEM1*) downregulates *rough sheath1* and *gnarley1* (*knox4*), two genes belonging to the *KNOX* family normally expressed in the shoot meristem (Kerstetter et al., 1994; Schneeberger et al., 1995; Foster et al., 1999). In mutant plants for *SEM1*, the expressions of these two *KNOX* genes are ectopically detected also in leaves (Scanlon et al., 2002). Thus, *SEM1* seems to be required to downregulate *KNOX* genes in lateral organs. However, the *sem1* mutant phenotype shows defects also in embryo and lateral root development, which are not seen in maize plants overexpressing *KNOX* genes, but in mutants with altered auxin transport. Indeed, auxin transport in *sem1* mutants is reduced to 20% in comparison to wild type plants. Thus, at least in maize shoots, overexpression of *KNOX* genes downregulates auxin transport. A similar effect, i.e. upregulation of *KNOX* gene expressions possibly related to a disturbed auxin transport, is shown later in my research in plants that ectopically express *LOLLO (LOL)*, a member of the Arabidopsis *LBD* gene family.

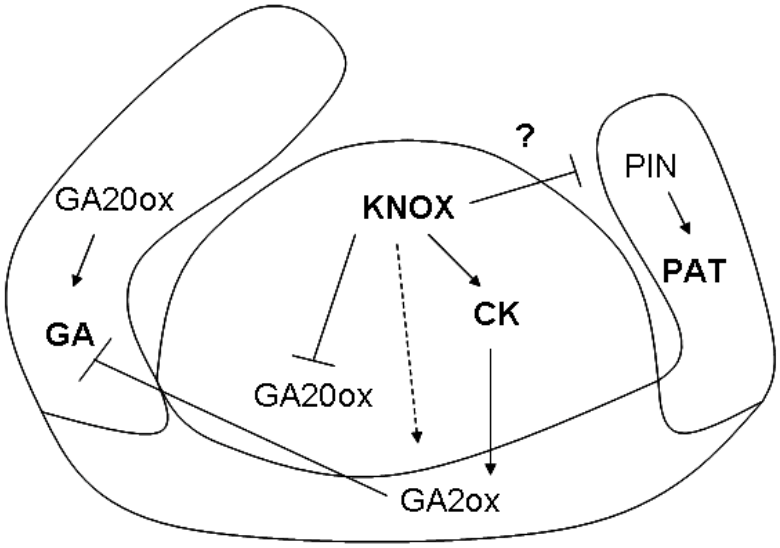


Fig. VIII. Model depicting interactions between KNOX proteins, GA, CK and polar auxin transport (PAT) in the shoot apex. KNOX proteins are expressed in the SAM, where they activate CK biosynthesis and repress GA20oxidase gene expression and hence GA biosynthesis, thus promoting meristem activity. CK also activates GA2ox expression, possibly stimulating GA deactivation. These interactions may confine active GA to the leaf. KNOX proteins may also activate GA2ox in a CK-independent manner. Ectopic KNOX expression may also downregulate PAT in organ primordia, possibly through the downregulation of PIN proteins.

1.9 Promotion of boundary identity

Meristems, boundaries and organ primordia do not only show specific gene expression patterns, but also different cell shapes, division and expansion rates (Dumais and Kwiatkowska, 2002; Kwiatkowska, 2004). In wild type SAM, meristem and primordia cells actively divide and expand, albeit at different rates. In boundary cells between meristem and organ primordia, a low expansion rate is detected, thus depicting boundary cells as not particularly active. *CUC3* is expressed in boundaries of many organs and could have a role, together with *CUC1* and *CUC2*, in the initiation of boundaries between the meristem and organ primordia. Another gene, *LATERAL ORGAN BOUNDARY* (*LOB*), may have a function in boundary maintenance (Shuai et al., 2002). *lob* loss of function leads to a fusion

between the lateral shoot stem and the subtending leaf pedicel (Lin et al., 2005). *LOB* expression is detected in a band of cells at the base of all aboveground lateral organs and secondary roots. *LOB* is the founder of the *LBD* family. All of the 43 Arabidopsis *LBD* members share the so-called LOB domain with yet unknown function (Iwakawa et al., 2002; Shuai et al., 2002). *LOB* expression is known to be positively regulated by both *KNAT1* and *AS2,* showing that meristem and organ primordia specific genes concur to promote genes expressed in the boundaries between them (Ori et al., 2000; Semiarti et al., 2001; Lin et al., 2003). Thus, boundaries may be required to maintain different cell fates in neighbouring areas. The characterization of a newly isolated member of the *LBD* gene family, *LOL/LBD30,* is described in my research and shows how this gene, expressed also in the boundary between meristem and organ primordia, integrates hormonal and KNOX gene signals to maintain a functional shoot apex.

2 Materials and Methods

2.1 Used materials

2.1.1 Chemicals

Chemicals were ordered in *pro analysis* quality from the following companies: *Amersham Life Science*, Braunschweig; *Biomol*, Hamburg; *Biozym*, Oldendorf; *Duchefa*, (NL); *Fluka*, Neu-Ulm; *Invitrogen* (Karlsruhe); *Merck-Eurolab*, Darmstadt; *Life Technologies* (Karlsruhe); *Pharmacia*, Freiburg; *Promega*, Heidelberg; *Roche Diagnostics GmbH*, Mannheim; *Roth*, Karlsruhe; *Serva*, Heidelberg; *Sigma*, Deisenhofen; *Clontech*, Heidelberg; *Gibco* (Karlsruhe); *New England Biolabs GmbH*, Frankfurt am Mein; *Fermentas GmbH*, St-Leon-Rot.

2.1.2 Enzymes

Enzymes were ordered from the following companies: *Invitrogen* (Karlsruhe); *New England Biolabs GmbH*, Frankfurt am Mein; *Roche Diagnostics GmbH*, Mannheim. All the enzymes were supplied and used with their buffers.

2.1.3 Buffers and Media

Buffers, solutions and media were prepared following the protocols from Maniatis, 1982. Starting from these standard protocols, variations were occasionally used.

2.1.4 Bacteria strains

The *E. coli* DH5α strain (*GIBCO/BRL*), DH10B strain (*Boehringer*) and XL1-BLUE strain (*Stratagene*) were used for plasmid amplification. The genotypes of these bacteria strains are available on the respective company catalogs.

Agrobacterium tumefaciens GV3101 strain was used for *Arabidopsis thaliana* and *Nicotiana tabaccum* infiltrations (Koncz C., 1986).

2.1.5 Basic Plasmids

pBluescript (Stratagene)

The pBluescriptKS+ vector was used for both sub-cloning steps and RNA *in vitro* transcription. It carries Ampicillin bacterial resistance.

pCRII-TOPO (Invitrogen, Karlsruhe)

The pCRII-TOPO vector was used for sub-cloning. It carries both Kanamycin and Ampicillin bacterial resistance.

pENTR/-D TOPO (Invitrogen, Karlsruhe)

This vector was used to create entry vectors suitable for GATEWAY recombination (*Invitrogen*). It carries a bacterial Kanamycin resistance. Several entry vectors (see Chapter 2.1.6) with the pENTR/-D TOPO backbone were processed via the LR GATEWAY recombination system to obtain plant expression vectors.

pGPTV-HPT-Asc

The pGPTV-HPT-Asc vector (donated by Prof. W. Werr, University of Köln) is a plant expression vector with a bacterial Kanamycin resistance as well as a plant Hygromycin resistance.

pGREENnos-BAR

pGREENnos-BAR is a plant expression vector. It contains a poly-linker from the pBluescriptSK vector, a bacterial Kanamycin resistance and Basta resistance in plants. It needs the helper-plasmid pJIC-Sa-Rep to be replicated in Agrobacterium. This binary vector system was constructed in

the John Innes Centre (UK). More information at htpp://www.pgreen.ac.uk as well as in Hellens et al., 2000.

pK373

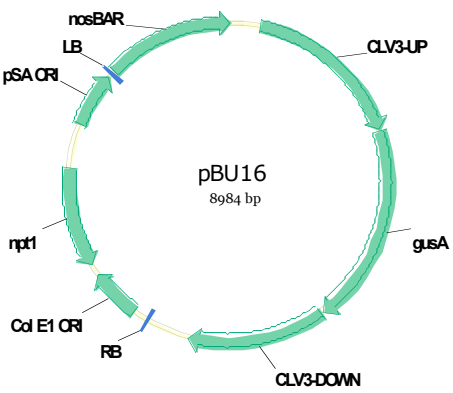
The pK373 vector (donated by Prof. W. Werr, University of Köln) was used for sub-cloning. Its backbone is from the pGEM3 vector (*Promega*, Heidelberg). pK373 additionally contains a 46bp long minimal promoter sequence (from the ubiquitous promoter CaMV35S), a uidA (GUS) gene and a nos-terminator sequence. It carries Ampicillin bacterial resistance.

pY22

Baits for the yeast one-hybrid system were cloned into the pY22 vector (donated by J. Lohmann, Tübingen). pY22 was used to clone several deletion fragments derived from the *CLV3* regulatory sequences. It can integrate in the *his-3* locus of the yeast genome. It carries bacterial Ampicillin resistance.

pBU16

U. Brand constructed the pBU16 vector. It consists of the *CLV3* upstream (CLV3-UP) and downstream (CLV3-DOWN) regulatory sequences, driving the expression of the *GUS* gene (uidA) It was cloned into the pGreen-nos-BAR backbone. pBU16 is the CLV3::GUS reporter gene used for the deletion analysis of the CLV3 regulatory sequences.



pBUdel1,pBUdel2,pBUdel3,pBUdel4,pBUdel5,pBUdel6,pBUdel7

I used these vectors, constructed by U. Brand, to quantify the activities of different fragments of the *CLV3* regulatory sequences. These vectors, based

on pBU16, carry different combinations of CLV3 upstream and downstream sequence deletions.

pUC-SPYCE/SPYNE

These vectors permit to transiently express proteins fused with the C-terminus or the N-terminus of the Yellow-Fluorescent-Protein (YFP). More details in (Bracha-Drori et al., 2004).

pSPYCE/SPYNE

These plant expression vectors permit to express proteins fused with the C-terminal or the N-terminal of the Yellow-Fluorescent-Protein (YFP). More details in (Bracha-Drori et al., 2004).

pBI-ΔGR (Lloyd et al., 1994)

This plant expression vector, based on the PBI121 vector, permits to express a transcription factor fused with the hormone binding domain of the rat glucocorticoid receptor. Activation of the –GR fusion protein occurs via spraying with the synthetic hormone Dexamethasone (Dex). This vector carries Kanamycin bacterial and plant resistance.

pGAD-T7 (Clontech)

This yeast transformation vector permits the expression, under the constitutive ADH1 promoter, of a fusion protein with the GAL4 activation domain (AD). It carries Ampicillin bacterial resistance and the LEU2 nutritional marker for selection in yeast. For more information: www.clontech.com

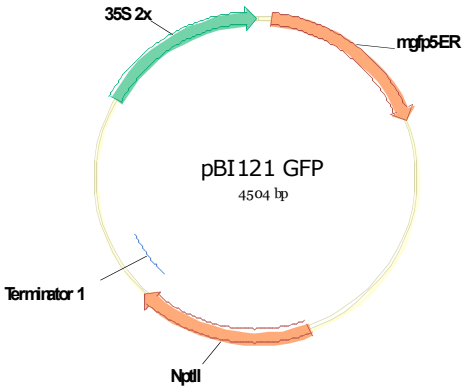
pGKB-T7 (Clontech)

This yeast transformation vector permits the expression, under the constitutive ADH1 promoter, of a fusion protein with the GAL4 binding domain (BD). It carries Ampicillin bacterial resistance and the TRP nutritional

marker for selection in yeast. This vector is used in yeast 2-hybrid screens. For more information: www.clontech.com

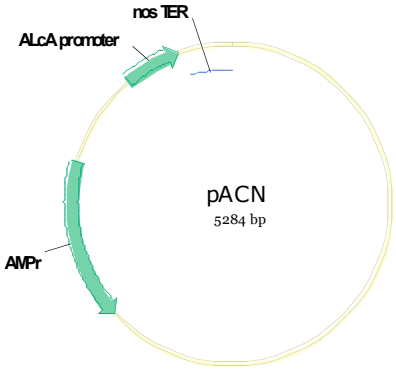
pBI121-GFP

This vector allows expression of a modified version of the Green - Fluorescent - Protein (m-GFp5-er), targeted to the endoplasmic reticulum under control of the ubiquitous 35SCaMV promoter. It carries a Kanamycin resistance.



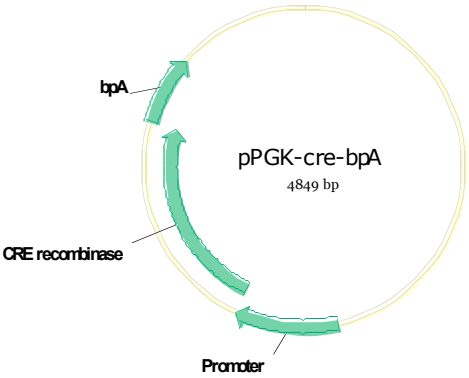
pACN

This vector is part of the ethanol switch AlcR/AlcA binary system. It permits to clone the gene of interest under control of the ethanol inducible promoter AlcA, where the transcriptional activator AlcR binds after ethanol induction.



pGK-CRE-bpA

This vector (donated by Prof. Z. Schwarz-Sommer) contains the CRE recombinase gene. The CRE enzyme recognizes and excises genomic DNA fragments flanked by two loxP sequences.



pMDC30

All the pMDC Gateway (Invitrogen) destination vectors were donated by Prof. Mark Curtis (http://www.unizh.ch/ botinst/Devo_Website/curtisvector/). All of them carry bacterial Kanamycin and plant Hygromycin resistance. pMDC30 permits to express the gene of interest in plants under control of a heat shock inducible promoter.

pMDC32

pMDC32 permits to express the gene of interest under control of a CaMV35S promoter in plants.

pMDC111

pMDC111 drives GFP expression in plants under control of the cloned promoter.

pMDC164

pMDC164 drives GUS expression in plants under control of the cloned promoter.

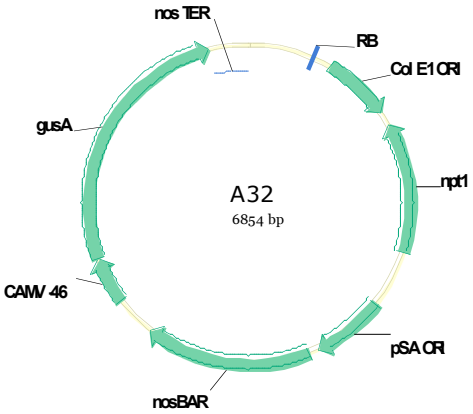
pMDC44

pMDC44 drives expression of a GFP fusion protein under control of the ubiquitous 35SCaMV promoter in plants.

2.1.6 Constructed plasmids

pA32

The pA32 vector, based on the pGREENnos-BAR backbone, carries the mp-uidA-ter cassette from the K373 vector (cloned *Hin*d III-*Not* I). This vector was used to isolate promoting/enhancer short regions in the CLV3 regulatory sequences.



pB4, pD4, pE4, pG2_1, pI4

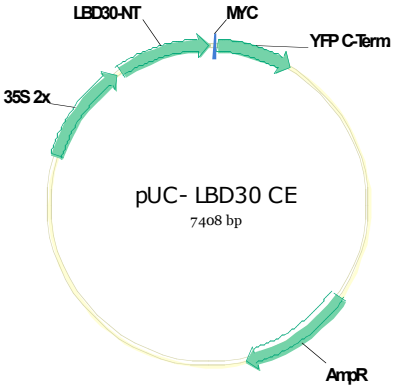
Each of these vectors contain, in front of a minimal promoter driving GUS expression, a short *Apa* I-*Cla* I cloned sub-region derived from the CLV3 putative enhancer present in pBUdel5. The backbone of these vectors is the pA32 vector. Each sub-region was previously cloned into the pCRII-TOPO vector for amplification and sequencing.

pX2, pY2

Each of these vectors, based on the pA32 backbone, carries a different sub-sequence derived from the CLV3 promoter fragment cloned into the pBUdel3 vector. Each of the approximately 180bp long sequences is cloned in front of a minimal promoter driving GUS expression (cloned *Apa* I-*Cla* I). The fragments were previously cloned into the pCRII-TOPO vector for amplification and sequencing.

pUC-SPYNE/SPYCE -LOL/ -B3/ -BROMO/ -AGO4 /-TXN

These vectors were used to confirm the possible interactions of LOL with its putative partners using a biolistic procedure: a B3-domain protein, a BROMO-domain protein, ARGONAUTE4 (AGO4) and Tritorax neighbor protein (TXN). LOL, B3 and BROMO sequences were cloned in *Bam*H I-*Xho* I restriction sites, AGO4 and TXN in *Bam* HI-*Spe* I.



pY22_Del2

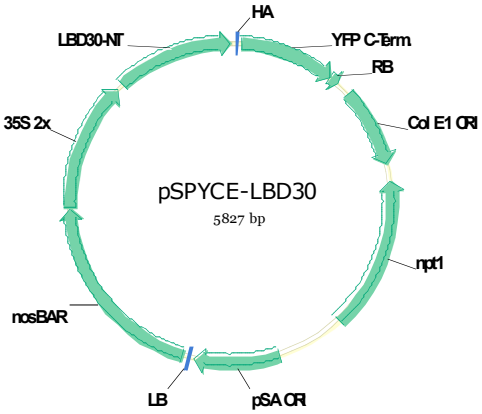
I constructed this vector so that Rebecca Kloppenburg could run a yeast one-hybrid screen against an Arabidopsis meristem cDNA bank, to find putative interactors with the Del1-Del2 CLV3 promoter region. The Del1-Del2 fragment is cloned into the *Xho* I site of the vector.

pDAD

This yeast transformation vector expresses a fusion protein between the *DONRÖSCHEN* (DRN) and the AD domain from the pGAD-T7 vector. The DRN sequence was cloned *Bam*H I-*Xho* I. Rebecca Kloppenburg used this vector to test if DRN can interact with sub-fragments of the CLV3 regulatory sequences in a yeast one-hybrid screen.

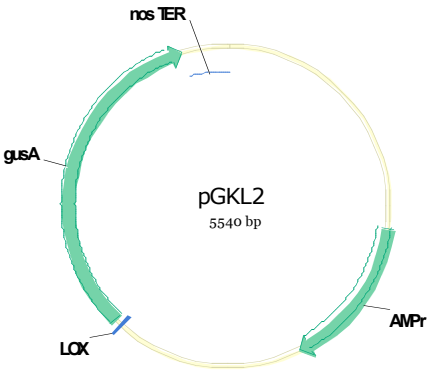
pSPYCE/SPYNE -LOL/ -B3/ -BROMO/ -AGO4 /-TXN

These vectors were used to test if LOL interacts *in vivo* with its putative partners isolated with a yeast two-hybrid screen analysis in Arabidopsis and tobacco leaf cells. LOL, B3 and BROMO sequences were cloned *Bam*H I-*Xho* I, AGO4 and TXN were cloned *Bam*H I-*Spe*I.



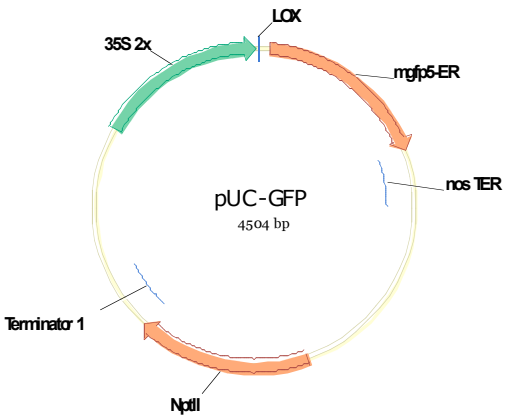
pGKL2

In this vector, with a pK373 backbone a loxP sequence is cloned using *Pst* I-*Nco* I. The loxP-GUS-ter cassette is part of the pQCLOX1 vector.



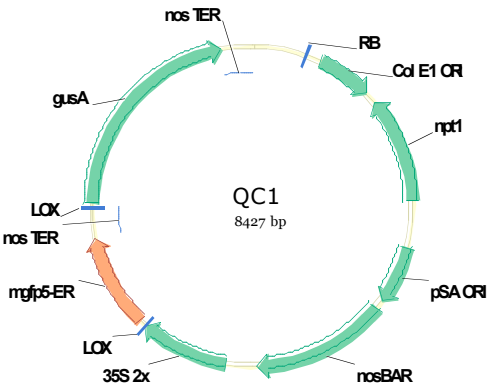
pUC-GFP

In this vector, based on the pBI121-GFP backbone, a loxP sequence is cloned in *Bam*H I-*Xba* I, and a *nos-ter* sequence in *Sst* I. The loxP-GFP-ter cassette is part of the pQCLOX1 vector.



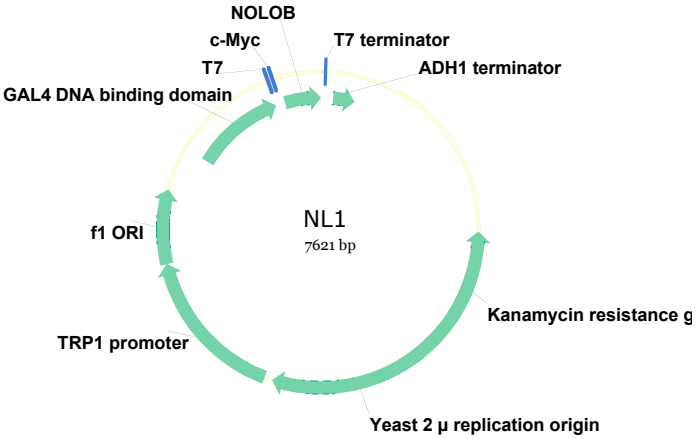
pQCLOX1

The *Eco*R I-*Sma* I (from pUC-GFP) and the *Sma* I-*Sst* 1 (from pGKL2) cassettes were cloned into the pGreen-nos-BAR plasmid, thus resulting in the pQCLOX1 (pQC1) vector. The 35SCaMV promoter can alternatively drive GFP or GUS expression, depending on the DNA excision event controlled by the CRE enzyme cloned in pALCA-CRE (see pag. 31)



pNL1

This vector was used by Rebecca Kloppenburg in a yeast two-hybrid screen to isolate LOL interaction partners. Its backbone is the PGKB-T7 vector. The NL1 sequence, i.e. the LOL sequence without LOB domain, was cloned *Nco* I-*Bam*H I.



pF1

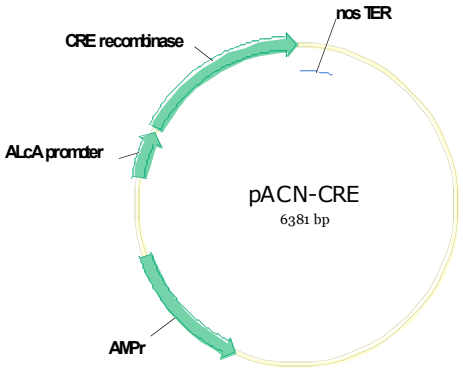
This vector was used in a yeast two-hybrid screen by Rebecca Kloppenburg to isolate LOL partners. Its backbone is the PGKB-T7 vector. F1, i.e. the full LOL sequence, was cloned *Nco* I-*Bam* HI.

pL1

This vector was used in a yeast two-hybrid screen by Rebecca Kloppenburg to isolate LOL interaction partners. Its backbone is the PGKB-T7 vector. L1, i.e. the LOB domain of LOL, was cloned *Nco* I-*Bam* HI.

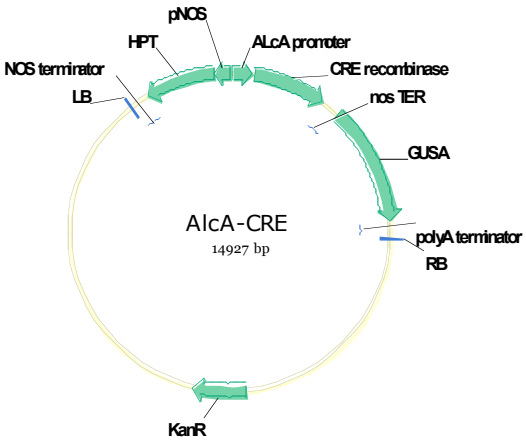
pACN-CRE

The CRE recombinase, excised from the pgk-CRE-bpa vector, was cloned using *Pst* I into the pACN vector under control of the AlcA promoter to obtain an ethanol inducible CRE expression.



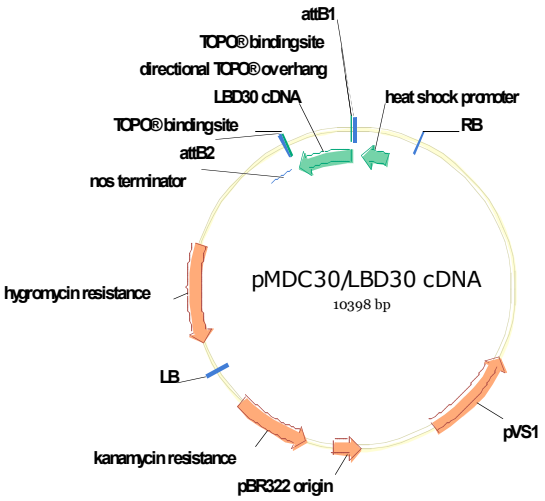
pAlcA-CRE

The AlcA-CRE cassette from the pACN-CRE vector was excised with *Nsi* I and cloned using *Pst* I into the plant expression vector pGPTV-HPT. Plants transgenic for the CLV3::*AlcR* vector were transformed with pAlcA-CRE to obtain an ethanol inducible system which permits to induce CRE expression only in the *CLV3* domain. pAlcA-CRE carries a bacterial Kanamycin resistance and a plant Hygromycin resistance.



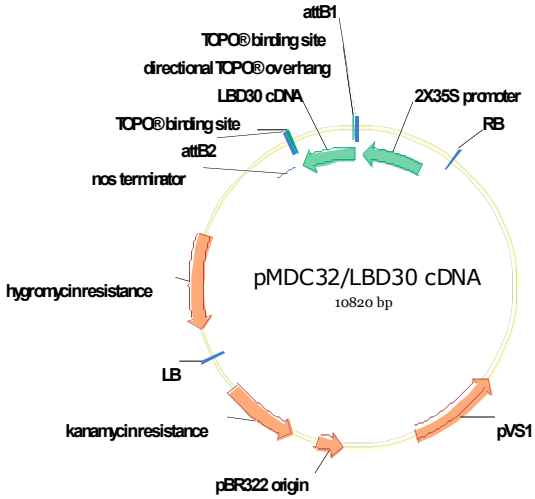
pMDC30 -LOL

This vector, obtained via the GATEWAY (Invitrogen) LR recombination reaction with a pENTR/-D TOPO vector containing the cDNA sequence of LOL (named pLOL-entry), was constructed to promote ectopic LOL expression under the control of a heat shock promoter.



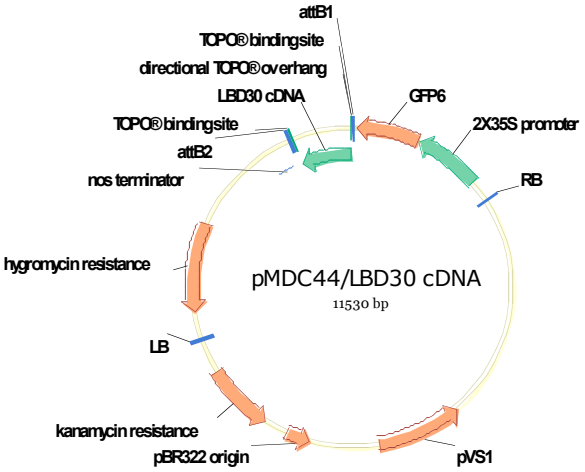
pMDC32 -LOL

This vector, obtained via the GATEWAY (Invitrogen) LR recombination reaction with the pLOL-entry vector, was constructed to promote LOL ectopic expression under control of the strong and ubiquitous promoter 35SCaMV.



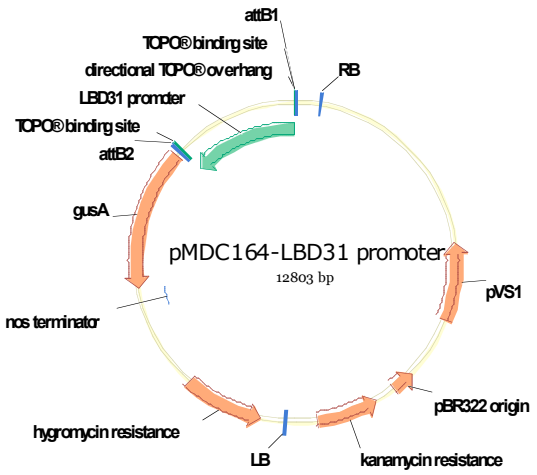
pMDC44 -LOL

This vector, obtained via the GATEWAY (Invitrogen) LR recombination reaction with the pLOL-entry vector, was designed to ectopically express a LOL-GFP fusion.



pMDC164 - LBD31

This vector, obtained via the GATEWAY (Invitrogen) LR recombination reaction, was constructed to promote GUS expression under control of the putative *LBD31* promoter. The *LBD31* promoter was previously cloned into the pENTR/-D TOPO vector (named pLBD31PR-entry).



pDel8

This vector, as all of the following pDelx vectors, is based on the pBU16 plasmid from Brand et al., 2002. This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-746; 3+595).

pDel10

pDel10 carries a deletion fragment of the CLV3 promoter (5-1487/-286; 5-154; 3+1256)

pDel13

This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-746; 3+965).

pDel14

This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-1166; 3+595).

pDel15

This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-1166; 3+965).

pDel16

This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-746; 3+275)

pDel17

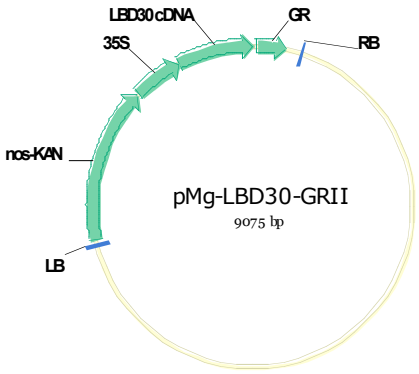
This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-746; 3+1)

pDel18

This vector carries a combination of deletion fragments of both *CLV3* upstream and downstream regulatory sequences (5-746; 3+275; 3+595/965)

pBI-LOL-GR

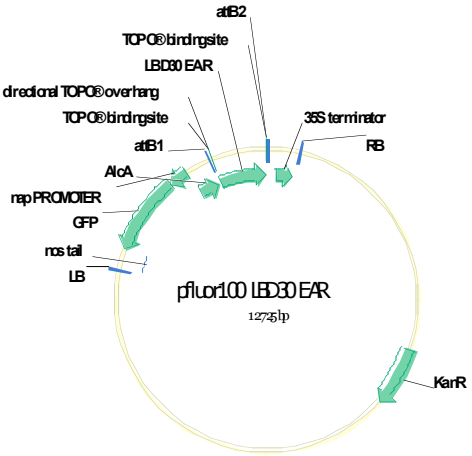
This plant expression vector promotes the ectopic expression of a LOL-GR fusion protein. pBI-LOL-GR is a pBI-ΔGR derivative. *LOL* cDNA was cloned *Spe* I-*Bam* HI into the *Xba* I - *Bam* HI sites of pBIΔ-GR.



pFullg, pDel2g, pDel3g, pDel4g, pDel6g, pDel7g and pDel8g

These vectors contain, instead of GUS, the *CLV3* genomic sequence. They were used to quantify the strength of different deletions of the *CLV3* regulatory sequences in wild type and *clv3-2* mutant plants. The *GUS* sequence was excised from pBU16, pBUDel2, pBUDel3, pBUDel4, pBUDel6, pBUDel7 and pDel8 and *CLV3* cloned using *Pst* I-*Not* I.

pETOH::LOL-EAR



In this pFluor100 GATEWAY (Invitrogen) plant destination vector, the LOL-EAR fusion, from pLOL-EAR, a pMDC32 vector ectopically expressing the LOL-EAR fusion (a dominant negative version of LOL) was cloned. An AlcA promoter drives LOL-EAR expression, while a Nap-promoter (expressed only in the seed coat) drives GFP (or, in other versions of the vector, YFP, CFP or RFP). Transgenic plants for this vector were selected by the fluorescence of their seed coats. This vector was kindly donated by Prof. Dr. Lucia Colombo (Milano University).

2.1.7 Oligonucleotides

The oligonucleotides used in this work for sequencing or amplification via PCR, RT-PCR or qRT-PCR, are listed in the following tables. Oligonucleotides were ordered from *Eurogentech*, Belgium; *Sigma*, Darmstadt; *Biotez*, Berlin and *Invitrogen*, Karlsruhe.

Name	Sequence	Description
LB401R	AAAAGTCGACATTTATCCTTCCCACCACATCAT	3´CLV3 enhancer deletion
LB252R	AAAAATCGATACCATAAATTTTAACGTATTA	3´CLV3 enhancer deletion
LB322R	AAAAATCGATTATTAAAATATCTTTTCAAGT	3´CLV3 enhancer deletion
LB402R	AAAAATCGATATTTATCCTTCCCACCACATCAT	3´CLV3 enhancer deletion
LB250R	AAAAGTCGACACCATAAATTTTAACGTATTA	3´CLV3 enhancer deletion
LB320R	AAAAGTCGACTATTAAAATATCTTTTCAAGT	3´CLV3 enhancer deletion
LB400R	AAAAGTCGACATTTATCCTTCCCACCACATCAT	3´CLV3 enhancer deletion
LBY26R	AAAAACTAGTGCGAGAAATGGGATCTCCTAT	3´CLV3 enhancer deletion
BLDEL6OS	GGGGGAGCTCATATAAAAAAATTGGTGATGGC GTAATAAC	3´CLV3 enhancer deletion
BLDEL6R	AAAACTCGAGATTCCAAAGCAAGTTAGATAT	3´CLV3 enhancer deletion
BLDEL4R	GGGGCTCGAGACCATAAATTTTAACGTATTA	3´CLV3 enhancer deletion
BL_D4D5aR	AAAACTCGAGCGAATCATCATCATCATTTT	3´CLV3 enhancer deletion
BL_D4D5bR	AAAACTCGAGCTACAAATGGTTGTCTTTGAC	3´CLV3 enhancer deletion
BL_D4D5cR	AAAACTCGAGTATTAAAATATCTTTTCAAGT	3´CLV3 enhancer deletion
LBGCLV3R	AAGCGGCCGCTCAAGGGAGC	3´CLV3 gene
LBCL3GR2	GGATCCTCAAGGGAGCTGAAAGTTGTT	3´CLV3 gene
LBCLV3R	TGCCTTCTCTGCTTCTCCAT	3´CLV3 gene
LBY26RHS	AAAAGTCGACGCGAGAAATGGGATCTCCTAT	3´CLV3 promoter deletion
LB3UR1	AAAAATCGATTCCTAAACGTGTATCATAGTT	3´CLV3 promoter deletion
LB3UR2	AAAAATCGATGCGTAAGCCTACAAGGGCGAG	3´CLV3 promoter deletion
LB3UR3	AAAAATCGATCTTTACTTTGGTAATGAAATG	3´CLV3 promoter deletion
LBY3R1	AAAAGAGTCTTTTTAGAGAGAAAGTGACTGA	3´CLV3 promoter deletion
LBY3R2	AAAACTCGAGTTTTAGAGAGAAAGTGACTGA	3´CLV3 promoter deletion
BLDEL1R	GGGGCTCGAGTATTTAGAAAAAAAAATGTAACC	3´CLV3 promoter deletion
BLDEL2R	GGGGCTCGAGTGATTTAGCTATAAATAAATTAA	3´CLV3 promoter deletion
LB–GUS2	AAAAAATGTAATGTTCTGCGACGCTCAC	3´GUS gene
LOL220PR	CTGCAGGTCCAAATAAACAAACATAC	3´LBD30
LOL220BR	GGATTCGTCCAAATAAACAAACATAC	3´LBD30
LB220BAM	AAAAGGATTCAAAGGACTTGTGTGGTAGAAA	3´LBD30
LB220SPE	AAAAACTAGTGTCCAAATAAACAAACATACG	3´LBD30
BLTP22GR	TCATTCTCGTTTTATCACTGACGAGGCAGAA	3´LBD30
BL20XRYFP	AAAACTCGAGTTCTCGTTTTATCACTGACGAGG	3´LBD30

	C	
LOL210PR	CTGCAGGTTCCTAATAAATGTCGCAA	3´LBD31
LOL210BR	GGATTCGTTCCTAATAAATGTCGCAA	3´LBD31
LB210BAM	AAAAGGATTCGTTACGTCTTTGACATAAAAG	3´LBD31
LB210HIN	AAAAAAGCTTTCCTAATAAATGTCGCAAAGG	3´LBD31
LB210SR2	CGACAGAAGAAATGAGTGGCACA	3´LBD31
BLTP21GR	TTATATTAAAGAAGATGGTCGGTATTTGCCTCC GGT	3´LBD31
LB251F	AAAAGTCGACCCTAATCTCTTGTTGCTTTAA	5´CLV3 enhancer deletion
LB252F	AAAAGGGCCCCCTAATCTCTTGTTGCTTTAA	5´CLV3 enhancer deletion
LB322F	AAAAGGGCCCTTTTACGTATAAAATGCAAAATA	5´CLV3 enhancer deletion
LB402F	AAAAGGGCCCCATTACGTTTGTTGCTGAAGTGA	5´CLV3 enhancer deletion
LB250F	AAAAAAGCTTCCTAATCTCTTGTTGCTTTAA	5´CLV3 enhancer deletion
LB320F	AAAAAAGCTTTTTTACGTATAAAATGCAAAATA	5´CLV3 enhancer deletion
LB400F	AAAAAAGCTTCATTACGTTTGTTGCTGAAGTGA	5´CLV3 enhancer deletion
LBY26F	AAAAGAATTCTGAACAAGTTCGTATAAGATC	5´CLV3 enhancer deletion
BLDEL6ON	GGGGGCGGCCGCATATAAAAAAATTGGTGATG GCGTAATAAC	5´CLV3 enhancer deletion
BLDEL6F	AAAACTCGAGATATAAAAAAATTGGTGATGG	5´CLV3 enhancer deletion
BLDEL4F	GGGGCTCGAGCCTAATCTCTTGTTGCTTTAA	5´CLV3 enhancer deletion
BL_D4D5aF	AAAACTCGAGTTTTACGTATAAAATGCAAA	5´CLV3 enhancer deletion
BL_D4D5bF	AAAACTCGAGTTCAATTGTCAATGCAAATA	5´CLV3 enhancer deletion
BL_D4D5cF	AAAACTCGAGATGATGTGGTGGGAAGGATA	5´CLV3 enhancer deletion
LBGCLV3F	AACTGCAGATGGATTCGAAG	5´CLV3 gene
LBCLV3F	TTTCCAACCGCAAGGTTATC	5´CLV3 gene
LBY26FHS	AAAAAAGCTTTGAACAAGTTCGTATAAGATC	5´CLV3 promoter deletion
LB3UF1	AAAAGGGCCCTGAACAAGTTCGTATAAGATC	5´CLV3 promoter deletion
LBY3F1	AAAAGAATTCTGAACAAGTTCGTATAAGATC	5´CLV3 promoter deletion
BLDEL1F	GGGGCTCGAGTCAGAAGTGTACTCCAGGTAT	5´CLV3 promoter deletion
BLDEL2F	GGGGCTCGAGCACAAATATTATATGTTTAAT	5´CLV3 promoter deletion
LOL220PF	CTGCAGAAGGACTTGTGTGGTAGAAA	5´LBD30
LOL220NF	GCGGCCGCAAGGACTTGTGTGGTAGAAA	5´LBD30
LB220KPN	AAAAGGTACCAAAGGACTTGTGTGGTAGAAA	5´LBD30
LB220XHO	AAAACTCGAGGTCCAAATAAACAAACATACG	5´LBD30
BLTP22GF	CACCATGAGCAGTAGCGGAAACCCTAGC	5´LBD30
BL20BFYFP	AAAAGGATCCATGAGCAGTAGCGGAAACCCT	5´LBD30
LOL210PF	CTGCAGCGTTACGTCTTTGACATAAA	5´LBD31
LOL210NF	GCGGCCGCCGTTACGTCTTTGACATAAA	5´LBD31
LB210XHO	AAAACTCGAGGTTACGTCTTTGACATAAAAG	5´LBD31
LB210KPN	AAAAGGTACCTCCTAATAAATGTCGCAAAGG	5´LBD31
BLTP21GF	CACCATGAGCGGAAGCACCACCGG	5´LBD31
BL10XF	AAAATCTAGACATGAGCGGAAGCACCACCGGT TGTGG	5´LBD31
BL20SF	AAAAACTAGTCATGAGCAGTAGCGGAAACCCTA	5´LBD31

	GCAGC	
LBAFD9	GAATTCCGGATTATCCATAATAAAAAC	*B. oleracea* CLV3 sequencing
LBBFD9	CTGCAGCCCTTGTAGGCTTACGCTATA	*B. oleracea* CLV3 sequencing
LBARD9	CTGCAGTGATTTAGCTATAAATAAATT	*B. oleracea* CLV3 sequencing
LBBRD9	CCCGGGTTTTAGAGAGAAAGTGACTGA	*B. oleracea* CLV3 sequencing
LBFUDOF	ATCGATCCTAATCTCTTGTTGCTTTAA	*B. oleracea* CLV3 sequencing
LBFUDOR	GAATTCATGTGTGTTTTTTCTAAACAA	*B. oleracea* CLV3 sequencing
LBBOL5	CCAATGTTCATGCACTTCCCATTC	*B. oleracea* CLV3 sequencing
LBBOL6	TAAATTGTATTTGAATGATACGGA	*B. oleracea* CLV3 sequencing
LBBOU1	AAAACGTAGAGTCTAAAAACAAGTTC	*B. oleracea* CLV3 sequencing
LBBOU2	AAAACTTGCAGCCTATAAATGATTGC	*B. oleracea* CLV3 sequencing
LBBOU3	AAAAATGGATTCGAGGACTCTGGTGC	*B. oleracea* CLV3 sequencing
LBBOD1	AAAAAGTGGGGTCCACAAAACGCTGA	*B. oleracea* CLV3 sequencing
LBBOD2	AAAAGCTGAACAGAGAGCAAAAACTC	*B. oleracea* CLV3 sequencing
LBL3L1	AATATCGTATCATATAGATT	*B. oleracea* CLV3 sequencing
LBL4L2	TATACTACAGTGTGCATGTT	*B. oleracea* CLV3 sequencing
LBBRD1	AGTGGGGTCCACAAAACGCTGA	*B. oleracea* CLV3 sequencing
LBBRD2	GCTGAACAGAGAGCAAAAACTC	*B. oleracea* CLV3 sequencing
BLCREISF	TCCAATTTACTGACCGTACACCAAAATTTGCCT	CRE in situ
BLCREISR-T7	GTAATACGACTCACTATAGGGCATCGCCATCTT CCAGCAGGC	CRE in situ
LBCRER	GAGTTGATAGCTGGCTGGTGG	CRE recombinase 3´
LBCREF	TGGGCCAGCTAAACATGCTT	CRE recombinase 5´
BL1HD2F1	AAA AGA ATT CTA CCT TCT CAT ATT TAG ATG CTA T	DEL2 for yeast 1-hybrid
BL1HD2R1	AAA ACT CGA GTA TAA AAC GGC AGG GGT AAT A	DEL2 for yeast 1-hybrid
BL1HD2R2	AAA ATC TAG ATA TAA AAC GGC AGG GGT AAT A	DEL2 for yeast 1-hybrid
BLDRNADR	AAAACTCGAGCCTATCCCCACGATCTTC	DONRÖSCHEN 3´
BLDRNADF	AAAAGGATCCGAATGGAAAAAGCCTTGAGA	DONRÖSCHEN 5´
LOX1A	CTAGTATAACTTCGTATAGCATACATTATACGAA GTTATC	HPLC purified, to create the LOX site
LOX1B	GATCGATAACTTCGTATAATGTATGCTATACGA AGTTATA	HPLC purified, to create the LOX site
LOX2A	AGCTTCCCGGGATAACTTCGTATAGCATACATT ATACGAAGTTATC	HPLC purified, to create the LOX site
LOX2B	CATGGATAACTTCGTATAATGTATGCTATACGA AGTTATCCCGGGA	HPLC purified, to create the LOX site
LOXA1	CTAGTATAACTTCGTATAGCATACATTATACGAA GTTATC	HPLC purified, to create the LOX site
LOXA2	GATCGATAACTTCGTATAATGTATGCTATACGA AGTTATA	HPLC purified, to create the LOX site
LOXB1	AGCTTCCCGGGATAACTTCGTATAGCATACATT ATACGAAGTTATC	HPLC purified, to create the LOX site

LOXB2	CATGGATAACTTCGTATAATGTATGCTATACGAAGTTATCCCGGGA	HPLC purified, to create the LOX site
LB22ISAT	GTAATACGACTCACTATAGGGCGTAAATCATATAGTTCTTAT	LBD30 3´ for in situ
LB22ISAT2	GTAATACGACTCACTATAGGGCCAGAACCACTGGTTCCCG	LBD30 3´ for in situ
LB22ISAF	CTTTCATATCTACAAGCACA	LBD30 5´ for in situ
LB22ISAF2	GAGTTAAATCCATGTATCTCAAAA	LBD30 5´ for in situ
LB22ISIF2	GAGCTGCCACAACCTCAAC	LBD30 5´ for in situ
BL210EAR	TCAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCAAGTATTAAAGAAGATGGTCGGT	LBD30 EAR fusion 3´
BL220EARR	TCAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCAAGTTCTCGTTTTATCACTGACG	LBD30 EAR fusion 5´
LBPR2203	AAAAAGCAGGCTGAGCCATTGGTTGCGTC	LBD30 promoter
LBPR2204	AAAAAGCAGGCTAAGATTGGACAAGAGCCGG	LBD30 promoter
LBPR2205	AAAAAGCAGGCTGCGGTGAGGAAAGGAG	LBD30 promoter
LBPR2R3	AGAAAGCTGGGTGTGGTGATTAGGGTTTTGAGA	LBD30 promoter
LBPR220F	AAAAAGCAGGCTTTCTCTTGTACTATTAGGCC	LBD30 promoter
LBPR220R	AGAAAGCTGGGTCTTCCTTTTCTACCACACAA	LBD30 promoter
LBPR220F2	AAAAAGCAGGCTAGAAATGAAATGGGAGCGTT	LBD30 promoter
LBPR220R2	AGAAAGCTGGGTCTTCCTTTTCTACCACACAAGTC	LBD30 promoter
BL22TOF2	CACCCACTCGCAAACGAGCCATTGGTTGCGTCA	LBD30 promoter
BL22TOR2	CTTCCTTTTCTACCACACAAGTCCTTTTTATTT	LBD30 promoter
BL22TOF3	CACCGACACATGGCGATCATATATACG	LBD30 promoter
BL20PRR3	GCTGCTAGGGTTTCCGCTACTGCTCAT	LBD30 promoter
BL22GRNT	TTCTCGTTTTATCACTGACGAGGCAGAA	LBD30 without stop codon 3´
BL20BNTR	AAAAGGATCCTCTTCTCGTTTTATCACTGACGAGGCAGAA	LBD30 without stop codon 3´
LB21ISAT	GTAATACGACTCACTATAGGGCAAGAAGATGGTCGGTATTTG	LBD31 3´ for in situ
BL_LOLISR	AATTAACCCTCACTAAAGGGAACAAAAGCTCATTCTCGTTTTATCACTGA	LBD31 3´ for in situ T3
LB21ISAF	GCTTACGTCCAAACTCAACT	LBD31 5´ for in situ
BL_LOLISF	ATGAGCAGTAGCGGAAACCC	LBD31 5´ for in situ
LBPR210F	AAAAAGCAGGCTCTAGTGCATATATTTCACAA	LBD31 promoter
LBPR210R	AGAAAGCTGGGTCTTTTATGTCAAAGACGTAA	LBD31 promoter
LBPR210F2	AAAAAGCAGGCTCCAATGGCTCGTTTGCGA	LBD31 promoter
LBPR210R2	AGAAAGCTGGGTCTTTTATGTCAAAGACGTAACGT	LBD31 promoter
BL21GRNT	TATTAAAGAAGATGGTCGGTATTTGCCTCCGGT	LBD31 without stop codon 3´
BL10BNTR	AAAAGGATCCTCTATTAAAGAAGATGGTCGGTATTTGCCTCCGGT	LBD31 without stop codon 3´

LBGFPF	CTGTCCACACAATCTGCCCTTT	mGFP5-er 5´
LBTERF	GGCGGCCGCTCTAGAACTAGTGGATCA	nos-ter
LBTERR	GGGATCCTCTAGAGTCCGCAAAAATCACCAG	nos-ter
BLTERSR2	AAAAGAGCTCCCCGGGGGATCAGCTTGCATGCCTGCAGGTCACT	nos-ter
BLTERSF2	AAAAGAGCTCGTCCGCAAAAATCACCAGTCTCTC	nos-ter
BLTERNF	AAAAGCGGCCGCGTCCGCAAAAATCACCAGTCTCTC	nos-ter
BLTERSR	AAAAGAGCTCGGATCAGCTTGCATGCCTGCAGG	nos-ter
RT_AIR3F	CTCGTTATCCTTCTTGGAGC	Real time PCR
RT_AIR3R	GGTCCAGGAATGTCATCCAT	Real time PCR
RT_LHYF	GACAAAGACTGCTGTTCAGA	Real time PCR
RT_LHYR	GATGCCTTTAACTTCAGCCT	Real time PCR
NP_LBD41F	GTGTAGTGAGGATTGTAGTA	Real time PCR
NP_LBD41R	CACTATTTCAGACTGATGAC	Real time PCR
RT_ARR7R	GTCACTATCAAATTCACCTTCA	Real time PCR
RT_ARR7F	CTAGGGCTTTGCAGTATCTT	Real time PCR
RT_NOP56R	TTAGGAAGATTCAACTCCAGAAA	Real time PCR
RT_NOP56F	ATGCTCTCAACCAAGTCAAC	Real time PCR
RT_EXP8R	AACCTTATTCCTCCTTTCTTCAT	Real time PCR
RT_EXP8F	TTCAGATCGCTCAGTATCGT	Real time PCR
RT_EXP1F	TTCAACGCATCGCTCAATAC	Real time PCR
RT_EXP1R	CTTATTCCTCCTCTTCTCACG	Real time PCR
RT_COL2F	CTGATGTCCATTGATGAAACGG	Real time PCR
RT_COL2R	GATTCTCCTCAGGAGCTCAC	Real time PCR
RT_EXP3R	GAATCTTATCCCTCCTATCTTCC	Real time PCR
RT_EXP3F	CAAGATCGGTCTATACCGTG	Real time PCR
RT_DFL1F	GTTGGCATCAGTTTCCTCTC	Real time PCR
RT_DFL1R	CTCAAGTCCTCTGTTCTAACC	Real time PCR
RT_AXR3F	CTTGTCCTAAAGATCCAGCC	Real time PCR
RT_AXR3R	ACGTTCTTCCGGTATGATCTC	Real time PCR
RT_CYCD32F	CTCTAATCGAAACCAAGCCA	Real time PCR
RT_CYCD32R	CACAGCAAGATACGTCAGAG	Real time PCR
RT_ARR16F	GTTCCTGTTGTGATAATGTCTTCAG	Real time PCR
RT_ARR16R	GCATAAACATTTGAGCTCCAC	Real time PCR
RT_PIN3F	TTCTATCTGATGCTGGTCTTGG	Real time PCR
RT_PIN3R	CCACAAGCGATTAATTTGGGT	Real time PCR
RT_ILL5F	TTGCTTTGAGGGCTGATATG	Real time PCR
RT_ILL5R	ATCTTCCCTGGAATCTTACTC	Real time PCR
RT_PIN7F	TTTCCGCAAGCAATTAATTTCG	Real time PCR
RT_PIN7R	TTTCTGATGCTGGTCTTGGT	Real time PCR
RT_CUCLF	AAGTTGATCTCAACAAGATTGAG	Real time PCR
RT_CUCLR	ACACAGAAGAAATACCATTCTTT	Real time PCR

RT_PIN4R	CCTCTCCACTATCAAGACCG	Real time PCR
RT_PIN4R	GCTAAGGAGATTCGGATGGT	Real time PCR
RT_CYCD31F	GGATTTCCTCAACAAATGCC	Real time PCR
RT_CYCD31R	AACTACTGATGGGAGGTACC	Real time PCR
RT_IAA4F	AAGAGATTGAATCCACTGGA	Real time PCR
RT_IAA4R	TAAGATCTAACTGGTGGCCA	Real time PCR
BL_CYCD3F	TAAAGTCGAAGAAACCCAAGT	RT-PCR
BL_CYCD3R	TAGTTGGAGGATTAGATCGTAG	RT-PCR
BL_PIN7F	TCGAATGTTGATCTCTGATCATA	RT-PCR
BL_PIN7R	GTGTAATCGGTAGTGCGATAAG	RT-PCR
BL_AXR3F	GAGAAGAGTGCTTGTCCTAA	RT-PCR
BL_AXR3R	ATATAATCGATACCACTTATCCTTT	RT-PCR
BL_EXP3F	GAATGCACACGCCACTTT	RT-PCR
BL_EXP3R	ATCTTATCCCTCCTATCTTCCGA	RT-PCR
BL_COL2F	TCAGGAGCTCACTACAACA	RT-PCR
BL_COL2R	GCACAACACTGATGTCCA	RT-PCR
BL_PIN3F	CCAGATCAATCTCACAACGG	RT-PCR
BL_PIN3R	ATGTAGTAAACCAGCGTGAT	RT-PCR
BL_ARR7F	ATAGTATTGTGGATCGTAAAGTCAT	RT-PCR
BL_ARR7R	TTCCTCTGCTCCTTCTTTGA	RT-PCR
BL_LB41F	GGTCCCAATCACCTTCGT	RT-PCR
BL_LB41R	AACCATAGATCGGATTCACAAT	RT-PCR
BL_EXP1F	GCACACGCCACATTCTAC	RT-PCR
BL_EXP1R	GTAAACCTTATTCCTCCTCTTCTC	RT-PCR
BL_ILL5F	CTGATATGGATGCACTTCCTAT	RT-PCR
BL_ILL5R	TCAAGAAAGTCAACTGTTGCATTA	RT-PCR
BL_NOP56F	TCTGCTCTCGATGCTCTC	RT-PCR
BL_NOP56R	TCTTCACCGAGGCATCAA	RT-PCR
BL_PIN4F	GAACAAGGTGCTAAGGAGAT	RT-PCR
BL_PIN4R	GGTAAGGCTATTAACATTCCAAA	RT-PCR
BL_LB42F	GGTCTTCTTAACCTCATCGAAT	RT-PCR
BL_LB42R	GACCACAAGCCTCGTACA	RT-PCR
BL_EFEF	AGCAATCACTATGGAGAAGATCA	RT-PCR
BL_EFER	ACTCTTGTACTTCCCATTGGTTA	RT-PCR
BL_CUCLF	GATTGAGCCTTGGGACTTAC	RT-PCR
BL_CUCLR	ATTGGAACCTTTGTACCATCG	RT-PCR
BL_ARR16F	GCTCAAGATCTCTTGTTGCAAA	RT-PCR
BL_ARR16R	CAACATCAGCAAGCTTCAAAG	RT-PCR
BL_AIR3F	TTCTTCTTGTTCACATGAGCT	RT-PCR
BL_AIR3R	TTGGATAGGTCCAGGAATGT	RT-PCR
BL_CYCDF	CCTCAAGTCCTCTGCTT	RT-PCR
BL_CYCDR	GTAACACTCCATTAACTCATCC	RT-PCR
BL_AS1SF	GAGCGGTCTAACGTTGTCCC	RT-PCR
BL_AS1SR	TCTGCTCTTCCCTAAGAGCT	RT-PCR
BLBPF	GCTCATCCTCACTACTCTACCCTCCTA	RT-PCR

BLBPR	GATTTGTTTCTGATCTAACCCCGTTGA	RT-PCR
BLKNAT2F	GCTTACTCCAAACCTACATCGATTGCC	RT-PCR
BLKNAT2R	AACGGCATGTTCTCCGAAGGCTTCCAA	RT-PCR
BLKNAT6F	GTATCCTCGCTTACTTCAAGCTTACAT	RT-PCR
BLKNAT6R	TTCCTCGGTAAAGAATGATCCACTAGAAT	RT-PCR
BLYAB3F	ACTTCTCATCTACGGACCAGCTCTGTT	RT-PCR
BLYAB3R	AACGTTGGCAGCTGAACCGTAAAACCC	RT-PCR
BL_AS2F	AAAAGGATCCATGGCATCTTCTTCAACAAA	RT-PCR
BL_AS2R	AAAACTCGAGTCAAGACGGATCAACAGTAC	RT-PCR
35sF	TCTATATAAGGAAGTTCATT	sequencing of 3´of CaMV35S
BL35SPRF	CCACTATCCTTCGCAAGA	Sequencing of CaMV35S promoter
BLGAL4F	GATGAAGATACCCCACCA	Sequencing of GAL4
LBGFPREV	AGTGAAAAGTTCTTCTCCT	Sequencing from GFP 3´
BLPACTR	GTGAACTTGCGGGGTTTT	Sequencing from PACT
LBLBB1	GCGTGGACCGCTTGCTGCAACT	Sequencing from T-DNA insertion
RBa1	TGGCCTCCGGACCAGCCTCC	Sequencing from T-DNA insertion
RBb1	GCACTTCAGGAACAAGCGGG	Sequencing from T-DNA insertion
BLLBB1V2	AAACCAGCGTGGACCGCTTGCTGCAACTCT	Sequencing from T-DNA insertion
BLYFPCR	CGTCCTCGATGTTGTGGC	Sequencing from YFP C-ter
BLYFPNR	CTCGACCAGGATGGGCAC	Sequencing from YFP N-ter
BLGFPLR	TGTTGCATCACCTTCACCCTCT	Sequencing GFP-LOX 3´
BLGFPLF	TGTCCACACAATCTGCCCTTTC	Sequencing GFP-LOX 5´
BL_STMISR	GTAATACGACTCACTATAGGGCTTCTGACAATT GAC	STM for in situ 3´
BL_STMISF	ATGGAGAGTGGTTCCAACAG	STM for in situ 5´
LB6145LP	TCTCAATTAGGATCACGGCACA	To check for T-DNA insertion in Salk line
LB6145RP	CCCACATGTGAACAGGTGATGA	To check for T-DNA insertion in Salk line
LB7808LP	TCACGGGCAAGAGCGTTAAGA	To check for T-DNA insertion in Salk line
LB7808RP	TCATGATGATGATTCCAGCCAGCC	To check for T-DNA insertion in Salk line
LB4730LP	TCACATTAAAATCACCGCCAAAA	To check for T-DNA insertion in Salk line
LB4730RP	TGTGAGAGACGCAGCCGTAGA	To check for T-DNA insertion in Salk line
LB7808RP	TCATGATGATTCCAGCCAGCC	To check for T-DNA insertion in Salk line
BL_BROR2	TGCTTCCTAAGTTTATCATCG	To check for T-DNA insertion in Salk line
BL_BROF	TGCCTCCTCAATTAGTCGAG	To check for T-DNA insertion in Salk line
BL_BROR	TTAACATTGGGCTTCTTTTGCTT	To check for T-DNA insertion in Salk line

BLQCLOX1	ATATCTCCTTGGATCGATAACTTCGTATAATGTATGCTATACGAAG	to remove the point mutation from the LOX
BLQCLOX2	CTTCGTATAGCATACATTATACGAAGTTATCGATCCAAGGAGATAT	to remove the point mutation from the LOX
BLY22F	CTGAGTTCCGACAACAATG	to sequence the clones inpY22
BLY22R	ATATGATCATGTGTCGTCGCA	to sequence the clones inpY22
BLWUSR3	AATGATCGTTAGCCGCCATC	WUSCHEL 3´
BLWUSSAR	GGGGGAGCTCGGATCCGCGTTCAGACGTAGCTCAAG	WUSCHEL 3´
BLWUSADR	GGGGCTCGAGCTAGTTCAGACGTAGCTCA	WUSCHEL 3´
BLWUSF3	CCCAGCTTCAATAACGGGAA	WUSCHEL 5´
BLWUSXHF	GGGGCTCGAGATGGAGCCGCCACAGCATCA	WUSCHEL 5´
BLWUSADF	AAAAGGATCCGAATGGAGCCGCCACAGCATCA	WUSCHEL 5´

2.1.8 Plants

Arabidopsis thaliana plants *(*ecotypes *Columbia* - *Col,* or *Landsberg erecta* - *Ler*) were grown on soil or 0.5 x Murashige and Skoog (Murashige T, 1962) medium supplemented with 1% (w/v) sucrose under either a 10-h-light/14-h-dark regime (short-day conditions) at 20°C or a 16-h-light/8-h-dark regime (long-day conditions) at 22°C. *Nicotiana tabaccum* plants were grown on soil at 22°C under long-day conditions. Mutant and transgenic *Arabidopsis thaliana* plants used in this work are listed below.

Transgene or mutation	**Genomic background**
/	*wus-1*
/	*stm-6*
/	*stm-5*
/	*stm-2*
LBD30-RNAi	*SALK_076504*
LBD31-RNAi	*SALK_020930*
/	*pin1*
DRN::GUS	*lol-D*
CLV3::GUS	*lol-D*
AS1::GUS	*lol-D*
UFO::GUS	*lol-D*
STM::GUS	*lol-D*

35S::LBD30-GR	SALK_023722
ALCA::LBD30-EAR	*35S::ALCR*
LOB::GUS	*lol-D*
dSpm/act	*lol-D*
as1-1	*lol-D*
bp	*lol-D*
ago4	*lol-D*
clv3-2	*lol-D*
pin1-1	*lol-D*
wus-1	*lol-D*
pA32	*Ler*
pB4	*Ler*
pD4	*Ler*
pE4	*Ler*
pG2_1	*Ler*
pI4	*Ler*
pDel8	*Ler*
pDel7-Ter	*Ler*
pY2	*Ler*
pX2	*Ler*
35S::DRN-GR / CLV3::GUS	*Ler*
pDel2g	*Ler*
pDel3g	*Ler*
pDel4g	*Ler*
pDel5g	*Ler*
pDel6g	*Ler*
pDel7g	*Ler*
pDel8g	*Ler*
pFullg	*Ler*
LOL-RNAi	*Ler*
/	*Ler*
35S::STM-GR	*Ler*
35S::LOL-GR	*Ler*
pMDC30-LOL	*Ler*
pMDC30-LBD31	*Ler*
pMDC32-LBD30	*Ler*
pMDC32-LBD31	*Ler*

pMDC44-LOL	*Ler*
pMDC44-LBD31	*Ler*
pMDC111-LBD31promoter	*Ler*
pMDC163-LBD31promoter	*Ler*
pDel14	*Ler*
pDel15	*Ler*
pDel16	*Ler*
pDel13	*Ler*
pDel10	*Ler*
pDel16	*Ler*
pDel17	*Ler*
pDel18	*Ler*
35S::LBD30-EAR	*Ler*
35S::LBD31-EAR	*Ler*
CYCB1::GUS	*Ler*
DR5::GUS	*Ler*
SCR::GFP	*Ler*
35S::LOL-GR	*DR5::GUS*
35S::LOL-GR	*DR5::GFP*
ago4	*35S::LOL-GR*
pA32	*Col-0*
pB4	*Col-0*
pD4	*Col-0*
pE4	*Col-0*
pG2_1	*Col-0*
pI4	*Col-0*
pBUDel1	*Col-0*
pDel8	*Col-0*
pBU16-DEL7-TER	*Col-0*
pY2	*Col-0*
pX2	*Col-0*
ALCA::WUS	*Col-0*
CLV3::ALCR	*Col-0*
CLV3::GUS	*Col-0*
UFO::GUS	*Col-0*
DRN::GUS	*Col-0*
STM::GUS	*Col-0*

AS1::GUS	*Col-0*
GL2:GUS	*Col-0*
SCR::GFP	*Col-0*
35S::PIN1	*Col-0*
DR5::GUS	*Col-0*
PIN1:GFP	*Col-0*
CYC1At::GUS	*Col-0*
BROMO-RNAi	*Col-0*
LBD31-RNAi	*Col-0*
LBD30-RNAi	*Col-0*
pCRE/loxP	*Col-0*
/	*Col-0*
pBU16-BAR-DEL2-DEL5	*clv3-2*
pBU16-DEL7-TER	*clv3-2*
pY2	*clv3-2*
pX2	*clv3-2*
/	*clv3-2*
pDel2g	*clv3-2*
pDel3g	*clv3-2*
pDel4g	*clv3-2*
pDel5g	*clv3-2*
pDel6g	*clv3-2*
pDel7g	*clv3-2*
pDel8g	*clv3-2*
pFullg	*clv3-2*
35S::WUS-GR	*CLV3::GUS*
ALCA::CRE	*CLV3::ALCR*
pDel8	*clv1-4*
pDel7-Ter	*clv1-4*
pY2	*clv1-4*
pX2	*clv1-4*
/	*bp1*
35S::LOL-GR	*bp1*
35S::LOL-GR	*as1*
/	*ago4*
pB4	*35S::WUS-GR*
pD4	*35S::WUS-GR*

pE4	*35S::WUS-GR*
pG2_1	*35S::WUS-GR*
pI4	*35S::WUS-GR*
pBUDel2	*35S::WUS-GR*
pBUDel3	*35S::WUS-GR*
pBUDel5	*35S::WUS-GR*
pBUDel6	*35S::WUS-GR*
pBUDel7	*35S::WUS-GR*
35S::DRN / CLV3::GUS	*35S::WUS-GR*
pA32	*35S::WUS-GR*
ago4	*35S::LBD30-GR*

Plants used in this work carrying a T-DNA insertion are listed in the following table. This seeds were ordered from the Nottingham Arabidopsis Stock Center (NASC) and from the T-DNA express center (The Salk Institute, La Jolla, California).

Line	**Insertion position / allele**
SALK_034730	*LOL* promoter
SALK_027320	*LOL* intron
SALK_024953	*LOL* intron
SALK_020930	*lol-1*
SALK_021150	*LBD31* exon
SALK_082957	*LBD31* 3´
SALK_067808	*LBD31* intron
SALK_076504	*LBD31* exon
SALK_023722	At5g65630
N3117	*as2-1*
N3118	*as2-2*
N3374	*as1-1*

2.1.9 Software

Microsoft Word, Excel and PowerPoint packets were used to organize experimental data. Adobe Photoshop v7.0 was used for image elaboration. Pearl Primer was used for primer design. pDRAW and Vector NTI (Invitrogen) were used for vector maps and sequence analysis. Databank gene researches were performed on TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/) and NCBI (http://www.ncbi.nlm.nih.org/). The RMAexpress data conversion tool was used to normalize Affymetrix microarray data. *GeneAMP 5700 SDS* was used to analyze qRT-PCR results. Using *DISKUS (Leica)*, the DNA content in trichome nuclei was quantified.

2.2 Genetic methods

2.2.1 Transgenic plant selection

Transgenic plants were selected by spraying with the BASTA (Bayer Crop Sciences, Monheim) herbicide or with antibiotics diluted in growth medium (Hygromycin 20mg/l or Kanamycin 50 mg/l). Seeds grown on plates were beforehand sterilised using 70% ethanol and 10% bleach (w/v). After germination surviving plants were transferred on soil. In some cases, seeds were plated on 1ml drops of growth medium (w/o Dexamethasone) in microtiter plates.

2.2.2 *Arabidopsis thaliana* transformation

To transform Arabidopsis plants the standard protocol from Bechtold and Pelletier, 1998 was followed. Plant transformation mediated by *Agrobacterium tumefaciens* can be optionally performed applying vacuum in order to facilitate bacteria infiltration into plant tissues. In some experiments, *Agrobacterium* infiltration was performed in leaf tissue only, both in

Arabidopsis and tobacco*,* with the aid of a syringe without needle. For leaf infiltration, Agrobacterium was prepared as follows: individual Agrobacterium colonies were grown for 20 hours in 5-ml cultures (Luria broth, 100 µg/ml rifampicin, 12.5 µg/ml tetracycline) at 30°C. This culture was used to inoculate a 50-ml culture (Luria broth, 20 µM acetosyringone/10 mM MES, pH 5.7/12.5 µg/ml tetracycline), which was grown for 16–20 hours at 30°C. The bacteria were pelleted by centrifugation, resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES, pH 5.7, 150 µM acetosyringone) to an OD_{600nm} of 0.5, and incubated at room temperature for a minimum of 3 h.

2.2.3 Cross-fertilization in *Arabidopsis thaliana*

To cross different Arabidopsis genotypes, pollen from mature flowers of the donor plant was released onto the stigmatic papillae of emasculated young flowers of the acceptor plant. Transgenic seeds were then collected from individual siliques.

2.2.4 Genetics for *LOL* characterization

Details of the tagging system through the activation of a transposable element are available on Schneider et al., 2005. The *lol-D* mutant plant identified in the mutagenized population carried a single *dSpm-Act* transposon insertion in *Columbia* genetic background. To generate double mutant lines, *lol*-D plants were crossed with *clv3-2/clv3-2*, *pin1/+* and *wus-1/+* mutant plants. The transmission of the *lol-D* dominant allele was followed in the different mutant backgrounds by using the BASTA resistance marker present in the dSpm-*Act* element. The resulting mutant phenotypes were analyzed amongst BASTA-resistant F3 progenies. A similar procedure was followed to generate STM::*GUS* and CLV3::*GUS* reporter gene plants in *lol-D* mutants.

2.3 Molecular biology methods

2.3.1 Isolation of nucleic acids

2.3.1.1 Preparation of plasmid-DNA

Plasmid-DNA extraction from *E.coli* or *Agrobacterium tumefacens* is performed following a modified *TELT Microquick method* protocol (Holmes and Quigley, 1981) or with the alkaline lysis QIAGEN Kits (Qiagen, Hilden).

2.3.1.2 Preparation of genomic DNA

To extract small amounts of plant genomic DNA, one or two Arabidopsis leaves are frozen in liquid nitrogen and mechanically homogenised. The isolation of genomic DNA from this material is performed as follows: homogenised leaf tissue is added to 400µL of DNA-extraction buffer (200 mM Tris/Cl pH 7,5; 250 mM NaCl; 25 mM EDTA; 0,5% SDS) and left at RT for 5 minutes. After a 2 min centrifugation step at 13.000 rpm, 300µL of the supernatant are transferred into a new Eppendorf tube and mixed with 300µL isopropanol. After a centrifugation for 5 min at 13.000 rpm, the supernatant is discarded and the pellet diluted in 200 µL TE buffer or water. For each PCR reaction, 2 µL of the DNA solution were used as a template.

2.3.1.3 Isolation of DNA-fragments

Isolation and purification of DNA fragments from agarose gels is performed using the *GFX DNA Purification* Kit (*Amersham*, Braunschweig). Purification of PCR products was performed with the QIAGEN Miniprep Kit (Qiagen, Hilden).

2.3.1.4 Isolation of total RNA from plant tissue

Isolation of total RNA from different plant tissues, mainly inflorescence or leaves, is performed following the Trizol extraction protocol. Frozen plant

material is ground in liquid nitrogen and collected in Eppendorf tubes. To each tube 1 ml of lysis buffer (Trizol) is quickly added , vortexed and left at RT for 5 min. After a centrifugation for 10 min at 13.000 rpm, the supernatant is collected in a fresh tube, 0.2 ml chloroform added, vortexed and left 3 min at RT. After a second 10 min long centrifugation at 13.000 rpm, the supernatant is collected in a fresh tube, supplemented with 500 µL isopropanol (to precipitate RNA), vortexed and left for 10 min at RT. After a third centrifugation for 10 min at 13.000 rpm (at 4°C), the pellet is washed with 1ml of 75% (v/v) ethanol, vortexed and centrifuged again for 5 min at 13.000 rpm (at 4°C). The tube is briefly left open to dry the pellet and 188µL of RNAse-free water are added to re-suspend the pellet. After a 10 min incubation at 60°C to facilitate the re-suspension, 10µL REACT buffer 2 (Invitrogen), 1µL RNAse inhibitor and 1µL (10U) DNAse (RNAse free) are added to degrade DNA. After a 30 min incubation at 37°C, 1 vol Phenol/Chloroform (1:1) is added to perform a phenol/chloroform extraction. After a 5 min long centrifugation at 13.000 rpm, the supernatant is collected in a fresh tube, 1 vol of Chloroform is added and the tube is vortexed. After a second 5 min long centrifugation at 13.000 rpm, the supernantant is collected in a fresh tube and 1 vol of 4M LiCl is added to precipitate RNA. Tubes are stored at 4°C overnight or for 1 hour. After a centrifugation for 10 min at 13.000 rpm (at 4°C), the RNA pellet is washed in 200 uL of 75% (v/v) ethanol and resuspended in 20-50 µL water (RNAse free).

2.3.1.5 Synthesis of cDNA

This protocol was followed to synthesize cDNA from total RNA. RNA starting quantity and quality is evaluated via spectrophotometric measurements. 3µg or 5µg RNA are used, depending if preparing cDNA for RT-PCR or qRT-PCR, in a volume smaller than 7µl. 1µl (0,5 µg/µl) of Oligo-dT-Primer or Random primers (Invitrogen) are added and filled with RNAse-free water to 8µl. After a 5 min incubation at 65°C, required for primer hybridization, 4µl of 5x first-strand buffer (Invitrogen), 2 µl 0,1M DTT, 5 µl dNTP-Mix (10 mM) and

1 ul (200U) SuperscriptTM II reverse transcriptase (Invitrogen) are added. After an incubation at 42°C for 50 min, required for cDNA synthesis, and 10 min incubation at 65°C for enzyme deactivation, the synthesized cDNA is diluted in 150 ul of TE buffer.

2.3.1.6 Synthesis of cRNA for Affymetrix micro array analysis

To synthesise cRNA for hybridization onto the *Affymetrix* microarray AtH1 chip, the *Affymetrix* protocol v.701023 rev.4, *Eukaryotic target preparation* section1, chapter1, at the points 2.1.9 and from 2.1.32 to 2.1.43. was followed. For more information: http://www.affymetrix.com/index.affx.

2.3.2 Molecular biology standard methods

All the molecular standard methods like DNA/RNA separation, nucleic acids concentration, DNA enzyme digestions, dephosphorylation of 5´ends, filling of protruding ends from DNA fragments, ligations, bacteria transformations, DNA sequencing, PCR reactions, phenol-chloroform extractions, ethanol precipitations, preparation of competent cell strains for transformations, heat-shock and electro- transformation and DNA gel electrophoresis were performed following the protocols from Maniatis, 1982 with minor modifications.

2.3.3 Non-radioactive in situ hybridization

The full in situ hybridization procedure, from plant tissue fixation, DIG-labeled RNA probe synthesis, to signal detection, was carried out following the protocol “Practical course in molecular and biochemical analysis of Arabidopsis, non-radioactive in situ hybridization”, a 1998 Embo course by Prof. Rüdiger Simon. For more information: www.roche-applied-science.com. *In situ* analyses were performed manually or with the aid of the *Insitu Pro V5* robot from Intavis AG, Cologne.

2.3.4 GATEWAY recombination

GATEWAY BP reaction and LR reaction kits were used to clone genes of interest into the appropriate plant destination vectors. The original *Invitrogen Gateway Manual* was followed. For more information: http://www.invitrogen.com

2.3.5 β–glucuronidase activity test with X-Gluc substrate (GUS-staining)

GUS staining in transgenic plants was performed following a modified protocol from (Sieburth and Meyerowitz, 1997). The β–glucuronidase enzyme cleaves the colorless substrate *X-Gluc* (5-Bromo-4-chloro-3-indolyl-β-D-Glucuron acid, Cyclohexylammonium salt, *Duchefa*), which then shows a intense blue color. Plant tissues were previously treated with cold 90% (v/v) aceton, then incubated 3-4 hours at 37°C in *X-Gluc* staining solution (50 mM $NaPO_4$, 5 mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 10 mM X-Gluc). Chlorophyll was removed using an ethanol series from 30% (v/v) to 100% (v/v) (each step for 1-3 hours at RT). Tissues were cleared with 50% to 100% (v/v) Roth-Istol (*Roth*), small tissues were treated as whole mounts, old tissues were fixed in paraffin and cut using a microtome (Fixative: 50% (v/v) ethanol, 5% (v/v) acetic acid, 3,7% (v/v) formaldehyde). Approximately 40 plants, 10-15 days old, were tested for each transgenic background. At this early developmental stage, plantlets produce few organs and the GUS staining signal in the meristem is more easily visible than in mature plants. GUS stainings in plants grown on microtiter plates occasionally gave a signal in the plant vasculature. This effect could be due to the absence of the treatment with 90% (v/v) cold acetone: plants stained *in vivo* could have transported some of the precipitated X-Gluc buffer into the vasculature.

2.3.6 β–glucuronidase activity test with MUG substrate

This experiment was performed following the *GUS activity assay in intact tissue protocol* from *Arabidopsis: a laboratory manual* (Weigel and Glazebrook, 2001). The results permitted to quantify GUS activities in different deletion constructs of the *CLV3* regulatory sequence vectors. 12 days old seedlings were incubated in 4-MUG buffer for 24 hours at 37°C. The β–glucuronidase expressed by the GUS reporter genes split MUG into the fluorescent 4-methyl umbelliferone (4-MU) and sugar. Fluorescence values were measured with a fluometer (BIORAD). For each of the 16 transgenic plant lines carrying a different deletion construct, between 20 and 70 seedlings were analyzed.

2.3.7 Chimeric constructs for LOL characterization

The *LOL* (At4g00220) and *LBD31* (At4g00210) coding sequences were amplified from cDNA by PCR with the primers 5'-CACCATG-AGCAGTAGCGGAAACCCTAGC-3' and 5'-TCATTCTCGTTTTA TCACTG-ACGAGGCAGAA-3' (for *LOL*) or 5'-CACCATGAGCGGAAGCACCACCGG-3' and 5'-TTATATTAAAGAAGATGGTCGGTATTTGCCTCCGGT-3' (for *LBD31*). cDNAs were reverse-transcribed from RNA extracted from inflorescences of *Col* wild type plants. After being sub-cloned into the pENTR/-D TOPO Gateway vector (Invitrogen), *LOL* and *LBD31* were cloned in the pMDC32 vector for overexpression analysis and in the pMDC44 vector for sub-cellular localization analysis.

To construct the CaMV35S::*LOL*-GR (*35S::LOL-GR*) vector, *LOL* cDNA was inserted using *Bam* HI-*Spe* I into the pBI-ΔGR vector (Lloyd et al., 1994), in frame with the hormone binding domain of the glucocorticoid receptor. *LOL* cDNA was amplified with the primers 5'-AAAAACTAGTCA-TGAGCAGTAGCGGAAACCCTAGCAGC-3' (forward) and 5'-AAAAGGATCCTCTTCTCGTTTTATCACTGACGAGG-CAGAA-3' (reverse).

To construct the *LBD31* promoter-GUS and the *LBD31* promoter-GFP vectors, a 1.5-kbp DNA sequence upstream of *LBD31* was amplified by PCR, using genomic *Col* DNA as a template, with the following primers: 5'-CACCGTATACAAGTAACGCTCCCATTTCATTT -3', 5'- CTTTTA-TGTCAAAGACGTAACGTTTTGT -3'. The *LBD31* putative promoter was inserted into the pENTR/-D TOPO Gateway vector. The promoter sequence was then cloned into the pMDC164 and into the pMDC111 plant destination vectors via the Gateway LR recombination reaction.

To construct the LOL/LBD31-EAR fusions, *LOL* and *LBD31* cDNAs were amplified with the following primers (underlined are the 36 bp of the EAR box): 5'- CACCATGAGCAGTAGCGGAAACCCTAGC -3', 5'-TCAAGCG AAACCCAAACGGAGTTCTAGATCCAGATCAAGTTCTCGTTTTATCACTGA CG-3' (for *LOL*); 5'-CACCATGAGCGGAAGCACCA-CCGG-3', 5'-TCAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCAAGTATTAAAGAA-GATGGTCGGT-3' (for *LBD31*). The cDNAs were then cloned into the pENTR/-D TOPO vector. Via LR recombination reactions with pMDC32, the 35S::LOL-EAR and 35S::LBD31-EAR vectors were constructed.

To construct the 35S::LOL-EAR-GR fusion, the LOL-EAR sequence was cloned into the pFLUO-YFP plant destination vector using the Gateway system.

2.3.8 Chimeric constructs for the deletion analysis of the CLV3 regulatory sequences

Fragments from the CLV3 downstream sequence in pBUdel5 were amplified with the following pairs of primers: LB252F/LB252R (250bp, B4); LB322F/LB322R (320bp, D4); LB402F/LB402R (320bp, I4); LB252F/LB402R (400bp, G2_1) and LB252F/LB322R (595bp,E4). Each amplicon was cloned into the PCR-II TOPO vector (*Invitrogen)*. The forward and reverse primers chosen for this amplification carry an *Apa* I and a *Cla* I restriction site, respectively (tested to be unique in the cloning vector), permitted to sub-

clone each amplicon into the pA32 vector. The new five reporter gene constructs were named pB4, pD4, pE4, pG2_1 and pI4. After checking for possible point mutations due to PCR amplification, wild type *Col* and *Ler* Arabidopsis plants were transformed with these vectors and assayed by GUS staining.

Fragments from the CLV3 promoter sequence in pBUdel3 were amplified with the following pairs of primers: LB3UF1/LB3UR1 and LB3UF1/LB3UR2. The first amplicon is 130 bp long and the second one is 190bp, assuming that the TATA box region of CLV3 is 156bp or 96 bp long, respectively. The two fragments were cloned in distinct PCR-II TOPO vectors. The forward and reverse primers chosen for this amplification carry an *Apa* I and a *Cla* I restriction site, respectively in order to sub-clone the amplicons into the pA32 vector. The new two reporter gene plasmids, named pX2 and pY2, after checking for possible point mutations due to PCR amplification, were used to transform wild type *Col* and *Ler* Arabidopsis plants.

The pDel10 vector lacks only the 190bp promoter region that is present in pY2. pDel10 required two cloning steps to be assembled. The two fragments flanking the Del10 deletion from the *CLV3* promoter were amplified via PCR on pBU16 . The first fragment was amplified with the primers LBAFD9 and LBARD9, the second one with LBBFD9 and UB_551up, the last primer is from U. Brand. The first 1177 bp long fragment was cloned into the *Eco* RI site of pBU13 (a pGreen-nosBar vector from U. Brand, which contains only the full downstream *CLV3* enhancer), named after this cloning pBU13A. The second 120 bp long fragment was cloned into the *Pst* I site of pBU13A, named after this cloning pDel10. pDel10 was sequenced to check for the correct directions of insertions of both of the two fragments.

In pDel8, pDel14, pDel14, pDel15 and pDel16, *CLV3* enhancer sequences were cloned using the *Not* I-*Sac* I restriction sites. In pdel17, the pBUdel5-pBUdel6 fragment was amplified with the primers BLDEL6OS and BLDEL6OS, then cloned into pBUdel2 previously cleaved with *Not* I-*Sac* I.

Different cloning steps were required to construct pDel18. The pBUdel2 *CLV3* promoter was cloned into pBUdel7, digested with *Eco* RI-*Pst* I to remove the *CLV3* full promoter region, and the vector thereafter named pDel18partial.The Del7-Del4 fragment was amplified by PCR with the primers BLDEL4F and BLDEL4R. The Del5-Del6 fragment was amplified from pDel17. The Del5-Del6 fragment was cloned into pDel18partial cleaved with *Not* I-*Sac* I, the Del7-Del4 fragment was cloned into the resulting vector digested with *Not* I. The cloning direction of this last fragment was checked by sequencing.

pDel19 was assembled starting from pBUdel7. The Del7-Del4 fragment was excised with *Not* I from the pDel18. The Del5-Del6 fragment was exized *Not* I - *Sac* I from pDel18. In pDel7, opened with *Not* I-*Sac* I, the Del5-Del6 fragment was cloned. The Del7-Del4 fragment was cloned into the resulting vector digested with *Not* I. The cloning direction of this last fragment was checked by sequencing.

2.3.9 Comparison between the CLV3 putative enhancer sequences in Arabidopsis thaliana and Brassica oleracea

CLV3 was found on the *Brassica oleracea* genome project web-site (http://tigrblast.tigr.org/euk-blast/eukdbsearch.cgi? db=bog1&file=bog_ seqs.ann). The sequence of the *Brassica oleracea CLV3* in this BAC clone was retrieved from the John Innes Center web-site (http://brassica.bbsrc.ac.uk/cgi-bin/ace/generic/tree/BrassicaDB?name= EM%3A BH5 64699&class=DNA). Using this sequence the primers LBBO - U1/-U2/-U3 LBBO –D1/-D2 were designed to amplify and sequence the CLV3 putative enhancer from genomic DNA extracted from *Brassica oleracea* inflorescences.

2.3.10 Cloning of the vectors required for yeast one- and two-hybrid screens

DRN was excised from the pMG-DRN-GR vector (constructed by Margit Grünewald) and cloned *Bam* HI-*Xho* I in the pGADT7 vector. pGADT7 is a vector suitable for yeast one-hybrid screens. The DRN-AD fusion was co-transformed into yeast with the Del2-Del3 and the Del4-Del5 fragments, previously cloned using *Xho* I into the pY22 yeast transformation vector.

2.3.11 Construction of an ethanol inducible system for permanent labeling of CLV3 expressing cells

The ethanol switch system, derived from the fungus *Aspergillum nidulans,* consists of an AlcR protein and an AlcA promoter. AlcR needs ethanol induction to become activated and thus to bind the AlcA promoter. This system, adapted for plants by Caddick et al., 1998, was used to induce CRE recombinase expression in the *CLV3* expression domain. CRE recombinase was excised with *Nsi* I from the pGK-CRE-A vector, and cloned into the compatible *Pst* I restriction sites in pACN. From the pACN vector, the *Hind* III cassette, containing the AlcA promoter, the CRE enzyme and a NOS-terminator, was cloned into the pGPTV-HPT plant transformation vector (Hygromycin resistant), named pPGTV-ALCA. The vector, sequenced to test the direction of the cloned *CRE* enzyme, was named ALCA::*CRE*.

Plants transgenic for CLV3::*ALCR* (donated by P. Laufs, INRA), were transformed with the ALCA::*CRE* vector. The T1 generation was selected on Hygromycin plates and sprayed with BASTA. To test if CRE expression is activated only after ethanol treatment, several inflorescences from CLV3::*ALCR* /ALCA::*CRE* transgenic plants were collected before and after ethanol induction, embedded in wax, sectioned and assayed via RNA *in situ* hybridization with a *CRE* antisense probe. The *CRE* RNA antisense probe was synthesized with the primers BLCREISF and BLCREISR. BLCREISR

carries a tail of 24 nucleotides, corresponding to the T7 RNA polymerase binding site. Ethanol treatment was carried out for 2 days with ethanol vapour. *In situ* results showed that CRE expression is activated only after ethanol induction.

2 loxP sites, flanking the GFP sequence in pQCLOX1, permit the CRE mediated excision event necessary to label cells expressing *CLV3*. These 2 loxP sites were engineered by the annealing of two pairs of oligos, LOX1A with LOX1B and LOX2A with LOX2B. The obtained LOX1 double strand sequence, flanked by *Bam* HI and *Xba* I sticky ends, was cloned into pB121-GFP at the 5' end of the GFP sequence. The new vector was named pUC-GFP. The LOX2 double strand sequence, flanked by *Hind* III-*Sma* I and *Nco* I, was cloned into the pK373 vector, at the 5' end of the GUS sequence. The new vector was named pKL2. pUC-GFP was digested with *Sst* I to clone a NOS-terminator sequence amplified from pK373 with the primers BLTERSF2 and BLTERSR2. The vector was named pLOX-GFP-TER. The *Sma* I-*Spe* I cassette from pKL2 and the *Eco* RI-*Sma* I cassette from pLOX-GFP-TER were both cloned into the plant transformation vector pGREEN-nos-BAR. The resulting vector, containing the 35SCaMV promoter driving the GFP gene (with its transcriptional terminator) flanked by two loxP sequences and GUS (with its transcriptional terminator) was called pLOX-full. This vector was sequenced and a point mutation was discovered in the LOX1 site. The Stratagene Quick Change Kit was used to perform a site directed mutagenesis to remove the point mutation. The primers BLQCLOX1 and BLQCLOX1 were designed and the Quick Change II Kit manual (*Stratagene*) was followed. The obtained vector was sequenced using the BL35SF primer and no point mutation was found. The new vector was named pQCLOX1 (pQC1).

2.4 Microscopy techniques

2.4.1 Light microscopy

The microscope used for this work is the *Axioskop* (*Zeiss*) with normal and Normasky optics. Pictures were shot with the *Zeiss Axiocam* digital camera and saved with the *Axio Vision* software.

2.4.2 Fluorescence microscopy

GFP expression analyses were performed with the *Leica* MZFL-III binocular and the GFP1, GFP3 (Plant GFP) and YFP filter set (Leica). Pictures were shot with a Pixelfly digital camera. A DAPI filter was used to quantify nuclei DNA contents in mutant and wild type trichomes.

2.4.3 Scanning electron microscopy

The LEO (Zeiss) scanning electron microscope was used for this work, together with the provided software. Prints of the analyzed Arabidopsis tissues were obtained following the protocol published in Kwiatkowska, 2004.

2.4.4 Confocal microscopy

The *Leica TCSNT* scanning confocal microscope and the *Leica Confocal Software* were used in this work to analyze root mutant phenotypes in Arabidopsis.

2.5 Histological techniques

2.5.1 Embryo fixation

The protocol for embryo fixation and subsequent microscope analysis is published in Apuya et al., 2002.

2.5.2 Trichome fixation

To separate trichomes from leaves, the protocol from Zhang and Oppenheimer, 2004 was followed. Trichomes were consequently fixed overnight in 70% (v/v) ethanol or in 4% (w/v) formaldehyde for further analyses.

2.5.3 DAPI staining of trichomes

This protocol was developed by the Ph.D. student Elena Galiana Jaime (Institute of Botany, University of Köln). Leaf tissue is fixed in 70% (v/v) ethanol overnight, and then incubated in a water solution containing 5 ug/ml DAPI for 20 minutes under vacuum at a pressure of 0,8 bars. Then the tissue is washed for 15 minutes in 70% (v/v) ethanol (if it was not previously fixed).

3 Deletion analysis of the *CLV3* regulatory sequences

3.1 Regulation of CLV3 expression

CLV3 has a central role in controlling meristem activity and stem cell identity, but little is known about the regulation of its expression. The aim of the deletion analyses of the *CLV3* regulatory sequences presented in my research is to identify *CLV3* regulatory motives that are required to promote or to repress (outside of the central zone of the meristem) the *CLV3* expression.

3.2 The CLV3::GUS reporter gene (pBU16)

U. Brand constructed in our lab the CLV3::*GUS* reporter gene, required to start the deletion analysis of the *CLV3* regulatory sequences. She found that a 1487 bp long sequence upstream of *CLV3*, the putative *CLV3* promoter, is not sufficient to promote gene expression. Therefore, she amplified an additional 1256 bp long sequence, from a region downstream of *CLV3*, a putative *CLV3* enhancer, and she cloned both of the two sequences in the plant transformation vector pGreen-nosBAR. In this vector, under control of the two *CVL3* regulatory sequences, she cloned the GUS sequence, and named this plasmid pBU16. She transformed the *Landsberg erecta* (*Ler*) ecotype of *Arabidopsis* with the pBU16 vector and tested the resulting transgenic plants with a GUS staining assay. She detected the signal of the CLV3::GUS reporter gene in the central zone of the meristem, in a comparable area to the *CLV3* RNA expression pattern (Fig. 1A-B). Therefore,

she could start the deletion analysis of the *CLV3* regulatory sequences (Brand et al., 2002).

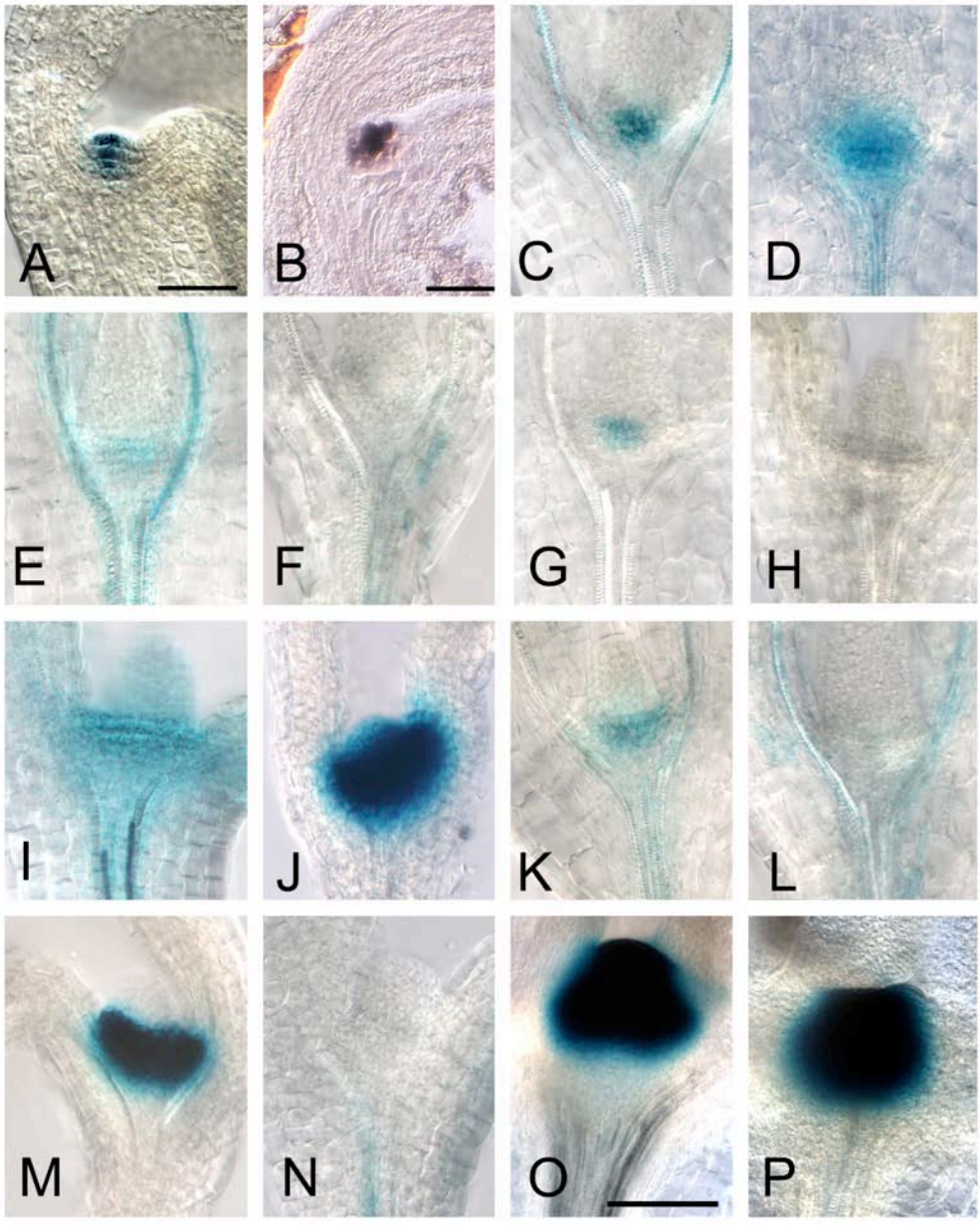


Fig.1. **GUS signals promoted by different deletion constructs of pBU16**. (A-B) Comparison of the expression domain of pBU16 and *CLV3* (RNA in situ hybridization) in the embryonic SAM. Both the signals are present in the three cell layers of the central zone of the meristem (Brand et al., 2002). (C) pBU16 signal in the SAM of 10 days old seedlings. (D) pBUdel2 GUS staining is broader than the reference pBU16. (E) In pBUdel3 plants, GUS staining is weaker but still present in the central zone of the meristem. (F) pBUdel5 does not promote the GUS staining signal. (G) pBUdel6 staining is comparable to pBU16. (H) pBUdel7 does not stain. (I-N) Analyses of the previous CLV3::GUS signals in 35S::WUS-GR plants, after Dex induction. All the transgenic plants respond to *WUS* expression up-regulation, except pBUdel5 and pBUdel7. (I) pBU16. (J) pBUdel2. (K) pBUdel3. (L) pBUdel5. (M) 20% of the induced pBUdel6 plants show a GUS staining similar to induced pBUdel2 plants. (N) pBUdel7. (O) Over-stainings of 35S::WUS-GR/35S::DRN/pBU16 plants before and (P) after Dex induction. Ectopic expressions of *WUS* and *DRN* are not sufficient to promote *CLV3* expression outside of the meristem. Scale bar: 50 μm.

3.3 Deletion analysis of the CLV3 regulatory sequences

U. Brand engineered nine different pBU16 deletion constructs: **pBUdel1**, **pBUdel2** and **pBUdel3** (deletions of the *CLV3* promoter); **pBUdel4**, **pBUdel5**, **pBUdel6** and **pBUdel7** (deletions of the putative enhancer region); **pBUdel3X4** and **pBUdel3X5**, later in this thesis named **pBUdel11** and **pBUdel12** (combination of promoter and enhancer deletion fragments). I repeated the GUS staining analyses performed by U. Brand to test the reproducibility of her results and to better quantify the GUS activities and expression patterns of these deletion constructs.

pBUdel1 lacks the first 321 bps of the *CLV3* promoter. Its GUS staining signal is comparable to the reference pBU16. The missing *CLV3* promoter region does not seem to play a role in the regulation of *CLV3* expression.

The pBUdel2 GUS signal is much stronger and broader in comparison to the pBU16 reference staining. The pBUdel2 construct lacks 761 bps at the 5' end of the *CLV3* promoter. GUS RNA *in situ* analysis on pBUdel2 Arabidopsis plants confirmed that the pBUdel2 reporter gene signal is broader than the reference pBU16 (Fig.2). So, the additional missing 420 bps, in comparison to pBUdel1, may contain a binding site for a negative regulator of *CLV3* expression.

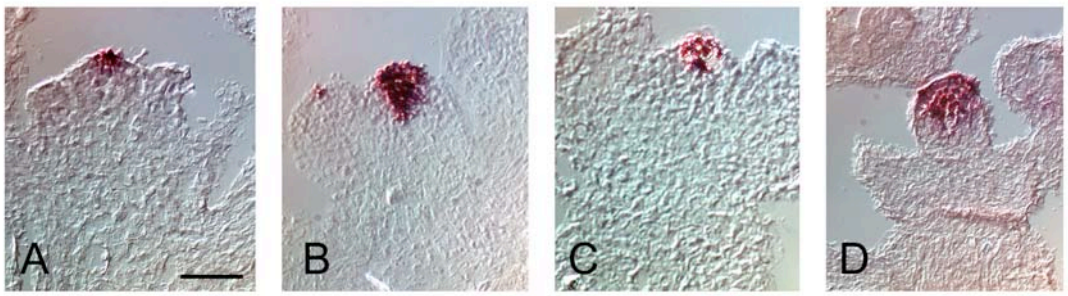


Fig.2. **Results from RNA *in situ* analysis with a GUS probe on mature plants which are transgenic for different CLV3::GUS deletion vectors.** (A) pBU16 GUS signal is detected in the stem cell domain. (B) pBUdel2 promotes GUS transcription in a broader and deeper region than the reference pBU16. (C) pBUdel6 GUS RNA is detected in the stem cell domain. (D) pDel8 signal in flower meristem of pDel8a plants: the signal is present in both the central and the peripheral zone. Scale bar: 30 μm

The pBUdel3 GUS signal is still present in the meristem's CZ but it is weaker than in the pBU16 reference. The additional 460 bps missing in the *CLV3* promoter of pBUdel3 compared to pBUdel2 could contain the binding site for a positive regulator of *CLV3* expression.

pBUdel4 shows no GUS staining in transgenic plants. The 275 bps left of the *CLV3* enhancer are not sufficient to promote GUS expression.

The pBUdel5 GUS signal was detected in the first layer of the embryonic SAM only in U. Brands experiments. Repeated experiments did not show GUS staining in any tissue. From this new result, the first 595 bps of the pBUdel5 enhancer sequence are considered not sufficient to promote GUS expression.

The **pBUdel6** staining pattern and intensity is comparable to the pBU16 reference. The 965 bps long *CLV3* putative enhancer, with the additional 370 bps in comparison to pBUdel5, seems to contain a binding site for a positive regulator of *CLV3* expression.

pBUdel7 lacks the full *CLV3* putative enhancer sequence, thus GUS expression is driven by the *CLV3* 5' region only. **I**n U. Brands experiments, the pBUdel7 signal was rarely visible in axillary meristems. I did not observe any GUS staining in pBUdel7 transgenic plants. However, *CLV3* expression is still faintly detectable via RNA *in situ* hybridization in *clv3-2* mutants, where *CLV3* expression is also driven only by the *CLV3* promoter, because a chromosome inversion removed the full *CLV3* putative enhancer sequence. The not detectable pBUdel7 GUS expression could be due to the absence of a transcription terminator sequence at the 3´ end of *CLV3*. Thus, the **pDel7-Ter** vector was constructed, cloning a nos-terminator sequence downstream of *CLV3*. However, also in pDel7-Ter transgenic plants a GUS staining was not visible. The weak *CLV3* expression in *clv3-2* mutants could be due to the presence of a new enhancer element close to *CLV3,* positioned behind the chromosome inversion. Sequencing of the *CLV3* 3' end region in the *clv3-2* mutant was not helpful to identify known enhancer sequences. Therefore, the

absent GUS signal in pDel7-Ter transgenic plants could be due to a GUS expression too low to be detected by the GUS assay.

The results from these repeated GUS staining analyses permitted to design a model for the regulation of *CLV3* expression where “positive” and “negative” regulatory sequences, both in the promoter and in the putative enhancer of *CLV3*, act in synergy to promote *CLV3* expression in the central zone of the meristem or to repress it in the meristem periphery. In summary: the **420 bps** long region belonging to the *CLV3* promoter, missing in pBUdel2, acts as a negative regulator of *CLV3* expression. The **460 bps** long *CLV3* promoter in pBUdel3 and the **690 bps** long *CLV3* enhancer regions present in pBUdel5 and pBUdel6 may contain binding sites for positive regulators of *CLV3* expression (Fig.1C-H, Fig3a).

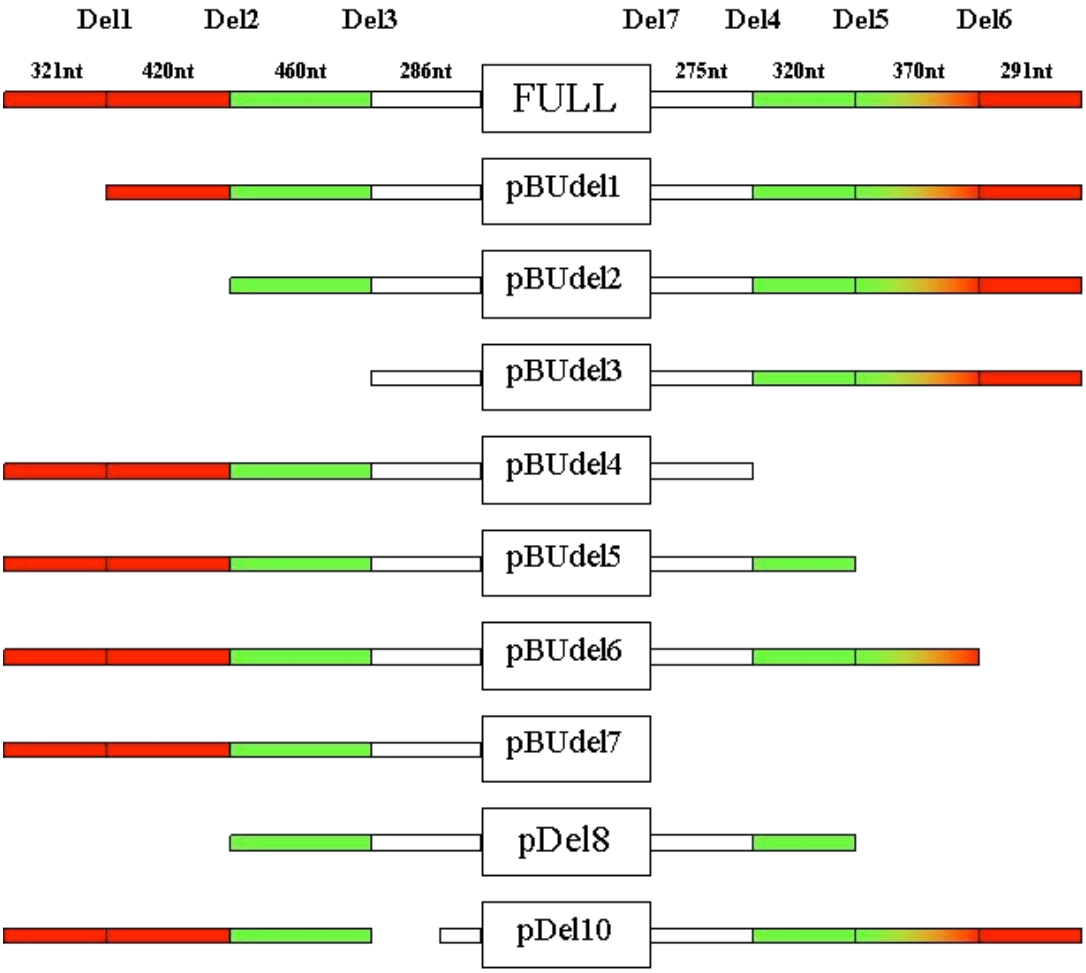


Fig.3a. **Summary of the deletion constructs prepared to analyze the *CLV3* regulatory sequences**. Fragments in red (5' end-Del1 / Del1-Del2 / Del6-3' end) contain binding sites for negative regulators of CLV3 expression. Fragments in green (Del2-Del3 / Del4-Del5) contain binding sites for positive regulators of *CLV3* expression. The fragment Del5-Del6 could have positive and negative influences on *CLV3* expression. pBUdel7 lacks the full downstream regulatory sequence. pDel10 lacks part of the promoter region which is present in pBUdel3. pDel8 is a combination of pBUdel2 and pBUdel5.

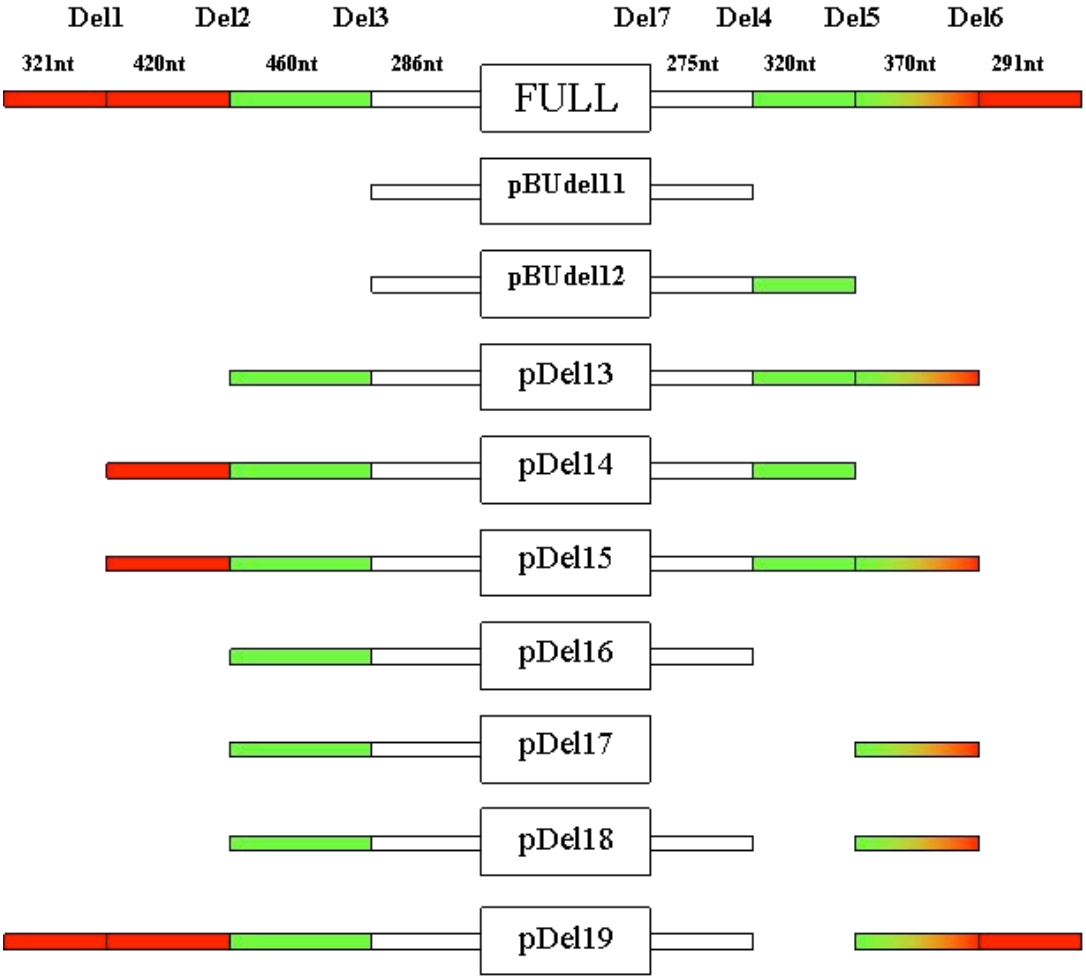


Fig.3b. **Summary of additional deletion constructs prepared to analyze the *CLV3* regulatory sequences**. pBUdel11 and pBUdel12 do not contain enough positive regulatory sequences to promote *CLV3* expression. pDel13 signal does not expand into the peripheral zone of the meristem or in organ primordia, as was detected in plants transgenic for pDel8, possibly because of the presence of the Del5-Del6 fragment, which acts as both positive and negative regulator of *CLV3* expression. pDel16, pDel17 and pDel18 stain outside of the CZ of the meristem and not in the stem cell domain. The pDel19 signal is weaker than the pBU16 reference, thus showing that Del4-Del5 is necessary to enhance *CLV3* expression in the central zone of the meristem.

3.4 Deletion analysis of the pBUdel5 enhancer region

U. Brand described the pBUdel5 enhancer region as the shortest fragment sufficient to promote *CLV3* expression (together with the full *CLV3* promoter). I further narrowed down this region to find which short motives may be

sufficient to enhance *CLV3* expression. Four different and partly overlapping sub-regions of the *CLV3* enhancer from pBUdel5, as well as the full length pBUdel5 enhancer, were cloned in front of a minimal promoter, driving GUS expression. The T1 generations of transgenic plants carrying these different reporter genes were selected using the BASTA herbicide. The GUS analysis conducted on these transgenic plants gave no positive results. Therefore, the putative enhancer sequences in the *CLV3* downstream fragment of pBUdel5 seem not sufficient to promote GUS expression. They probably require a synergistic interaction with other sequences present in the *CLV3* promoter (Fig. 4).

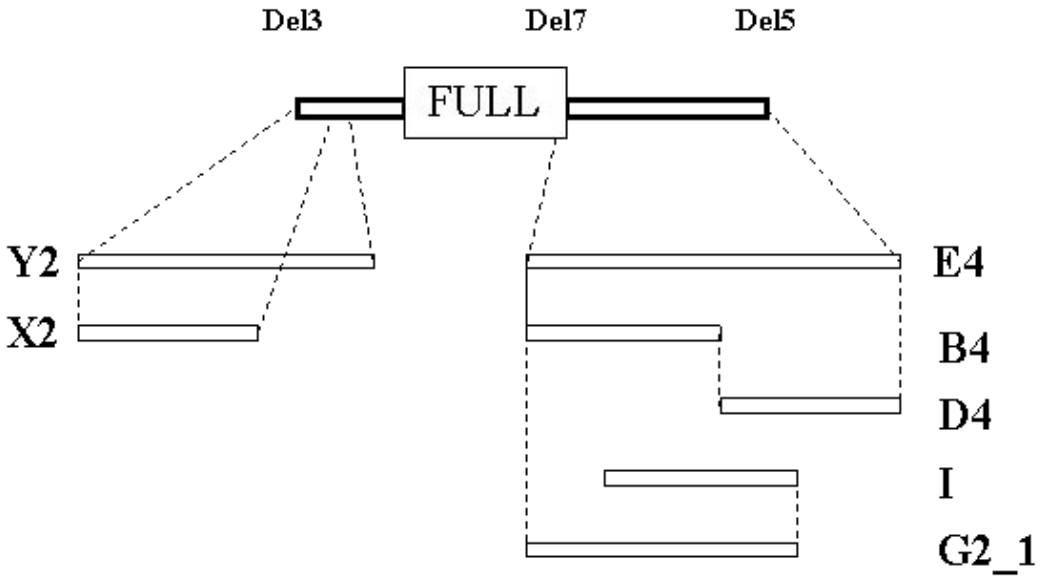


Fig.4. **Scheme of pBUdel3 promoter and pBUdel5 enhancer deletion analyses**. None of the sub-fragments amplified from these *CLV3* regulatory sequences, cloned in front of a 35S minimal promoter driving GUS expression, could promote gene expression.

3.5 Deletion analysis of the pBUdel3 promoter fragment

pBUdel3 carries a 286 bps long *CLV3* promoter region, plus the full *CLV3* downstream enhancer. Plants transgenic for pBUdel3 show a weak GUS staining in the central zone of the meristem. I further narrowed down this short promoter to find which motives are necessary for *CLV3* expression. Via PCR, two sequences between the 5' end of the pBUdel3 CLV3 promoter and the *CLV3* putative TATA box sequence were amplified and cloned in front of a minimal promoter driving GUS expression. The T1 generations of each transgenic plant were selected with the BASTA herbicide. Also in this case, GUS analyses gave no positive results. None of the pBUdel3 promoter sub-regions was sufficient to promote GUS expression. Therefore, the TATA box region of the *CLV3* promoter possibly contains not only the binding site for the general transcriptional machinery, but also for specific, yet unknown, *CLV3* transcriptional activators, required to promote *CLV3* expression. Alternatively, the transcriptional factors that possibly bind to the pBUdel3 *CLV3* promoter may require the combinatorial activity of other factors binding to the *CLV3* putative enhancer, to promote gene expression (Fig.4).

3.6 Searching for conserved motives in the CLV3 putative enhancers of different Brassicaceae

The *CLV3* regulatory sequences seem to contain some redundant positive and negative elements, which act in a combinatorial way to promote *CLV3* expression. I aligned the *Arabidopsis thaliana* and the *Brassica oleracea CLV3* putative enhancer sequences to find out which motives are conserved and thus probably necessary for the regulation of *CLV3* expression. The Arabidopsis *CLV3* genomic sequence was blasted against the Brassica genome and thus the *Brassica oleracea CLV3* gene, 73% identical to the

Arabidopsis *CLV3*, was identified. Using this sequence, suitable primers to amplify and sequence the *CLV3* putative enhancer of *Brassica oleracea* (var. *Broccoli)* were designed. The alignment of the two *CLV3* putative enhancer sequences from Arabidopsis and Brassica gave a 61,4% identity value, which, together with the high scattering of identity, did not permit to isolate any particular motive. Therefore, no significant conserved sequences in the *CLV3* putative enhancer were identified (Appendix A).

3.7 WUSCHEL responsive regions in CLV3 regulatory sequences

The *WUS* homeobox gene encodes for the so-called organizing center factor, which promotes stem cell identity and *CLV3* expression in the central zone of the meristem. *WUS* and *CLV3* expression domains are probably not overlapping, thus no direct interaction between them is expected. However, some regions of the *CLV3* regulatory sequences could contain the binding sites for other transcriptional factors downstream of *WUS*. Therefore, to find which of the *CLV3* regulatory sequences contain *WUS* sensitive motives, I tested which of the *CLV3* reporter genes previously analyzed are still sensitive to *WUS* upregulation. U. Brand transformed plants which are inducible for *WUS* overexpression (35S::*WUS-GR)*, by spraying with Dexamethasone (Dex), with the following reporter genes: pBu16, pBUdel2, pBUdel3, pBUdel5, pBUdel6, pBUdel7. The GUS analyses she performed were repeated to confirm and better quantify her results. 10 days after germination under long day conditions, approximately 50 plants each transgenic background, grown on medium with or without Dex, were GUS assayed. The GUS staining pattern detected in transgenic plants grown without Dex was comparable to the previous results, except for a GUS signal visible in the seedling vasculature, probably due to the *in vivo* staining

procedure (see Materials and Methods). GUS expression patterns were then analyzed in induced transgenic plants.

Induced **pBU16** plants show a broader blue staining in the meristem and no signal in other tissues compared to non-induced pBU16 plants. The induced *WUS* overexpression promotes stem cell identity, a larger stem cell population and a consequent broader *CLV3* expression in the meristem. But, as known from previous experiments, *WUS* overexpression is not sufficient to promote ectopic expression of *CLV3*.

In induced **pBUdel2** plants, the GUS signal is broader and more intense than in the induced pBU16 reference plants. The whole meristem, not only the central zone, is stained. pBudel2, which lacks a *CLV3* negative regulatory sequence, strongly responds to WUS induction, and thus still contains a WUS sensitive element.

pBUdel3 plants are still inducible by *WUS* overexpression as their GUS staining is stronger than in pBUdel3 plants grown without Dex.

pBUdel5 and **pBUdel7** transgenic plants, even if induced, do not show GUS staining. Both of these reporter genes probably lack important regulatory sequences to promote GUS expression. The staining visible in the vasculature of pBUdel5 plants is an artifact, as no signal was detected on pBUdel5 plants grown on soil (see Material and Methods).

20% of the **pBUdel6** seedlings show a strong induction signal, comparable to the one detected in induced pBUdel2 plants. In 80% of the induced pBUdel6 plants, GUS staining is comparable to the induced pBU16 reference. The occasionally high WUS sensitivity of pBUdel6 plants could be due to a missing *CLV3* regulatory sequence that negatively controls *CLV3* expression after up-regulation of the *WUS* signal.

Except pBUdel5 and pBUdel7, all the reporter genes positively reacted to up-regulation of *WUS* expression. Therefore, the supposed high redundancy of the *CLV3* regulatory sequences did not permit to isolate a single *WUS* responsive region (Fig. 1I-N).

3.8 WUS and DRN control CLV3 expression

The *DONRÖSCHEN* (*DRN*) transcription factor could be, like WUS, a positive regulator of *CLV3* expression. In *drn-D* dominant mutant plants, *CLV3* is upregulated at first and detectable in both central and peripheral zones of the shoot meristem, and later, when leaf production in the mutant meristem arrests, *CLV3* expression is shifted down to the organizing center area (Kirch et al., 2003). *DRN* expression partly overlaps with the *CLV3* domain in the CZ of the meristem, but it is also present in young organ primordia. DRN belongs to the AP2/ERF protein family. In Arabidopsis, three proteins belonging to this family have been characterized: APETALA2 (AP2), responsible for flower organ development, AINTEGUMENTA (ANT), involved in embryo development, and TINY, which affects hypocotyl elongation and fertility (Jofuku et al., 1994; Klucher et al., 1996; Wilson et al., 1996). The AP2 binding domains of these three different proteins bind to DNA sites that share the common core sequence 5´- CCGAC -3´. This short motive is present also in the *CLV3* regulatory sequences, in the Del4-Del5 fragment, which was shown, at least by U. Brands experiments, to be a positive regulator of *CLV3* expression in the stem cell domain.

To assay if DRN and WUS are direct or indirect activators of *CLV3* expression, a time window analysis was set up. The pBU16 reporter gene signal was quantified in 35S::WUS-GR/pBU16 and 35S::DRN-GR/pBU16 plants at different time points: 0 (before Dex induction), 3, 9, 12, 24 and 96 hours after induction (h.a.i). A change in expression of the reporter gene in both the mutant plants was visible only 96 h.a.i.. These results might indicate that neither *DRN* nor *WUS* are direct activators of *CLV3*. However, the GUS staining assay could be not sensitive enough to show weak variations in gene expression intensity (Fig.5).

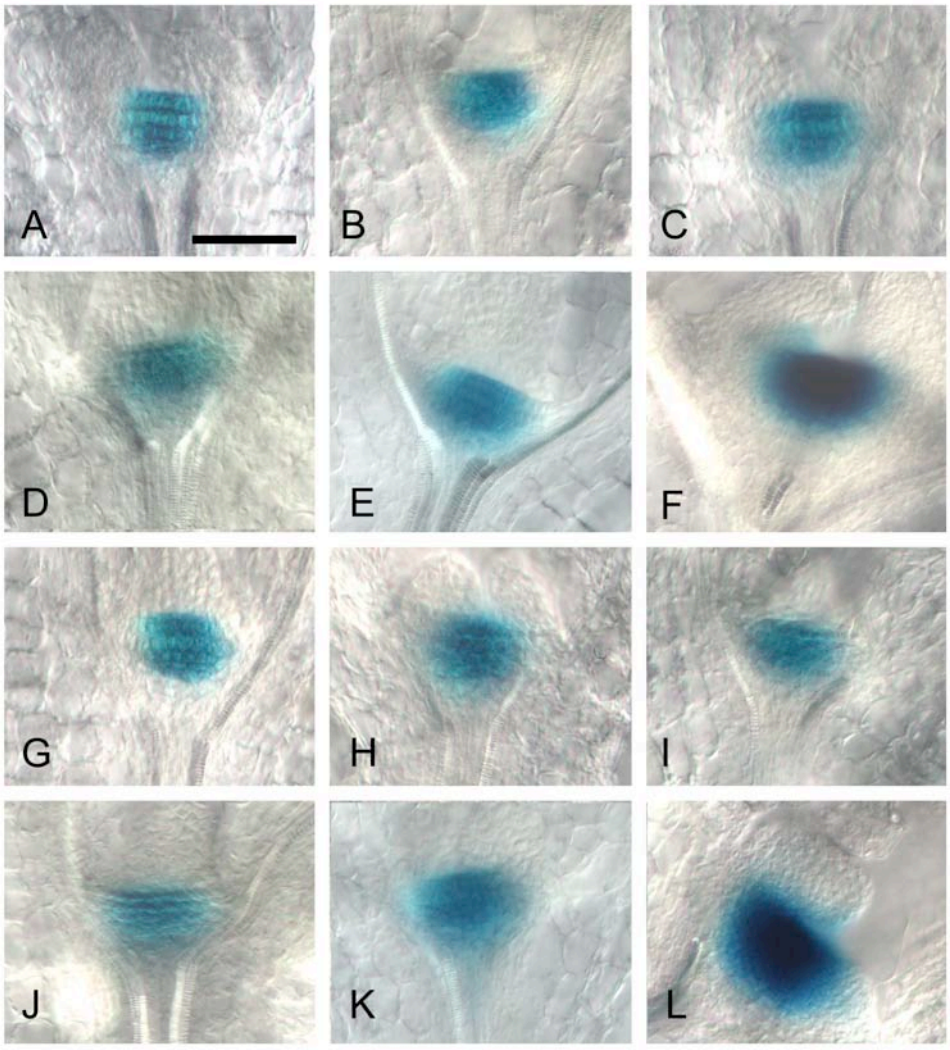


Fig.5. **Time window analysis of pBU16 expression levels in 35S::WUS-GR** (A-F) **and 35S::DRN-GR plants** (G-L). GUS staining was performed at 0, 3, 9, 12, 24, 96 hours after induction (h.a.i). In both the transgenic plants, CLV3::GUS signal intensity does not change before 96 h.a.i. From these results, DRN and WUS seem to be indirect activators of *CLV3* expression. Scale bar: 50 μm.

3.9 DRN may be a direct activator of CLV3

To better quantify weak differences in GUS expression levels, I switched from the GUS staining analysis to *GUS* RNA quantification. In 35S::WUS-GR/pBU16 and 35S::DRN-GR/pBU16 plants, transcriptional levels of GUS RNA were quantified via RT-PCR, before and after Dex induction. To understand if WUS or DRN are direct or indirect activators of *CLV3*, I treated

the transgenic plants with Dex and Cycloheximide (Cyc), a general inhibitor of protein translation.

Before induction, a high amount of WUS or DRN–GR fusion protein is produced in plant cells and stored in their cytoplasm. After Dex induction, the -GR fusion protein changes its conformation and enters the cell nucleus. If plants are at the same time treated with Cyc, I can tell if the –GR fusion is a direct or indirect activator of *CLV3* by quantifying the GUS transcription product (Samach et al., 2000). In fact, if after Cyc treatment and Dex induction CLV3::*GUS* RNA levels increase, no translation of additional proteins is needed in the *CLV3* transcription activation pathway: the –GR fusion protein is a direct activator of *CLV3*. If *GUS* RNA levels remain unchanged after Cyc and Dex treatment, then at least one additional protein "X" is required to activate *CLV3* expression. Thus, the –GR fusion is an indirect activator of *CLV3*.

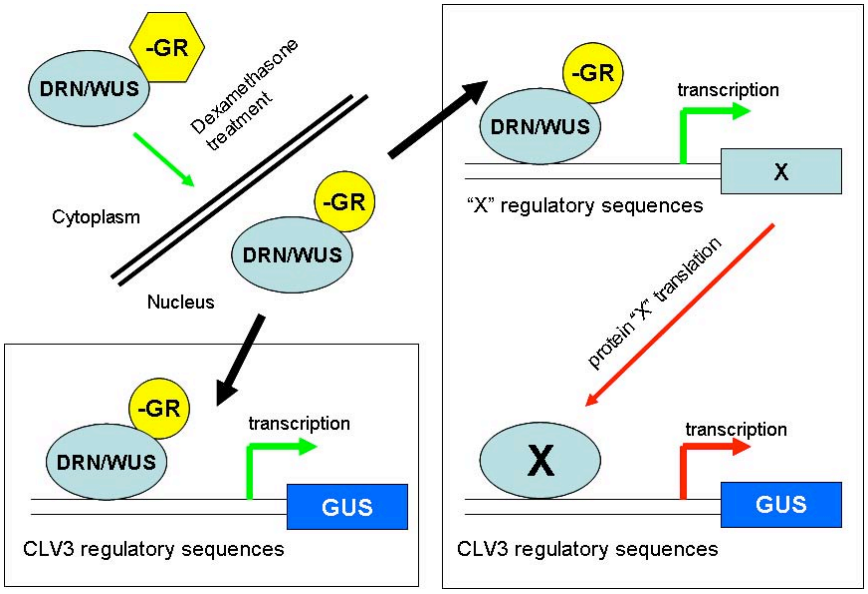


Fig.6. **Direct (left) or indirect (right) activation of *CLV3* expression by the WUS/DRN–GR fusion proteins.** The –GR fusion protein (top left) after Dex treatment changes its conformation and enters the cell nucleus. If the fusion protein is a direct activator of CLV3, CLV3::GUS transcription is enhanced even in the presence of Cyc (bottom left). Alternatively, the fusion protein activates the transcription of a gene "X", which is translated into the protein "X" (top right). Then, "X" binds the *CLV3* promoter and promotes the transcription of GUS (bottom right). Red arrows indicate the steps inhibited by Cyc.

3,5 hours after the Cyc plus Dex treatment, seedlings were collected, frozen in liquid nitrogen, total RNA was extracted and GUS RNA amplified via RT-PCR. In both of the transgenic plants, Cyc, but not water, slightly increased GUS RNA stability. In 35S::*WUS-GR* plants, the 3,5 hours long treatment was not sufficient to upregulate CLV3::*GUS* expression, with or without Cyc. From this result, *WUS* is not considered to be a direct activator of *CLV3*. In 35S::*DRN-GR* plants, the 3,5 hours long induction promoted GUS expression with or without Cyc. Therefore, *DRN* may be a direct activator of *CLV3* (Fig. 7A,B).

To quantify the upregulation of GUS expression after Dex induction, I performed a quantitative RT-PCR (qRT-PCR). In 35S::*DRN-GR* plants, GUS expression is increased 60 fold after Cyc plus Dex treatment. In 35S::*WUS-GR* plants, GUS expression is raised only by the secondary effect of Cyc. Furthermore, *CLV3* RNA levels were measured, but not such a big induction was detected. The difference between GUS and *CLV3* induction levels could be explained by a possible higher GUS mRNA stability. Therefore, results of the qRT-PCR analyses confirmed that *DRN,* but not WUS, could be a direct activator of *CLV3* expression (Fig.7D,E).

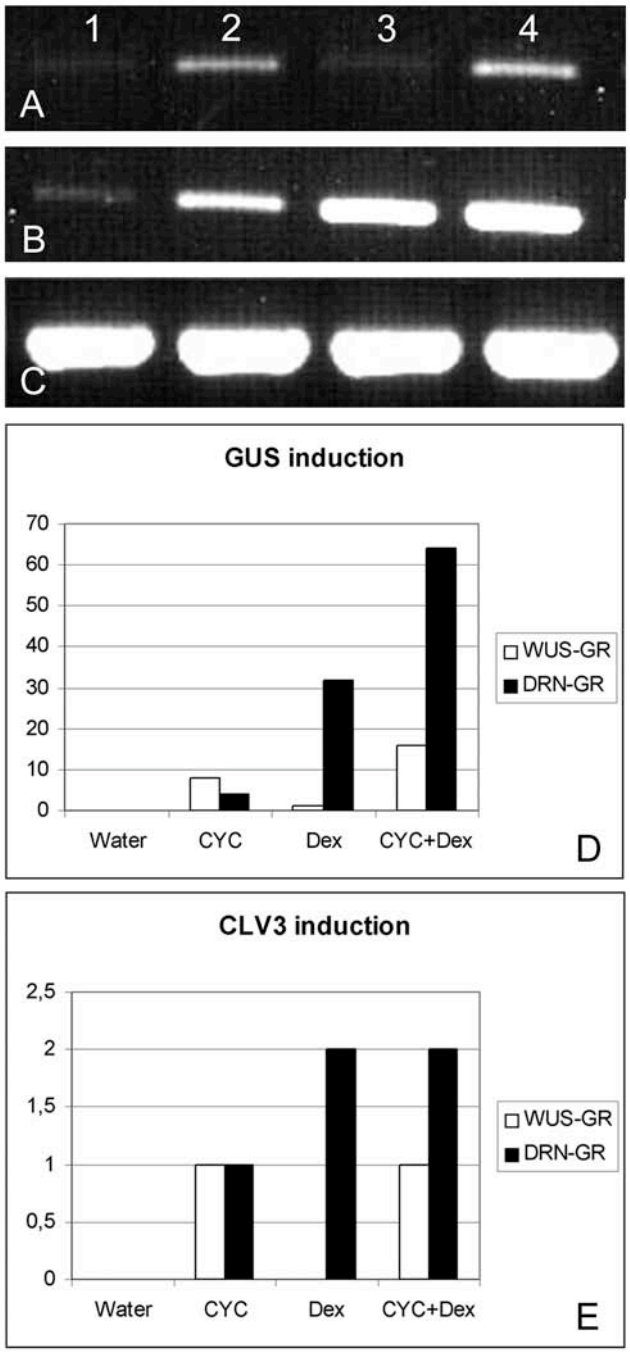


Fig.7. **GUS RNA quantification analyses in 35S::WUS-GR (A) and 35S::DRN-GR (B) plants showed that only DRN may be a direct activator of *CLV3* expression**. RT-PCR amplification on GUS RNA was performed after water mock test (1), Cyc treatment (2), Dex induction (3) and Dex + Cyc treatment (4). Only in 35S::DRN-GR plants, GUS transcription after Dex and Dex + Cyc treatment is strongly upregulated. The panel (C) shows RT-PCR using primers to the Cytochrome b5 gene as a control. qRT-PCR showed that GUS transcription is 60 fold upregulated by DRN ectopic expression (D), while *CLV3* is only 2 fold upregulated (E). No considerable effects on *CLV3* and *GUS* RNA expressions are detected in 35S::WUS-GR plants.

3.10 Unknown meristem factors are required to promote CLV3 expression

WUS and DNR, possibly indirectly and directly, positively control *CLV3* expression. To test if both of them are sufficient to activate *CLV3*, the CLV3::*GUS* staining pattern was analyzed in the triple transgenic plants 35S::*WUS-GR;35S::DRN;pBU16* before and after Dex induction. Although a 16 hours long GUS staining assay was performed to detect even weak GUS signals outside of the meristem, no ectopic CLV3::GUS expression was visible. Therefore WUS and DRN are not sufficient to promote *CLV3* expression in differentiated tissues, even if they positively regulate *CLV3* expression in the central zone of the meristem. Additional unknown meristem factors are necessary to ectopically induce *CLV3* expression. Alternatively, negative regulators of *CLV3*, expressed outside of the stem cell domain, are not down-regulated by the ectopic expressions of both *WUS* and *DRN* (Fig.1O-P).

3.11 Combinatorial CLV3::GUS deletion constructs

To understand which different combinations of the “positive” and “negative” elements present in the *CLV3* regulatory sequences are required to promote or repress *CLV3* expression, I engineered 11 new deletion constructs.

pDel8 (5-746 / 3+595) is a combination of pBUdel2 and pBUdel5. The *CLV3* enhancer of pBUdel2 was substituted with the shorter *CLV3* enhancer of pBUdel5. The pDel8 signal is rarely detected in transgenic plants. In some cases, GUS staining is larger than the pBU16 reference and present not only in the central zone but also in the peripheral zone of the meristem and in young organ primordia. In other cases, pDel8 signal is comparable to the pBU16 reference, except for an additional staining visible at the base of flower pedicels. pDel8 seems to lack regulatory sequences necessary both to

promote and restrict *CLV3* expression, as its signal is not always detectable and occasionally expands outside of the stem cell domain.

pDel10 (5-1487 to -286 and 5-154 to -1 / 3+1256) was constructed to assay if the promoter region (putative TATA box excluded) present in pBUdel3 is necessary for *CLV3* expression. The pDel10 GUS signal is present in the central zone of the meristem, in a pattern comparable to the pBU16 reference (data not shown). Thus, the *CLV3* promoter fragment required in pBUdel3 for *CLV3* expression is not necessary in pDel10. This is an additional evidence for the presence of redundant elements in the *CLV3* regulatory sequences.

pBUDel11 (5-286 / 3+275) and **pBUDel12** (5-286 / 3+595) do not stain in any tissue. pBUDel11 is a combination of pBUdel3 and pBUdel4. pBUDel12 is a combination of pBUdel3 and pBUdel5. Possibly none of these two vectors carry *CLV3* regulatory sequences sufficient to promote GUS expression.

The **pDel13** (5-746 / 3+965) reporter gene is a combination of pBUdel2 and pBUdel6. The *CLV3* enhancer of pBUdel2 was substituted with the shorter pBUdel6 *CLV3* enhancer. pDel13 GUS staining, stronger than pBU16, is due to the absence of the Del1-Del2 fragment, which contains a negative regulator of *CLV3*. However, the pDel13 staining is weaker than pDel8. I suppose that the Del5-Del6 enhancer fragment, present in pDel13 but not in pDel8, contains not only positive but also negative *CLV3* regulatory motives.

pDel14 (5-1166 / 3+595) is a combination of pBUdel1 and pBUdel5. The pBUdel1 *CLV3* full enhancer was substituted with the shorter pBUdel5 *CLV3* enhancer. pBUdel5 did not stain, or only weakly in the embryonic SAM, as shown in previous experiments done by U. Brand; pBUdel1 stained like the pBU16 reference. pDel14 shows a signal weaker than the pBU16 reference, but detectable also in non-meristematic tissues like pollen anthers. As pBUdel5 does not stain in adult plants, contrary to pDel14, the region missing in the pBUdel1 promoter must carry a negative regulatory sequence which absence allows pDel14 to promote GUS expression.

pDel15 (5-1166 / 3+965) is a combination of pBUdel1 and pBUdel6. The pBUdel1 *CLV3* full enhancer was substituted with the pBUdel6 *CLV3* enhancer. pBUdel6 and pBUdel1 show a GUS signal comparable to the reference pBU16. However, pDel15 signal in the meristem seems to be stronger than the pBU16 reference (this will be confirmed with the GUS quantification assay in chapter 3.12), and it is additionally detectable in flower anthers and nectaries. This result confirms that pBUdel1 promoter, and possibly pBUdel6 enhancer, lack negative regulatory sequences.

pDel16 (5-746/3+275) is a combination of pBUdel2 and pBUdel4. The pBUdel2 full *CLV3* enhancer was substituted with the pBUdel4 short *CLV3* enhancer. pDel16 confirms that the pBUdel2 promoter lacks a negative regulatory sequence. In fact, in contrast to pBUdel4, the pDel16 signal is detectable, and it is present in pollen anthers, in carpels and at the base of flower pedicels. However, pDel16 does not stain in the central zone of the meristem. Thus, the combination of pBUdel2 promoter and pBUdel4 enhancer is not sufficient to promote *CLV3* expression in its normal pattern.

pDel17 (5-746 / 3+595 to 965) was constructed to assay if the Del5-Del6 region is sufficient to promote *CLV3* expression in the wild type pattern in combination with the pBUdel2 *CLV3* promoter. The pDel17 signal, like pDel16, is not present in the meristem, but it is strong in pollen anthers and at the base of flower pedicels. The Del4-Del5 region, missing also in pDel16, seems to be required to promote *CLV3* expression in the meristem.

pDel18 (5-746 / 3+275 and 3+595 to 965) was constructed to test if the Del4-Del5 region is necessary, together with the pBUdel2 *CLV3* promoter, to promote *CLV3* expression in the central zone of the meristem. pDel18 shows the same signal detected in plants transgenic for pDel17: no staining is visible in the meristem, but it is present in anthers and at the base of flower pedicels. Thus, the Del4-Del5 region seems to be indeed required to promote *CLV3* expression in the central zone of the meristem. All the constructs that lack the Del4-Del5 fragment and have the short *CLV3* promoter of pBUdel2 (pDel16, pDel17 and pDel18) show no signal in the meristem. To test if the

absence of the Del4-Del5 fragment is sufficient to impair the *CLV3* expression pattern in the central zone of the SAM, pDel19 was constructed.
The **pDel19** vector (5-1486; 3+275 and 3+595 to 1256) lacks only the Del4-Del5 enhancer sequence, in comparison to pBU16. Rarely, pDel19 transgenic plants show an ubiquitous GUS staining after a 4 hours long GUS assay (4 hours is the incubation time set for all the previously described GUS analyses). Most of the pDel19 plants show a signal comparable to the pBU16 reference only after a longer 16 hours incubation. The delayed GUS signal promoted by pDel19 indicates that the Del4-Del5 fragment has an important role in enhancing *CLV3* expression in the central zone of the meristem. However, Del4-Del5 cannot be considered the unique element necessary to promote *CLV3*, as a signal, even if delayed, is present in the shoot meristems of pDel19 transgenic plants, in contrast to pDel16, pDel17 or pDel18. Thus, the Del4-Del5 fragment may act in combination with other fragments of the *CLV3* regulatory sequences to promote *CLV3* expression.
These combinatorial deletion analyses permitted to identify new putative regulatory sequences. The **321 bps** long fragment missing in the CLV3 promoter of pBUdel1 possibly contains a binding site for a negative regulator. In the **420 bps** long Del1-Del2 promoter fragment the presence of a negative regulatory sequence is confirmed. An enhancer/patterning element of *CLV3* expression is present in the **198 bps** long Del4-Del5 *CLV3* enhancer fragment, which function is probably redundant with motives present in the 5' end-Del2 or in the Del6-3' end fragments. The **370 bps** long Del5-Del6 *CLV3* enhancer fragment seems to contain both positive and negative regulatory sequences (Fig.8, Fig. 3b, Fig.9).

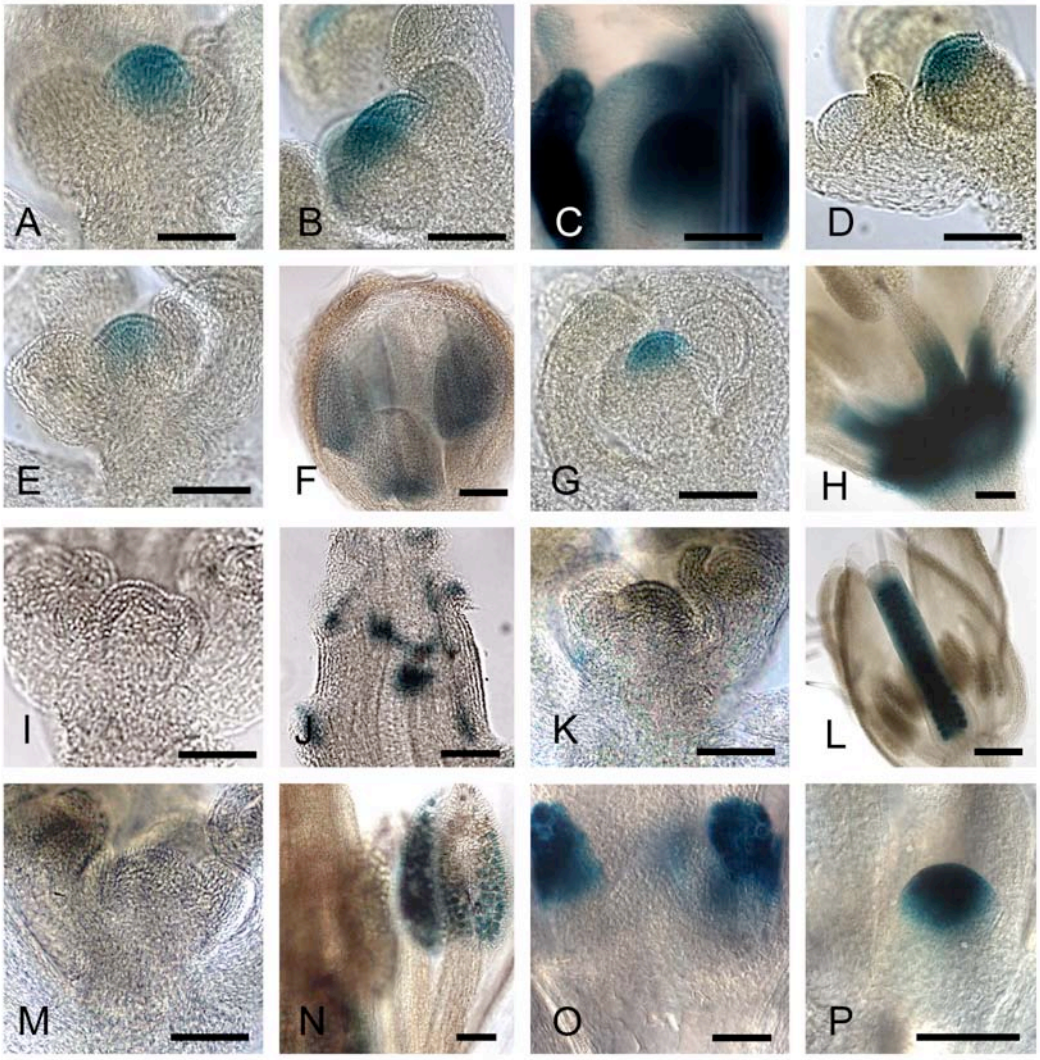


Fig.8 **GUS staining analyses on plants transgenic for combinatorial CLV3::GUS deletion constructs**. Approximately 3 weeks old plants were GUS assayed to visualize possible stainings also in differentiated tissues outside of the central zone of the meristem. (A) pBU16 reference (B-C) pDel8 occasionally shows strong GUS staining in organ primordia. (D) pDel13 signal is detectable only in the meristem. (E-F) pDel14 signal is weaker than the reference pBU16 in the meristem, but present also in flower anthers. (G-H) pDel15 stains in the CZ of the meristem and additionally in flower anthers and nectaries. (I-J) pDel16 shows no GUS signal in the SAM, but only at the base of flower pedicels (K-L) pDel17 signal is not present in the meristem but only in flower carpels, probably in ovules. (M-N) pDel18 signal is visible only in flower anthers and at the base of flower pedicels. (O) The pDel19 signal is absent in the SAM 4 hours after GUS incubation, but occasionally visible in organ primordia. (P) The pDel19 signal, 16 hours after incubation, is detected in the SAM–overstaining. Scale bar: (A-G, I, L-P) 50μm; (H, J, N) 200μm

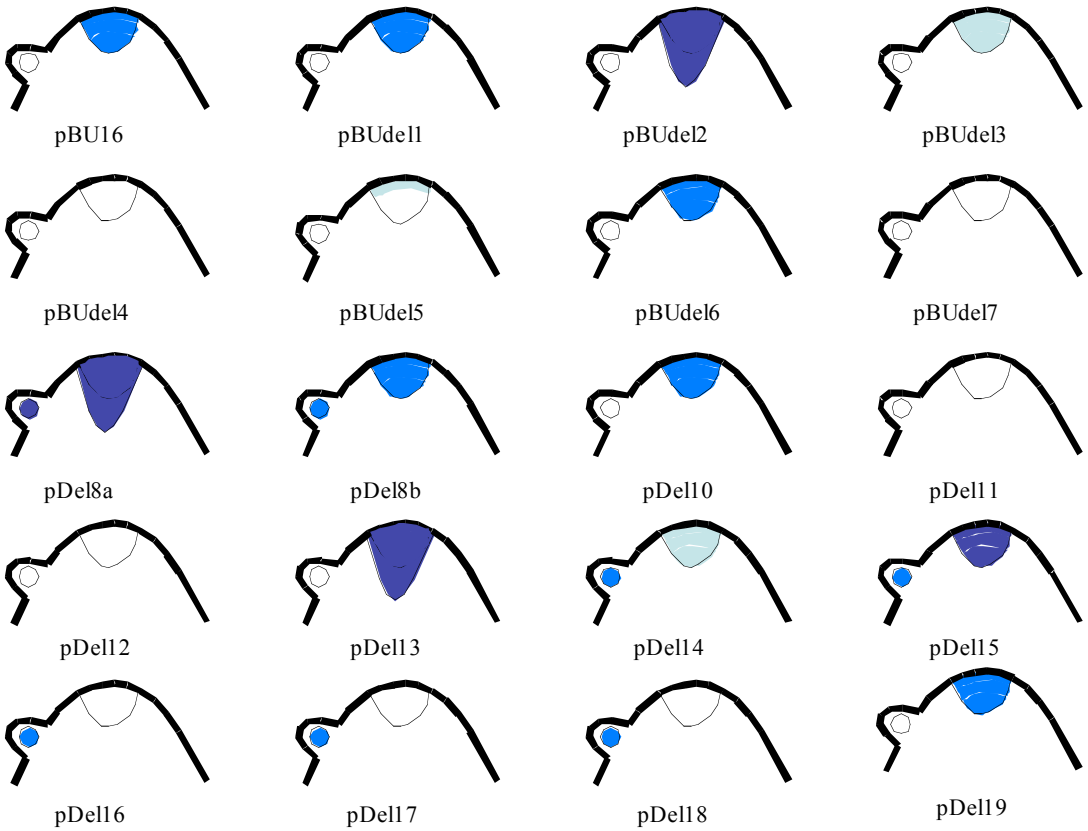


Fig.9. **Summary of the expression patterns of the analyzed CLV3::GUS deletion constructs**. The transgenic Arabidopsis plants staining in the same pattern and with similar intensity to the pBU16 reference are pBUdel1, pBUdel6, pDel10, pDel19 (after a 16 hours long incubation in the GUS assay). pBUdel2, pDel8a, pDel13 and pDel15 show a stronger and sometimes broader signal than the reference pBU16. pBUdel4, pBUdel7, pDel11, pDel12 do not show any GUS staining. pBUdel5 (in U. Brand experiments), pBUdel3 and pDel14 show a weak GUS staining in the meristem. pDel8a-b, pDel14,15,16,17,18 show ectopic *CLV3* expression in differentiated organs.

3.12 GUS expression quantification

The “positive” and “negative” functions found in the *CLV3* regulatory sequences were speculated by GUS staining intensities and patterns estimated only by eye. To better quantify the strengths of the different reporter gene expressions, I performed a GUS assay using the 4-MUG substrate (4-methyl umbelliferone glucuronide) instead of X-Gluc. 4-MUG is converted to fluorescent 4-MU by the GUS enzyme, thus giving the possibility to more accurately assay GUS enzymatic activities via a fluorescence measurement. The measured raw fluorescence values of 20 to 70 seedlings

for each transgenic background were graphed on a 4-MU standard curve, so that fluorescence values were converted to β–glucuronidase activities expressed in nmol of 4-MUG transformed to fluorescent 4-MU per hour. The fluorescence given by *Col* wild type seedlings was measured as a negative control, thus obtaining the threshold for a possible background fluorescence signal. Depending on the calculated enzymatic activity, plants transgenic for the different reporter genes were organized into four different groups.

In **Group1** (GUS activity between 135 and 295 nmol/hour) are the plants that show an average value close to the *Col* basal level. These plants are pDel16, pDel17 and pDel18, which were not staining in the SAM but only in flowers (flowers are not yet produced in the tested young seedlings), pBUdel4, pBUdel7 and pBUdel5, which did not show GUS staining even in the previous experiments.

In **Group2** (GUS activity between 354 and 407 nmol/hour) are pBUdel3 and pDel14, weakly staining in previous X-Gluc GUS assays. Also pDel19 plants are in this group, because of their delayed staining.

In **Group3** (GUS activity between 1057 and 2330 nmol/hour), together with the reference pBU16, are pBUdel1, pBUdel6 and pDel15. The higher enzyme activities of pDel1, pDel6 and of their combinatorial reporter gene pDel15, compared to pBU16, shows that pDel1 and pDel6 lack sequences which negatively regulate *CLV3* expression.

In **Group4** (GUS activity between 3070 and 4318 nmol/hour) are pBUdel2, pDel8 and pDel13. All these constructs lack the Del1-Del2 negative regulatory sequence.

Interestingly, all the combinatorial deletions carrying the *CLV3* promoter region of pBUdel2 showed a higher GUS enzyme activity than the pBU16 reference. The only exception are plants transgenic for pDel16, a combination of pBUDel2 and pBUDel4, probably because the *CLV3* enhancer fragment present in pBUdel4 is not sufficient to promote any signal in the meristem. These results are additional and independent evidences that in the Del1-Del2 *CLV3* promoter fragment a binding site for a negative

regulator of *CLV3* expression is present. All the results shown in these experiments are comparable to the previous results obtained in the GUS assays with the X-Gluc substrate. The weak signals of pBUDel3 and pDel14 confirm that the *CLV3* promoter consists of redundant positive and negative regulatory sequences, which act in a combinatorial way to control *CLV3* expression levels (Fig.10).

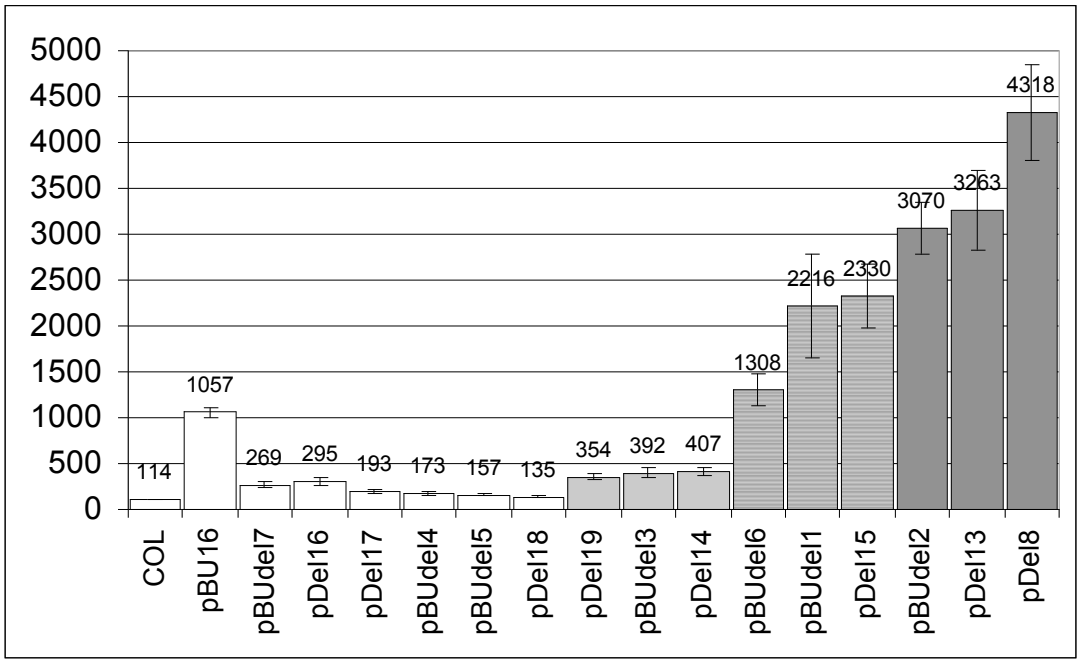


Fig. 10 **Summary of GUS staining quantification results**. pBU16 is the CLV3::*GUS* reference construct. In pBUdel7, pBUdel4 and pBUdel5 no GUS staining and no GUS activity were detected. pBUdel16, pBUdel17, pBUdel18 showed no GUS staining in the CZ of the meristem but only in flower organs, and thus no GUS activity is detected in the 12 days old seedlings analyzed in this experiment. pBUdel3, pDel14 and pDel19 have weak GUS activities. In pBUdel6, the GUS activity is similar to the pBU16 reference. pBUdel1 GUS activity is almost 2 fold stronger in comparison to pBU16, and similar to pDel15. pBUdel2, pDel13 and pDel8 showed a stronger and broader GUS signal, and 3 to 4 fold higher GUS activity.

3.13 Identification of putative regulators of CLV3 expression

From the deletion analyses previously shown, the Del1-Del2 and the Del4-Del5 fragments are supposed to contain binding sites for transcriptional

factors that regulate *CLV3* expression. Del1-Del2 and Del4-Del5 sequences were cloned in a vector suitable to conduct a yeast one-hybrid screen against a library of genes expressed in the meristem of Arabidopsis. Rebecca Kloppenburg performed the yeast one-hybrid screen. She fished out the GLABRA2 (GL2) homeodomain factor with the Del1-Del2 fragment, and the SERRATE (SE) zinc finger protein with the Del4-Del5 fragment. These results are discussed later.

3.14 The CLV3/WUS loop is buffered

CLV3 controls the size of the stem cell population through the *CLV3/WUS* feedback loop. To understand how sensitive the *CLV3/WUS* loop is, i.e. what are the *CLV3* expression levels necessary to modulate *WUS* expression and thus to promote changes in the stem cell population, I constructed a tool to express *CLV3* at different intensities. The GUS sequence was substituted with the *CLV3* genomic sequence in the following reporter gene vectors: pBU16, pBUDel2, pBUDel3, pBUDel4, pBUDel6, pBUDel7 and pDel8, which were then named pFullg, pDel2g, pDel3g, pDel4g, pDel6g, pDel7g and pDel8g. Each of these new constructs, depending on which *CLV3* regulatory fragments it carries, expresses *CLV3* at different levels.

To detect if these different *CLV3* expression levels promote variations in the stem cell population of Arabidopsis, the size of the stem cell domain was not directly measured. The number of carpels per flower is known to change in consequence of an alteration in the meristem size. A wild type plant produces two carpels per flower. The *clv3-2* mutant, with a large stem cell domain, produces approximately six carpels per flower. A plant overexpressing *CLV3*, with a small central zone in the meristem, produces only one carpel or no carpel per flower. Thus, to “measure” the meristem size in transgenic plants expressing *CLV3* at different levels, the carpel number per flower was counted and compared to the wild type reference.

Wild type plants transformed with pFullg, pDel2g, pDel3g, pDel4g, pDel6g, pDel7g and pDel8g had a constant number of two carpels per flower. Therefore, in wild type plants, none of the *CLV3* promoting vectors could alter the *CLV3/WUS* feedback loop, which in consequence appears to be quite robust. I then used these vectors to transform *clv3-2* mutant plants. In *clv3-2* mutants, *WUS* expression is not under control of the endogenous *CLV3*. Thus, these mutant plants could be more sensitive to the *CLV3* expressions promoted by my constructs.

Plants carrying **pDel4g** and **pDel7g** have 6 carpels per flower: the *clv3-2* mutant phenotype was not rescued, as expected, because pBUdel4 and pBUdel7 did not promote GUS activity in previous experiments.

Some plants transformed with **pDel8g** failed to complement the *clv3-2* mutants, other were fully rescued. Occasionally, a *wus*-like phenotype was visible: flowers developed no carpels and no stamen. The presence of different phenotypes in these transgenic plants is explainable with the fact that pDel8 GUS signal was not frequently detectable, and sometimes it was also present in the peripheral zone of the meristem. The rare *clv3-2/*pDel8g plants that phenocopy *wus* mutants could express *CLV3* also outside of the central zone of the meristem, thus strongly repressing *WUS*.

clv3-2/**pDel6g** plants, like *clv3-2*/**pFullg**, are fully rescued: each flower has on average two carpels.

The most interesting result comes from the comparison between *clv3-2* mutants transgenic for pFullg, **pDel2g** and **pDel3g**. The quantification experiment in *chapter 3.12* showed that the *CLV3* regulatory sequences cloned in these three vectors drive GUS expression with different strengths: pBU16 promoted a GUS activity of approx. 1000 nmol/hours, pBUdel2 of approx. 3000 nmol/hours, pBUdel3 of approx. 390 nmol/hours. Thus, *CLV3* expression levels driven by pDel2g and pDel3g could be almost 10 fold different. However, the *clv3-2* mutant plants transformed with these vectors have a constant number of two carpels per flower. This result show that even if *CLV3* expression is promoted at different levels, at least in a certain range,

the size of the stem cell population is maintained constantly by the CLV3/WUS loop (Fig.11).

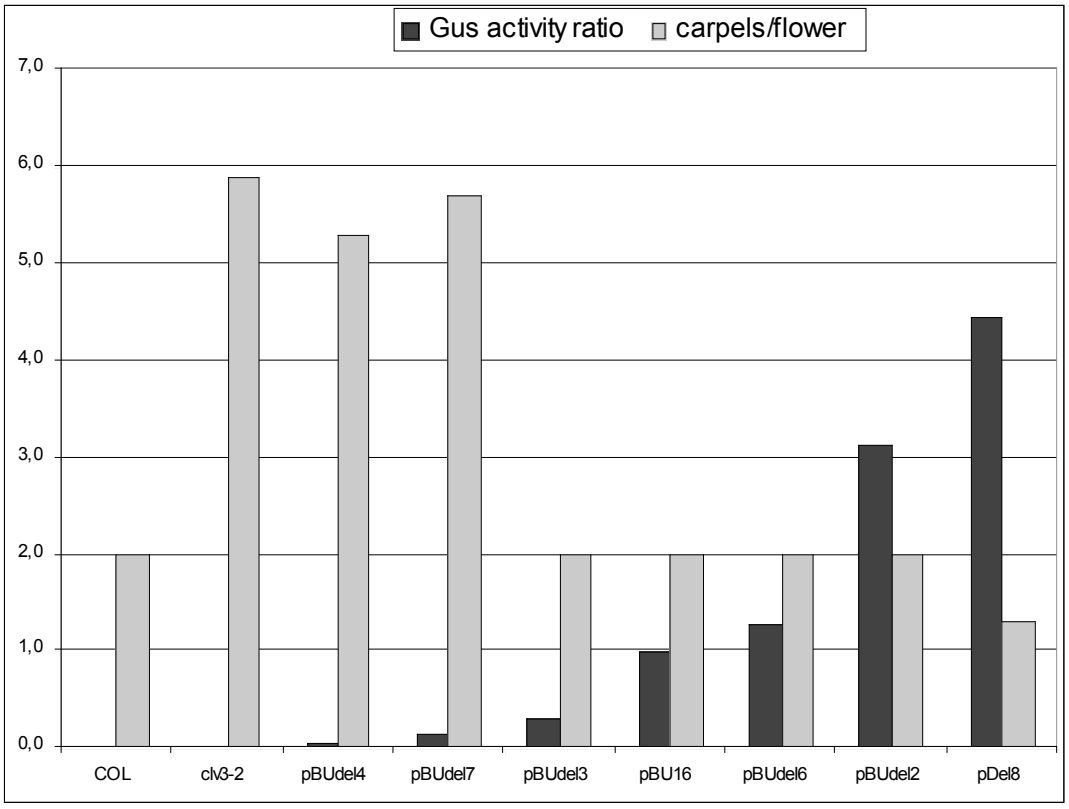


Fig. 11. **Comparison between the number of carpels per flower (Col N=2; *clv3-2* N= approx. 6) in *clv3-2* mutant plants complemented with different *CLV3::CLV3* deletion constructs (grey columns).** Black columns represent GUS activity ratios, relative to pBU16, of each CLV3::GUS deletion. The not rescued plants (5<N<6) pBUdel4 and pBUdel7 show a GUS activity of nearly zero. Interestingly, the *clv3-2* plants complemented with pBU16, pBUdel3, pBUdel6 and pBUdel2 have the same number of carpels per flower (N=2), even if GUS enzyme activity of the relative constructs is up to 10 fold different. The occasional *wus*-like phenotype of *clv3-2*/pDel8 plants reveals how a stronger *CLV3* signal can alter the size of the stem cell population (N=1.3).

4 Stem cell clonal analysis

4.1 Clonal analysis of CLV3 expressing cells

Previous cell clonal analyses on Arabidopsis SAM showed that a small number of stem cells is located in the central zone of the meristem (Steeves and Sussex, 1989; Lyndon, 1998). CLV3 expression is also detected only in the central zone of the meristem, even in Arabidopsis plants with a larger or smaller stem cell population, respectively, like in *clv* or *wus* loss of function mutants as reviewed in Waites and Simon, 2000. On the basis of these results, *CLV3* is considered a gene expressed only in stem cells, a stem cell marker. With the following experiment, I try to directly verify if *CLV3* expression is indeed active in stem cells or in already differentiated cells. A stem cell produces a large progeny in comparison to a differentiated cell. This difference in size is used in the following experiment to assay if stem cells express *CLV3*.

4.2 An ethanol inducible system to track the progenies of CLV3 expressing cells

To mark the progenies originated by *CLV3* expressing cells, I used an ethanol inducible system that, once activated, promotes only in these cells a DNA excision event and a consequent permanent GUS labelling.

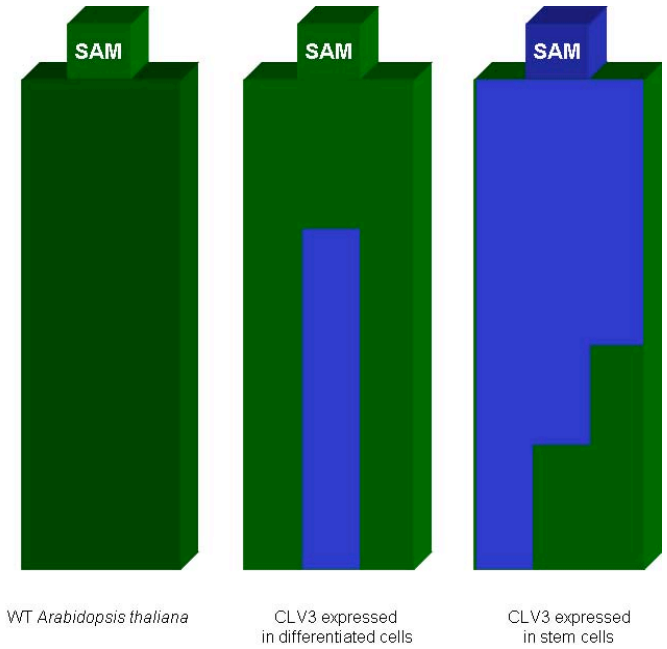


Fig.12. **Schematic representation of three Arabidopsis plants**. The first from the left, where no blue labelling is visible, is a wild type Arabidopsis plant. In the other two plants, the progeny of cells expressing *CLV3* is labelled. If *CLV3* is not expressed in stem cells, the labelling is present in a small amount of cells (middle plant). If *CLV3* is expressed in stem cells, large labelled sectors, starting from the central zone of the SAM, are visible (plant on the right).

The DNA excision event is obtained with a *CLV3*-promoter-driven expression of the CRE recombinase. The CRE enzyme recognizes and excises genomic fragments flanked by two loci of cross-over (loxP) originally isolated from the P1 bacteriophage. As continuous expression of CRE recombinase is known to disturb Arabidopsis development, an AlcR/AlcA ethanol inducible system was used to promote pulses of CRE expression in the *CLV3* domain (Coppoolse et al., 2003). The two *CLV3* regulatory sequences were used to drive AlcR expression. CRE was cloned under control of the AlcA promoter. AlcR, after ethanol treatment, binds to the AlcA promoter and activates CRE transcription. In this way, after ethanol treatment for 6 hours, transgenic plants express CRE in the CLV3 domain for approximately an additional 32 hours (Deveaux et al., 2003). Arabidopsis plants carrying the transgenes required for the ethanol switch system (CLV3::AlcR/AlcA::CRE) were assayed via *RNA* in situ hybridization with a *CRE* probe, before and after

ethanol induction. CRE expression was successfully detected exclusively after ethanol induction and only in the *CLV3* domain. Plants selected for the presence of the ethanol switch system were then transformed with the transgene required to permanently label the *CLV3* expressing cells. This transgene consists of a 35SCaMV promoter, which alternatively drives GFP expression or, only after a CRE/loxP excision event, GUS expression (35S::loxP-GFP-loxP-GUS, named pQCLOX1). Plants containing both the CRE and the loxP transgenes were selected and ethanol induced.

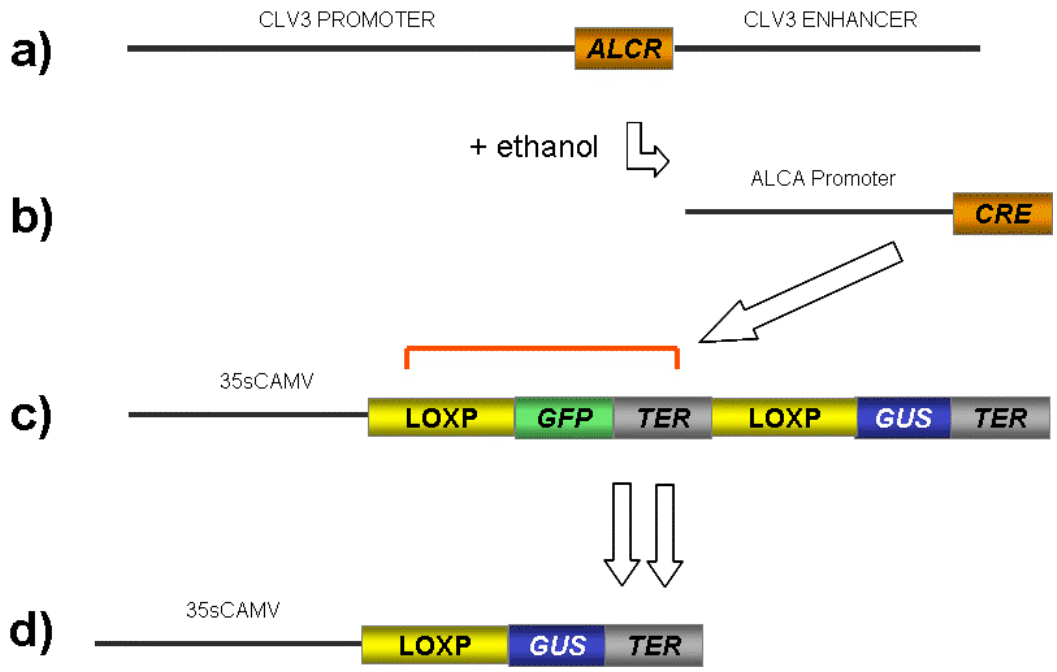


Fig.13**. Schematic representation of the ethanol-inducible Cre/loxP system**. a) AlcR is expressed under control of the *CLV3* regulatory sequences in the *CLV3* domain. b) After ethanol induction, AlcR binds to the AlcA promoter, which drives CRE expression. c) CRE recognizes the two loxP fragments, and excises the loxP-GFP-TER cassette (marked in red). d) The 35S promoter, which was driving GFP expression before the excision event, now drives GUS expression in the progeny of CLV3 expressing cells.

Three days after the treatment, inflorescences of these plants were incubated for 16 hours in the GUS assay buffer and showed a strong GUS staining in the *CLV3* domain (Fig. 14E,G). This result demonstrates that indeed the

labelling process of *CLV3* expressing cells is activated. I repeated the GUS assay after four days, to quantify a possible size variation of the labelled sectors. This time no GUS staining was visible in the *CLV3* domain, except for a weak signal in the L1 layer of the SAM, which suddenly disappeared (Fig. 14H). A second ethanol induction was lethal for the transgenic plants. I can't explain why the GUS staining fainted away one week after the first ethanol induction. One reason could be that the 35SCaMV promoter, as we noticed in previous experiment, is not strongly active in undifferentiated cells (data not shown). Recently, even pulses of CRE expression were shown to impair stem cells development (confidential data), thus leaving no possibilities to successfully repeat this experiment.

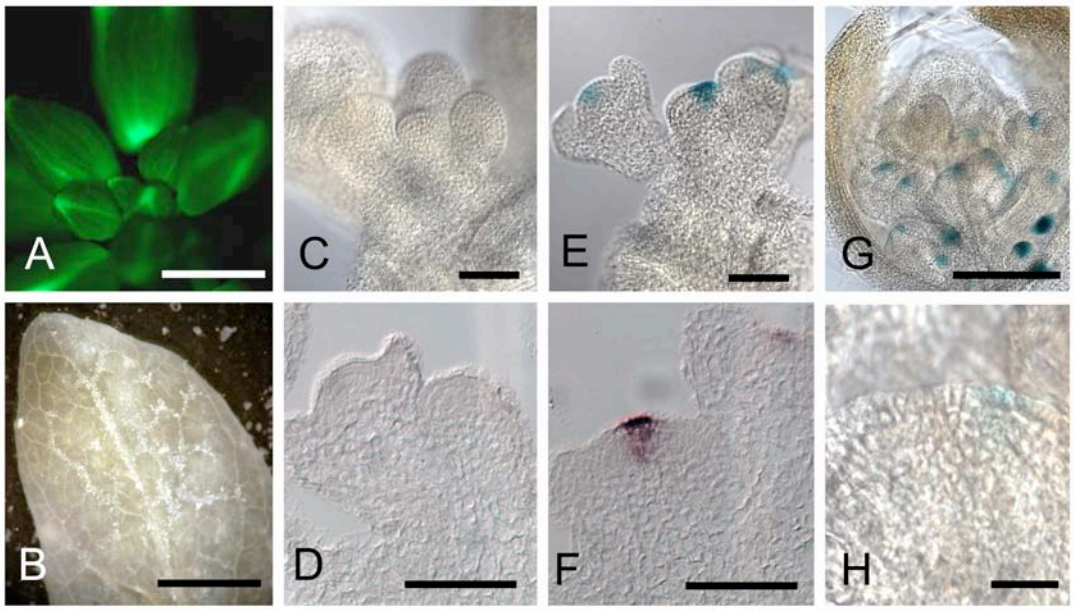


Fig.14 **Results of the clonal analysis on *CLV3* expressing cells**. Transgenic plants were tested for the presence of the transgenes necessary for the activation of the CRE/loxP excision event. (A) CRe/loxP plants show GFP fluorescence in every tissue (here the inflorescence). (B) Before ethanol induction, GUS staining is neither visible in differentiated tissues (here one leaf), nor in the shoot apex (C). (D) RNA in situ hybridization shows that CRE is not transcribed before, but only after (F) ethanol induction, in the expression domain of *CLV3*. (E,G) 3 days after ethanol induction, GUS staining is visible in the central zone of SAMs and FMs, both in the shoot apex as well as in the lateral shoots. (H) One week after the first ethanol induction, GUS staining was occasionally detected only in a couple of cells in the L1 layer of the SAM. Scale bar: (A, B) 4mm; (C-F) 50 μm; G 300 μm; H 10 μm.

5 Discussion

Maintenance of not differentiated stem cells is crucial for plants, which have to produce organ primordia throughout their whole life. The size of the stem cell domain is controlled by the tuning of the stem cell derived *CLV3* signal, and by the resulting regulation of the WUS stem cell identity factor. To understand how the size of the stem cell population is maintained and regulated during Arabidopsis development, the *CLV3* regulatory sequences were analyzed to identify control elements for *CLV3* expression.

5.1 CLV3 regulatory sequences contain redundant elements

The identification of a single, unique sequence motive necessary and sufficient to promote *CLV3* expression was not successful. Instead, *CLV3* regulatory sequences appear to be composed of multiple redundant elements, which positively or negatively control *CLV3*. In the *CLV3* downstream regulatory region, the Del4-Del6 fragment is required to enhance the *CLV3* signal. A smaller sequence, the 198 bps long Del4-Del5 element, seems to be necessary to control *CLV3* transcription in the stem cell domain. I analyzed *in silico* the presence of putative binding sites in the *CLV3* regulatory fragments. Interestingly, an AP2/ERF binding site is present in the Del4-Del5 fragment. The *DONRÖSCHEN* (*DRN*) transcription factor encodes a protein with an AP2/ERF binding domain. DRN, as shown from my previous experiments, could be a direct activator of *CLV3*. However, a yeast one-hybrid assay performed by Rebecca Kloppenburg showed that DRN alone cannot interact with the Del4-Del5 fragment. DRN could require a meristem-specific partner protein for binding to the *CLV3* regulatory sequences. A yeast two-hybrid assay could shed some light on the composition of this hypothesized DRN heteromeric complex. A similar

complex could also bind the Del2-Del3 *CLV3* promoter fragment, which was shown to act as a positive regulator of *CLV3* expression. In contrast, the 420 bps long *CLV3* regulatory sequence of Del1-Del2, which acts as a negative regulator of *CLV3* expression, does not contain AP2/ERF binding sites. Several results indicate that the Del1-Del2 fragment contains a binding site for a factor that represses *CLV3* outside of the CZ. a) The pBUdel2 reporter gene, which lacks the Del1-Del2 fragment, shows a stronger and broader GUS signal in comparison to the pBU16 reference. b) In RNA *in situ* analysis, the GUS RNA probe on plants transgenic for pBUdel2 marked a deeper and larger region than the central zone of the meristem. c) All the combinatorial *CLV3* reporter genes that lack the Del1-Del2 fragment showed enhanced GUS activity in comparison to the pBU16 reference.

Interestingly, both the Del4-Del5 and Del1-Del2 elements possibly contain redundant elements required to promote the *CLV3* expression pattern in the stem cell domain. All reporter genes lacking both the Del1-Del2 and the Del4-Del5 fragments (pDel16, pDel17, pDel18) do not express GUS in the stem cell domain, but only in differentiated tissues such as flower organs.

5.2 Putative direct regulators of CLV3 expression

When the Del1-Del2 fragment was used as bait in a yeast one-hybrid screen, the homeodomain of GLABRA2 (GL2) was isolated. GL2 is a transcriptional factor involved in trichome and root development, but nothing is known so far about its possible function in the SAM (Rerie et al., 1994; Szymanski et al., 1998). In the shoot apex, staining of a GL2::*GUS* reporter gene was found only in stipules (data not shown). Thus, GL2 is unlikely to interact with *CLV3* regulatory sequences in the central zone of the meristem, and thus cannot be considered as a regulator of *CLV3* expression. However, the GL2 homeodomain shares 62% amino acid identity with the *Arabidopsis thaliana* MERISTEM LAYER DOMAIN1 protein (AtML1), another member of the GL2

homeodomain protein family. *AtML1* is expressed in the Arabidopsis epidermal layer and in the L1 layer of the shoot apex, and could therefore interact with the *CLV3* promoter *in vivo* (Lu et al., 1996; Sessions et al., 1999). However, *atml1* loss of function does not alter the SAM structure, thus it is difficult to think that AtML1 has an exclusive role in controlling *CLV3* expression. The aphenotypic *atml1* mutant could be caused by redundancy to other genes involved in L1 layer maintenance, e.g. the closely related factors *PROTODERMAL FACTOR1* and *PROTODERMAL FACTOR2* (Abe et al., 1999; Abe et al., 2003). *PDF1* and *PDF2* expressions are limited to the L1 layer of vegetative, inflorescence and floral meristems and to the protoderm of organ primordia. *pdf2* loss of function is aphenotypic, but *atml1/pdf2* double mutant plants lack the epidermis and fails to survive after germination. A model where expressions of *AtML1* and *PDF2* equally contribute to the maintenance of the L1 layer by the formation of homo- and heterodimers is hypothesized in Abe et al., 2003. The possible interactions between these L1 layer factors and the *CLV3* regulatory sequences could be assayed via a yeast three-hybrid screen.

AtML1 could act as a *CLV3* negative regulator as part of a heterodimeric complex with other unknown factors that are specific for the peripheral zone of the meristem. However, besides the repressing activity, Del1-Del2 is also supposed to be required (together with Del4-Del5) to activate *CLV3* expression in the central zone of the meristem. Thus, if AtML1 binds the Del1-Del2 fragment, AtML1 could also have a positive function in the regulation of *CLV3* expression. AtML1 could initiate a signal that promotes *CLV3* expression in the L1 layer. *DRN*, expressed in the L1/L2 CZ layers, could focus and enhance this signal in the CZ. The *WUS* signal, expanding in all directions from the organizing center in the L3 layer, would overlap in the meristems CZ with *AtML1* and *DRN* signals, and the synergistic action of all these three factors could promote *CLV3* expression in the stem cell domain. A GUS assay could be performed on plants transgenic for pBU16 that ectopically express *AtML1* (or other genes expressed in the L1 layer), *DRN*

and *WUS*, in order to verify if *CLV3* is still expressed only in the central zone of the meristem or also in differentiated cells. To isolate other transcriptional factors controlling *CLV3* expression, new yeast one-hybrid screens are in progress with different baits from the *CLV3* regulatory sequences.

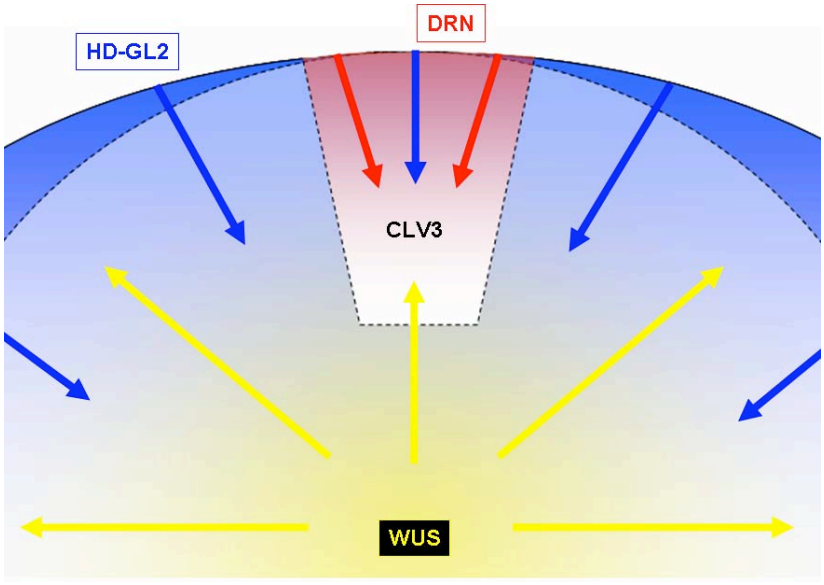


Fig.15 **Schematic representation of *CLV3* induction in the SAM**. The WUS signal, coming from the organizing center in the L3 layer, the AtML1 signal, originated in the L1 layer, and the DRN signal, present in the first two layers of the central zone of the meristem, overlap in the stem cell domain and promote *CLV3* expression.

Rebecca Kloppenburg recently showed via a yeast one-hybrid screen that the transcription factor SERRATE (SE) can interact with the Del4-Del5 fragment. *se* mutant plants do not properly initiate cotyledons and post-embryonic lateral organs and occasionally their meristems are arrested (Clarke et al., 1999; Prigge and Wagner, 2001). SE encodes a protein with a single zinc finger and it is transcribed in shoot meristems and in emerging organ primordia. The synergistic genetic interaction between *SE* and *FASCIATION1* (*FAS1*), which encodes the largest subunit of chromatin assembly factor I, made Prigge et al. suggest that SE regulates changes in

gene expression via chromatin modification. Taken together, these results indicate that SE could have a role in activation of *CLV3* in the stem cell domain, maybe by chromatin remodelling.

5.3 Redundant WUS responsive elements in the CLV3 regulatory sequences

The WUS protein is unlikely to interact with the *CLV3* regulatory sequences directly, as was shown in this research work. Furthermore, *WUS* and *CLV3* are not expressed in the same cells. The hypothesis of a non-cell autonomous effect is rejected, as ectopic expression of *WUS* is not sufficient to activate *CLV3* expression in any cell (Brand et al., 2002). In addition, *WUS* is expressed in ovules, whereas *CLV3* is not (Gross-Hardt et al., 2002). Thus, other factors that may be localized to specific domains of the plant, like in the meristem tip, are likely to be required for interaction with WUS.
Both WUS and DRN are positive regulators of *CLV3* expression, but even their simultaneous overexpression is not sufficient to promote *CLV3* expression outside of the central zone of the meristem. Probably *CLV3* needs the synergistic action of some other yet unknown *CLV3* promoting factors. Alternatively, expression of *CLV3* in differentiating cells may be restricted by limited accessibility of the chromatin at the *CLV3* locus.

5.4 Robustness of the CLV3/WUS loop

The *CLV3/WUS* feedback loop is not easily disturbed by alterations in *CLV3* expression levels. Even a 10-fold change in the *CLV3* expression level was shown to be not sufficient to affect the stem cell number in shoot or floral meristems. Not all the components involved in the *CLV3/WUS* loop are known so far. The presence of buffered or limiting steps during the

transmission of the signal responsible for either *WUS* downregulation or stem cell identity promotion could weaken the strength of the *CLV3* signal. CLV1 is probably not the only CLV3 receptor: other receptor complexes, less sensitive to *CLV3* signal, could be activated only after *CLV3* levels have increased beyond a certain threshold, thus resulting in a strong downregulation of *WUS* transcription.

5.5 Conclusion

By the deletion analyses of the *CLV3* promoter and putative enhancer, different fragments from the *CLV3* regulatory sequences, required to promote or repress *CLV3* expression, were isolated. These results permitted to hypothesize a combinatorial model of the regulation of *CLV3* expression. Due to high redundancy, single motives necessary and sufficient to promote *CLV3* in its own pattern were not isolated. However, a negative regulator of *CLV3* expression (the Del1-Del2 fragment) and an expression enhancer, possibly interacting with DRN and SE (the Del4-Del5 fragment) were identified. These two fragments will be used as baits to isolate, by yeast one-hybrid screens, new transcriptional factors regulating *CLV3* expression in the stem cell domain and in differentiated cells. Due to its importance in tuning the size of the stem cell population, *CLV3* expression is probably fine-tuned by many different pathways, possibly including also high-level transcription control systems like DNA methylation and chromatin remodeling. Some of the identified regulatory regions could be required for the recruitment of histone modifying or DNA methylating enzymes at the *CLV3* locus. Current chromatin immuno precipitation (ChIP) experiments indicate that *CLV3* is indeed subject to chromatin modification and repression in non-stem cells.

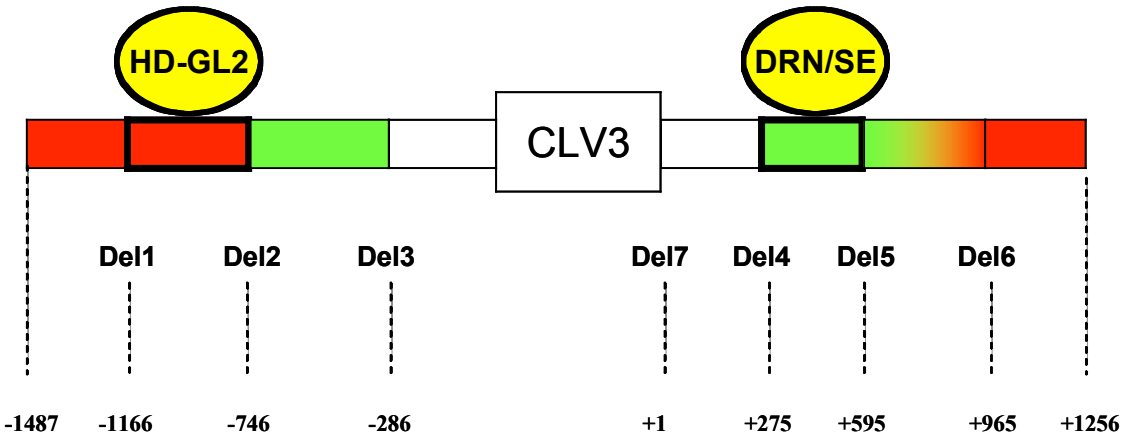


Fig.16 **The CLV3 regulatory sequences**. In red or green are the fragments containing binding sites for factors which negatively or positively control *CLV3* expression, respectively. Fragments in bold are thought to contain binding sites for factors necessary to promote *CLV3* expression in the stem cell domain, i.e. members of the HOMEODOMAIN GLABRA2 (HD-GL2) protein family, DRN and SE.

6 Characterization of *LBD30* (*LOLLO*)

6.1 Isolation and phenotype of the LOLLO-D (lol-D) mutant

The *lol-D* mutant phenotype was identified after an activation tagging mutagenesis in *Arabidopsis thaliana* (Schneider et al., 2005). The used tag was a modified Spm transposable element (dSpm-Act), carrying four copies of the 35SCaMV enhancer and resistance to the BASTA herbicide. A T-DNA containing the dSpm-Act transposon plus the gene encoding the transposase was inserted into the genome of Arabidopsis (*Columbia* ecotype) via *Agrobacterium tumefaciens* infection. The T1 generation was selected with BASTA, the T2 generation phenotypes were analyzed and the *lol-D* mutant was isolated because of its aberrant leaf shape, which resembled the leaves of the lettuce variety *LOLLO BIANCO* (Fig. 18A,B). During early development, *lol-D* plants are indistinguishable from wild type plants. Approximately 10-20 days after germination *lol-D* mutant plants start to produce leaves which become gradually shorter (wild type length 3.5 cm +/- 0.17 (s.e.), n=46; *lol-D* length 2.4 cm +/- 0.11 (s.e.), n=43), darker green and lobed. To verify if cell fate is altered in the aberrant *lol-D* leaves, adaxial and abaxial sides of the mutant leaves were analyzed via scanning electron microscopy: leaf cell size, stomata number and cell identity are comparable to wild type (Fig. 18L-M'). The blade-to-petiole ratio is as well progressively increased in *lol-D* mutants (wild type: 1.6; *lol-D*: 3.7) (Fig. 17). Petiole length and a general reduction in organ size give the *lol-D* mutant plant a bushy phenotype. Lateral shoots are occasionally produced and develop properly. After an average of 13 wild type flowers per plant (12.7 +/- 0.64 (s.e.); n=12), *lol-D* inflorescences produces defective flowers with bent carpels, uni-valved pistils and occasionally underdeveloped flower organs, especially petals and

stamens (Fig. 18C-F). However, mutant plants are not sterile. Approximately six weeks after germination, a small, radialized pin-like organ is visible in the SAM of *lol-D* mutants. Scanning electron microscopy revealed that this pin structure is the arrested shoot meristem. In the peripheral zone of the mutant meristem, occasionally an additional smaller pin is visible, possibly the remainder of an aborted flower. Before termination, *lol-D* meristems often produced aberrant flower buds lacking the adaxial sepals (Fig. 18G-K). As only 60-70% of all *lol-D* plants show the described mutant phenotypes, the *lol-D* mutation is not considered fully penetrant.

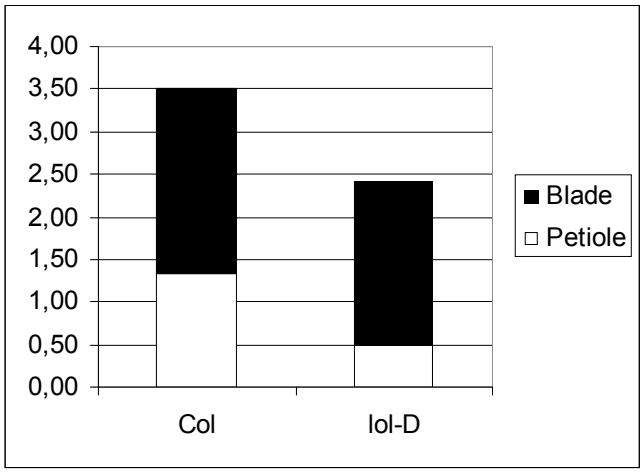


Fig. 17. **Blade-to-petiole ratio**. Wild type leaves are 1.4 fold longer than leaves in *lol-D* mutants. Wild type petioles are almost 2 fold longer than mutant petioles.

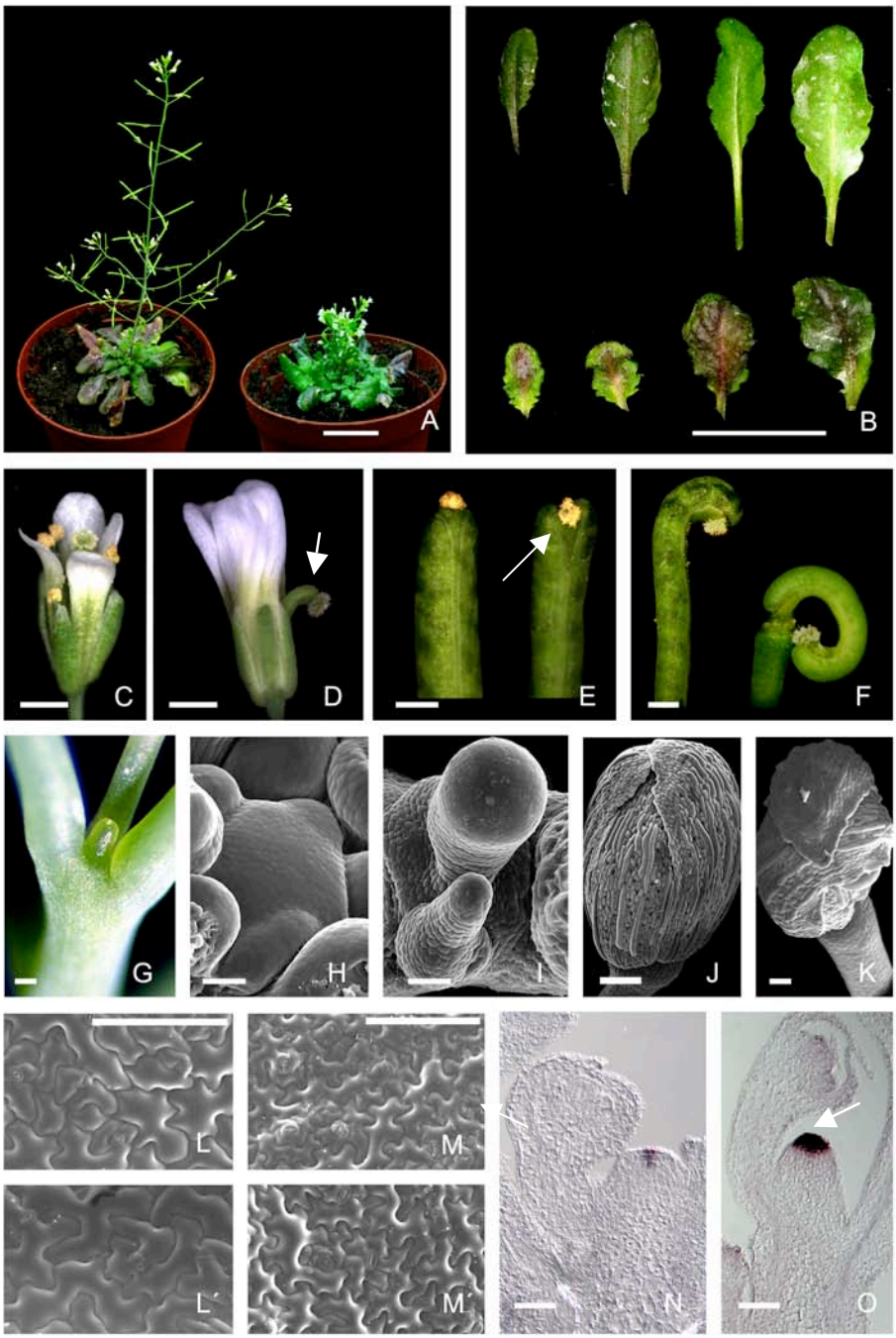


Fig.18. **The *lol-D* mutant phenotype**. (A) 5 weeks old *Col* wild type (on the left) and *lol-D* mutant (on the right) plants. (B) Both young and mature *lol-D* leaves (bottom row) are shorter than wild type leaves (upper row). (C) Wild type Arabidopsis flower (D) *lol-D* mutant flower with bent pistil (arrow). (E) Siliques in *lol-D* mutants are enlarged at their distal side (arrow). (F) Last flowers produced by *lol-D* mutant plants show an extreme pistil bending, which is even stronger in uni-valved pistils. (G) The arrested shoot meristem of a 6 weeks old l*ol-D* plant, surrounded by flower pedicels. (H) Scanning electron microscope picture of a wild type meristem (courtesy of Dr. Kwiatkowska). (I) A *lol-D* arrested meristem flanked by a probably aborted flower. (J) A wild type flower bud. (K) The last flower produced by *lol-D* mutant plants lacks the adaxial sepal (arrow). (L, M) Adaxial and abaxial sides of leaves from wild type plants are comparable to (L', M') adaxial and abaxial sides of leaves from *lol-D* mutant plants. (N,O) RNA in situ analysis shows the *CLV3* antisense probe signal in the central zone of the shoot meristem of a wild type plant. (O) The same probe evidences a broader domain in the shoot meristem of a *lol-D* mutant plant. Scale bar: 2 cm (A,B); 600μm (C-F); (G) 50 μm; (H,I,N,O) 30 μm; (J,K) 80 μm; (L,M) 100 μm.

6.2 Molecular analysis of the lol-D mutant

The position of the dSpm-Act transposon in the *lol-D* genome was identified via inverse PCR and sequencing (Schneider et al., 2005). The dSpm-Act transposon is inserted in a presumably not-transcribed area on the fourth chromosome, between the *At4g00210* and *At4g00220* coding sequences. This area, approximately 3kbp long, contains the putative promoter sequences of these two genes, which have opposite transcriptional orientations (Fig. 19). Northern blot and RT-PCR analyses on total RNA extracted from *lol-D* leaves revealed that *At4g00220*, and not *At4g00210*, is upregulated in *lol-D* mutant plants (data not shown). By RNA in situ hybridizations on *lol-D* tissue sections, I detected transcripts of *At4g00220* throughout the plant (Fig. 20P). Interestingly, *At4g00210* and *At4g00220*, this last named *LOLLO (LOL)*, encode for *LBD31* and *LBD30*, two transcription factors belonging to the *LATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) gene family (Shuai et al., 2002). The founding member of this family, *LOB*, is expressed in the boundary regions that separate young organ primordia from meristems. By expressing LOL fused with GFP, LOL was shown to be localized in cell nuclei (data not shown).

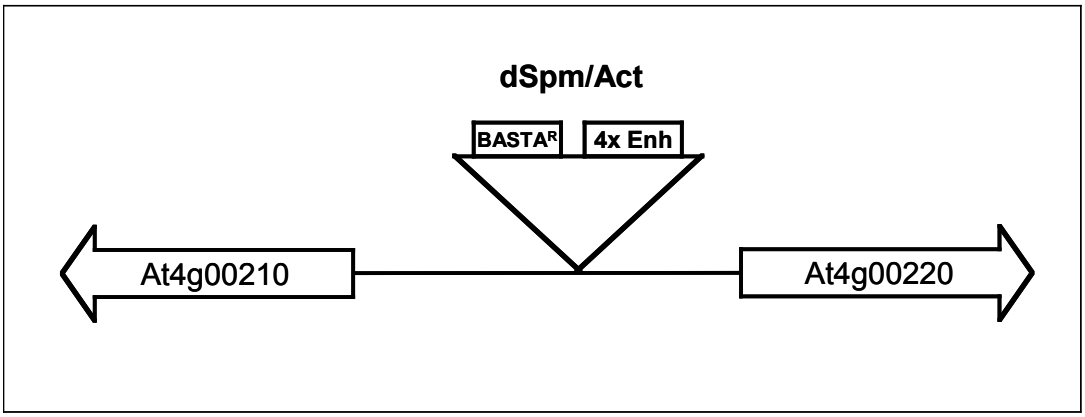


Fig. 19. **The dSpm/Act transposon is inserted between *At4g00210* and *At4g00220***. The insertion locus is the putative promote region of both of these genes. The modified transposon carries a BASTA resistance and four copies of the 35SCaMV enhancer sequence (4xEnh).

6.3 LOL and LBD31 expression patterns

The *LOL* expression pattern was analyzed in wild type plants via RNA in situ hybridization (Fig. 20A-O). *LOL* transcripts are initially found at the sites of organ primordia formation in the peripheral zone of the shoot meristem. As visible on longitudinal sections along the inflorescence apex, *LOL* is expressed in a roughly triangular domain of 4-6 cells width and 3-4 cells depth. When organ primordia emerge, the *LOL* signal is present in the adaxial side of organ primordia. Cross-sections revealed that *LOL* RNA is present at the tip of developing flower organ primordia and that it is later restricted to the boundaries between meristem and initiating flower primordia. However, *LOL* RNA is not detected in the boundary between late stage 2 flower primordia and the inflorescence meristem. In flowers, *LOL* RNA is first detected when sepal primordia are initiated. At stage 3, when sepals are separated from the floral meristem, *LOL* is expressed in a narrow band about 2 cells wide and 3 cells deep that marks the boundary between sepals and meristem. *LOL* expression is not detected later than stage 4 in meristem/organ boundaries. During carpel development, *LOL* is weakly expressed in ovules. I did not detect *LOL* transcript in any stage of embryo development, but RT-PCR from embryonic total RNA revealed weak *LOL* expression (data not shown).

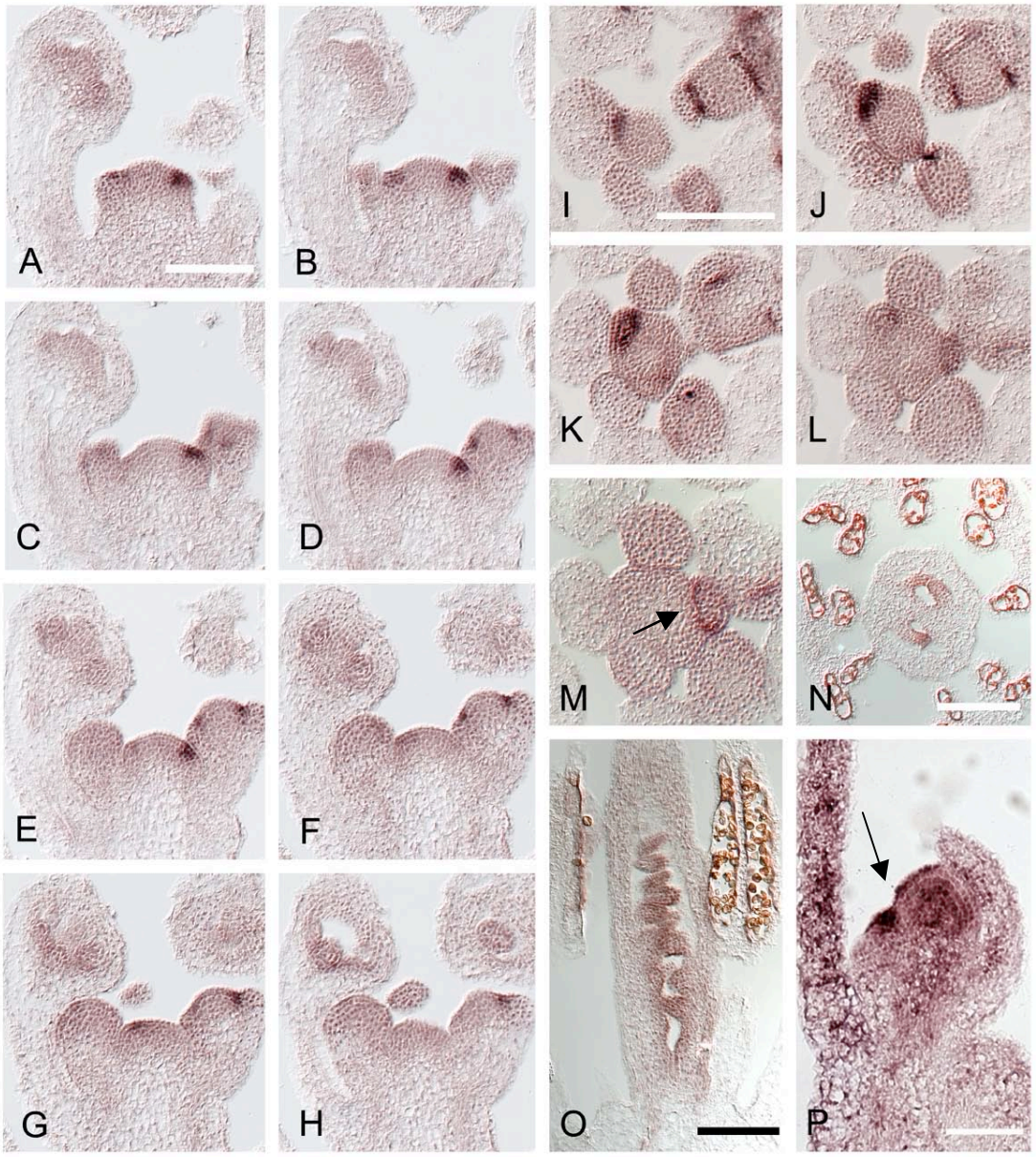


Fig.20 **RNA in situ hybridization with a *LOL* antisense probe**. (A,H) Consecutive longitudinal sections of shoot apices of wild type Arabidopsis. *LOL* signal is present at the adaxial side of the emerging organ primordia. *LOL* is also expressed in young flowers, between floral meristems and petal primordia. (I-M) Consecutive cross sections of shoot apices of wild type Arabidopsis. *LOL* expression is detected in the boundaries between floral meristems and sepal primordia. *LOL* signal is also present in flower primordia and in the boundaries between meristem and flower primordia (black arrow). (N) Cross and (O) longitudinal sections of a mature silique: LOL is expressed in ovules. (P) *LOL* RNA in situ analysis on sections of the shoot apex of *lol-d* mutant plants. *LOL* is ectopically expressed, and strongly detected in the boundary between meristem and organ primordium (arrow) but also in every other tissue. Scale bar: (A-M) 100 μm; (N,O,P) 50 μm.

LOL and *LBD31* expression patterns appear comparable in the shoot meristem. By RNA in situ hybridization, the *LBD31* signal was detected in young organ primordia and in the boundaries between organ primordia and

meristems (Fig. 21A-D). To assay *LOL* and *LBD31* expression patterns in the whole plant, I tried to amplify the putative promoter of each gene via PCR. A fragment, approximately 1.5 kbps long, upstream of *LBD31* was used to drive the GUS reporter gene. The LBD31::*GUS* signal is visible at the boundaries between meristem and flower organs, at the base of flower pedicels, in leaf vasculature and in hydathodes. Further analyses on LBD31::GUS expression pattern are in process (Fig. 21E-G). The amplification of the putative *LOL* promoter was not yet successful.

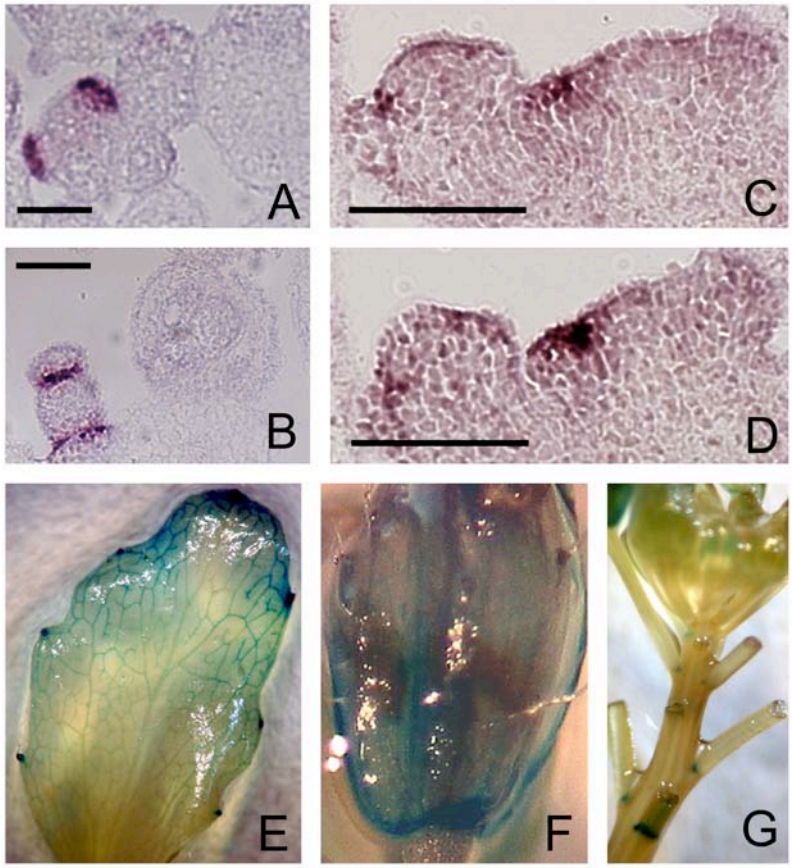


Fig. 21 **Expression pattern of *LBD31* in wild type Arabidopsis**. (A,B) Cross sections of inflorescence and floral meristems. *LBD31* is expressed in young organ primordia and in the boundaries between sepals and floral meristems. (C-D) Contiguous longitudinal sections of the shoot apex. *LBD31* is expressed in the boundary between meristems and organ primordia. (E, F, G) LBD31::*GUS* signal is visible in leaf vasculature and hydathodes, at the bases of petals and flower pedicels.

6.4 Gene expression analysis in lol-D mutants

The meristem arrest of *lol-D* mutants could be due to a failure of stem cell maintenance. To characterize the developmental defects of *lol-D* mutant plants, the expression patterns of genes involved in meristem function were analyzed. The *CLV3* signal was investigated in the mutant meristem via RNA in situ hybridization. *CLV3* expression was detected in a region far broader than the stem cell domain in wild type plants, extending to the presumptive PZ of the mutant meristem (Fig. 18N-O). Thus, the stem cell population is not lost in the *lol-D* shoot meristem, but on the contrary seems to be increased, suggesting a delayed exit of cells from the central to the peripheral zone. A similar phenotype is present in the *pin-formed1* (*pin1*) mutant meristem. PIN1, a putative auxin efflux facilitator, canalizes auxin to the meristem PZ and organizes peaks of auxin concentration that promote organ primordia initiation (Reinhardt et al., 2003). In *pin1* plants, rosette leaves are often fused and the pin-shaped inflorescence produces flowers with a strong temporal delay in comparison to wild type. Application of ectopic auxin on *pin1* mutant meristem promotes the formation of organ primordia, but the same experiment gave no results on the pin-like meristem of *lol-D* plants, indicating that *lol-D* mutants are less sensitive to exogenous auxin.

To further characterize the *lol-D* mutant phenotype, two GUS reporter genes for *STM* and *AS1* were introduced into *lol-D* mutant plants. *STM* is required for meristem maintenance and identity: in wild type background, it is expressed only in meristematic tissue and down-regulated at sites of organ formation. *AS1* is normally expressed in initiating organs, but excluded from the meristem due to *STM* activity. Thus, the complementary expressions of these two reporter genes allow to identify meristematic and organogenic tissues, respectively. *Col* Arabidopsis plants carrying the STM::*GUS* reporter gene were crossed with *lol-D* plants and the resulting F1 generation selected with the BASTA herbicide. STM::*GUS* signal was detected not only in the arrested meristem, but also in the medial ridge zone of bent carpels and in a

spotted pattern possibly in the placenta (Fig. 22D-E). No STM::*GUS* staining is normally visible in wild-type carpels, probably because *STM* expression is too weak to be detected with a GUS assay (Fig. 22C). Thus, *STM* expression is enhanced in *lol-D* mutant plants. Interestingly, *STM* was particularly up-regulated in the medial ridge of uni-valved pistils: this could indicate that the initiation of the second carpel failed because *STM* expression was maintained at high levels in *lol-D* plants. The AS1::GUS signal in *lol-D* mutants was comparable to wild type. *AS1* was still excluded from *STM*-expressing cells, indicating that the regulatory interactions between *AS1* and *STM* are still maintained in *lol-D* mutant plants (Fig. 22F-G).

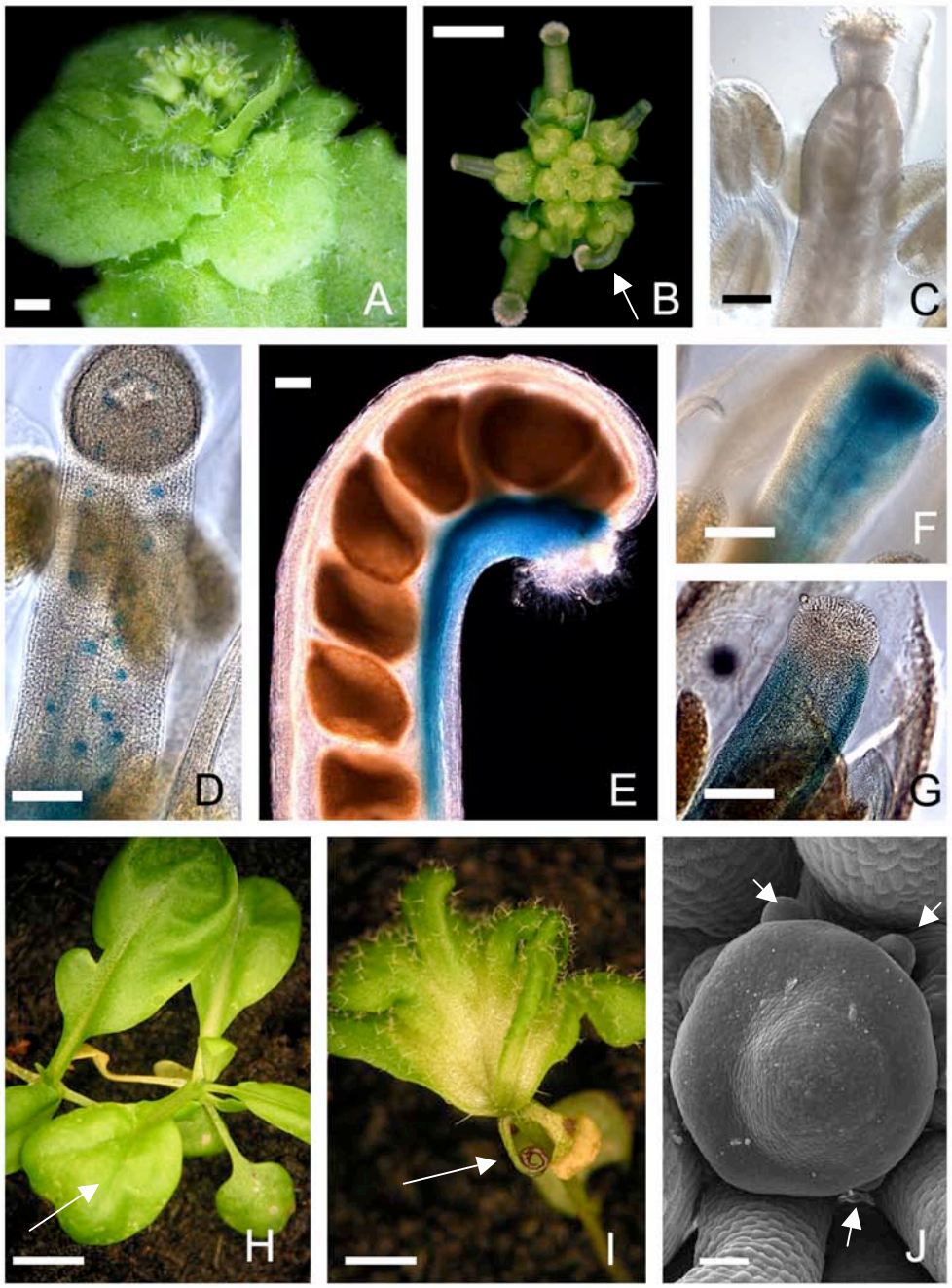


Fig.22 (A,B) *LOL* overexpression phenotype. (A) 3 weeks old 35S:*LOL* plants are small, have lobed leaves and are sterile. (B) Particular of the inflorescence of 35S::LOL mutants: the arrow points to a bent pistil. Mutant flowers do not develop petals or stamens. (C) STM::GUS signal in *Col* wild type plants: no staining is visible in the pistil. (D,E,F) STM::*GUS* signal in *lol-D* mutant plants: blue staining is possibly in the placenta, close to ovules, and in the medial ridge of carpels.(G) AS1::GUS expression in *lol-D* pistils. The signal is present in carpel valves but not in the medial ridge, where *STM* is expressed. (H) Two weeks old *pin1* mutants: the arrow points at the fusion between two leaves. (I) *pin1* x *lol-D* double mutants show extreme leaf fusion: all the rosette leaves are fused in one lobed organ. The arrow points at a not polarized organ produced by the mutant meristem. In (J) the arrested meristem of *clv3-2/lol-D* double mutants, surrounded by flower pedicels is shown. At the base of the meristem (arrows) three arrested organ primordia are visible. Scale bar: (A,B) 2 mm; (C) 200 μm; (D) 400 μm; (E) 80 μm; (F,G) 300 μm; (H,I) 1 cm; (J) 50 μm.

6.5 LOL genetic interactions

The failure to induce organ formation in the arrested *lol-D* meristem by providing auxin indicated that *lol-D* mutants are compromised in auxin sensing or signal transduction. The phenotypic similarity between *pin1* and *lol-D* arrested meristems suggested to analyze the *pin1/lol-D* double mutants. *lol-D* mutant plants were crossed with *pin1/+* heterozygote plants to assay if *LOL* has a role in the regulation of auxin activity. *pin1/lol-D* plants showed a strong mutant phenotype: all the rosette leaves were fused in a unique lobed organ, the inflorescence did not elongate and occasionally the shoot meristem produced a short radialized organ (Fig. 22H,I). Because the *lol-D* genotypic background strongly enhanced the *pin1* mutant phenotype, *LOL* seems to have a role in auxin distribution or sensing during plant development.

CLV3 RNA in situ hybridization showed that the arrested *lol-D* meristem contains an increased *CLV3* expressing cell population. To assay if the feedback loop between *WUS* and *CLV3* is affected in *lol-D* mutants, I analyzed double mutant combinations. *clv3-2* plants were crossed with *lol-D* mutant plants to verify if the broader stem cell population in *lol-D* arrested meristems depends on *CLV3* expression. *Clv3-2* single mutants accumulate stem cells in shoot apical and floral meristems, resulting in fasciation, over-production of floral organs and partial indeterminacy of floral meristems. Five weeks after germination, *clv3-2/lol-D* double mutant plants formed lobed rosette leaves, flowers with an increased carpel number and a larger inflorescence meristem that terminated in an expanded, pin-like structure with arrested organ primordia (Fig. 22J). From this additive double mutant phenotype, *lol-D* meristem arrest does not seem to be caused by a strong up-regulation of *CLV3.* Thus, in *lol-D* mutant plants, *WUS* is possibly still expressed and promoting stem cell identity. Consistent with this, the *wus*-like phenotype of *lol-D/wus* double mutants revealed that *WUS* activity is still required in *lol-D* mutants (data not shown).

6.6 Overexpression of LOL phenocopies the lol-D mutant phenotype

To confirm that the *lol-D* mutant phenotype is exclusively due to the expression enhancement of *LOL*, I analyzed the mutant phenotype of transgenic plants overexpressing either *LOL* or *LBD31.*The *LOL* and *LBD31* cDNAs were cloned under control of the 35SCaMV ubiquitous promoter (35S::*LOL* and 35S::*LBD31*). The T1 generations were selected for the presence of the transgenes and, in the case of 35S::*LOL* plants, only six resistant plants were isolated. The low number of transgenic plants, approximately 1/5 of the average usually obtained with a Gateway (Invitrogen) plant transformation vector, could be due to a lethal effect caused by *LOL* overexpression. Surviving 35S::*LOL* plants developed small and lobed leaves, had defective stem elongation and flowers with occasionally bent carpels. In addition, sepals and stamens were missing, and the mutant plants are therefore sterile (Fig. 22A,B). 35S::*LOL* plants showed a phenotype comparable to *lol-D* mutants*,* even though much stronger, which is probably due to the high *LOL* expression driven by the 35SCaMV promoter. Cell identities in leaves of 35S::*LOL* plants were analyzed by scanning electron microscopy on both adaxial and abaxial leaf sides, but no alterations were found in comparison to wild type. Interestingly, plants overexpressing *LBD31* did not show a mutant phenotype, confirming that *lol-D* mutants are caused only by the expression enhancement of *LOL*.

The deleterious effects of *LOL* overexpression in the 35S::*LOL* plants cause difficulties in performing further analyses. To better characterize the consequences of *LOL* overexpression, I fused the LOL protein to the hormone binding domain of the glucocorticoid receptor (35S::LOL-GR). Four transgenic plants were identified to respond to Dexamethasone (Dex) induction. Single inductions were sufficient to phenocopy 35S::*LOL* plants,

including shoot meristem termination. Repetitive inductions with Dex on young transgenic plants caused in 10% of the cases premature arrest of organ development and subsequent plant death, indicating that gross *LOL* misexpression interferes with cellular processes essential for plant survival.

6.7 LOL misexpression interferes with cell cycle regulation

LOL inducible mis-expression causes alterations in trichome development. 48 hours after Dex induction, the cell volume of trichomes is enlarged, although the spike structure is left unaltered (Fig. 24A,B). This transient effect could be due to a failure in controlling trichome nuclei endoreduplication, thus causing a cell size enlargement. Trichomes from *Col* wild type and induced 35S::LOL-GR plants were fixed and stained with 1 μM YO-YO1 solution to measure the nuclei area (Fig. 24C,D), revealing that mutant nuclei are almost double the size than wild type (35S::LOL nuclei area = 278 μm^2 +/- 21.78 (s.e.),n=40; *Col* nuclei area = 169 μm^2 +/- 8.01 (s.e.),n=40). The additional quantification of DNA content through DAPI fluorescence in *Col* and induced 35S::LOL-GR trichomes showed that, while in wild type plants trichome nuclei are 32C, in the mutant plants they are 128C, meaning mutant nuclei undergo two additional rounds of endoreduplication (Fig. 23). Thus, induced overexpression of *LOL* transiently interferes with cell cycle regulation in trichomes. Furthermore, in *LOL* mis-expressing plants, aberrant cell proliferation at the sites of lateral root formation and premature arrest of root hair differentiation were observed (Fig. 24E,F).

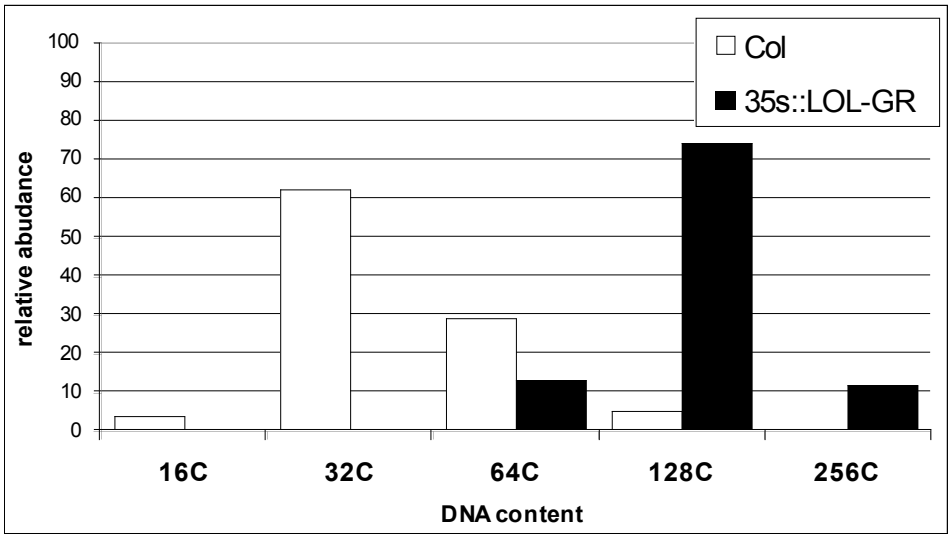


Fig. 23 **Relative abundance of DNA copies in trichomes from wild type and induced 35S::LOL-GR plants**. In wild type, 60% of the trichome nuclei contain 32 DNA copies, while in *LOL* mis-expressing mutants more than 70% of the trichome nuclei contain 128 DNA copies. Thus, trichomes from 35S::LOL-GR induced plants undergo two more endoreduplication cycles.

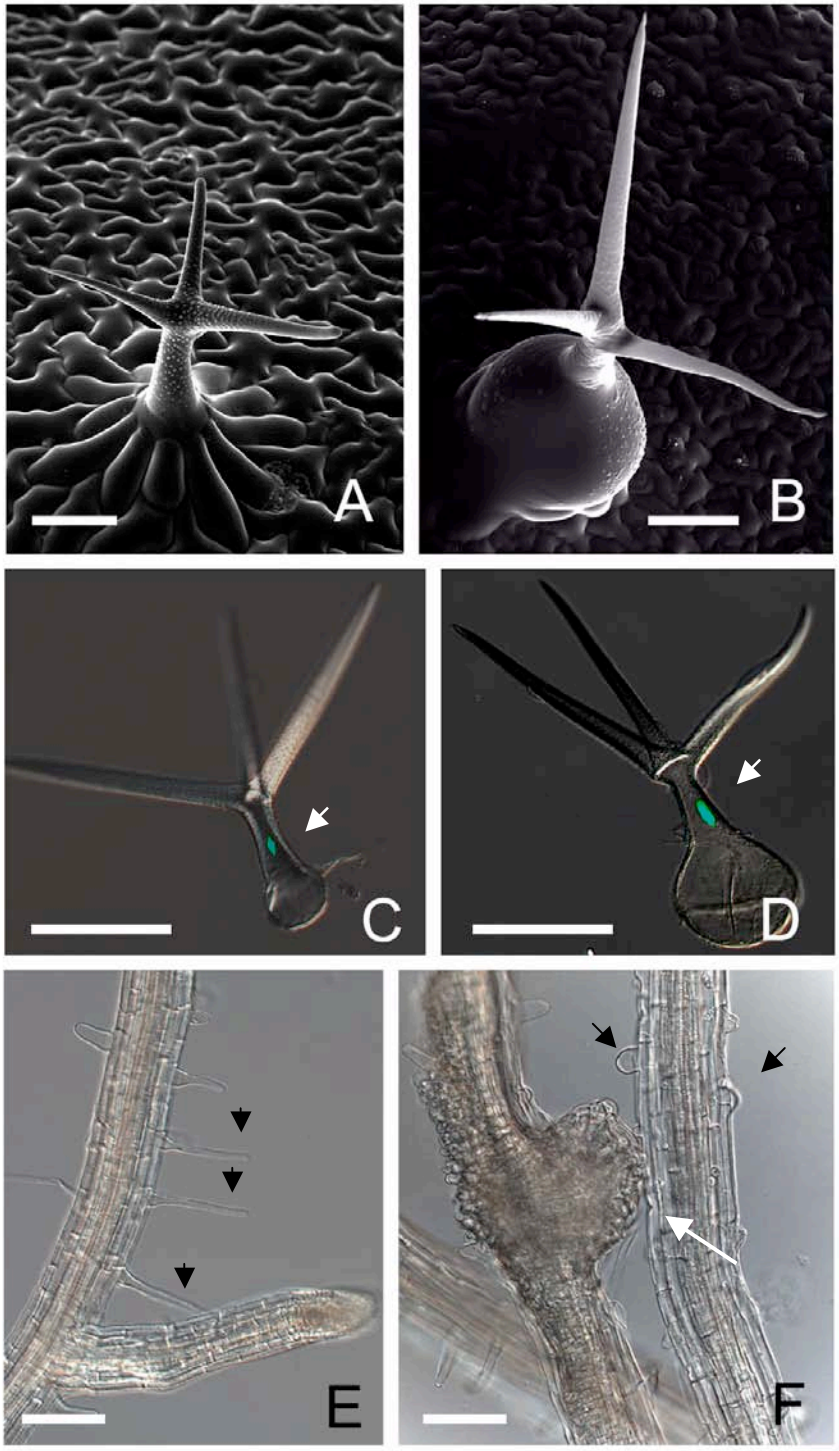


Fig. 24. (A) Scanning electron microscope (S.E.M.) picture of a wild type trichome. (B) S.E.M. picture of a "ball-shaped" mutant trichome from induced 35S::LOL-GR plants. (C-D) Overlapping of Normasky and fluorescence pictures, evidencing nuclei in wild type and mutant trichomes (arrows) (E) Wild type lateral roots grown on medium containing 1 μM Dex: root hairs are developed (black arrows). (F) *LOL* mis-expressing plants have altered lateral root production (white arrow) and root hairs are only initiated (black arrows). Scale bar: (A,B) 100 μm; (C,D) 200 μm; (B,C) 30 μm.

6.8 LOL is required for embryo development

To investigate the role of *LOL* during plant development, I analyzed the mutant line SALK_020930 that carries a T-DNA insertion in the second exon of *LOL* (allele named *lol-1*) (Alonso et al., 2003). Plants obtained from the Arabidopsis stock centre were shown to be heterozygous for the *lol-1* allele. However, genotyping by PCR revealed no homozygous plants in the progeny after selfing of *lol1/+* plants, indicating that *LOL* function is required for development to the seedling stage. Indeed, analysis of the siliques from *lol-1/+* plants showed that approximately ¼ of the embryos arrested in early stages of development (647 wild type:194 mutant, Fig. 25A). The ratio between developed and arrested embryos is 3,3:1. Deviation from the 3:1 ratio, typical of zygotic lethality, is not significant (X^2 value = 1,675; 0.5<P<0.10). To understand at which developmental stage the mutant embryos are arrested, siliques from *lol-1/+* heterozygous plants were analyzed. In each silique, approximately 25% of all the embryos (the *lol-1/lol-1* homozygotes) were delayed or arrested in development compared to their siblings. The first difference to wild type development was observed at the octant stage, when mutant embryos consisted of only 3 or 4 cells, not even correctly distributed. Mutants were able to develop until the late globular stage, but failed to initiate cotyledon primordia: their cell division was abnormal and proliferation delayed. However, occasionally the suspensor consisted of multiple cell rows, indicating that cell division was not impaired (Fig. 25B-G').

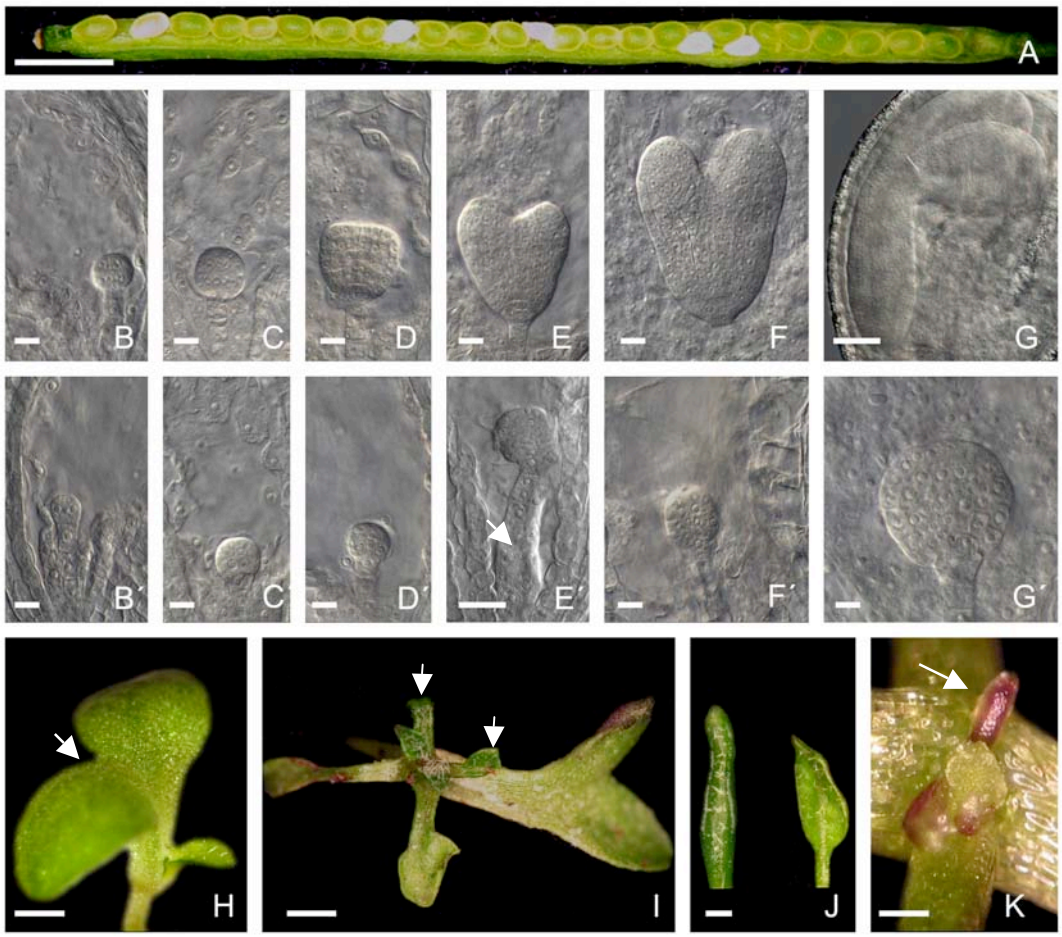


Fig.25 (A-G') Phenotype of *lol-1/lol-1* homozygous mutants. (A) An open, mature silique from a *lol-1/+* heterozygous plant. Wild type/heterozygous embryos are green, while *lol-1/lol-1* homozygous arrested embryos are white. (B-G) Stages of wild type embryo development: (B,C) early and late globular stage, (D) protodermal stage, (E) heartstage, (F) torpedo stage and (G) walking stick stage. (B'-G') Development of *lol-1/lol-1* mutants arrests before the initiation of cotyledon primordia. (E') Rarely, in *lol-1/lol-1* mutant embryos the suspensor contains more than 1 cell row (arrow). (H-J) In *lol-DN* mutants, cotyledons are partially fused. Leaves do not properly expand . (K) Occasionally, *lol-DN* mutant plants do not produce leaves but only small radialized filaments (indicated by the arrow). Scale bar: (A,H,I) 2 mm; (J,K) 300 μm; (B-F, B'-D', F'-G') 15 μm; (G) 100 um; (E') 30μm.

6.9 Overexpression of a dominant negative version of LOL (lol-DN)

The lethality of *lol-1/lol-1* homozygous embryos does not permit to further analyze *LOL* function in mature plants. Therefore, a *lol*-dominant negative version (*lol-DN*) was cloned, fusing the ETHYLENE-RESPONSE-FACTOR

ASSOCIATED AMPHIPHILIC REPRESSION DOMAIN (EAR) at the C-terminus of the LOL protein. The EAR domain is a 12 aa long sequence conserved in different repressors of transcription. The fusion of this short domain to a transcription factor acts as a dominant repressor: it suppresses expression of specific target genes, even in the presence of redundant transcription factors, with resultant dominant loss-of-function phenotypes (Hiratsu et. al, 2003). From previous experiments, *LOL* is supposed to encode a transcription factor: LOL protein is localized in the cell nucleus, as shown by a LOL-GFP fusion, and LOL fusion with the GR receptor is sensitive to Dex induction. The conversion of LOL into a transcription repressor would lead to the repression of LOL target genes, and therefore it could phenocopy a hypomorphic or amorphic mutant. The majority of the obtained *lol-DN* transgenic plants (n=60) appeared wild type. However, approximately 20% of the seedlings showed a partial fusion of the cotyledons, and produced small, not expanding, upwards curled leaves with thick blade margins. Some of the mutant plants, instead of aberrant leaves, produced filamentous structures without proximal-distal polarity (Fig. 25H-K). Most of the mutant plants did not reach the flowering stage, wilted and died. I analyzed by scanning electron microscopy the epidermal leaf structure. Adaxial cells of young wild type leaves are variable in size and shape, while abaxial cells are more elongated and strongly interdigitized. Cells on both surfaces of the narrow *lol-DN* mutant leaves resembled wild type adaxial cells, indicating a failure in establishing the abaxial cell fate (Fig. 26A-E).

The differences between *lol1/lol1* and *lol-DN* mutant phenotypes could be explained as follows: either *lol-DN* mutants are “leaky”, i.e. they allow the expression of some LOL target genes that permit embryo development, or the observed *lol-DN* mutant seedlings weakly express the transgene, as plants with a stronger phenotype died before the seedling stage. Indeed, the transformation frequency using the 35S::lol-DN transgene was lower than expected with similar transformation vectors. To avoid the possible deleterious effect of *lol-DN* embryonic expression, I constructed an ethanol

inducible version of *lol-DN* (Etoh::lol-DN). Ethanol induction of Etoh::lol-DN plants during the vegetative development did not produce any effect. A repeated ethanol treatment during the reproductive stage occasionally caused arrest in embryo development (experiment in progress). Thus, mis-expression of lol-DN during embryo development seems to phenocopy homozygosis for the *lol-1* allele.

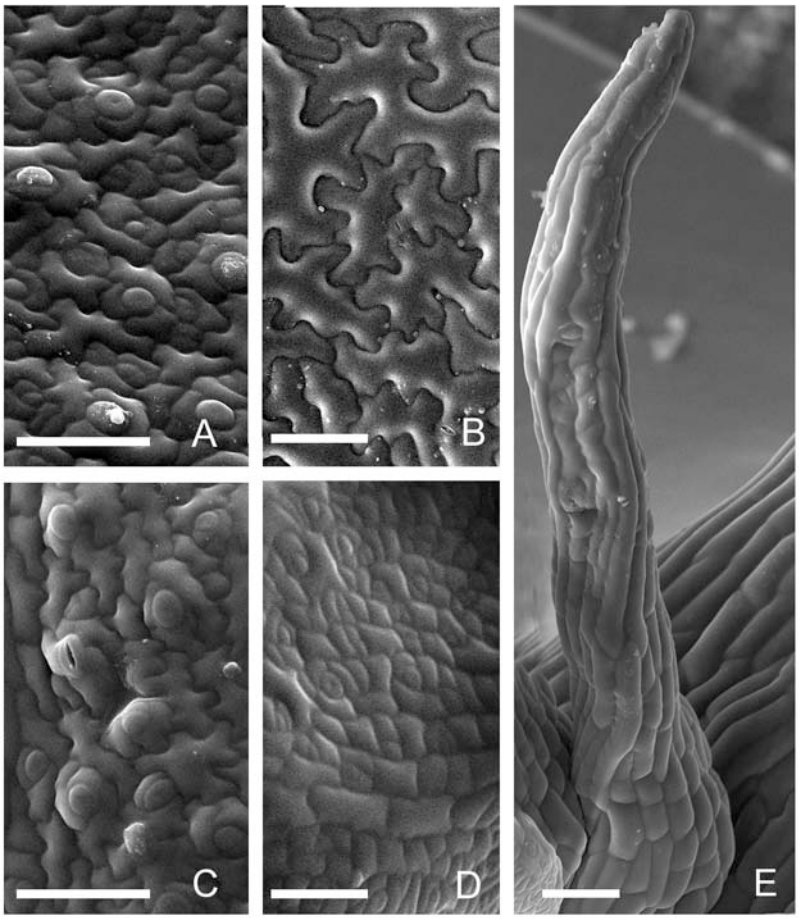


Fig. 26 (A-E) Scanning electron microscope analysis of *lol-DN* mutant plants. (A,B) Adaxial and abaxial sides of wild type young leaves. (C,D) Adaxial and abaxial sides of *lol-DN* young mutant leaves: cells of the abaxial side lost their identity. (E) A filamentous organ, possibly adaxialized. Scale bar: (A,C,E) 50 μm; (B,D) 100 μm; (E,F) 50 μm.

6.10 LOL interaction partners

To find putative partners of LOL, a yeast two-hybrid screen was performed by Rebecca Kloppenburg. The LOB domain of LOL was used as bait against a cDNA library from Arabidopsis meristem genes. The LOB domain fished out the B3-domain protein encoded by *At2g24700*, the PAZ/PIWI domain protein ARGONAUTE4 (AGO4), known to be involved in short interference RNAs (siRNAs) maturation and DNA methylation (Zilberman et al., 2003), and the GENERAL TRANSCRIPTION FACTOR GROUP E7 (GTE7), a BROMO domain protein, whose family is involved in control of gene expression through chromatin remodelling (Prymakowska-Bosak et al., 2003).

To confirm the results of the yeast two-hybrid screen, I tried to detect *in vivo* both in Arabidopsis and in tobacco plants the interactions between LOL and its putative partner, using the split-YFP method (Bracha-Drori et al., 2004). The proteins to be tested were alternatively fused to the C-terminal or the N-terminal domain of a split YFP protein, and co-expressed in plant cells. If the putative partners interact, a fluorescence signal is emitted from the reconstituted YFP protein. This system was initially tested on co-bombarded onion cells (biolistic method), but no positive results were obtained, except for a possible LOL-LOL homodimeric interaction. The fusion proteins were then co-expressed in Arabidopsis leaves, by infiltration with *Agrobacterium tumefaciens*, thus to avoid potential problems due to the biolistic technique. Indeed, the co-expressions of LOL-LOL, LOL-B3, and LOL-GTE7, but not of LOL-AGO4, generated positive signals. However, cell size in Arabidopsis leaves does not allow to easily distinguish a real positive signal from a possible background fluorescence. The co-expression experiments were then repeated in *Nicotiana tobaccum* leaves. Tobacco cells have bigger nuclei than Arabidopsis cells, thus facilitating the detection of a fluorescent signal. Except for a putative LOL-LOL homodimeric interaction, the other results were not confirmed (Fig. 27). As no positive controls to test the quality

of these experiments were available, the obtained results have to be confirmed, e.g. via in vitro co-immuno precipitation analysis.

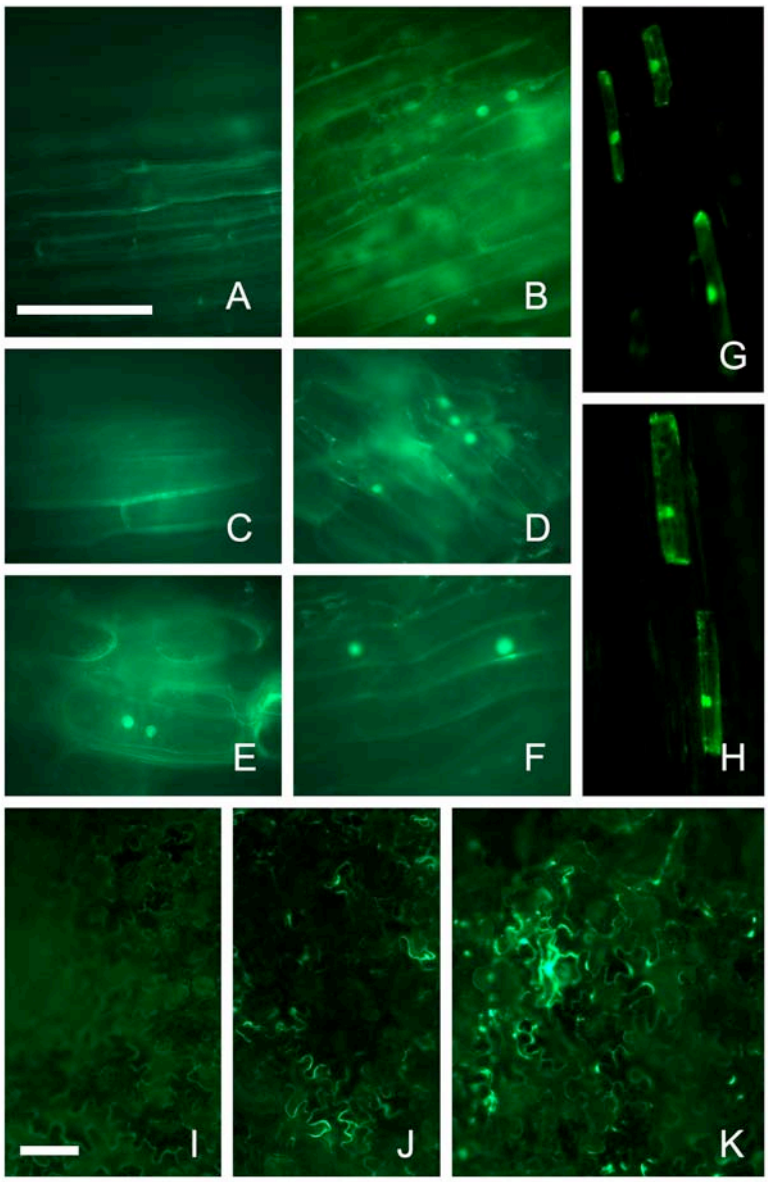


Fig.27**. Co-expression experiments results**. (A) No signal is present in Arabidopsis cells if the empty YFP-C and YFP-N vectors are co-expressed (negative control). (B) Co-expression of LOL-YFP-C and LOL-YFP-N promotes YFP fluorescence in cell nuclei. (C) Co-expression of LOL-YFP-N and AGO4-YFP-C, or vice versa, promotes no YFP fluorescence. (D, E, F) Interactions between LOL-YFP-N and B3-YFP-C / BROMO-YFP-C, or LOL-YFP-C and B3-YFP-N were positive. (G) Co-bombardment of LOL-YFP-C and LOL-YFP-N promotes a fluorescent signal also in onion cells. (H) This signal is comparable to the 35S::GFP nuclei fluorescence (positive control). All the co-expression experiments were repeated in tobacco leaves. (I) background fluorescence in not infiltrated leaves; (J) background fluorescence in leaves infiltrated with empty YFP-C and YFP-N vectors; (K) Fluorescence visible after co-expression of LOL-YFP-N and LOL-YFP-C. The other interactions visible in Arabidopsis leaves were not confirmed in tobacco. Scale bar: (A,F) 10 μm; (I,K) 50 μm.

6.11 STM, KNAT1 and PIN1 are LOL target genes

From previous results, genes belonging to the *KNOX* family seem to be up-regulated in *LOL* mis-expressing plants. The lobed leaf phenotype of *lol-D* mutants is also visible in plants overexpressing *STM* and *KNAT1*. The STM::GUS staining is enhanced in *lol-D* mutant plants. The aberrant flowers in 35S::LOL plants phenocopy flowers of plants induced for *STM* mis-expression (data not shown). Thus, *STM* and *KNAT1* are two candidate genes possibly regulated by *LOL*. Since the *pin1/lol-D* double mutant showed an enhanced organ fusion, expressions of genes involved in auxin transport and signalling could be indeed altered by *LOL* mis-expression. Thus, additional candidate genes possibly regulated by LOL may be members of the PIN family. *PIN3*, *PIN4* and *PIN7* have a primary role during embryo and root development. *PIN1* is known to be expressed throughout the plant, in young organ primordia, in the vasculature and in the L1 layer of the shoot meristem. Therefore, *STM*, *KNAT1* and *PIN1* RNA expression levels were initially analyzed in 35S::LOL-GR transgenic plants. 35S::LOL-GR plants were induced, and tissues for RNA extraction were separately collected from inflorescences or leaves of 16 days old plants at different time points: 0, 3, 9, 12 and 25 hours after induction (h.a.i.). Quantitative reverse transcriptase PCR (qRT-PCR) revealed a 2-fold upregulation of *STM* expression 25 h.a.i. in inflorescences, while RNA levels of *KNAT1* and *PIN1* remained virtually unchanged. Stronger effects of the *LOL* mis-expression were detected with RNA extracted from leaves. Three h.a.i., *PIN1* RNA levels rapidly decline, and only residual expression is detected until 25 h.a.i.. Both *KNAT1* and *STM* are weakly expressed in leaves before induction. However, their RNA levels increased 10 to 20 fold, respectively, within 25 h.a.i., reaching 40% of the *PIN1* pre-induction expression level (Fig. 28). Thus, LOL could be involved in auxin transport control by negatively influencing *PIN1* expression levels. *KNAT1* and *STM* upregulations, delayed in time if compared to *PIN1* downregulation, could be a consequence of defective auxin transport.

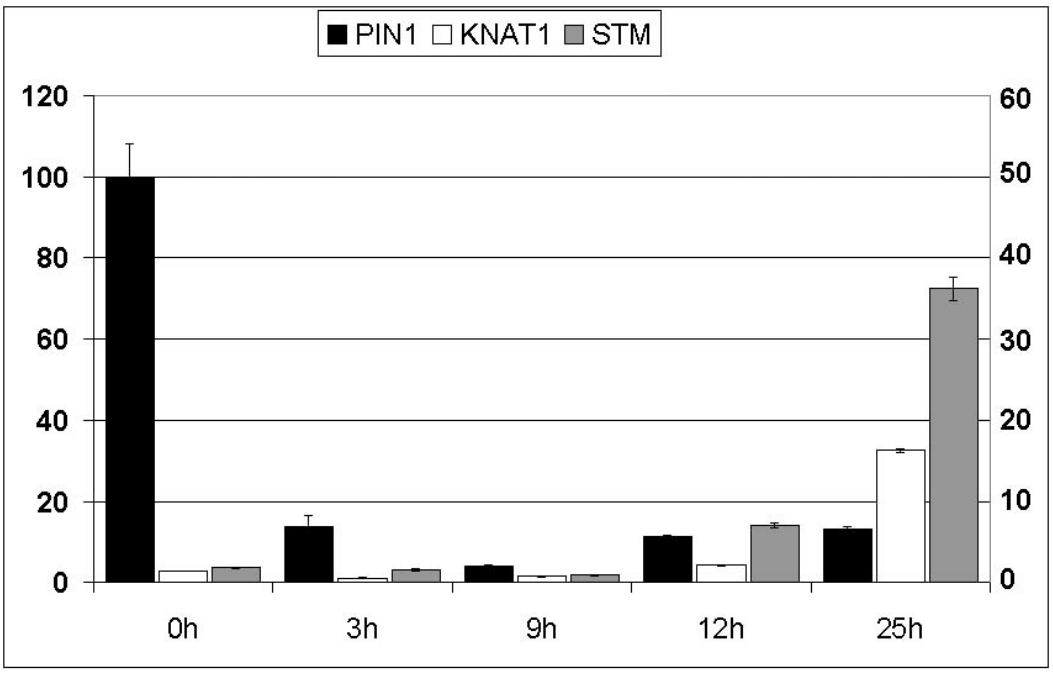


Fig. 28. ***STM, KNAT1* and *PIN1* induction ratios** (primary Y axis related to *PIN1* expression, secondary Y axis related to *KNAT1* and *STM* expressions). 0 hours after induction (h.a.i.), before Dex spraying, *STM* and *KNAT1* are weakly expressed in leaf tissues, while *PIN1* expression is detected. PIN1 expression level is arbitrarily set to 100%. Only 3 h.a.i., and until 25 h.a.i., *PIN1* expression remains strongly downregulated. 25 h.a.i., *KNAT1* and *STM* are upregulated in leaf tissue, respectively to approximately 20% and 40% of PIN1 pre-induction expression level.

6.12 Searching for additional LOL target genes

To isolate new LOL target genes, total RNA was extracted from 35S::LOL-GR leaves at 0 and 25 h.a.i, and changes in gene expression levels were analyzed via the Affymetrix micro array technology. The induction ratio thresholds for down- and up- regulated genes were set to 0.51 and 2.00, respectively. 187 genes and 290 genes were found down- or up-regulated after induction of LOL mis-expression. Genes that were annotated and possibly involved in plant development were sub-grouped depending on their role in the following table.

Name	Induction ratio	AT_number
Ribosome sub-units		
RPL35B	0,39	*At2g39390*
RPL90D	0,41	*At4g10450*
RPL23aB	0,45	*At3g55280*
RPL37A	0,45	*At1g15250*
NOP56	0,45	*At1g56110*
RPS17C	0,47	*At3g10610*
RPL34A	0,47	*At1g26880*
RPL36A	0,49	*At2g37600*
L36-rel	0,49	*At5g20180*
RPL26B	0,49	*At5g67510*
RPL35aB	0,50	*At1g41880*
RPL35aA	0,51	*At1g07070*
Ethylene pathway		
TINY	0,21	*At1g21910*
EBF2	0,23	*At5g25350*
ERS1	0,29	*At2g40940*
TINY-like	0,32	*At1g68840*
EFE	0,35	*At1g05010*
Eth-R	0,35	*At5g03730*
Eth-ind	0,37	*At3g04720*
ERF_B-2	0,38	*At3g16770*
ERS2	0,44	*At1g04310*
AP2-like	0,48	*At4g39780*
AP2-like	0,48	*At1g01250*
AP2-like	0,49	*At1g25560*
Root development		
AXR3	0,36	*At1g04250*
ARR16	0,46	*At2g40670*
Expansins		
EXP8	0,21	*At2g40610*
EXP1	0,30	*At1g69530*
EXP3	0,32	*At2g37640*
EXP11	0,35	*At1g20190*
Auxin efflux facilitators		
PIN7	0,15	*At1g23080*
PIN3	0,32	*At1g70940*
PIN4	0,38	*At2g01420*
NAC domain		
CUC2-like	0,08	*At5g39610*
NAP	0,28	*At1g69490*
NAM-like	0,45	*At5g63790*
NAM-like	0,46	*At2g33480*
NAM-like	0,47	*At2g17040*
Auxin storage		
IAR3	2,77	*At1g51780*

DFL1	20,80	*At5g54510*
Root development		
AIR3	2,62	*At2g04160*
Cell cycle		
CYCD3;1	2,30	*At4g03270*
CYCD3;2	2,93	*At4g34160*
LBD family		
LBD41	5,05	*At3g02550*
LBD42	2,01	*At1g68510*

Tab. 2. **Affymetrix micro array analysis.** Summary of genes down- and up- regulated after *LOL* mis-expression. Genes were grouped depending on their (putative) functions.

Many genes encoding **ribosomal sub-units proteins** were found to be down-regulated, including the *NUCLEOLAR PROTEIN56* (*NOP56)-like* enzyme, which promotes rRNA maturation in humans (Watkins et al., 2002). The reasons for such general down-regulation are unknown.

Interestingly, ***PIN7***, ***PIN4***, and ***PIN3*** expressions were downregulated. PIN4 and PIN3 mainly control auxin transport during root development. PIN7 is involved in the establishment of embryo polarity (Friml et al., 2003). The downregulation of 50% of the PIN family members, including PIN1 (detected in the qRT-PCR analysis), indicates that *LOL* mis-expression could alter the auxin distribution pattern.

EXP1, ***EXP3***, ***EXP8*** *and* ***EXP11*** belong to the *EXPANSIN* (*EXP*) family. *EXP* genes promote cell wall expansion after auxin-driven induction. *EXP1* is expressed in leaves, *EXP3* in roots, leaves and internodes, *EXP8* in embryos (Cosgrove et al., 2002; Li et al., 2002; Li et al., 2003). In line with a possible disturbed auxin transport in *LOL* mis-expressing plants, six other genes, encoding putative auxin inducible proteins with unknown function, are down-regulated.

Many genes involved in the **ethylene pathway** are also down-regulated, including the *ETHYLENE FORMING ENZYME* (*EFE*), which is a key factor in ethylene biosynthesis (Gomez-Lim et al., 1993). Three ethylene receptors

(*ETHYLENE RESPONSE SENSOR1* (*ERS1), ETHYLENE RESPONSE SENSOR2 (ERS2)* and *ETHYLENE RECEPTOR* (*Eth-R)*), two proteins of the ethylene response pathway (*EIN3-BINDING F-BOX2* (*EBF2*) and *ETHYLENE RESPONSE FACTOR (ERF_B-2*)) and five not yet annotated AP2 domain proteins (also ethylene inducible) are also downregulated. Ethylene and auxin signalling pathways are convergent, thus auxin transport alteration in *LOL* mis-expressing plants could influence as well the expression of ethylene response factors (Li et al., 2004).

Two genes specifically expressed in **roots** are down-regulated: *ARABIDOPSIS RESPONSE REGULATOR16* (*ARR16)* encodes a protein phosphatase 2C (PP2C), involved in root vasculature formation; *AUXIN RESISTANCE3 (AXR3/IAA17)* encodes an auxin inducible protein that controls root hair initiation. Another gene expressed in roots is up-regulated: *AUXIN INDUCED ROOTS3 (AIR3)* encodes for a subtilisin-like endoprotease involved in lateral root development (Neuteboom et al., 1999). Mis-regulation of these genes could influence the root hair and lateral root development in *LOL* mis-expressing plants.

Five members of the **NAM family** are down-regulated, but none of them is yet characterized. One of them, *CUC2-like*, is strongly down-regulated. *CUC1*, *CUC2* and *CUC3* expression levels resulted to be unaltered.

LBD41 and ***LBD42*** are up-regulated. Both of them belong to the *LBD* Class II proteins, which lack the putative coiled coil domain at the C-terminal end, present in LBD Class I proteins. No *LBD* Class II gene function is known yet. From RT-PCR analysis, *LBD41* appears to be ubiquitously expressed, particularly in rosette leaves and roots (Shuai et al., 2002).

Two genes controlling cell cycle regulation are up-regulated: ***CYCD3;1*** and ***CYCD3;2***. *CYCD3;1* is involved in cell division regulation and *CYCD3;2* is not yet characterized (Riou-Khamlichi et al., 1999; Dewitte et al., 2003). Their upregulation could promote the two additional endoreduplication cycles detected in trichomes of plants mis-expressing *LOL*.

*DWARF IN LIGHT1 (**DFL1**)* was identified as a dominant mutation displaying shorter hypocotyls in light grown plants when compared to wild type seedlings. This protein is similar to the *GH3* auxin inducible gene from pea (Nakazawa et al., 2001). *dfl1-D* plants showed resistance to exogenous auxin treatment, inhibition of lateral root, shoot and hypocotyl growth. For the similarities between their dominant mutant phenotypes, *DFL1* and *LOL* could share some functions required for a normal plant development.

In addition to PIN1 down-regulation, *IAA-ALANINE RESISTANT3 (**IAR3**)* up-regulation in *LOL* mis-expressing plants could play a negative role in auxin distribution. *IAR3* promotes amide-linked conjugates of auxin, i.e. it controls the formation of putative storage or inactivation forms of auxin. Up-regulation of *IAR3* could lead to excessive auxin sequestration and consequent downregulation of auxin inducible genes.

I performed qRT-PCR and RT-PCR analyses to confirm the induction ratios of some of the *LOL* target genes identified via the Affymetrix micro array analysis. Induction ratios were calculated on the different RNA expression levels measured at 0 and 25 h.a.i. The micro array results were confirmed. The following table contains the genes assayed by at least two different experiments.

	qRT-PCR induction ratio (fold)	*Affymetrix microarray induction ratio*	*RT-PCR amplification trend*
PIN1	-7,7		down
PIN3	-8,9	0,32	
PIN4	-7,6	0,38	
PIN7	-18,1	0,15	
EXP1	-2,9	0,30	
EXP3	-4,1	0,32	
ARR16	-3,1	0,46	

AXR3	-13,1	0,36	Down
AIR3	2,1	2,62	Up
CUC2-like	-60,1	0,08	
LBD41	17	5,05	
LBD42	6,9	2,01	
CYCD3;1	4,6	2,30	Up
CYCD3;2	9,2	2,93	Up
DFL1	15,6	20,80	
KNAT1	12,6		Up
STM	20,2		Up

Tab. 3. **Summary of RNA quantification analyses performed on putative *LOL* target genes after induction of *LOL* mis-expression**. In grey background are the downregulated genes, in white background the upregulated ones.

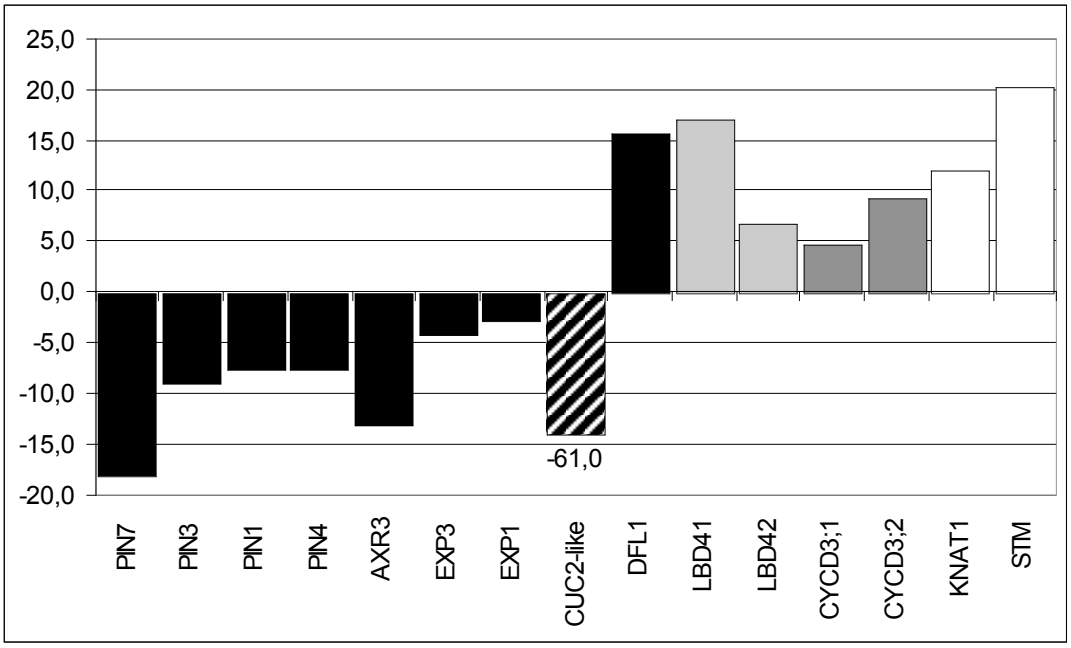


Fig. 29 **RNA induction ratios of putative LOL target genes measured via qRT-PCR**. Auxin inducible genes are in black. Two not yet characterized *LBD41* and *LBD42* genes, as well as two *CYCD3* genes are up-regulated. The homeobox genes *KNAT1* and *STM* are up-regulated. A not yet characterized member of the *CUC* gene family, *CUC2-like*, is strongly down-regulated.

7 Discussion

7.1 The lol-D mutant phenotype is caused by enhancement of LOL expression

The *lollo* dominant mutant (*lol-D*) was isolated in an activation tagging mutagenesis (Schneider et al., 2005). The *lol-D* mutant phenotype is due to the insertion of a dSpm-Act transposon in the putative promoter regions of two genes belonging to the *LATERAL ORGAN BOUNDARY* (*LBD*) family: *LBD30,* later named *LOLLO (LOL),* and *LBD31*. The dSpm-Act transposon, which carries four copies of the 35SCaMV enhancer element, could enhance both *LBD31* and *LOL* expression. However, Northern blot, RT-PCR and RNA in situ analyses showed that only *LOL* is ectopically expressed in *lol-D* mutants.

It is known that DNA super-coiling permits enhancer action upon a promoter that is distant even more than 2.500 bp (Liu et al., 2001). Thus, both genes could be activated by the 35S enhancers. However, insulator sequences, which protect genes from the effects of adjacent enhancers or silencers, could limit the interaction of the 35S enhancers with their flanking genes (Chung et al., 1993). As the Arabidopsis genome is densely packed with genes, a high number of insulator sequences could be required to avoid unwanted activity of enhancers on close-by localized genes (Bevan et al., 1998). To date, the only known insulator sequence in plants is 5'-GAATATATATATTC-3' (Susheng, 2004), which is not present in the shared promoter region of *LOL* and *LBD31*. Alternatively, the 35S enhancers could activate only certain promoters because of “promoter preference”, a phenomenon until now known only from animal systems (Ohtsuki et al., 1998).

7.2 KNOX gene expression is upregulated in lol-D mutant plants

lol-D mutant plants were isolated because of their leaf mutant phenotype. This phenotype is not visible during early stages of development, and the first 6-10 leaves appear wild type. Mutant phenotypes of *lol-D* plants are probably caused by an accumulation effect: also the flower mutant phenotype becomes visible only after the production of a certain number of wild type flowers. 10-20 days after germination, *lol-D* plants produce leaves that are lobed, shorter and have a higher blade-to-petiole ratio in comparison to wild type. Lobed or serrated leaves are also produced by plants overexpressing *KNOTTED1-LIKE HOMEOBOX* genes (*KNOX*),or plants that are mutant for *ASYMMETRIC LEAVES1* or *ASYMMETRIC LEAVES2* (*AS1*, *AS2*). *KNOX* genes, like *SHOOTMERISTEMLESS* (*STM*) or *KNOTTED1-LIKE in ARABIDOPSIS THALIANA* (*KNAT1*), are involved in meristem maintenance, while *AS1* and *AS2* are required for the symmetric development of lateral organs. Ectopic expression of *KNAT1* or loss of *as1* or *as2* promotes formation of lobed leaves and ectopic meristems (Ori et al., 2000). Thus, mis-expression of *KNAT1* confers (partial) meristem identity to organ primordia.
Interestingly, the phenotype of lobed leaves in transgenic plants over-expressing *STM* or *KNAT1* can be suppressed by exogenous application of the phytohormone gibberellic acid (GA) (Hay et al., 2002). GA biosynthesis is normally active in organ primordia and not in meristems, where *STM* down-regulates the expression of GA20ox, the key enzyme for GA production. Mutant plants ectopically expressing *STM* phenocopy plants impaired in GA production or plants that are GA insensitive: both have short leaves with short petioles. Thus, the down-regulation of GA activity could be one of the causes for the *lol-D* mutant phenotype. I tried to rescue the *lol-D* leaf phenotype by ectopic application of GA3, an active form of GA, but without

success. This result leads to two possibilities: the smaller organs in *lol-D* plants are not a consequence of defects in GA biosynthesis, or *lol-D* plants are insensitive to GA treatment (e.g., defective in GA signal transduction).

The supposed effects of *LOL* overexpression on *KNAT1* and *STM* were quantified in induced 35S::LOL-GR plants. In inflorescences, strong variations in *KNOX* gene expression levels before and after *LOL* mis-expression were not detected, possibly because of the high basal expression of *KNOX* genes in flower meristems. *STM* and *KNAT1* were found to be up-regulated in leaves 10 to 20 fold compared to wild type plants. Thus, *KNOX* gene up-regulation in *lol-D* mutants could promote the formation of lobed leaves. In contrast to 35S::KNAT1/STM plants, no ectopic meristems are formed in *lol-D* mutants. It is possible that high-level expressions of *KNAT1* and/or *STM* are required for ectopic meristem induction. In *lol-D* mutants, *KNAT1* and *STM* are probably expressed at too low levels. Indeed, in *lol-D*/STM::GUS plants, no GUS staining was detected in leaves, and GUS activity was enhanced only in tissues were *STM* is normally expressed, e.g. in the medial ridge of the carpel and possibly in the placenta tissue.

Enhanced expression of *STM* could be also the cause of the flower mutant phenotypes visible in all the transgenic plants mis-expressing *LOL*. Pistil bending, extreme in uni-valved pistils of *lol-D* plants, could be due to asymmetric development of the two carpels. 35S::*LOL* transgenic plants, which ectopically express *LOL* from embryo development onwards, do not produce wild type flowers, but only defective flowers that lack both sepals and stamens. Interestingly, petal/stamen mis-development is visible also in plants where *STM* overexpression is induced in later stages of plant development (data not shown). Thus, enhanced *STM* expression in consequence of *LOL* mis-expression could not allow development of differentiated tissue in flowers, but in contrast promote meristem cell identity.

7.3 LOL is expressed in organ anlagen and in boundaries between young organ primordia and meristems

LOL expression was detected via RNA *in situ* hybridization. The 43 genes belonging to the *LBD* gene family share a 25% to 82% identity throughout the LOB domain (Shuai et al., 2002). Thus, RNA *in situ* analysis with a *LOL* probe containing the *LOB* sequence could have resulted in detection of unspecific signals. However, this was not the case, as the in situ hybridization signals obtained with full-length LOL RNA antisense probe and with a shorter version lacking the LOB domain have comparable patterns. *LOL* is expressed in primordia anlagen, in young organ primordia and in their boundaries with the meristem. *LOL* could be involved in the initiation of the primordium or in the establishment of its adaxial/abaxial identity, as *LOL* seems to be mainly expressed on the adaxial side of primordia. Later in development, *LOL* could be required to maintain a boundary between meristem and organ primordia. In flowers, a similar expression pattern is visible between young sepal primordia and floral meristems. *LOL* is not expressed in old organ primordia. *LOL* could regulate the expression patterns of genes belonging to the PIN family in young organ primordia, were auxin transport is required for a regular development of a symmetrical leaf blade. Interestingly, *as1* and *as2* mutants, which phenotypically resemble *lol-D* plants, were recently shown to be affected in auxin distribution (Zgurski et al., 2005).

Initially from young primordia, and then from their boundaries with the meristem, *LOL* could indirectly promote *KNOX* gene expression. Alternatively, up-regulation of *STM* and *KNAT1* in *lol-D* plants could be a consequence of altered auxin transport. Evidences for this connection between auxin transport and expression regulation of *KNOX* genes come from researches on other plant models. In maize, for example, *SEMAPHORE1* (*SEM1*) is required for the negative regulation of a subset of

maize *KNOX* genes (Scanlon et al., 2002). In *sem1* mutant plants, ectopic expression of *KNOX* genes in leaf and endosperm tissue was detected and auxin transport was found to be significantly reduced. Thus, possibly also a reduction in auxin transport, due to the down-regulation of several *PIN* genes, could alter the expressions of *KNOX* genes in Arabidopsis.

7.4 lol-D mutants are disturbed in auxin transport or sensing

lol-D inflorescence meristems arrest approximately five weeks after germination. The last flowers produced lack the adaxial sepal, or are just filamentous organs without proximal-distal polarity. The shape of the terminated *lol-D* meristem is reminiscent of the *pin1* mutant inflorescence. *PIN1* encodes an auxin efflux facilitator, and *pin1* mutant plants were shown to have a reduced auxin transport (Okada et al., 1991). PIN1 organizes auxin peaks in the peripheral zone of the meristem, which promotes organ primordia formation (Reinhardt et al., 2003). In *pin1* mutants, the inflorescence appears “naked” as no organ primordia are initiated and no flowers are produced. Only in later stages of development, some defective flowers are formed on the flanks of the shoot apex. Before transition to the reproductive stage, the SAM of *pin1* mutants is still able to initiate leaves, but they are often fused. Ectopic application of auxin on *pin1* inflorescences promotes organ primordia formation. However, no such effect was visible on *lol-D* arrested meristems. Therefore, *lol-D* mutant meristems appear auxin insensitive. *pin1/lol-D* double mutants show an enhanced *pin1* phenotype, with all rosette leaves fused in one strongly lobed organ. Thus, enhanced expression of *LOL* seems to negatively control auxin transport or sensitivity to auxin. I analyzed via qRT-PCR and RT-PCR if *PIN1* expression is down-regulated in plants mis-expressing *LOL*. *PIN1* RNA levels were found to be seven-fold down-regulated within 3 hours after induced *LOL* overexpression.

Interestingly, also the expression levels of other *PIN* genes, *PIN3*, *PIN4* and *PIN7* were down-regulated 4 to 18 fold. In consequence, auxin transport in *LOL* mis-expressing plants could be severely affected. To detect *in planta* if expression/localization of PIN proteins is altered in *lol* loss of function mutants, *lol-1* homozigous plants were crossed with plants transgenic for PINs reporter genes, expressing either GUS or GFP fused with PIN1, PIN3, PIN4 and PIN7. This experiment is still in progress. To directly assay if auxin distribution is altered in *LOL* mis-expressing plants, auxin signals could be tracked *in vivo* using radioactively labelled auxin (Rashotte et al., 2003).
Although shoot apices in *lol-D* mutants are arrested in growth, they still express *STM* (data not shown), indicating that they kept their meristem identity. RNA in situ hybridizations with a *CLV3* antisense probe revealed that stem cells are still present in shoot apices of *lol-D* mutants, and that they form even larger populations than in wild type. *WUS* expression should be present in *lol-D* mutant background, because stem cell identity is still promoted. Indeed, *wus/lol-D* double mutant plants show an additive phenotype, thus indicating that *WUS* expression is still required in *lol-D* mutant plants. Thus, *lol-D* meristem arrest does not seem to be caused by alterations in the *CLV3/WUS* feedback loop. Possibly, *lol-D* meristems terminate because of a disturbed auxin transport that does not permit organ initiation.

7.5 35S::LOL plants strongly phenocopy lol-D mutants

To test if the *lol-D* mutant phenotype is due only to the enhanced expression of *LOL*, I analyzed the phenotype of 35S::*LOL* and 35S::*LBD31* plants. Transgenic plants for *LBD31* did not show a mutant phenotype, confirming that *lol-D* phenotypes are due to *LOL* mis-expression only. Consistent with this, 35S::*LOL* plants phenocopy *lol-D* mutants. Transformation rates of plants infiltrated with the 35S::*LOL* vector were low. Possibly, *LOL* over-

expression is lethal for embryo development or interferes with seed germination. The cumulative down-regulation of *PIN1, PIN3, PIN4 and PIN7* measured via qRT-PCr in *LOL* mis-expressing plants, could cause altered auxin distribution during embryo development, and thus interfere with early pattern formation. Indeed, *PIN7* is known to be essential in the establishment of embryo polarity (Friml, 2003; Friml et al., 2003), and *pin1pin3pin4pin7* quadruple mutants show an arrest of embryo development (Blilou et al., 2005).

7.6 A role for LOL in cell cycle regulation?

To avoid the deleterious effects of continuous *LOL* overexpression, I constructed the inducible 35S::LOL-GR vector. Induced 35S::LOL-GR plants phenocopied 35S::*LOL* plants, but prolonged Dex inductions on young transgenic seedlings caused plant death. Plant lethality in consequence of gene mis-expression is a rare event, which was previously described, e.g. in the case of altered expression of cell cycle regulators. Overexpression of ICK, a protein that inhibits cyclin-dependent-kinases (CDKs), causes arrest of cell growth and consequently plant death (Schnittger et al., 2003). Mis-regulation of the cell cycle could cause some of the novel mutant phenotypes observed in induced 35S::LOL-GR plants. Measurements of nuclear DNA content showed that the enlarged trichomes formed in *LOL* mis-expressing plants underwent two additional endoreduplication cycles, thus reaching a DNA copy number of 128 (128C). In wild type plants, trichomes switch from normal mitosis to undergo four rounds of endoreduplication to reach a DNA copy number of 32 (32C). Several genes are known to act as negative regulators of endoreduplication. Trichomes in plants mutant for *KAKTUS* (*KAK*) are 64C. However, *kak* and all the other mutants known to affect endoreduplication in trichomes show also aberrations in cell branching, a

mutant phenotype which was never observed in 35S::LOL-GR induced plants (El Refy et al., 2003).

Affymetrix micro array analysis showed that *LOL* mis-expression causes the upregulation of two D-type cyclin genes, *CYCD3;1* and *CYCD3;2*. Cyclin-dependent kinases (CDKs) and their cyclin partners (CYC) control cell cycle progression by regulating the transitions between G1 to S and G2 to M phases. Overexpression of *CYCD3;1* in trichome cells have been shown to promote DNA replication (up to 80C), but also cell divisions, which were not detected in trichomes of induced 35S::LOL-GR plants (Schnittger et al., 2002). *CYCD3;2* is not yet known to have a function in trichome development (Swaminathan et al., 2000). Up-regulation of both *CYCD3;1* and *CYCD3;2* in *LOL* mis-expressing plants may be responsible for the two additional endoreduplication cycles and thus for the observed trichome mutant phenotype.

Interestingly, the expression of *CYCD3* genes is enhanced by up-regulation of *KNOX* genes. *KNOX* gene function is known to be mediated by cytokinin (CK), a phytohormone involved in control of cell division and meristem function genes (Jasinski et al., 2005). High levels of CK are known to promote *CYCD3* expressions (Riou-Khamlichi et al., 1999). Therefore, *STM* and *KNAT1*, which were shown to be up-regulated in *LOL* mis-expressing plants, could promote trichome endoreduplications through the CK pathway.

Another evidence that *LOL* mis-expression may interfere with cell cycle regulation is the abnormal cell proliferation observed at the sites of lateral root initiation in the mutant plants. Thus, as in wild type plants *LOL* is also expressed in boundaries, where cells have a different division rate in comparison to meristem and organ primordia cells, *LOL* could regulate/repress cell divisions at these sites (Dumais and Kwiatkowska, 2002; Kwiatkowska, 2004).

7.7 lol loss of function causes embryo lethality

Approximately 25% of the progeny from the selfing of *lol-1/+* heterozygote plants is arrested in development during early embryo stages. Occasionally, *lol-1/lol-1* homozygous mutants are already affected at the 8-cell stage, and planes of cell divisions are not organized as in their wild type siblings. Most of the *lol-1/lol-1* embryos reached the octant stage, but failed to proceed to the heart stage. Cell division is active in mutant embryos, which occasionally produce suspensors with multiple rows of cells, but cotyledon primordia are not initiated. In wild type embryos, vasculature, meristem and proto-dermal tissues are initiated at the late globular stage. In *lol-1/lol-1* mutant embryos, these types of cells are not recognized, indicating that *LOL* is required for patterning of the early embryo. *LOL* expression was not detected in embryos via RNA in situ hybridization, but via RT-PCR and also in the public microarray expression analysis database (AtGENEXPRESS). The failure to detect *LOL* RNA by in situ hybridization could indicate that *LOL* is expressed at low levels in embryos. Several Arabidopsis mutants show embryo arrest development: approximately 250 “EMB” genes, required for normal embryo development, were isolated until now, and their functions involve every aspect of cell development (Tzafrir et al., 2004).

Interestingly, quadruple mutations for *pin1pin3pin4pin7* or down-regulated expressions of *CYCD* genes are embryo lethal. *LOL* was shown to be possibly required for the regulation of *PIN* and *CYCD* gene expression. Therefore, the arrested development in *lol-1* homozygous embryos could be explained with mis-expression of genes belonging to the *PIN* or the *CYCD* family.

7.8 Is LOL involved in the establishment of adaxial / abaxial leaf polarity?

The embryo arrest in *lol-1/lol-1* homozygous mutants does not permit to analyze *LOL* function at later stages of development. Therefore, I engineered a dominant negative version of LOL (LOL-EAR). Chimeric repressors that include the EAR motif dominantly suppress the expression of their target genes (Hiratsu et al., 2003). Plants overexpressing the LOL-EAR fusion (*lol-DN*) occasionally showed cotyledon fusion, a mutant phenotype that may confirm *LOL* involvement in the formation of boundaries. The adaxialization of the abaxial side of leaves in *lol-DN* mutants implies that some of the target genes of *LOL*, which are repressed in *lol-DN,* are required for abaxial cell identity. Genes belonging to the *KANADI* (*KAN*) and *YABBY* (*YAB*) family control cell identity in the abaxial side of leaves (Siegfried et al., 1999; Eshed et al., 2001). *KAN* and *YAB* genes act antagonistically to genes belonging to the *HD-ZIPIII* family, which in contrast are required to establish the adaxial cell identity (McConnell et al., 2001). Plants mutant for *KAN* or *YAB* genes produce adaxialized lateral organs, due to the consequent expanded expression of *HD-ZIPIII* genes. Thus, *LOL* either promotes *KAN* and *YAB* gene expressions, or, through alternative and yet unknown pathways, inhibits the expression of *HD-ZIPIII* genes in the abaxial side of leaves. *LOL* seems to be normally expressed on the adaxial side of organ primordia. Thus, if the adaxializing effect seen in *lol-DN* mutants is not an artefact, *LOL* should act non-cell-autonomously to promote abaxial cell identity. *LOL* could act, for example, through the regulation of auxin transport. Interestingly, double mutant plants for *auxin response factor3/auxin response factor4* (*arf3/arf4*) also produce adaxialized mutant leaves (Pekker et al., 2005). ARF proteins are transcription factors that mediate responses to auxin, i.e. they activate downstream target genes in consequence of auxin signalling. Expression

levels of *ARF3 and ARF4* should be assayed in *lol-DN* mutant plants, thus to understand if loss of abaxial cell identity in the mutant leaves is connected to an altered auxin transport.

7.9 LOL forms homodimeric complexes

The yeast two-hybrid screen performed by Rebecca Kloppenburg isolated putative interacting partners of the LOL protein. A B3-domain protein, the GENERAL TRANSCRIPTION FACTOR E7 (GTE7) bromo-domain protein and ARGONAUTE4 (AGO4) were identified. The B3-domain, a DNA-binding motif unique to plants, is characteristic of several transcriptional factors, which are often involved in embryo development. LEAFY COTYLEDON2 (LEC2), for example, is a B3-domain protein required for suspensor maintenance, specification of cotyledon identity, and progress through the full embryonic development (Stone et al., 2001). The function of the B3-domain protein isolated in our two-hybrid screen is not yet known, but its interaction with LOL could be required in early stages of embryo development.

The bromo-domain is found in many chromatin-associated proteins. While GTE7 has not been characterized yet, the related protein GTE6 was recently shown to promote AS1 expression, and thus to be involved in initiation of leaf symmetry (Chua et al., 2005).

AGO4 directs chromatin modifications through promotion of maturation and targeting of long siRNAs. *ago4-1* mutant plants show decreased DNA and histone methylation (Zilberman et al., 2003). Known targets of AGO4 activity are *FLOWERING TIME LOCUS A* (*FWA*), one of the several genes promoting flowering in Arabidopsis (Koornneef et al., 1991), and *SUPERMAN* (*SUP*), which control cell proliferation in floral meristems (Sakai et al., 1995). The scarcity of information on partners and on additional targets of AGO4 does not permit to speculate on a possible function of the putative LOL-AGO4 complex.

It is tempting to argue that LOL regulates target gene expression by controlling chromatin structure via interactions with AGO4, GTE7 and the B3-domain transcription factors. I tried to assay the interactions between LOL and its putative partners *in vivo* by using the split-YFP system. The obtained results evidenced the possible formation of a LOL-LOL homodimeric complex. Interactions between LOL, AGO4, B3-domain and GTE7 proteins have to be confirmed by in vitro pGST-pull down analyses.

7.10 Putative LOL targets

Affymetrix micro array analysis was performed to identify LOL target genes. Interestingly, four genes of the *PIN* family are down-regulated in *LOL* mis-expressing plants: *PIN1*, *PIN3*, *PIN4* and *PIN7*. PIN1 has a pivotal role in auxin transport in Arabidopsis: auxin transport activity in *pin1* mutants is decreased to approximately 10% in comparison to wild type plants (Okada et al., 1991). Normal level of auxin transport activity is required in the inflorescence for flower primordia initiation and in general for cell expansion and differentiation (Reinhardt et al., 2003). *PIN3* is mainly required for root development. PIN3 positioning in the cell membrane is controlled by gravity sensing: PIN3 redirects auxin flux to control asymmetric root growth (Friml et al., 2002a). *pin4* mutants are defective in the establishment and maintenance of auxin gradients, fail to canalize externally applied auxin, and display various patterning defects in both embryonic and seedling roots (Friml et al., 2002b). PIN7 establishes the apical-basal auxin activity gradient in early stages of embryo development, thus controlling the specification of apical embryo structures (Friml et al., 2003). Therefore, down-regulation of *PIN* gene expression could explain many of the mutant phenotypes described in *LOL* mis-expressing plants.

Several genes involved in the ethylene signal transduction pathway are down-regulated, as well as many AP2 domain genes, whose expression is promoted by ethylene. Interestingly, ethylene and auxin pathways are known to be interdependent and to control common target genes. Ethylene inducible genes, for example, can compensate the down-regulation of auxin inducible genes, thus to maintain a correct developmental plan (Gomez-Lim et al., 1993). In 35S::LOL-GR induced plants, this compensation could be absent because even a key factor in ethylene biosynthesis, the *ETHLENE FORMING FACTOR* enzyme (*EFE*), seems to be down-regulated.

The reduction in organ size observed in *LOL* overexpressing plants could be due to the down-regulation of genes belonging to the *EXPANSIN* (*EXP*) family. *EXP* genes promote cell expansion by controlling the relocation of cellulose fibers in the cell walls. Auxin is known to play a role in the cell wall expansion: the "acid growth" theory proposes that auxin-induced cell wall acidification is an essential component of cell expansion, as reviewed in Rayle and Cleland, 1992. *EXP* genes are differentially regulated by environmental and hormonal signals, and hormonal regulatory elements have been found in their promoter regions (Lee et al., 2001). Down-regulation of *PIN* expression in *LOL* mis-expressing plants could result in altered auxin accumulation in young organs, and therefore altered expression of auxin-regulated genes. Thus, the down-regulation of the four expansins *EXP1, EXP3*, *EXP8* and *EXP11* could be a consequence of auxin mis-distribution.

Several genes encoding NAC domain proteins were down-regulated. Amongst them is the CUC2-like gene, with yet an unknown function. Because of its sequence similarity to *CUC2* and its strong down-regulation in *LOL* mis-expressing plants, *CUC2-like* could be an object for further researches.

To verify the consistency of these results obtained by micro array analysis, *in planta* experiments have to be assayed. Many of these putative *LOL* target genes are auxin inducible. If *LOL* mis-expression indeed down-regulates

many components of the *PIN* family, overexpression of *PIN* genes in *lol-D* plants could partly rescue the mutant phenotype. This work is in progress.

7.11 Conclusion

The putative transcription factor *LOL*, expressed in primordia anlagen and in the boundaries between meristem and young organ primordia, affects the expression of genes belonging to *KNOX* and *PIN* families. *STM* and *KNAT1* were found to be up-regulated, while four PIN proteins, PIN1, PIN3, PIN4 and PIN7 were down-regulated in *LOL* overexpressing plants. *LOL*, through the regulation of *KNOX* gene expression, could control the cell cycle of boundary cells. *LOL* could regulate auxin transport in young organ primordia via the down-regulation of *PIN* expressions. Many of the mutant phenotypes described in this research in *LOL* mis-expressing plants as well as in *lol-1* homozygous mutants could be explained with a disturbed auxin distribution or sensing. To understand if indeed *LOL* is involved in the control of auxin transport, *in planta* auxin transport assays have to be performed. Interestingly, the until now isolated putative interaction partners of LOL promote DNA and histone methylation, thus indicating that possibly LOL controls its target genes by acting on chromatin remodelling.

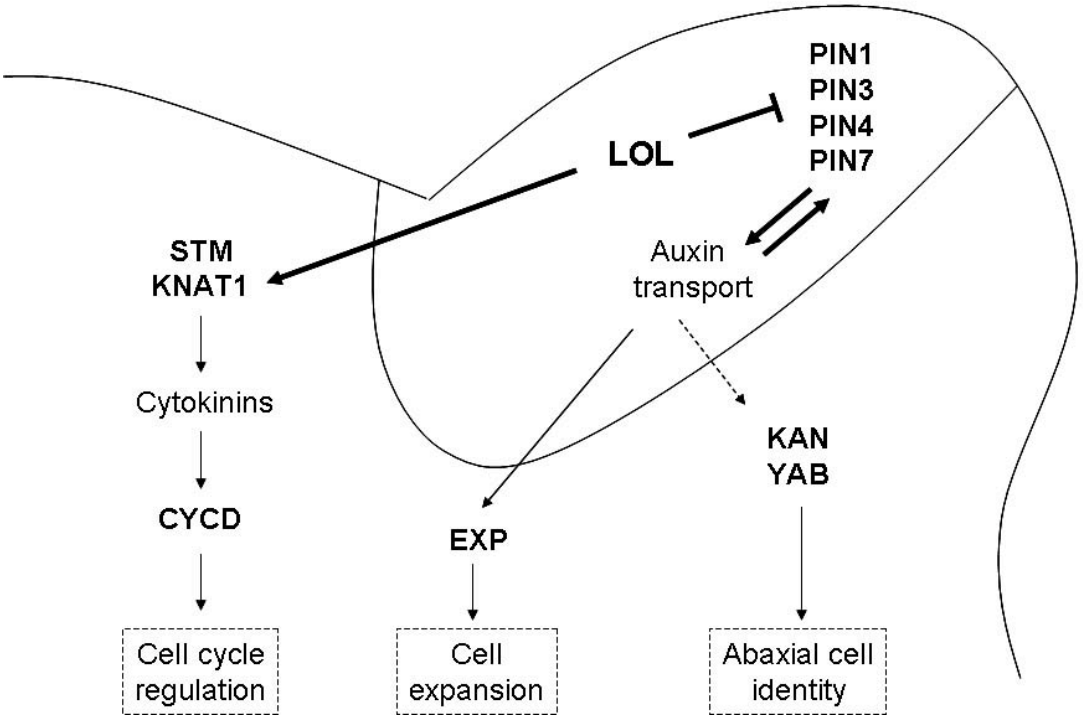


Fig.30. LOL is expressed in the adaxial side of young organ primordia and in the boundaries between meristems and organ primordia. LOL could control cell cycle regulation through the up-regulation of *KNOX* genes. LOL may control auxin transport in young organ primordia, therefore regulating cell expansion and cell identity in lateral organs.

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APPENDIX A

The alignment between the *CLV3* enhancer sequences from *Arabidopsis thaliana* and *Brassica oleracea* shows a 61,4 % of identity.

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At_CLV3_Enh    (1) CCTAATCTCTTGTTGCTTTAAATTATTTCATATTGTAAATTACTTTCTGC
Bo_CLV3_Enh    (1) GCTAAGGACT-GTCCCTTCAGGACCTGACCCTTTGCACCATCATGTGAAC

At_CLV3_Enh   (51) TTTATCGGTTTTACCATTTCGGGAGTCTTTTTTGTGTGCAATCTGTTTCG
Bo_CLV3_Enh   (50) CCCC-CAAGAAAGCCACGAACCGACTCTCATATCCCTTAAACCTCCTTTC

At_CLV3_Enh   (101) TTTGGTAAGCTTGTAGTTTCATGAAAGTG--AATGTAAGATATGCATTAC
Bo_CLV3_Enh    (99) TTTTGT----TTTTTTTTTTATGTATTTCGTATTGTAAATTAACTCTGCT

At_CLV3_Enh   (149) GTTTGTTGCTGAAGTGAATGTAAGATACGCACTATTATATCTCATGATTT
Bo_CLV3_Enh   (145) GTTTAATC--GGTTTCATGTTGAGGGATTTTCTGTTTTATC-CCTCACTT

At_CLV3_Enh   (199) TCTAAGAAAACCCTCTTAAAACGAAGATGTCTATAGCATTACGTTTCTAT
Bo_CLV3_Enh   (192) TCCCTAAGGTTGTGATGATAGTTAATGTCAGATAAGCACCACTTTTGT-T

At_CLV3_Enh   (249) TTCCATATAATACGTTAAAATTTATGGTTTTTACGTATAAAATGCAAAAT
Bo_CLV3_Enh   (241) TCCCATGGA-TCTATTTAGGANCCGCATTTTGATGTATTGGCCACAAATT

At_CLV3_Enh   (299) AAAGACACAAGTATATCTCCAAAGCAATGTACCGTTGGGAAAATTTATTA
Bo_CLV3_Enh   (290) AAAAACTTCTTCAAG---ACAATATTATATGCCGTTGGGGAA-TGTATTA

At_CLV3_Enh   (349) GTACGTTTTCAATTGTCAATGCAAATAATTAATGGATGTGATAGTCACAA
Bo_CLV3_Enh   (336) GTACTTTTGCGATTGTCAAGGCAGCTAATTAATGGAAATGACAGT----A

At_CLV3_Enh   (399) TTAAACATACAATAATAAAAATGATGATGATGATTCGATGATGTGGTGGG
Bo_CLV3_Enh   (382) TGATATATAG--TAATGATGATGATGA-GTTGAT---ATGATGTTGTGGG

At_CLV3_Enh   (449) AAGGATAAATTAAACCGACTTTGGGGCAGTGACAGGCAGTGTCAGTGTCA
Bo_CLV3_Enh   (426) AAGTATAAATGGAATCG-------G-CAGTGACAGGCAGTGTCAGTGTCA

At_CLV3_Enh   (499) AAGACAACCATTTGTAGTCACTATTTCTATCGAAGGTTGCAAATTGAATG
Bo_CLV3_Enh   (468) AAGATGACCATTTGTAGTCACAACTTGTATCGAAGGCTG---NTNCCGTT

At_CLV3_Enh   (549) GTGGAGGAG--TATCAAAACGACAC-ACATACTTGAAAAGATATTTTAAT
Bo_CLV3_Enh   (515) GTGGAGGAGAGTAACCATACGACACCACATATGGTTTTTAATAGTATGAA

At_CLV3_Enh   (596) AATATAAAAAAATTGGTGATGGCGTAATAACAAACCTAGAG--CTAATTA
Bo_CLV3_Enh   (565) AATATCGATA---TTGTGATGGCATAATAGCTAACCTAGAGAGCTAATTA

At_CLV3_Enh   (644) TTATCCTTAATGATACCAAATCTATATGATACGATATTTGTTTTAAA-AA
Bo_CLV3_Enh   (612) TTATCCTTAATGATACCAAATCTATATGATACGATATTATTGGTATAGAA

At_CLV3_Enh   (693) GAGTAAAG--ACTGACACTTGAGATGTGACACTGGCGATTTCGCTCACGT
Bo_CLV3_Enh   (662) GAGTAAAGTGACTGACACTTGAGATGTGACACTGGCGATTTCGCTCACGT

At_CLV3_Enh   (741) CACCACTTTTCCCACCTCAAATAACGCTTACGGCTTTATCCATTAATTCT
Bo_CLV3_Enh   (712) CACCACTTTGCCCTCCTCAAAGATCGCTTACGGCTTTATCCATTCACTTT

At_CLV3_Enh   (791) ----------AAGTATAATTTTAAGTGTATTTTTTCT-------TGCC
Bo_CLV3_Enh   (762) TCCGTATCATTCAAATACAATTTAAATTGTATTTTTATTAAATCGGTGTT

At_CLV3_Enh   (822) AAATTCAAATATATC----TTACTAAATG-GATGAACATTATAA-AATTG
Bo_CLV3_Enh   (812) ATAAGAAAAT-TATCACTATTATTACAGGCGTTCCATGTTCTTATAATTG

At_CLV3_Enh   (866) TTATCAAAACCATTAAATGTTCTTAT-AATTTCTTTCGTTCCTCCAATGT
Bo_CLV3_Enh (861) CTGGAAATAATACTAGA-GTTATCGTCAATTTCGTTCTTTCCTCCAGTGT
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At_CLV3_Enh   (915) CATCCCAAGACTTTTTGACCTAATATATGATATATCTAACTTGCTTTGGA
Bo_CLV3_Enh   (910) CATCCCAACACTTGTTTTGGTGTAACGAGAATTTCCATACGTACTTTAAA

At_CLV3_Enh   (965) ATCGTATGACATATATCTTCAAATACATATTTCGTATTTTTTTTTCACGA
Bo_CLV3_Enh   (960) ATCA-AGCAGATATATTTCCATATCCTTTTTTTGGAAACAGAAACAAGAA

At_CLV3_Enh  (1015) AAACTAATTTAGAAAG-TAGAAAACCAGCT----------
Bo_CLV3_Enh (1009) AATCTATATTATAATGATTGAGTTTTTGCTCTCTGTTCAGC
```

Fig. A1. Aligment obtained with a gap opening penalty value of 15 and and a gap extension penalty of 6.66 (standard values in Vector NTI).

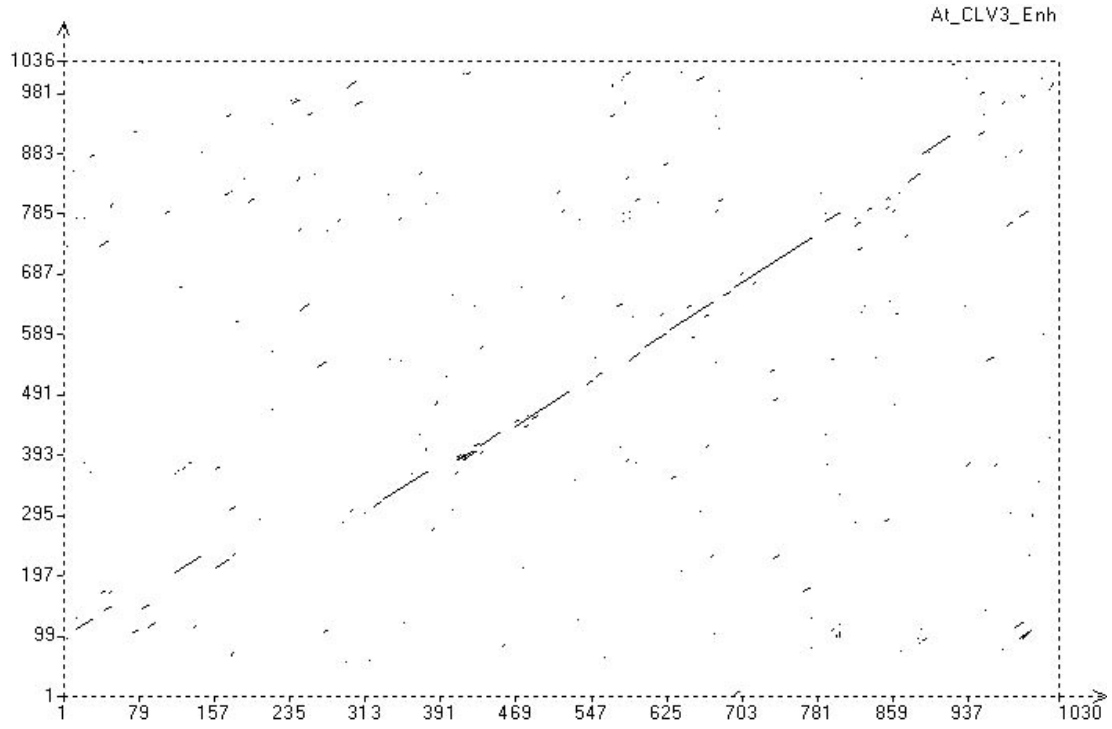

Fig. A2. Dotplot obtained by the comparison of the *CLV3* enhancer sequences from *Arabidopsis thaliana* and *Brassica oleracea.* Stringency value is set to 25, window value to 14.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig und ohne unzulässige Hilfe angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und in Stellen der Arbeit , einschließlich Tabellen und Abbildungen, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Fall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht worden ist, sowie dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der geltenden Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Rüdiger Simon betreut worden

Düsseldorf, im November 2005

Abstract

The shoot apical meristem (SAM) of higher plants has to maintain the stem cell population during plant life by keeping the balance between cell proliferation in the central zone and cell differentiation in the peripheral zone. *CLAVATA3* (*CLV3*) and *LOLLO (LOL)* are two *Arabidopsis thaliana* genes involved in the regulation of stem cell fate and lateral organ differentiation. *CLV3* has a central role in controlling SAM activity and stem cell identity, but little is known about the regulation of its expression. *LOL*, a newly isolated member of the *LATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) gene family, expressed in organ anlagen and in the boundaries between meristem and organ primordia, can act to integrate gene activities and hormonal signalling to control organ development.

Deletion analysis of CLV3 regulatory sequences

By deletion analyses of the *CLV3* promoter and downstream putative enhancer, different fragments of the *CLV3* regulatory sequences, required to promote or repress *CLV3* expression, were isolated. On these results, a combinatorial model of *CLV3* expression regulation is hypothesized. Due to high redundancy, single motives necessary and sufficient to promote *CLV3* in its own pattern were not isolated. However, a negative regulator of *CLV3* expression in the *CLV3* promoter and a positive regulator in the *CLV3* putative enhancer were identified. These two fragments were used as baits to isolate, by yeast one-hybrid screens, transcription factors regulating *CLV3* expression in the stem cell domain and in differentiated cells. Results are discussed. Due to its importance in controlling the stem cell population, *CLV3* expression is probably finely regulated by many different pathways, possibly including also high-level transcription control systems like DNA methylation and chromatin remodelling. Some of the identified regulatory regions could be required for the recruitment of histone modifying or DNA methylation

enzymes at the *CLV3* locus. Current chromatin immuno precipitation (ChIP) experiments indicate that *CLV3* is indeed subject to chromatin modification and repression in non-stem cells.

Characterization of LOL

lol-D mutant plants, identified after an activation tagging mutagenesis, are shown to ectopically express *LOL*, a putative transcription factor belonging to the *LBD* gene family. Among the few already characterized *LBD* genes are *LATERAL ORGAN BOUNDARY (LOB)* and *ASYMMETRIC LEAVES2 (AS2)*, involved in maintenance of lateral organ boundaries and specification of organ primordia, respectively (Shuai et al., 2002; Lin et al., 2003). *LOL*, expressed in primordia anlagen and in the boundaries between meristem and young organ primordia, seems to affect the expression of genes belonging to the *KNOTTED-LIKE HOMEOBOX (KNOX)* and *PIN-FORMED (PIN)* families. *KNOX* genes are required to maintain meristem identity; PIN proteins facilitate the polar transport of the phytohormone auxin, required for organ initiation and cell differentiation/expansion. In *LOL* mis-expressing plants, the development of lateral organs is affected during the vegetative stage: leaves are lobed and organ size is reduced. Later, during the reproductive stage, organ production arrests. The leaf mutant phenotype may be promoted by an up-regulation of *KNOX* gene expression, while organ production arrest could be due to altered auxin transport. Indeed, two *KNOX* genes were found to be up-regulated, while four *PIN* genes were down-regulated in *LOL* overexpressing plants. *LOL*, through the regulation of *KNOX* gene expression, could control the cell cycle of boundary cells. *LOL*, via the down-regulation of *PIN* expressions, could regulate auxin transport in young organ primordia. To characterize the *LOL* function, *lol-1* homozygous mutants were isolated. *lol* loss of function causes embryo lethality. Overexpression of a *lol-DN* dominant repressor promotes partial cotyledon fusion and lateral organ adaxialization. Interestingly, quadruple mutants for *pin1pin3pin4pin7* are also

embryo lethal, while mutant plants for *arf3/arf4*, two auxin response factors, have also adaxialized leaves. Therefore, also in plants where *LOL* is not expressed, or where its dominant negative version is ectopically expressed, mutant phenotypes could be caused by altered auxin transport. In line with this, Affymetrix micro array analysis showed that several auxin inducible genes are down-regulated in *LOL* mis-expressing plants. To understand if indeed *LOL* is involved in the control of auxin transport, *in planta* auxin transport assays have to be performed. Interestingly, the putative LOL interaction partners until now isolated promote DNA and histone methylation, thus indicating that possibly LOL controls its target genes by acting on chromatin remodelling.

Kurzfassung

Die Aufgabe des Sprossapikalmeristems (SAM) höherer Pflanzen besteht darin, die Stammzellpopulation während des Lebens der Pflanze zu erhalten, indem es die Balance zwischen Zellproliferation in der zentralen Zone und Zelldifferenzierung in der peripheren Zone erhält. Die beiden Gene *CLAVATA3* (*CLV3*) und *LOLLO* (*LOL*) aus *Arabidopsis thaliana* sind in die Regulierung des Stammzellschicksals und die Differenzierung von Seitenorganen involviert. *CLV3* spielt eine zentrale Rolle in der Kontrolle der SAM Aktivität, aber wenig ist über die Regulierung seiner Genexpression bekannt. *LOL*, ein neu isoliertes Mitglied der *LATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) Genfamilie, wird in den Organanlagen und an den Grenzen zwischen Meristem und Organprimordien exprimiert, wo es hormonelle und genetische Signale zur Erhaltung eines funktionalen Sprossapex integriert.

Deletionsanalyse von CLV3 regulatorischen Sequenzen

Durch Deletionsanalysen am *CLV3* Promoter und dem putativen Verstärkerelement in der 3´ Region, wurden verschiedene Fragmente der *CLV3* regulatorischen Sequenzen isoliert, die entweder die *CLV3* Expression verstärken oder reprimieren. Aufgrund dieser Resultate wurde ein kombinatorisches Modell für die Regulierung der *CLV3* Genexpression aufgestellt. Aufgrund der hohen Redundanz konnten einzelne Motive, die notwendig und hinreichend für die *CLV3* Expression im eigenen Muster sind, nicht isoliert werden. Dennoch wurden ein negativer Regulator in der CLV3 Promoter Region und ein positiver Regulator im *CLV3* putativen Verstärkerelement identifiziert. Diese beiden Fragmente wurden als Köder in einer Hefe-Ein-Hybrid Durchmusterung eingesetzt, um *CLV3* Expression regulierende Transkriptionsfaktoren in der Stammzelldomäne und in differenzierten Zellen zu finden. Die Ergebnisse hierzu werden diskutiert. Wegen der wichtigen Rolle von CLV3 in der Kontrolle der

Stammzellpopulation wird die *CLV3* Genexpression wahrscheinlich durch viele verschiedene Wege feinreguliert, vielleicht auch durch höher geordnete transkriptionelle Kontrollsysteme wie DNA Methylierung und Chromatin Remodellierung. Einige der identifizierten regulatorischen Regionen könnten für die Rekrutierung von Histon-modifizierenden oder DNA-methylierenden Enzymen am *CLV3* Lokus benötigt werden. Zur Zeit durchgeführte Chromatin-Immunopräzipitationsexperimente (ChIP) weisen darauf hin, dass *CLV3* tatsächlich einer Chromatinmodifizierung und Repression in Nicht-Stammzellen unterliegt.

Charakterisierung von LOL

lol-D Mutanten wurden durch eine Aktivierungsmarkierungsmutagenese identifiziert und überexprimieren *LOL*, einen putativen Transkriptionsfaktor, der zur LBD Genfamilie gehört. Unter den schon charakterisierten LBD Genen sind *LATERAL ORGAN BOUNDARY* (*LOB*) und *ASYMMETRIC LEAVES2* (*AS2*), welche an der Aufrechterhaltung von Organgrenzen und der Initiation von Organprimordien beteiligt sind (Shuai et al. 2002; Lin et al., 2003). LOL wird in Primordienanlagen und an der Grenze zwischen Meristem und jungen Organprimordien exprimiert und scheint die Expression von Genen der *KNOTTED-LIKE HOMEOBOX* (*KNOX*) und *PIN-FORMED* (*PIN*) Familien zu beeinflussen. *KNOX* Gene werden für die Aufrechterhaltung der Meristemidentität benötigt; PIN Proteine unterstützen den polaren Transport des Phytohormons Auxin, das für die Organinitiation und Zelldifferenzierung/expansion benötigt wird. In *LOL* fehlexprimierenden Pflanzen ist die Entwicklung der Organe während der vegetativen Phase betroffen: Blätter sind gelappt und die Organe sind verkleinert. Später während der reproduktiven Phase hört die Organproduktion ganz auf. Die Blattphänotypen der Mutante könnte durch eine stärkere Expression der *KNOX* Gene erklärt werden, wohingegen das Stoppen der Organproduktion auf einen veränderten Auxintransport zurückgeführt werden könnte.

Tatsächlich werden in *LOL* überexprimierenden Pflanzen zwei *KNOX* Gene stärker exprimiert, und vier PIN Gene werden weniger stark exprimiert. *LOL* könnte über die Regulation der *KNOX* Genexpression den Zellzyklus der Grenzzellen kontrollieren. *LOL* könnte darüberhinaus über die verminderte Expression der *PIN* Gene den Auxintransport in jungen Organprimordien regulieren. Um die *LOL* Funktion weiter zu charakterisieren, wurden *lol-1* homozygote Mutanten isoliert. Der *lol* Funktionsverlust führt zur Embryolethalität. Die Überexpression von *lol-DN* dominantem Repressor fördert eine partielle Kotyledonenfusion und die Adaxialisierung der Organe. Interessanterweise zeigen vierfach Mutanten für *pin1pin3pin4pin7* ebenso Embryolethalität, während *arf3/arf4* (auxin response factor) Mutanten auch adaxialisierte Blätter zeigen. Deswegen können Phänotypen in Pflanzen, die *LOL* nicht exprimieren oder eine dominant negative Form von *LOL* exprimieren, durch veränderten Auxintransport bedingt sein. Auch in Affymetrix Microarrays konnte gezeigt werden, dass einige Auxin-induzierte Gene in *LOL* fehlexprimierenden Pflanzen herunterreguliert sind. Um weitere Erkenntnisse zu erlangen inwiefern *LOL* in der Kontrolle des Auxintransports involviert ist, müssen *in planta* Auxintransportuntersuchungen durchgeführt werden. Interessanterweise unterstützen die bis jetzt isolierten drei putativen LOL Interaktionspartner DNA und Histonmethylierung, was darauf hinweist, dass LOL möglicherweise seine Zielgene durch Chromatinremodellierung kontrolliert.

Curriculum vitae

DATE OF BIRTH	April 1st, 1976
PLACE OF BIRTH	Milano, Italy
EDUCATION	1990-1995: High school degree at *Scientific Lyceum A. Einstein,* Milano.
	1996-2001: Attended the *University of Milano* courses in Biological Sciences, with Bio-molecular specialization.
	March 2000-July 2001: Diploma work in Dr. Martin Katers laboratory. Title: "AGL11 an ovule specific gene in *Arabidopsis thaliana* functional analysis and molecular regulation of its expression".
	July 2001: Graduated in Biological Science (Bio-molecular specialization).
	From September 2001: PhD student work in Prof. Dr. Rüdiger Simons laboratory. Title: "Regulation of stem cell fate and organ development in *Arabidopsis thaliana*". In the *Institute of Developmental Biology* at the University of Cologne until July 2003; in Institute for Genetics of the Heinrich-Heine-University in Düsseldorf from August 2003 onwards.