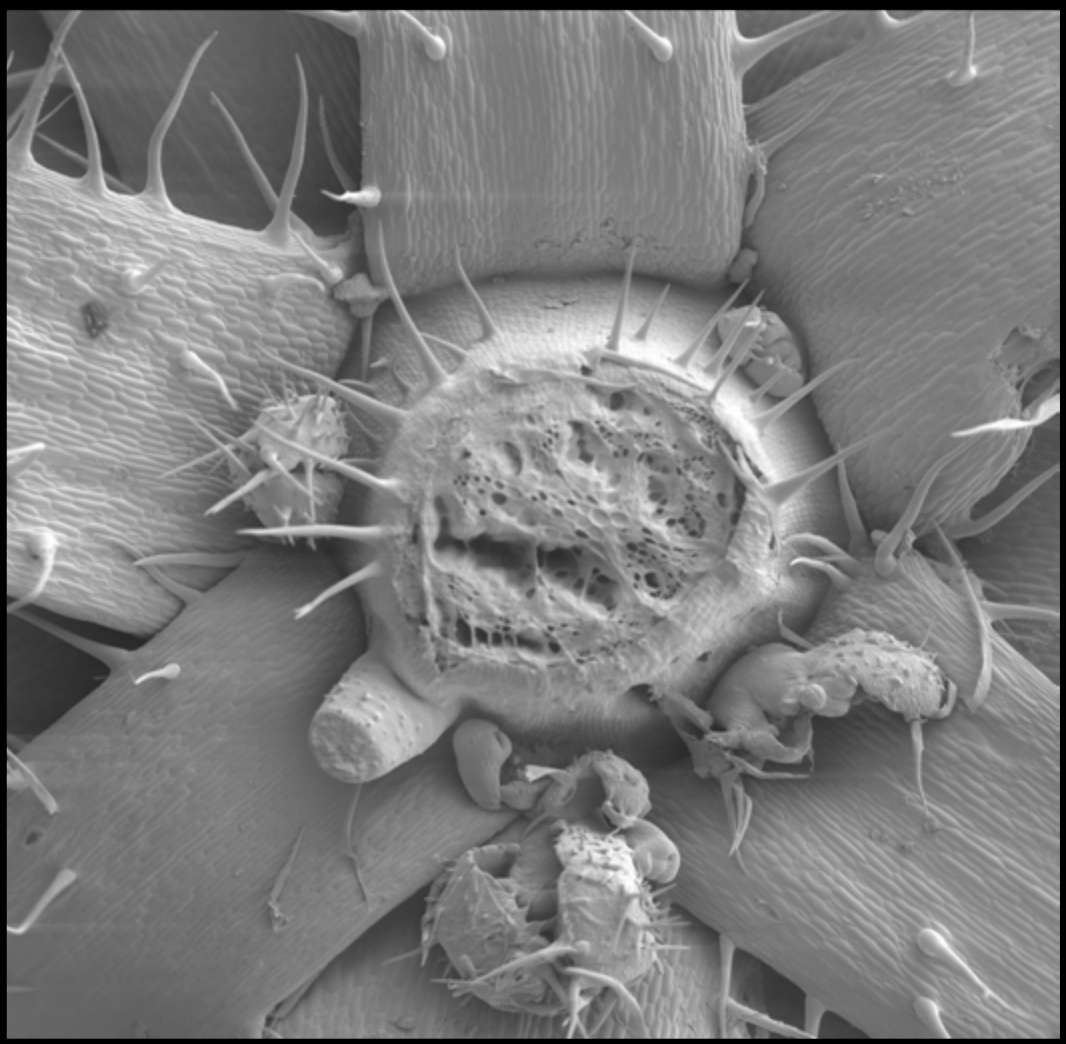
Genetic Analysis of Axillary Meristem Development in *Arabidopsis*:

Roles of *MIR164*, *CUC1*, *CUC2*, *CUC3* and *LAS*, and identification of novel regulators.



Inaugural – Dissertation zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln
Vorgelegt von Smita Raman Komnedath aus Arimpur, Indien
Köln 2006

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Smita Raman Komnedath

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Köln 2006

Berichterstatter:	Prof. Dr. Klaus Theres
	Prof. Dr. Wolfgang Werr
Tag der mündlichen Prüfung:	12.02.2007

For my mother.

CONTENTS

1 INTRODUCTION

The basic body plan of higher plants is laid down during embryogenesis by the establishment of the apical-basal axis defined by the root apical meristem (RAM) at the one end and the shoot apical meristem (SAM) at the opposite end. The RAM gives rise to the root system, whereas the SAM produces the various aerial organs of a plant through the establishment of a series of developmental modules called phytomeres (Steeves and Sussex, 1989; Sussex and Kerk, 2001). A phytomere consists of an internode, a node subtended by a leaf and a secondary meristem formed in the axil of each leaf (Lyndon, 1990). These secondary meristems are called lateral/axillary meristems and they subsequently recapitulate the SAM function by initiating several leaf primordia, resulting in the formation of axillary buds. Depending on their position along the shoot axis, the developmental phase of the plant or environmental factors, these buds either remain dormant or grow out to form secondary axes of growth (McSteen and Leyser, 2005). These secondary axes of growth are in the form of branches or flowers, and determine the adult morphology and the reproductive success of a plant. They are also the major source of evolutionary and environmental diversity in shoot architecture in higher plants.

1.1 SAM IS THE PRIMARY SEAT OF SHOOT GROWTH IN PLANTS

1.1.1 *KNOX* GENES CONTROL THE ESTABLISHMENT AND MAINTENANCE OF THE SAM

Since the initiation and development of all aerial organs is ensured by the continuous activity of the SAM, its establishment and maintenance is of prime importance in plant development. Studies on mutants defective in SAM development in maize have revealed that gain-of-function mutations in, or ectopic expression of, *KNOTTED 1 (ZmKN1)* develops ectopic meristems on the adaxial surface of leaf blades (Smith *et al.,* 1992; Sinha *et al.,* 1993). Subsequent loss-of-function studies have demonstrated that *ZmKN1* is essential for shoot meristem formation and maintenance (Kerstetter *et al.,* 1997; Vollbrecht *et al.,* 2000). *Arabidopsis* encodes four class I *KN1*-related homeobox (KNOX) genes (Bharathan *et al.,* 1999; Reiser *et al.,* 2000; Semiarti *et al.,* 2001), namely *KNOTTED 1-like homeobox protein 1/BREVIPEDICELLUS (KNAT1/BP), KNAT2, KNAT6 and SHOOTMERISTEMLESS (STM*). Whereas sequence comparison shows *KNAT1* as the *Arabidopsis* orthologue of *KN1* (Bharathan *et al.,* 1999; Reiser *et al.,* 2000), *STM* has closer functional similarity to *KN1* on the basis of similar null mutant phenotypes and expression patterns (Long *et al.,* 1996; Vollbrecht *et al.,* 2000). Mutants harbouring the strong loss of function alleles *stm-1, stm-5* and *stm-11* lack a functional SAM at the base

of the cotyledons, and the weak *stm-2* mutants are characterised by the formation of a short-lived SAM. Thus, *STM* is essential for initial shoot meristem formation and for the subsequent maintenance of SAM organisation (Barton und Poethig, 1993; Long *et al.,* 1996; Clark *et al.,* 1996; Endrizzi *et al.,* 1996). However, mutations or disruptive insertions in *KNAT1, KNAT2* or *KNAT6* do not normally affect shoot meristem development or function (Byrne *et al.,* 2002; Douglas *et al.,* 2002, Venglat *et al.,* 2002; Dean *et al.,* 2004), suggesting that the critical KNOX function for SAM development is provided by *STM*. Transcripts of *STM* are detected in the SAM and the interprimordial regions, but are notably absent in the leaf primordia (Long and Barton, 2000; Grbic and Bleecker, 2000). *STM* and *KNAT1* are closely related, as determined by phylogenetic analysis of nucleotide and amino-acid sequence, and it has been postulated that they arose following an ancient gene duplication event (Bharathan *et al.,* 1999; Reiser *et al.,* 2000). Overexpresion of *KNAT1* results in the formation of ectopic meristems on leaf blades reminiscent of the *kn1* mutants (Chuck *et al.,* 1996). Expression of *KNAT1* and *KNAT2*, but not of *STM*, is repressed in the leaf primordia by the Myb gene *ASYMMETRIC LEAVES1* (AS1, Byrne *et al.,* 2000; Ori *et al.,* 2000). Loss of function *stm* mutants can be rescued by knocking out *AS1* (Byrne *et al.,* 2002). This rescue is provided by the redundant activity of *KNAT1* which is de-repressed by loss of *AS1* function.

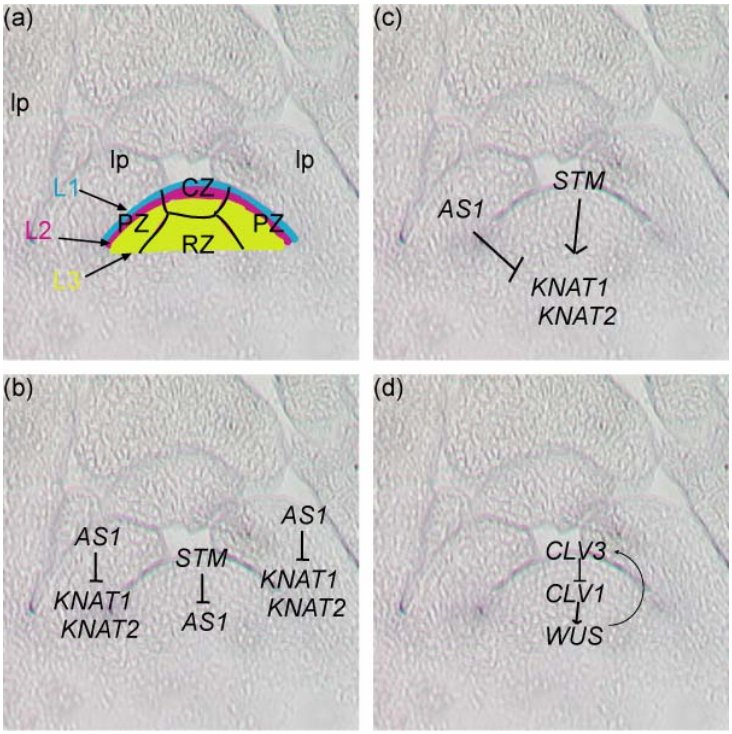


Fig.1.1-1: *AS1-KNOX* and *CLV-WUS* interactions regulates the SAM maintenance. (a) Schematic representation of division of the SAM into 3 layers and 3 zones. (b) Model of interactions among *STM, AS1* and *KNAT1/2* proposed by Byrne *et al.,* (2000). (c) Alternative model for interactions among *STM, AS1* and *KNAT1/2* (Byrne *et al.,* 2002). (d) Stem cell regulation in the SAM via the CLV-WUS feedback loop. lp = leaf primordial, CZ = central zone, PZ = peripheral zone, RZ = rib zone. (Adapted from Carles and Fletcher, 2003; Doerner, 2003).

This functional compensation of *STM* by *KNAT1* has been shown to depend on the BEL-like homeodomain transcription factor, *BELLRINGER/PENNYWISE* (Byrne *et al.,* 2003; Smith and Hake, 2003). These proteins have been shown to physically interact with the class I KNOX proteins, and genetic studies indicate these interactions are essential for normal KNOX function in the SAM. On the other hand *AS1* expression in the SAM is

repressed by *STM* (Byrne *et al.,* 2002) which is reflected by the complementary patterns of transcript accumulation of *STM* and *AS1* in the SAM and the leaf anlagen, respectively. Suppression of *AS1* activity by *STM* permits the expression of *KNAT1* and *KNAT2* in the SAM, thus allowing the activation of the SAM (Fig. 1.1-1b). However, Byrne *et al*. (2002) have shown that *stm1 as1 knat1* triple mutants lack a SAM and are indistinguishable from the *stm1* single mutant. This suggests an alternative model, whereupon *STM* and *AS1* might competitively regulate *KNAT1* directly, with the activating function of *STM* titrating out the repression by *AS1* (Fig. 1.1-1c). Whether such interactions are direct or indirect is not yet known.

1.1.2 *WUSCHEL-CLAVATA* FEEDBACK MECHANISM REGULATES STEM CELL MAINTENANCE IN THE SAM

The SAM in *Arabidopsis* is organised into an outer tunica, consisting of the L1 and L2 cell layers, and an inner corpus, both of which contribute towards organ formation and growth (Fig. 1.1-1a). Cell divisions in the L1 and L2 layers occur anticlinally (perpendicular to the surface of the meristem), so that the progeny cells remain in their layer of origin. The organization into tunica and corpus can be combined with an overlapping organisation into zones. These zones are characterised by specific cell sizes, cytoplasmic architecture, cell division rates and gene expression patterns. At the meristem summit is the central zone defined by a small group of slowly dividing cells (Fig. 1.1-1a). Cells of the central zone divide considerably slowly when compared to those in the peripheral zones of the meristem. The L1, L2 and L3 layers in this central zone are bestowed with a stem cell identity due to the expression of *WUSCHEL (WUS)* in the underlying sub-apical layers (Mayer *et al.,* 1998). *WUS* encodes a homoeodomain transcription factor and the loss-of-function *wus* mutants are unable to maintain a functional SAM (Laux *et al.,* 1996). *WUS* positively controls stem cell fate in the meristem. In the cells where *WUS* is expressed, it also activates expression of *CLAVATA3 (CLV3)*, a small diffusible peptide that binds and activates *CLV1/CLV2* receptor complex in the deeper cell layers to trigger an auto feedback response in which *WUS* is downregulated (Brand *et al.,* 2000; Schoof *et al.,* 2000; Fig. 1.1-1d). *CLV1* encodes a leucine rich repeat (LRR) receptor kinase (Clark *et al.,* 1993) and *CLV2* encodes an LRR receptor-like protein without a kinase domain (Jeong *et al.,* 1999). Contrary to the *wus* mutants, the *clv1, clv2* and *clv3* mutants are characterised by accumulation of undifferentiated cells in the centre of the shoot and floral meristems, resulting in a massive increase in their size, thus indicating a negative control of stem cell fate. All the three *CLV* genes are expressed in the meristem in the central zone above the *WUS* expression domain. All *clv* mutants fail to repress *WUS* activity, leading to stem cells accumulation (Schoof *et al.,* 2000), whereas, increasing the CLV3 signal by ectopic expression causes a repression of *WUS*-dependent stem cell promotion, and the size of the stem cell population is reduced (Brand *et al.,* 2000). Also, Double mutant combinations of *wus* and *clv* are almost indistinguishable from the *wus*

single mutant, consistent with the model that *WUS* is a target for negative regulation by the *CLV* genes to maintain a constant number of stem cells in the central zone of the SAM (Laux *et al.,* 1996; Schoof *et al.,* 2000).

1.1.3 *CUP-SHAPED COTYLEDON* GENES ARE NEEDED FOR FORMATION OF THE SAM

The *CUP-SHAPED COTYLEDON 1 (CUC1), CUC2* and *CUC3* genes in *Arabidopsis* belong to the family of the NAC domain proteins (Aida *et al.,* 1997; Takada *et al.,* 2001; Vroemen *et al.,* 2003). Whereas development in single mutants is almost normal, any combination of double mutants of the *CUC* genes shows severe boundary mis-specification leading to cotyledon fusions and resulting in cup-shaped structures which does not contain a SAM (Aida *et al.,* 1997; Vroemen *et al.,* 2003). In *cuc1 cuc2* double mutant embryo, *STM* expression is not detected, demonstrating that these *CUC1* and *CUC2* are activators of *STM* (Aida et al., 1999; Takada et al., 2001). All the three *CUC* genes are expressed in a narrow strip of cells in the boundary domain around the organ primordia. During embryognesis, *CUC1* and *CUC2* expression coincides with the onset of *STM* expression (Aida *et al*., 1997). *CUC3* expression domain largely overlaps that of the other two *CUC* genes. However *CUC3* is expressed earlier and its expression is strongest in the epidermal cell layer, whereas *CUC1* is expressed only weakly in the epidermis and *CUC2* is not expressed in the epidermis at all (Aida *et al.,* 1997; Takada *et al.,* 2001; Vroemen *et al.,* 2003). It has been shown that *CUC1* and *CUC2,* but not *CUC3*, are under the post-transcriptional regulation of a microRNA, miR164 (Rhoades *et al.,* 2002; Kasschau *et al.,* 2003).

1.1.4 HORMONAL REGULATION OF STEM CELLS IN THE SAM

In addition to their centralised position, the group of stem cells in the SAM shows a characteristic placement along the proximal/distal axis. Complex patterns of synthesis, transport and accumulation of the plant hormone auxin has been shown to determine this precise positioning of the stem cells. In the SAM, relatively low levels of auxin is a prerequisite for normal patterning, which seems to be mediated through the expression of the *CUC1* and *CUC2* genes and the subsequent activation of *STM* activity (Jenik and Barton, 2005). Localised increases in auxin levels in the SAM is shown to trigger initiation of lateral organ primordia, and this is accompanied by a downregulation of *KNOX* genes in the founder cells of the primordia (Benkova *et al.,* 2003; Reinhardt *et al.,* 2003).

The phytohormones cytokinins have classically been known as the antithesis of auxin. Tissue culture studies have shown that high cytokinin to auxin ratios favour the formation of shoots, while low ratios favour roots (Skoog and Miller, 1957). The role of cytokinin signalling in homoeostatic regulation of the SAM was revealed by the analysis of maize *ABPHYL* gene which encodes an A type cytokinin response regulator that typically

suppresses cytokinin reponse. The loss-of-function mutants of this gene are characterised by formation of an enlarged meristem and change of the alternate phyllotaxy to a decussate pattern (Giulini *et al.,* 2004). Thus *ABPHYL* may act to limit cytokinin-mediated cell proliferation of the SAM. This is supported by observations that direct application of cytokinin results in enhanced growth of the SAM. Furthermore, overexpression of *KNOX* genes in the SAM leads to a rapid increase in cytokinin levels through activation of *AtIPT7*, a gene encoding an isopentenyl transferase which mediates the cytokinin biosynthesis (Yanai *et al.,* 2005). Also, the misexpression of *AtIPT7* in the SAM from the STM promoter could partially suppress the *stm* loss-of-function phenotype, providing the functional significance of increase in cytokinin in the maintenance of the SAM.

The tobacco *KNOX* gene *NTH15* has been shown to directly inhibit the transcription of GA-20 oxidase, an enzyme involved in a crucial step of biosynthesis of GA, thus causing suppression of gibberellic acid (GA) activity in the SAM (Sakamoto *et al.,* 2001). The application of exogenous GA suppresses the phenotype of misexpression of *KNOX* genes, giving further proof that *STM* exerts a negative influence on the GA biosynthesis and thus on the cell differentiation (Hay *et al.,* 2002). Additionally, a mutation in the *SPINDLY* gene, a constitutive activator of the GA signal transduction, leads to an enhancement of the *stm-2* mutant phenotype (Hay *et al.,* 2002).

1.2 AXILLARY MERISTEMS REGULATE PLANT ARCHITECTURE

The SAM regulates growth in plants indeterminately. The number, timing and form of the organs produced are moderated by the environmental and genetic status of the plant. This allows for developmental plasticity in plants, such that they can adapt their morphology as required. The production and activity of axillary meristems (AMs) greatly enhances this plastic potential. Axillary meristems form in the axils of leaves on the adaxial side of the region where the leaf joins the stem (Figure 1.1-2; Grbic and Bleecker, 2000; Long and Barton, 2000). They develop secondary axes of growth and allow the continued elaboration of plant morphology.

There occurs also plasticity in the production and activation of axillary meristems. Axillary meristems can be formed throughout the life cycle of the plant, but they are usually produced and activated later than the leaves in whose axils they are found. Once formed, they essentially recapitulate the function of the SAM by initiating several leaf primordia, resulting in the formation of axillary buds.

These buds grow out or remain dormant depending on their position along the shoot axis, the developmental phase of the plant or environmental factors (Grbic and Bleecker, 2000; Gregory and Veale, 1957; McIntyre, 1977). The majority of axillary buds grow out to form lateral branches, which generally repeat the pattern of development seen in the primary shoot, including the production of leaves, flowers, axillary meristems, and eventually, lateral branches. In some plants, for instance *Pisum sativum*, accessory meristems are formed, which are initiated on the adaxial side between the axillary shoot and the leaf (Fig. 1.1-2).

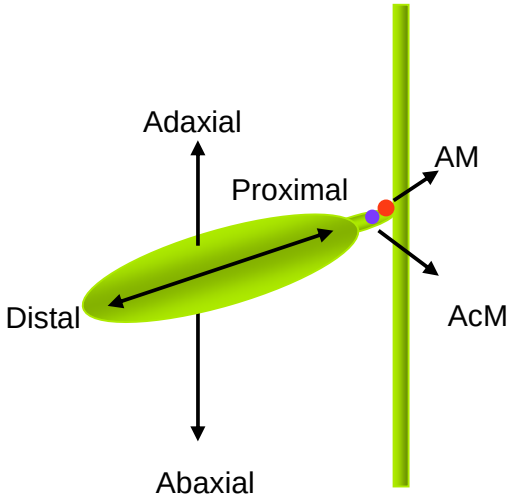


Fig. 1.1-2: Schematic representation of the axil of a leaf. The axil is the region between leaf and stem, on the adaxial surface. The red circle represents the site of the axillary meristem (AM). The blue circle represents the site of the accessory meristem (AcM) which is sometimes formed in plants. (Adapted from Bennett and Leyser, 2006).

1.2.1 Ontogeny of axillary meristems

To date there are two opposing models explaining the origin of axillary meristems. The first suggests that axillary meristems initiate *de novo* separately from the SAM in leaf axils after organogenesis of the subtending leaf (Snow and Snow, 1942). This hypothesis is supported by the observation that in the axils of leaves formed early in development there is no morphologically distinguishable axillary meristem at the time of leaf initiation. Additionally, sometimes in the axils of younger cauline leaves in *Arabidopsis* accessory meristems are initiated (Grbic and Bleecker, 2000), suggesting a *de novo* initiation of these meristems. An alternative model suggests that axillary meristems derive from groups of meristem cells that detach from the SAM at the time of leaf initiation and never lose their meristematic identity (Garrison, 1955; Sussex, 1955). Evidence for this hypothesis comes from species such as tomato and the younger leaf axils of *Arabidopsis*, where axillary meristems are evident very early in organogenesis, and are clonally more closely related to later leaves than the subtending leaves (Johri and Coe, 1983; McDaniel and Poethig, 1988). Further support for this model also comes from the focussed expression pattern of the SAM marker *STM* in the axils of leaves immediately preceeding the appearance of a morphological axillary meristem. This characteristic *STM* expression in leaf axils is missing in *Arabidopsis* mutants like *revoluta (rev)* and *lateral suppressor (las-4)* .which show defects in axillary shoot formation.

1.2.2 Spatial and temporal specification of axillary meristems

Axillary meristems are formed only on the adaxial side of organs, including ectopically adaxialised tissue in various mutants (Long and Barton, 2000; McConnell and Barton, 1998). The adaxial tissue is uniquely competent to express meristematic genes (Sinha *et al.,* 1993). Mutations in the *PINHEAD* and *ARGONAUTE1* genes, which result in failure of correct adaxial domain specification, also show deficiencies in formation of all meristems (Lynn *et al.,* 1999; Kidner and Martienssen, 2004), suggesting a role of these genes in connecting meristem fate to adaxiality. The HD-ZIP genes *PHABULOSA, PHAVOLUTA* and *REVOLUTA* function to specify adaxiality within the shoot (Emery *et al.,* 2003). Loss-of-function *rev* mutants often lack axillary meristems in the axils of organs (Talbert *et al.,* 1995), whereas gain-of-function *phb-1-d* mutants often produce ectopic axillary meristems on the adaxialised sides of organs (McConnell and Barton, 1998). Also, the *phb, phv rev* triple mutants, that are completely abaxialised, have no shoot meristems (Emery *et al.,* 2003).

The temporal specification of axillary meristem initiation varies in different species. For instance, in tomato axillary meristems are formed immediately in all leaf axils. On the other hand, in *Arabidopsis*, not all axillary meristems develop at the same time: axillary meristems are more pronounced in the older leaves of short-day grown plants and in the youngest cauline leaves (Grbic and Bleecker,2000). It can be inferred from the latter that either floral transition promotes axillary meristem development in young leaves or the newly formed inflorescence meristem represses axillary meristem development more weakly than the SAM (Grbic and Bleecker, 2000). This implies a role for auxin in the development of axillary meristems. Hence, a combination of intrinsic (age, hormonal balance) and environmental (nutrient status, photoperiod) factors appear to control the time window of initiation of axillary meristems.

1.2.3 Genetic regulation of axillary meristem development

Several mutants with compromised axillary meristem initiation, which reveal the underlying genetic regulators of this process, have been identified in various plant species, including *Arabidopsis*, tomato, rice and maize (Schmitz and Theres, 2005). The *LATERAL SUPPRESSOR* genes in *Arabidopsis* (*LAS,* Greb *et al.,* 2003) and tomato (*Ls,* Schumacher *et al.,* 1999) encode a putative transcription factor belonging to the VHIID subfamily of the plant-specific GRAS family. These genes specifically regulate the initiation of axillary meristems during the vegetative phase of development. This phenotype is the result of the cells in the axils of leaves losing their meristematic competence. Transgenic copies of the *Arabidopsis LAS* can rescue the phenotype of tomato *ls* mutants, showing that these two genes indeed have an evolutionarily conserved function (Greb *et al.,* 2003). Mutations in *MONOCULM1*, the rice orthologue of

LAS, result in lack of tiller formation (Li *et al.,* 2003). Additionally, the *moc1* mutant also has drastically reduced branching in the inflorescence. *LAS* is expressed in all organ primordia, from very early in organogenesis, and becomes localized to a proximal area of the adaxial side, the presumptive axil (Greb *et al.,* 2003).

The allelic *blind* and *torosa* mutants in tomato display a strong reduction in axillary meristem formation during vegetative and reproductive development (Mapelli and Kinet, 1992; Schmitz *et al.,* 2002). Often no sympodial shoot is formed in these mutants and the plant terminates with a single inflorescence. In addition, the number of floral meristems produced is greatly reduced, and flowers are often fused. Molecular analysis has demonstrated that the *Blind* gene encodes a transcription factor of the R2R3 MYB class. In *Arabidopsis*, the *Blind*-homologous *REGULATOR OF AXILARY MERISTEMS (RAX)* genes control the formation of axillary meristems in overlapping zones along the shoot axis (Müller *et al*. 2006; Keller *et al.,* 2006). *RAX1* expression is seen in a circular domain at the adaxial center of the boundary between the SAM and the leaf primordial. However, the precise mechanism of the function of these MYB proteins in promoting axillary meristem formation is not yet known.

Many mutants with defective axillary meristem development have been discovered in the grasses. In maize, the *barren inflorescence2 (bif2)* mutant is characterised by complete suppression of axillary meristem formation in the inflorescence (McSteen and Hake, 2001). Two rice mutants with axillary meristem defects are *lax panicle (lax)* and *small panicle (spa)*. The *lax* mutant lacks lateral spikelets and rachis branches; whereas the *spa* mutant lacks primary rachis branches only at lower nodes (Komatsu *et al*., 2001; Komatsu *et al*., 2003a). The *lax spa* double mutants are completely devoid of branching in the inflorescence, and additionally have a greatly reduced tiller number, caused by a defect in axillary meristem formation. This observation is supported by the absence of *OsSH* (the rice *STM* orthologue) expression is in the presumptive axillary meristems (Komatsu *et al*., 2003b). Hence these two rice genes act redundantly to regulate axillary meristem development. *LAX* encodes a bHLH transcription factor (Komatsu *et al*., 2003a), whereas *SPA* is yet to be cloned. The expression of *LAX* in a narrow band of cells between the SAM and the developing axillary meristem suggests that it might act non-cell autonomously to amplify a meristem promoting signal arising from the SAM, or conversely might prevent an inhibitory signal from affecting the axillary meristem (Komatsu *et al*., 2003a). The maize orthologue of *LAX* was found to be the *barren stalk1 (ba1)* gene. The *ba1* mutant is devoid of vegetative as well as reproductive branches and is reminiscent of the *lax spa* double mutant (Gallavotti *et al*., 2004). Additionally, *ba1* mutants also lack female inflorescences.

Among the most well known negative regulators of axillary meristem development is the *Teosinte branched1 (Tb1)* gene of maize. It is the founding member of the TCP family of

transcription factors, and the *tb1* mutant is highly tillered (Doebley *et al*., 1995, 1997). The overexpression of the rice orthologue of this gene, *OsTb1*, results in plants with reduced branching, whereas mutants in *OsTb1 (fine culm1)* cause increased branching (Takeda *et al*., 2003). The maize *branched silkless (bd1)* mutant forms branches in place of spikelets (Colombo *et al*., 1998), and is similar to the rice *frizzy panicle (fzp)* mutants (Komatsu *et al*., 2001; Komatsu *et al*., 2003b). These mutations are caused in orthologous genes that code for ERF (AP2/EREBP) family of transcription factors.

1.2.4 Phytohormones and axillary meristem development

The auxin response mutants, *auxin resistant 1 (axr1)*, do not show any deviation in axillary bud formation when compared to the wild-type plants (Stirnberg *et al*., 1999). However, *axr1 las-4* double mutants produce less/more secondary rosette branches than *las-4* single mutants, which suggesting a role for auxin as a negative regulator of axillary meristem formation (Greb *et al*., 2003). The *supershoot (sps)* mutant of *Arabidopsis* has increased cytokinin levels and produces multiple numbers of axillary buds per axil (Tantikanjana *et al*., 2001). This observation suggests a role for cytokinin in axillary meristem initiation. Circumstantial evidences indicate that gibberellins play a role in regulating axillary meristem formation. The *LAS* gene of *Arabdidopsis* and *LAX* gene of rice are speculated to downregulate GA signaling in axillary meristem (Benett and Leyser, 2006).

1.3 Aim of this work

In *Arabidodpsis*, the GRAS gene *LATERAL SUPPRESSOR (LAS)* has been identified as a primary regulator of axillary meristem development (Greb *et al*., 2003). The aim of this work was to elucidate the mechanism of *LAS* function in leaf axils. The spatial and temporal specification of *LAS* expression for its function was analysed using misexpression studies. A possible role of *LAS* function in suppressing GA signaling in leaf axils was examined by testing for complementation of *las-4* branching defect in mutant plants expressing a *GAI-ΔDELLA* transgene from the *LAS* promoter. A further aim of this study was to identify novel regulators of axillary meristem initiation. To this end, an EMS mutagenesis screen for suppressors of the *las-4 max1-1* branching defect was conducted and three suppressor of *las-4 (sol)* loci were characterised. Futhermore, the role of *CUP-SHAPED COTYLEDON1 (CUC1), CUC2* and *CUC3*, the NAC domain transcription factors which have an overlapping expression to *LAS* in leaf axils, in the development of axillary meristems was examined, along with any potential role of miR164, the post-transcriptional regulator of *CUC1* and *CUC2*.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The chemicals used during this work, and their degree of purity, were as certified by the following suppliers:

Merck KgaA, Feinchemikalien und Laborbedarf Deutschland, Darmstadt
Invitrogen GmbH, Karlsruhe, Germany
Carl Roth GmbH, Karlsruhe, Germany
Difco Laboratories, Detroit/Michigan, USA
DuchEFA, Haarlem, The Netherlands
Sigma Chemical Co., St.Lois, USA
Biozym, Hess.Oldendorf, Germany
Amersham Pharmacia Biotec, Braunscheig, Germany
Roche, Basel, Switzerland
New England BioLabs GmbH, Schwalbach/Taunus, Germany
MBI Fermentas GmbH, St. Leon-Rot, Germany
Operon, Cologne, Germany

2.1.2 Antibodies

Anti-Digoxigenin-AP Fab-Fragments (from sheep), Roche, Basel, Switzerland

2.1.3 Buffers and Culture media

Stock of the following buffers and culture media were prepared as described by Sambrook und Russell (2001):

BSA, Denhardt's Solution, PBS, PCR Buffer, SDS, SSC, SSPE, TAE, TBE, TE, LB, YEP

All media, buffers and aqueous solutions were made with highly purified Milli-Q-water (Millipore Waters GmbH, Neu-Isenburg). Whenever required, the solutions were autoclaved for 20 min at 121°C.

2.1.4 Enzymes

Enzymes used during the course of this work were from following suppliers:

Invitrogen GmbH, Karlsruhe, Germany
New England BioLabs GmbH, Schwalbach/Taunus, Germany
MBI Fermentas GmbH, St. Leon-Rot, Germany

2.1.5 Oligonucleotides

Invitrogen, MBI Fermentas, Operon and Sigma were the major suppliers of the following oligonucleotides used during this study.

Name	Primer sequence in 5' to 3' orientation
AE42-1522R	CATCCTAGGCATGGTACCTTGAAACGATAGAAAAAGATG
AtLsATGKpnIF	CATGGTTACCATGCTTACTTCCTTCAAATCCTC
AtLs3568SpeIR	CATACTAGTTCATTTCCACGACGAAACGG
AtLsATGXhoIF	CATCTCGAGATGCTTACTTCCTTCAAATCCTC
AtLsORFApaIR	CATGGGCCCTCATTTCCACGACGAAACGG
AtLs3070R	AACACAATTGACGGCAATGG
AtLs2599F	CAGTGTATGCAAAGAACAGTTC
AtLs2230R	CATCCTAGGTACCTTGAAACGATAGAAAAAGATG
AtLs2349F	ACCTCCGTCGTCTTCTTTTC
AtLs2593muR	TGGTTCGAAACAAGAACTAGT
attBKnat1PrF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGATCTAGAGCCCTAGGATTTGA
attBKnat1PrR	GGGGACCACTTTGTACAAGAAAGCTGGGTACCCAGATGAGTAAAGATTTGAG
attBSTMPrF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGTGTTTGATTCGACTTTTGT
attBSTMPrR	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTCTCTTTCTCTCACTAGTA
attBUFOPrF	GGGGACAAGTTTGTACAAAAAAGCAGGCTGAATTCTCTGTTTTAATTGCCCCA
attBUFOPrR	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGCTGAAAAATGAAAAGA
FDH-2725-R	TCAGCCGAAAGTTATGTTTCTTT
FDH-1-F	GCCAACACAACTCCTTATAAG
GAI-ATG-AvrII-F	CATCCTAGGATGAAGAGAGATCATCATCATTC
GAI-ATG-Acc65I-F	CTA GGTACC ATGAAGAGAGATCATCATCATCA
GAI-STOP-AvrII-R	CATCCTAGGCTAATTGGTGGAGAGTTTCCA
GAI-130-EcoRI-F	CATGAATTCGCTGATGTTGCTCAGAAACTC
GAI-78-EcoRI-R	CATGAATTCGCCGTTACCGTCGTCTTCTT
GAI-8191-F	GTGACGCGATTCTCAATCAG
GAI-8711-F	AGGTCTTCAATGGCCGGCGC
GAI-9121-F	CGTTGCATTATTACTCGACG
GFP-1-XbaI-F	CATTCTAGAATGAGTAAAGGAGAAGAACTTTTCACTGGA
GFP-stop-SmaI-R	CATCCCGGGTTATTTGTATAGTTCATCCATGCCATGTGT
GJ2148-F	CACATGCTATAGCTAGTGTG
HTH-2928-R	TATCTTGCTACGAATTTGTCTAC
HTH-1-F	GAGAAGCAACACACAAGACA
Las-1-XbaI-F	CATTCTAGAATGCTTACTTCCTTCAAATCCTCTAGC
Las-stop-SmaI-R	CATCCCGGGTCATTTCCACGACGAAACGGAGA
LCR1f	GTGGGTCATTTAGTCATAGATCTCTCT
LCR2f	CCACCTGAGCCATCTCTATTTTCG
LCR3f	GCTAAGAAACAAGGTCTCGTCACTG
LCR4f	CGCGGTGGCTTTCGATCGAG
LCR5f	GACACGTGGCGATGATGTGGC
LCR6f	CGGTGGTG ACGGGGCATAAG
LCR7r	GCAGCCAACCAACCGACGAC
LCR-1-F	GTGGGTCATTTAGTCATAGAT
LCR-2480-R	GAAACCTTTAGAATAAGATTTATTAAATC
Max2-1F	GGAGAATTTTTGACGCCATT
Max2-1R	GTGGTGGCCAATAATCAAGC
Max1-SNP-F	GACAAGAAGTCTTTTGAGTC
Max1-SNP-R	TGAAAGAGGTACCGGGAACA
MIR164A12	GCTAGTCAAGAACAACGAAAAA
MIR164A13	TATAAAGATTTAATCAACCGTC
MIR164A18	TTTCGTTGTTCTTGACTAGCTCTTC

MIR164A532f	TGGAGAAGCAGGGCACGTGCA
PlasmidF	CACGACGTTGTAAAACGACGGCCAG
pGPTV-rev2	TCCATAAAACCGCCCAGTC
pGPTV-FOR	CCGCAACGATTGAAGGAGCC
pGPTV-REV	AATAGCCTCTCCACCCAAGC
p35S-F	CGCAAGACCCTTCCTCTATA
t35S-R	CCTTATCGGGAAACTACTCACACAT

SSLP markers used for molecular mapping:

Name	**Primer sequence in 5’ to 3’ orientation**
F21M12-F	GGC TTT CTC GAA ATC TGT CC
F21M12-R	TTA CTT TTT GCC TCT TGT CAT TG
ciw12-F	AGG TTT TAT TGC TTT TCA CA
ciw12-R	CTT TCA AAA GCA CAT CAC A
ciw1-F	ACA TTT TCT CAA CCT TAC TC
ciw1-R	GAG AGC TTC TTT ATT TGT GAT
nga280-F	CTG ATC TCA CGG ACA ATA GTG C
nga280-R	GGC TCC ATA AAA AGT GCA CC
nga111-F	TGT TTT TTA GGA CAA ATG GCG
nga111-R	CTC CAG TTG GAA GCT AAA GGG
ciw2-F	CCC AAA AGT TAA TTA TAC TGT
ciw2-R	CCG GGT TAA TAA TAA ATG T
ciw3-F	GAA ACT CAA TGA AAT CCA CTT
ciw3-R	TGA ACT TGT TGT GAG CTT TGA
nga1126-F	CGC TAC GCT TTT CGG TAA AG
nga1126-R	GCA CAG TCC AAG TCA CAA CC
nga168-F	TCG TCT ACT GCA CTG CCG
nga168-R	GAG GAC ATG TAT AGG AGC CTC G
ciw11-F	CCC CGA GTT GAG GTA TT
ciw11-R	GAA GAA ATT CCT AAA GCA TTC
nga162-F	CAT GCA ATT TGC ATC TGA GG
nga162-R	CTC TGT CAC TCT TTT CCT CTG G
ciw4-F	GTT CAT TAA ACT TGC GTG TGT
ciw4-R	TAC GGT CAG ATT GAG TGA TTC
nga6-F	TGG ATT TCT TCC TCT CTT CAC
nga6-R	ATG GAG AAG CTT ACA CTG ATC
ciw5-F	GGT TAA AAA TTA GGG TTA CGA
ciw5-R	AGA TTT ACG TGG AAG CAA T
ciw6-F	CTC GTA GTG CAC TTT CAT CA
ciw6-R	CAC ATG GTT AGG GAA ACA ATA
ciw7-F	AAT TTG GAG ATT AGC TGG AAT
ciw7-R	CCA TGT TGA TGA TAA GCA CAA
nga1107-F	GCG AAA AAA CAA AAA AAT CCA
nga1107-R	CGA CGA ATC GAC AGA ATT AGG
CTR1-F	CCA CTT GTT TCT CTC TCT AG
CTR1-R	TAT CAA CAG AAA CGC ACC GAG
ciw8-F	TAG TGA AAC CTT TCT CAG AT
ciw8-R	TTA TGT TTT CTT CAA TCA GTT
PHYC-F	CTC AGA GAA TTC CCA GAA AAA TCT
PHYC-R	AAA CTC GAG AGT TTT GTC TAG ATC
ciw9-F	CAG ACG TAT CAA ATG ACA AAT G
ciw9-R	GAC TAC TGC TCA AAC TAT TCG G
ciw10-F	CCA CAT TTT CCT TCT TTC ATA
ciw10-R	CAA CAT TTA GCA AAT CAA CTT

Molecular markers for identification of mutant alleles:

Allele	Marker	Polymorphism
las-4	AtLs3070R+AtLS2599F AtLs2593muR+AtLS2349F	AtLs3070R+AtLS2599F produces a 471 bp Wt product, AtLs2593muR+AtLS2349F produce a 244 bp *las-4* band.
max1-1	Max1SNPF+Max1SNPR	CAPS marker producing a 125 bp wt and 85 bp *max1* product on digestion with AluI.
max2-1	Max2-1F+Max2-1R	CAPS marker prodcing a 206 bp wt and 172 bp max2 product on digestion with TasI.

2.2 EXPENDABLES

The following were the main suppliers of laboratory expendables used during this work:

Incubation tubes and Petri-dishes: Greiner Lobortechnik; Eppendorf-Netheler-Hinz GmbH, Hamburg; Sarstedt AG & Co, Nümbrecht.

Kits for DNA and RNA extraction and purification: Qiagen, Hildesheim

X-ray films: BioMax MR-1, Kodak Co., New York

Nylon membranes: HybondTM-N+, Amersham International plc.

PVDF membranes: Macherey-Nagel GmbH & Co.KG, Düren

2.3 ORGANISMS

2.3.1 BACTERIA

Specific DNA fragments inserted into vector backbones for cloning were transformed using the following chemical competent cells (Hanahan, 1983) or electrocompetent cells from Invitrogen GmbH:

DH5alpha: F-*end* A1 *hsd*R17 (rk-, mk+) *gyr*A96 *rel*A1 *sup*E44 L- *rec*A1 80dlacZM15 D (*lac*ZY AargF) U196

DB3.1: F-*gyr*A462 *end* A1 D(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rsp*L20(Smr) *xyl*-5 l- *leu mtl*-1

Agrobacterium tumefaciens was used for transformation of plants.

GV3101: Virulence Plasmid: pMP90 (Koncz und Schell, 1986)

Selection markers: Rifampicin, Gentamycin and Kanamycin

2.3.2 Plants

The experiments described in this work were carried out on model plant *Arabidopsis thaliana*, belonging to the ecotypes Columbia, Landsberg, Wassilewskija, Zurich-0 and Enkheim-2. Seeds for the different ecotypes were obtained from the Nottingham Arabidopsis Stock Centre (NASC).

The following *Arabidopsis thaliana* mutants were used during the course of this work:

Mutant	Allele	Type of mutation	Genetic background	Source
cup-shaped cotyledon1	*cuc1-1*	semi-dominant allele; carries an endogenous transposon (Tag1) and a maize transposon (Activator AC).	L*er*	NASC N3869
	5mCUC1	Dominant allele; resistant to miR164 regulation due to presence of five additional mismatches in the miRNA binding site.	Ws	Mallory *et al.,* 2004
cup-shaped cotyledon2	*cuc2-1*	semi-dominant allele; carries an endogenous transposon (Tag1) and a maize transposon (Activator AC).	L*er*	NASC N3870
	CUC2g-m4	Dominant allele; resistant to miR164 regulation due to presence of four additional mismatches in the miRNA binding site.	Ws	Nikovics *et al.,* 2006
cup-shaped cotyledon3	*cuc3-2*	Null allele; T-DNA insertion in the second exon.	Ws	Versailles T-DNA line; Vroemen *et al.,* 2003.
more axillary shoots1	*max1-1*	Recessive allele; Line V367 from the Arabidopsis Information Service mutant collection.	Introgressed from En-2 to Col.	NASC N754 (O .Leyser)
mir164a	*mir164a-4*	Null allele; T-DNA insertion causing a 24 bp deletion downstream from the miRNA164a.	Col	SALK SM333570
mir164b	*mir164b-1*	Null allele; T-DNA insertion in the hairpin precursor of miR164b.	Col	SALK N636105
lateral suppressor	*las-4*	Null allele; frameshift arising from deletion of 20 nucleotides and addition of a single nucleotide in the begining of the ORF.	Col	Greb *et al.,* 2003.
	las-11	T→ G change at base 393 in the ORF resulting in the introduction of a stop codon.	L*er*	Y. Eshed

2.3.3 Vectors

2.3.3.1 *E. coli* vectors

For insertion and cloning of DNA fragments into *Escherichia coli* the following vectors were used:

pGEM4Z - Vector for cloning and transcription of DNA fragment under the T7 Promotor (Promega).

pGEM-T - Vector for cloning of PCR products and their transcription under the T7 Promotor (Promega).

pDONR201 - Vector for cloning of DNA-Fragmenten for use in Gateway System (Invitrogen).

pGW-nos - Vector for cloning of DNA-Fragmenten for use in Gateway System (Gateway Technologies).

2.3.3.2 Plant vectors

For transformation of *A. thaliana*, the following binary vectors were used:

pGW-nos - modified ppGII0229 containing R1-R2 sites from Gateway vector in modified pGreen backbone (An *et al.,* 2004) with Helper plasmid pSoup (Hellens *et al.,* 2000)

pGPTV-Bar-*AscI* - GUS carrying binary plant transformation vector (Überlacker *et.al,*1996).

2.4 Computer programmes and databases

DNA sequence analyses were accomplished using the University of Wisconsin GCG software (Genetics Computer Group, 1997) and Clone Manager software package. The databases of National Center for Biotechnology Information (NCBI), Bethesda, USA and the Arabidopsis Information Resource (TAIR) (Huala *et al.,* 2001) were used for DNA sequence searches and comparisons.

2.5 Methods

All general molecular biology laboratory methods not mentioned here are as described by Sambrook und Russell (2001).

2.5.1 Isolation of Genomic DNA

Isolation of genomic DNA from plants for genotyping and segregation analyses was done using the quick-prep protocol (Edwards *et al.,* 1991). High quality genomic DNA for mapping, cloning and genotyping was extracted using the *DNeasy® 96 Plant Kit* (Qiagen, Hilden) and *BioSprint® 96* automated DNA extraction apparatus (Qiagen, Hilden).

2.5.2 Isolation of Plasmid DNA

Plasmid DNA from bacteria and agrobactria were isolated using the *Plasmid Mini and Plasmid Midi* kits (Qiagen, Hilden).

2.5.3 Purification of PCR products

PCR products were cleaned using *Quiaquick PCR Purification* kit (Qiagen, Hilden).

2.5.4 Isolation of RNA from plants

RNeasy Plant Mini Kit (Qiagen, Hilden) was used for isolation of total RNA from plants. Subsequently, the RNA was submitted to DNase digestion and cleaned by use of the *Protocols for RNA cleanup* (Qiagen, Hilden).

2.5.5 cDNA synthesis / RT-PCR

For first strand cDNA synthesis, *SuperscriptTM II Reverse Transcriptase* (Invitrogen, Karlsruhe) was used to transcribe the isolated RNA according to manufacturer's protocol. Approximately 1.5µg of RNA was used for this reaction. 1 to 2µl of the synthesized cDNA was used subsequently for a PCR.

2.5.6 Polymerase Chain Reaction

Unless otherwise specified, PCR reactions were set as following: 5 µl 10xPCR Buffer, 1.5 µl of 50 mM MgCl, 0.5 µl dNTP (25 mM of each nucleotide), 0.2 µl *Taq*-Polymerase and 1 µl of each Primer (10 pmol/µl) in a 50 µl reaction made up with H_2O. 20 ng of DNA was used as starting DNA template. The *Taq* polymerase was synthesized according to the protocol standardized by Pluthero (1993).

Unless specified otherwise, reactions were accomplished using the following PCR programme in a T3 Thermocycler by Biometra or the Biozym Multicycler PTC 225:

1. Denaturation at 95°C for 3 min
2. Denaturation at 94°C for 20 sec
3. Annealing at 60°C for 30 sec
4. Extension at 72°C for 1 min (for each 1 Kb of DNA to be amplified)

5. Extension at 72°C for 5 min
6. Cooling down to 20°C

The steps 2 to 4 were generally cycled 34 times.

For the amplification of DNA fragments for cloning, a *Pfu* polymerase (Invitrogen, 2.5 U/µl), which exhibits a synthesis speed of 500 BP/MIN and possesses a 5' to 3'-exonuclease activity, was used. The PCR products thus amplified do not have 3'-dA-nucleotid overhang.

2.5.7 Design and cloning of misexpression constructs

i. pSR30 (Gateway Destination vector containing *LAS* ORF)
1.3 KB *LAS* ORF was amplified from genomic DNA using AtLsATGXhoIF + AtLsORFApaIR primer pair, and cloned into the multiple cloning site (MCS) of a modified Gateway destination vector, pGW-nos (An *et al.,* 2004) containing a pGRERN backbone as an XhoI-ApaI fragment downstream of the attR-attR cassette, to produce pSR30.

ii. pSREV1 (*STM::LAS*)
A 4.54 Kb *STM* promoter sequence preceeding the ATG was amplified using attBSTMPrF + attBSTMPrR primer pair and cloned into a gateway donor vector, pDONR207, with BP clonase reaction. The entry clone thus formed, pENT-STM, was cloned into pSR30 with LR clonase reaction, to produce the expression vector pSREV1 (*STM::LAS*).

iii. pSREV2 (KNAT1::LAS)
A 1.48 Kb *KNAT1* promoter sequence preceeding the ATG was amplified using attBKnat1PrF + attBKNat1PrR primer pair and cloned into a gateway donor vector, pDONR207, with BP clonase reaction. The entry clone thus formed, pENT-KNAT1, was cloned into pSR30 with LR clonase reaction, to produce the expression vector pSREV2 (*KNAT1::LAS*).

iv. pSREV3 (*UFO::LAS*)
A 3.79 Kb *UFO* promoter sequence preceeding the ATG was amplified using the attBUFOPrF + attBUFOPrR primer pair and cloned into a gateway donor vector, pDONR207, with BP clonase reaction. The entry clone thus formed, pENT-UFO, was cloned into pSR30 with LR clonase reaction, to produce the expression vector pSREV3 (*UFO::LAS*).

v. pSR47 (*LAS::GAI*)

A 1.5 Kb LAS 5' promoter sequence was amplified from pAE42 (Eicker, 2005) using the primer pair PlasmidF + AE42-1522R and cloned back into XhoI + Avr II digested pAE42, to produce pSR40 vector. *GAI* ORF amplified from pTG27a (Greb, 2003) using GAI-ATG-Acc65IF + GAI-STOP-AvrIIR primer pair and cloned into Acc65I + AvrII digested SR40 as an Acc65I-AvrII fragment, to produce pSR41, which is a *LAS::GAI* construct in an assembly vector. This vector was introduced into a pGJ2148 (modified pGEM) binary vector as a PstI-BsphI fragment, to form pSR47 transformation vector.

vi. SR65 (*LAS::GAI-ΔDELLA*)

Genomic *gai-1* ORF was amplified using GAI-ATG-Acc65IF + GAI-STOP-AvrIIR primer pair and cloned into Acc65I + AvrII digested SR40 as an Acc65I-AvrII fragment, to produce pSR49, a *LAS::GAI-ΔDELLA* construct in an assembly vector. This vector was introduced into a pGPTV-Bar-AscI binary vector as an AscI fragment, to form pSR65 plant transformation vector.

2.5.8 RNA *IN-SITU* HYBRIDIZATION

i. Description of probes used

The *LAS* probe contained the nucleotides 2 to 1348 relative to the ATG (Greb *et al.,* 2003) and the *RAX1* probe was specific for the third exon and contained the nucleotides 934 to 1436 relative to the ATG (Müller *et al.,* 2006). Both these probes were cloned into pGEM vector in antisense orientation relative to the T7 promoter. Linearised plasmids were used as templates for probe synthesis with T7 RNA polymerase.

ii. Preparation of tissue sections and hybridization of probes

Sample preparations and in situ hybridizations of 8-mm sections were done as described by Coen *et al.* (1990) with slight modifications. 0.03% Tween-20 was added to the fixative, and dewatering of the fixed material was done without NaCl. Plant material was embedded in Paraplast+ (Kendall) in the ASP300 tissue processor (Leica). Probes were not hydrolyzed. After the color reaction, slides were mounted in 30% glycerol and photographed using differential interference contrast microscopy.

2.5.9 DNA SEQUENCING

DNA sequencings were accomplished by the MPIZ service unit Automatic DNA Isolation and Sequencing (ADIS) using Applied Biosystem (Weierstadt) *Abi Prism 377 and 3700 Sequenzer* by means of *BigDye-terminator chemistry*.

2.5.10 Incubation conditions for bacteria

E. coli were incubated in LB medium at 37°C over night (Sambrook and Russell, 2001) and Agro bacteria in YEP medium at 28°C for 3 days. Bacteria were plated on respective solid medium containing 1 % agar. Liquid cultures were incubated at 200 RPM on a shaker.

2.5.11 Bacterial transformation and selection

Transformations of vectors in *E.coli* were carried out by heat-shock treatment of chemical competent cells as described by Hanahan (1983). In cases where heat-shock transformations were inefficient, electro-transformations were performed using electro-competent cells (ElectroMAX DH5alpha-E Cells, Invitrogen) as described by Dower *et al.* (1988). Agro bacterial competent cells were transformed using approximately 1µg of template DNA. Subsequently, the cells were incubated for 5 minutes each on ice, in liquid nitrogen and at 37°C. After the addition of 800µl YEP, the cells were incubated on a shaker at 28 °C for 3 hours, and then plated out on solid YEP medium. Depending upon the selection marker of the transformed vectors, Ampicillin (200µg/ml), Kanamycin (50µg/ml), Gentamycin (25µg/ml), Rifampicin (100µg/ml), Chloramphenicol (50µg/ml), Streptomycin (50µg/ml) or Tetracyclin (5µg/ml) were added to the medium.

2.5.12 Plant transformation and selection

The agro bacteria mediated transformations of *A. thaliana* were performed according to the Floral Dip method by Clough and Bent (1998). For the selection of transgenic plants in the T1, 250 mg/l Glufosinat (BASTA®, Hoechst) was sprayed on the plants 14, 16 and 18 days post germination. For segregation analysis in the T2, the same concentration was sprayed once after the identification of the phenotypes.

2.5.13 Growth conditions for plants

A. thaliana seeds were stratified at 4°C for 2-3 days before sowing, so as to obtain uniform germination rate. Plants were grown at a daytime temperature of 20 - 25°C and a night temperature of 10 - 15°C in the greenhouse, or in a growth chamber (Mobylux GroBanks, CLF Plant Climatics, Germany) at a daytime temperature of 23°C and a night temperature of 18°C. Plants were either grown to maturity in short days (8 h light, 16 h darkness) or long days (16 h light, 8 h darkness), or were grown for the first 28 days in short day conditions, and then shifted to long day conditions to facilitate bolting and flowering. In case of T1 transgenic plants subjected to BASTA selection, the trays sown with seeds were first placed in a cold chamber at 4°C for a few days and then directly shifted to long day conditions (16 h light, 8 h darkness).

2.5.14 Crossing of plants

Unless otherwise mentioned, reciprocal crosses were made in each case. To prepare the pistil as pollen acceptor for crosses, young inflorescences were selected. The meristem and any open flowers were removed from the selected inflorescence. Un-open floral buds were priced open with fine forceps, and sepals, petals and stamen were carefully removed. The naked unfertile stigma was subsequently allowed to visibly develop for two days. Young, recently opened flowers of the pollen donor were dusted over the prepared stigma. The stigmata were inspected for the presence of yellow pollen dust. The F1 of the crosses were examined in each case for the occurrence of the wild-type phenotype. In the case of homozygous sterile mutations, heterozygous plants were selected for crossing from a segregating population. The heterozygosity of the plant was confirmed either by a molecular marker or by phenotypic analysis of its descendants.

2.5.15 Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was performed with assistance from Rolf-Dieter Hirtz on a DSM 940 (Zeiss). Tissue were first frozen in liquid nitrogen and subsequently coated with a gold layer under vacuum.

3 RESULTS

3.1 ROLE OF *CUP-SHAPED COTYLEDON* GENES AND *MICRORNA164* IN THE REGULATION OF AXILLARY MERISTEM DEVELOPMENT

Lateral (axillary) meristems are initiated in the axils of leaf primordia. It has been suggested that the identity of leaf axils is established by an overlap of specific transcription patterns defined by the SAM and the leaf primordia. In *Arabidopsis*, the axillary region is established during the early stages of leaf primordium development and is marked by the expression of a specific set of genes. Transcripts of *LAS, RAX1, RAX3,* and *LATERAL ORGAN BOUNDARY (LOB)* accumulate in similar domains in the developing leaf axil (Greb *et al.,* 2003; Müller *et al.,* 2006; Keller *et al.,* 2006; Shuai *et al.,* 2002), where axillary meristems will form. Expression of these genes may be a prerequisite for the initiation of new meristems.

Another set of genes that show a very characteristic expression in the axils of developing leaves are the *CUP-SHAPED COYLLEDON 1 (CUC1), CUC2* and *CUC3* genes (Greb, 2003; Vromen *et al.,* 2003; Keller *et al.,* 2006; Hibara *et al.,* 2006). The *CUC* genes are members of the plant specific family of NAC domain putative transcription factors (Ernst *et al.,* 2004; Olsen *et al.,* 2005), which consists of about 100 members in *Arabidopsis* (Riechmann *et al.,* 2000; Duval *et al.,* 2002). *CUC1* and *CUC2* are known to function redundantly in the establishment of cotyledon- and floral-organ boundaries and in the initiation of the SAM through the regulation of *STM* expression (Aida *et al.,* 1997; Long *et al.,* 1996 and Aida *et al.,* 1999). The discovery of a third *CUC* gene in *Arabidopsis*, *CUC3* (Vroemen *et al.,* 2003), which encodes a protein with a high homolgy to CUC1 and CUC2, uncovered an additional level of redundancy in the function of these genes. Any combinations of *cuc1-1, cuc2-1*, and *cuc3-2* lead to the formation of cup-shaped cotyledons and arrested growth. This renders it difficult to uncover redundant functions of these genes in shoot development. In *Antirrhinum majus*, however, single mutations in the *CUPULIFORMIS* gene, an orthologue of *CUC1* and *CUC2* of *Arabidopsis*, not only causes striking fusion phenotypes in nearly all shoot organs, but also frequently displays absence of axillary shoot formation from the leaf axils (Weir *et al.,* 2004). This observation hints at a possible role for *CUC* genes in axillary meristem development.

microRNA164 (miR164) post-transcriptionally regulates *CUC1* and *CUC2* expression by cleavage of their transcripts (Rhoades *et al.,* 2002; Kasschau *et al.,* 2003). The constitutive overexpression of miR164 phenocopies the *cuc1 cuc2* double mutant by downregulating *CUC1* and *CUC2* transcript accumulation (Laufs *et al.,* 2004; Mallory *et al.,* 2004). Plants expressing miR164-resistant versions of *CUC1* (Mallory *et al.,* 2004; Baker *et al.,* 2005) or *CUC2* (Laufs *et al.,* 2004; Nikovics *et al.,* 2006) show severe

alterations in embryonic, vegetative and floral development due to an enlargement of various boundary domains. miR164 is encoded by three genes: *MIR164A, MIR164B* and *MIR164C* (Reinhart *et al.,* 2002; Bonnet *et al.,* 2004; Jones-Rhoades and Bartel, 2004; Wang *et al.,* 2004).

In this study, the role of the *CUC* genes in regulating axillary meristem development was analysed. Plant harbouring null alleles of the three *CUC* genes were characterised for axillary bud formation. Overexpression of miR164 in wild-type and *cuc3-2* mutant backgrounds was analysed to uncover redundant roles of *CUC1* and *CUC2* in the regulation of axillary shoot formation. Analysis of *mir164* knockout mutants and transgenic plants carrying miR164-resistant alleles of *CUC1* and *CUC2* demonstrated that miR164 regulation of *CUC1/CUC2* transcript accumulation is required to achieve a normal shoot branching pattern.

3.1.1 Analysis of the pattern of axillary meristem formation in knock-out mutants of *CUC1, CUC2 and CUC3*

Axillary meristems develop in the boundary zone between the SAM and leaf primordia overlapping with the expression domains of the *CUC* genes. To assess the role of the three *Arabidopsis CUC* genes in the development of lateral meristems, the patterns of axillary bud formation in plants homozygous for the null alleles *cuc1-1, cuc2-1* and *cuc3-2* were analysed in short- and long-day growth conditions. The *cuc1-1* and *cuc2-1* mutants displayed no changes in axillary bud development compared to the L*er* controls in either growth conditions (Fig. 3.1-1a). Axillary bud formation was observed in these mutants in the axils of all leaves during vegetative as well as reproductive phases of development. *CUC1* and *CUC2* have been shown to function redundantly in controlling organ boundary formation and initiation of the SAM (Aida *et al.,* 1997; Long *et al.,* 1996). However, the early growth arrest in *cuc1 cuc2* double mutants (Aida *et al.,* 1997) rendered it impossible to examine the pattern of shoot branching in these double mutants.

Stereomicroscopic analysis of leaf axils of *cuc3-2* revealed that in comparison to the Wassilevskija (Ws) wild-type, the mutants display a strong reduction in the number of axillary buds originating from the axils of rosette leaves (Fig. 3.1-1b). Regardless of growth conditions, most of the rosette leaves formed in the early and middle phase of vegetative development did not support the formation of axillary buds. However, depending upon the growth conditions, a varying number of axillary buds developed from the rosette leaf axils formed late in vegetative development (Fig. 3.1-1g, 3.1-1l and 3.1-1m). *cuc3-2* homozygous plants grown to maturity in short days displayed the most severe branching defect.

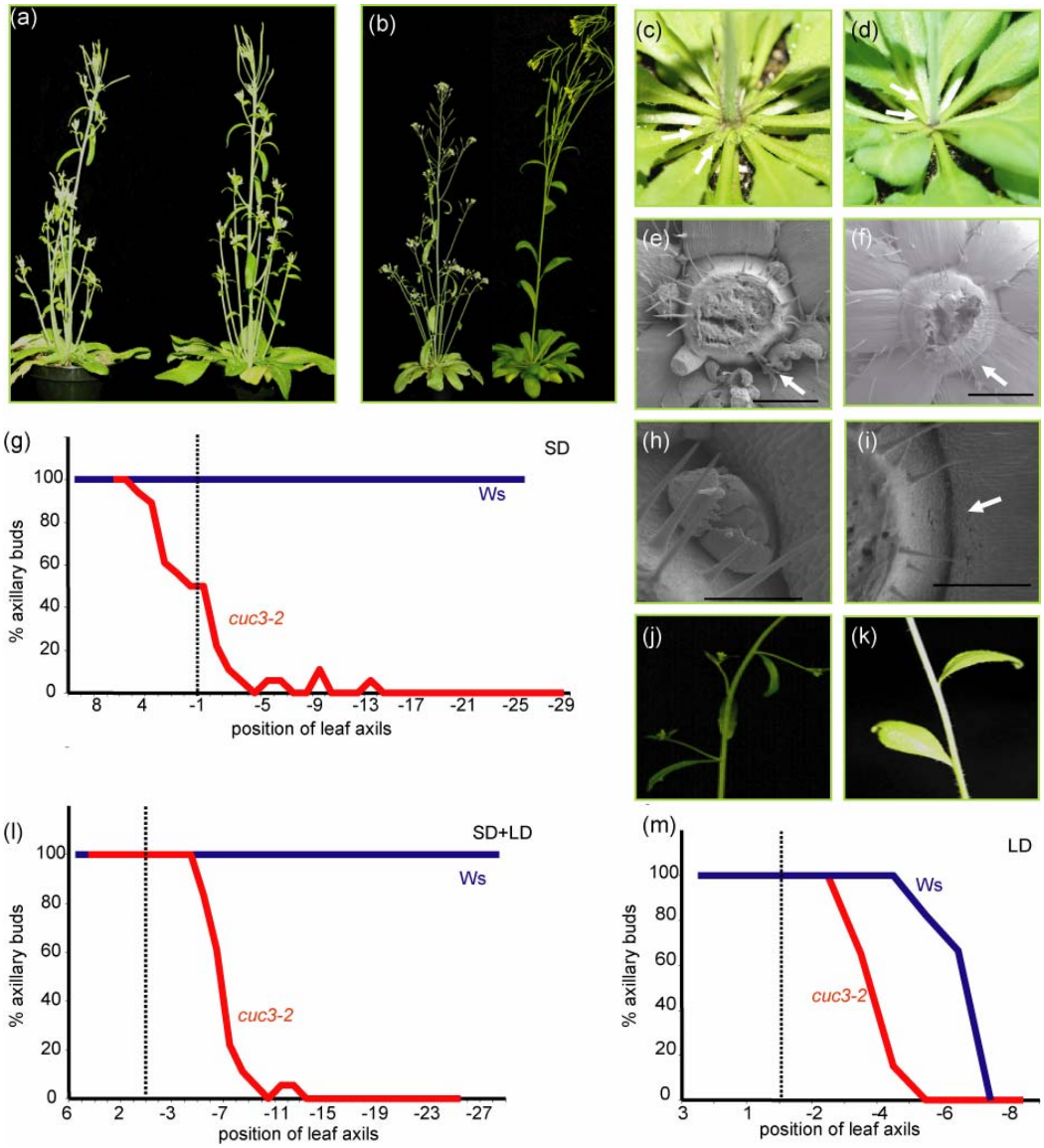


Fig. 3.1-1: Growth habit and branching pattern of *cuc1, cuc2 and cuc3*. (a) Growth habit of *cuc1-1* (left) and *cuc2-1* (right) grown to maturity in SD. (b) Growth habit of a Ws wild-type (left) and a *cuc3-2* mutant (right) grown to maturity in SD. (c and d) Close-ups of rosette leaf axils of a Ws wild-type plant (c) and the *cuc3-2* (d) mutant. (e and f) SEM micrographs of the top of the rosette in a Ws wild-type plant (e) and the *cuc3-2* (f) mutant. The scar of the excised main bolt is seen in the middle. Bars = 25 μm. (h and i) SEM micrograph of the axil of a single rosette leaf in a Ws (h) and a *cuc3-2* (i) plant. (j and k) Close ups of cauline leaf axils of a Ws wild-type plant (j) and the *cuc3-2* (k) mutant. (g, l and m) Graphic representation of axillary bud formation in *cuc3-2* plants in comparison with Ws wild-type plants. Plants were grown either to maturity in short day conditions (g), or for 34 days in short photoperiods and subsequently for 15 days in long photoperiods to induce flowering (l), or to maturity under long day conditions (m). Leaf axils of plants were examined under a binocular microscope. Genotypes are indicated next to the graphs. The dotted line represents the position of the youngest rosette leaf. To the right of the dotted line are positions of progressively older rosette leaves, and to the left are positions of progressively younger cauline leaves. The percentage values indicate the proportion of plants analysed (n=20) that formed an axillary bud in a specific position along the shoot axis.

These plants were characterised by an almost complete block in axillary bud formation during vegetative development extending acropetally into the early cauline leaf axils (Fig. 3.1-1d, 3.1-1g and 3.1-1k). Defects in axillary bud formation in cauline leaf axils were not observed in *cuc3-2* mutants grown under short day conditions and shifted to long days to

induce flowering, or grown to maturity in long photoperiods (Fig. 3.1-1l and 3.1-1m). Under both these conditions, also the topmost rosette leaves always supported the formation of axillary buds (Fig. 3.1-1l and 3.1-1m). Closer inspection of the empty leaf axils using a stereomicroscope and scanning electron microscopy (SEM) did not uncover any morphological structure resembling an axillary bud (Fig. 3.1-1f and 3.1-1i). This indicated that the barren leaf axils were due to a failure in axillary meristem initiation rather than to a defect in axillary bud outgrowth. These results suggest that the *CUC3* gene plays an important role in the genetic control of axillary meristem formation in a day-length dependent manner.

3.1.2 Analysis of the effect of overexpression of *MIR164A* and *MIR164B* on the formation of axillary buds

The activity of six *Arabidopsis* NAC-domain genes is predicted to be regulated by miR164 (Schwab *et al.,* 2005). Among these are the genes *CUC1* and *CUC2* (Laufs *et al.,* 2004; Mallory *et al.,* 2004), but not *CUC3*. To uncover a function of *CUC1* and/or *CUC2* in the process of axillary meristem formation that might be masked by *CUC3* activity, the shoot branching pattern of transgenic lines harbouring a *2x35S::MIR164A* or *2x35S::MIR164B* construct, and thereby overexpressing miR164 (Laufs *et al.,* 2004), were characterised in a Ws or a *cuc3-2* background. Similar to what has been previously reported by Laufs *et al.,* (2004) and Mallory *et al*. (2004), one *2x35S::MIR164A* and two *2x35S::MIR164B* transgenic lines analysed in the Ws background displayed fusions of cotyledons, sepals and stamens. However, in both short and long photoperiods, overexpression *of MIR164A* or *MIR164B* in Ws plants did not lead to any deviation from the wild-type branching pattern, neither in the vegetative phase (Fig. 3.1-2b) nor in the reproductive phase (Fig. 3.1-2c) of development.

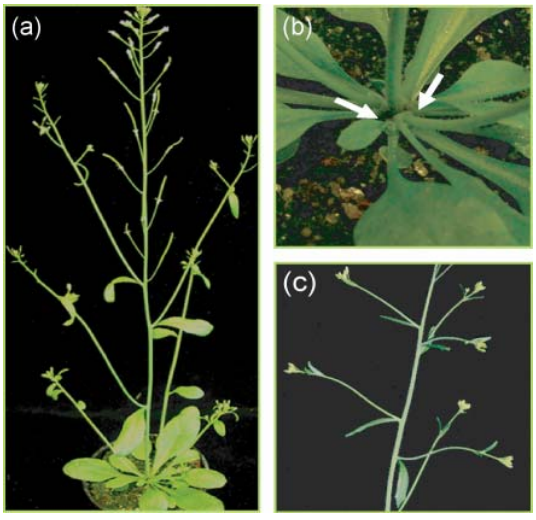


Fig. 3.1-2: Habitus and axillary shoot formation of *2x35S::MIR164B* plants in Ws wt background. (a) 30 day old LD transgenic plant harbouring the *2x35S::MIR164B* construct. (b) Close up of the rosette leaf axils of a 30 d LD *2x3S::MIR164B* plant showing lateral shoot formation (arrows). (c) Close up of cauline leaf axils of a *2x35S::MIR164B* plant developing axillary shoots.

This indicated that the reduction of *CUC1* and *CUC2* activity resulting from miR164 overexpression (Laufs *et al.,* 2004) in these lines did not affect axillary meristem development.Two independent populations of plants homozygous for *cuc3-2* and segregating for *2x35S::MIR164A* (A) or *2x35S::MIR164B* (B), grown in short photoperiods for 30 days and then shifted to long days to induce flowering, were analysed. In both populations, formation of cup-shaped cotyledons (Fig. 3.1-3a) was observed in less than one quarter of the plants (population A: 10/50, population B: 6/48). PCR analysis demonstrated that these seedlings contained the *2x35S::MIR164* T-DNA. The majority of these plants initiated a new SAM in the hypocotyl region below the base of the cotyledons (Fig. 3.1-3b).

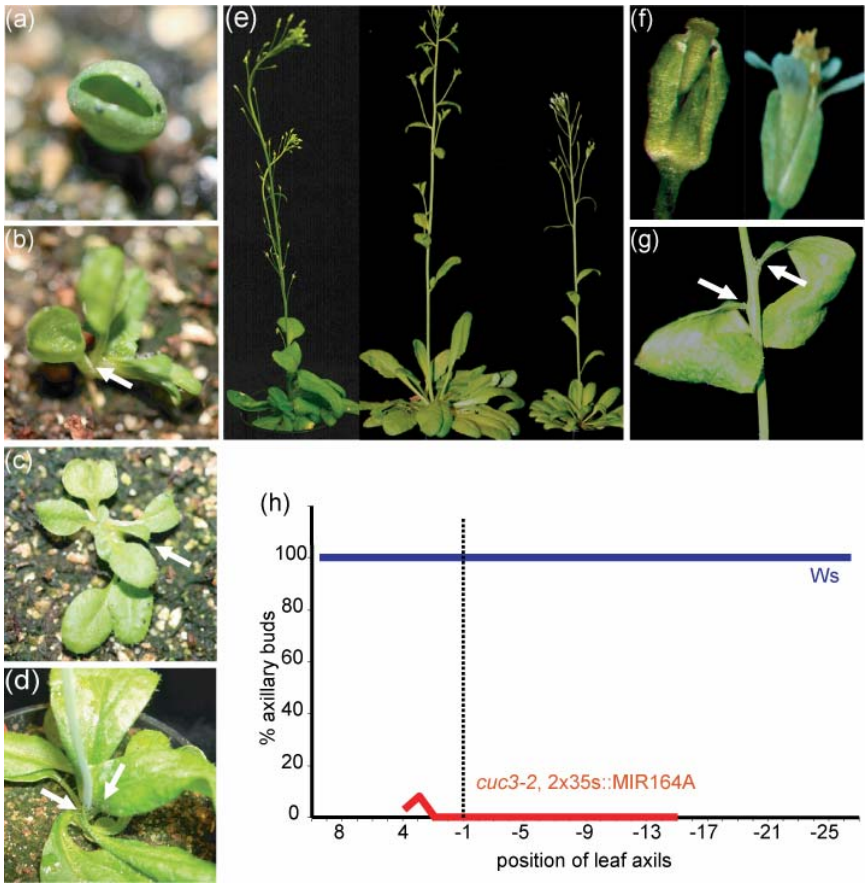


Fig. 3.1-3: Habitus and axillary shoot formation in a *cuc3-2; 2x35S::MIR164A/+* segregating population. (a) 10d old *cuc3-2; 2x35S::MIR164A* plant in SDs showing cup-shaped cotyledon. (b) 20d old *cuc3-2; 2x35S::MIR164A* plant in SDs showing formation of new shoot apex (arrow) at the hypocotyl. (c) 30d old *cuc3-2; 2x35S::MIR164A* plant in SDs developing a rosette (arrow). (d) Rosette leaves fused to main shoot and displaying empty axils (arrows) and distorted phylotaxis in 65d old *cuc3-2; 2x35S::MIR164A* plants in SD. (e) Adult growth habit of the two classes of *cuc3-2; 2x35S::MIR164A* plants which recover from a cup-shaped cotyledon phenotype (left) and which do not display a cup-shaped cotyledon (right) compared to Ws (centre) grown for 65d in SD. (f) Flower from a *cuc3-2; 2x35S::MIR164A* plants which recover from a cup-shaped cotyledon phenotype (left) compared to a Ws wilt-type flower (right). (g) Empty leaf axils (arrows) and curled cauline leaves in 65d old *cuc3-2; 2x35S::MIR164A* plants in SD. (h) Graphical representation of axillary bud formation in the axils of *cuc3-2; 2x35S::MIR164A* plants which recover from a cup-shaped cotyledon phenotype and Ws wild-type plants (n=20), grown to maturity in SDs.

This new SAM then produced rosette leaves (Fig. 3.1-3c) and developed into a flowering shoot (Fig. 3.1-3e, left). However, during vegetative (Fig. 3.1-3d) as well as reproductive (Fig. 3.1-3g) development, these plants failed to develop axillary buds in most of their leaf axils. Some plants produced a single axillary shoot in a cauline leaf axil (Fig. 3.1-3h). In addition to the defect in axillary meristem initiation, these plants were characterised by a reduction in the number of cauline leaves, fusion of leaf petioles to the main axis, distorted phyllotaxis, curled cauline leaves, fused flowers, immature siliques, and dark green colour (Fig. 3.1-3d, 3.1-3e, 3.1-3f, 3.1-3g and 3.1-3h). These plants also produced a low number of rosette leaves (Fig. 3.1-3h). Accordingly, they also flowered slightly earlier than the Ws wild-type. The remaining plants of the two populations (Fig. 3.1-3e, right) were characterised by a shoot branching pattern similar to the *cuc3-2* mutant, but did not show cup-shaped cotyledons, fusions of leaf petioles or a distorted phylotaxis. Molecular analysis using PCR-based markers revealed that these plants segregated for the respective *2x35S::MIR164* construct.

This finding indicated that the *2x35S::MIR164* construct influenced the severity of the *cuc3-2* branching defect in a dose-dependent manner. The enhancement of the *cuc3-2* branching defect by overexpression of *MIR164A* or *MIR164B* suggested a redundant role for *CUC1* and/or *CUC2* in the control of axillary meristem formation.

3.1.3 Analysis of plants expressing miR164-resistant forms of *CUC1* and *CUC2*

To further elucidate the function of *CUC1* and *CUC2* in axillary bud formation, miR164-resistant *CUC2* and *CUC1* lines, *CUC2g-m4* (Nikovics *et al.,* 2006) and *5mCUC1* (Mallory *et al.,* 2004) respectively, were analysed. In transgenic *CUC2g-m4* plants the miR164 binding site in the *CUC2* gene, driven by its endogenous promoter sequence, contains four additional mismatches compared to wild-type *CUC2*, thus insulating it from miR164 regulation and leading to accumulation of high levels of *CUC2* mRNA (Nikovics *et al.,* 2006). Two independent *CUC2g-m4* transgenic lines were analysed for their shoot phenotypes. During the reproductive phase, *CUC2g-m4* plants frequently developed clusters of cauline leaves (Fig. 3.1-4a and 3.1-4b) with highly reduced internodes. These plants are characterised by serrated leaf margins (Fig. 3.1-4a, 3.1-4d and 3.1-4f) and are very reminiscent of leaves observed in *mir164a* mutants (Nikovics *et al.,* 2006). Most interestingly, microscopic analysis revealed that *CUC2g-m4* plants developed accessory side-shoots in the axils of cauline leaves (Fig 3.1-4b, 3.1-4c) and rosette leaves (Fig 3.1-4e). These additional side-shoots developed in the zone between the primary side-shoot and the leaf and appeared later in development than the primary side-shoot. During vegetative development in short photoperiods, accessory buds were formed predominantly in the axils of late rosette leaves (Fig. 3.1-4g). In the reproductive phase, the formation of accessory buds was found to be enhanced more in early cauline leaf

axils than in late cauline leaf axils (Fig. 3.1-4g). Formation of accessory buds was also observed in *CUC2g-m4* plants grown in long photoperiods (Fig. 3.1-4h). Accessory side-shoots were also found at a low frequency in cauline leaf axils of the Ws wild-type (Fig. 3.1-4g and 3.1-4h).

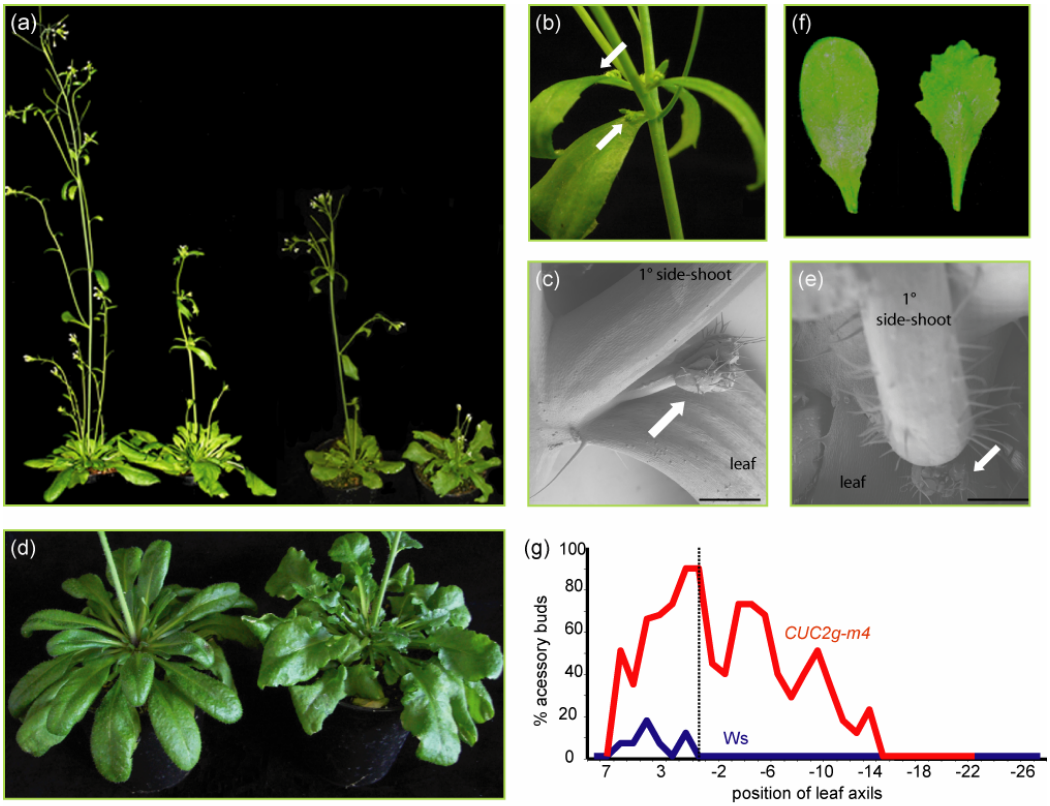


Fig. 3.1-4: Habitus and branching pattern in *CUC2g-m4*. (a) 65d short day grown Ws wild-type (right) and three *CUC2g-m4* plants (left). (b) Close-up of a *CUC2g-m4* shoot showing highly shortened internodes and accessory side-shoots in the axils of cauline leaves (arrows). (c) SEM micrograph of a *CUC2g-m4* cauline leaf axil showing an accessory bud (arrow). Bar = 125 μm. (d) Comparison of the rosettes of Ws (left) and *CUC2g-m4* (right) plants grown for 65d in short days. (e) SEM micrograph of a *CUC2g-m4* rosette leaf axil showing an accessory bud (arrow). Bar = 250 μm. (f) Serrated leaf margins in a *CUC2g-m4* rosette leaf (right) compared to a Ws rosette leaf. (g and h) Graphic representation of accessory bud formation in leaf axils of *CUC2g-m4* and Ws plants (n=20) grown either for 30 days in short photoperiods and subsequently for 35 days in long day conditions (g) or grown to maturity in long days (h).

Four independent *5mCUC1* transgenic lines were analysed for characteristic shoot phenotypes. The number of mismatches between the *CUC1* RNA and miR164 was increased from three in wild-type to eight in *5mCUC1* by the introduction of five silent mutations in the miR164-complementarity sequence of *CUC1*, and the construct was transformed into wild-type plants under the control of the native CUC1 5' and 3' regulatory sequences (Mallory *et al.,* 2004). The habitus of *5mCUC1* lines was not very different

from wild-type plants. In a very low percentage of plants, the internodes between the older cauline leaves were shortened to produce an aerial-rosette phenotype (Fig. 3.1-5a). However, unlike in the miR164-reistant *CUC2* transgenic plants, no deviation in leaf blade morphology was observed in *5mCUC1* lines.

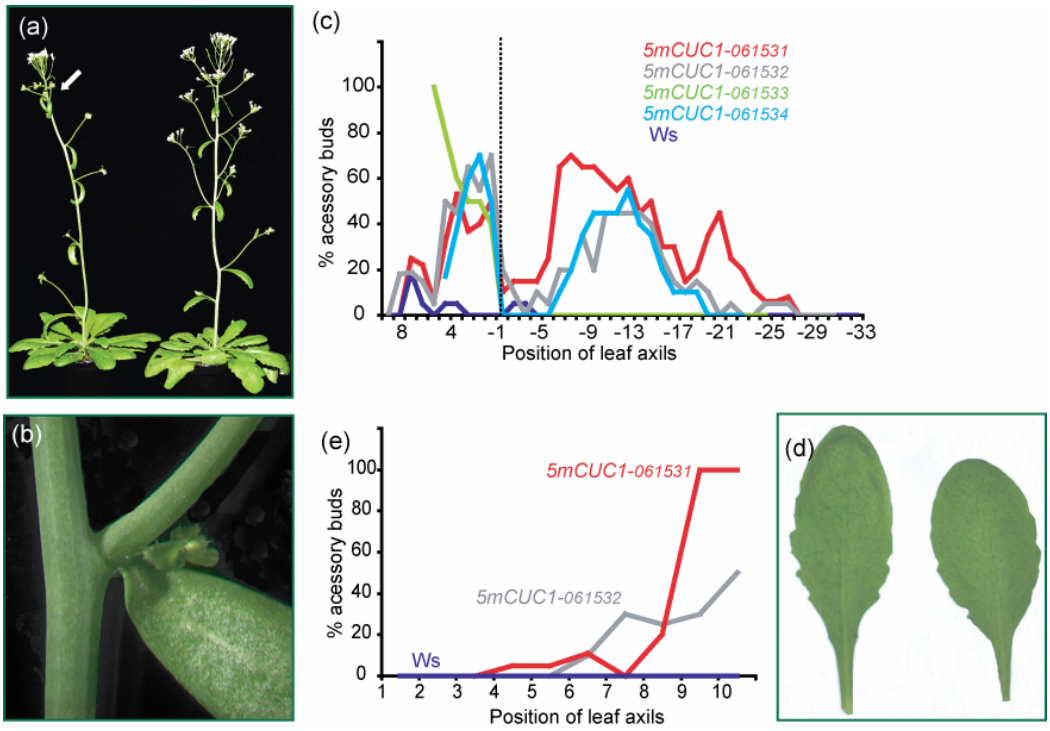


Fig. 3.1-5: Habitus and branching pattern in *5mCUC2g*. (a) Habitus of a *5mCUC2g* (left) and a Ws (right) plant grown for 42d in SD and then shifted to LD to induce flowering. (b) Close-up of a *5mCUC2g* node showing accessory bud (arrow) in the axils of a cauline leaf. (c) Graphic representation of accessory bud formation in leaf axils of *5mCUC2g* and Ws plants (n=20) grown for 42 days in short photoperiods and subsequently for 15d under long day conditions (d) Comparison of a *5mCUC2g* (left) and a Ws (right) rosette leaf. (e) Graphical representation of the proportion of cauline leaves subtending a flower instead of an axillary shoot in two *5mCUC2g* transgenic lines (061531 and 061532). n=20.

The leaf margins in these plants were not more serrated than in the Ws wild-type (Fig. 3.1-5d). Formation of accessory buds was, however, observed in a large number of cauline leaf axils of *5mCUC1* plants (Fig. 3.1-5b). Closer inspection under a stereomicroscope revealed that accessory buds were formed in most of the older cauline leaf axils and a low percentage of younger cauline leaf axils in all the four transgenic lines (Fig. 3.1-5b, 3.1-5c). In the rosette leaf axils, formation of accessory buds was more prominent during the middle phase of vegetative development in three of the four *5mCUC1* lines characterised (Fig. 3.1-5c). In one *5mCUC1* line (061533), no accessory bud formation was observed during vegetative development (Fig. 3.1-5c). Apart from accessory bud formation, in two of these *5mCUC1* lines (061531 and 061532), many of the cauline leaf axils supported the formation of a flower instead of an axillary shoot (Fig. 3.1-5e). This tendency was higher in the axils of younger cauline leaves than in older cauline leaves. Thus, misregulation of *CUC1* and *CUC2* activities by making them

resistant to miR164 regulation results in the formation of accessory buds, albeit in different zones in the rosette. Accessory bud formation is more prominent in the late and middle phases of vegetative development in *CUC2g-m4* plants; whereas in *5mCUC1* plants formation of accessory buds are more pronounced in the middle zone of rosette leaf axils. Taken together with the earlier observation that no axillary meristems are formed in *cuc3-2, 2x35S::MIR164* plants, these results show that activities of both *CUC1* and *CUC2* genes are redundantly required for axillary meristem development.

3.1.4 Analysis of axillary bud formation in *mir164* mutants

Since interference in the miR164 regulation of *CUC1* and *CUC2* led to accessory side-shoot formation, the role of miR164 in axillary meristem development was further investigated by studying loss of function mutants of the *MIR164* genes. Three loci have been predicted to code for miR164. These are *MIR164A, MIR164B* and *MIR164C* (Reinhart *et al.,* 2002; Bonnet *et al*. 2004; Jones-Rhoades and Bartel, 2004; Wang *et al.,*

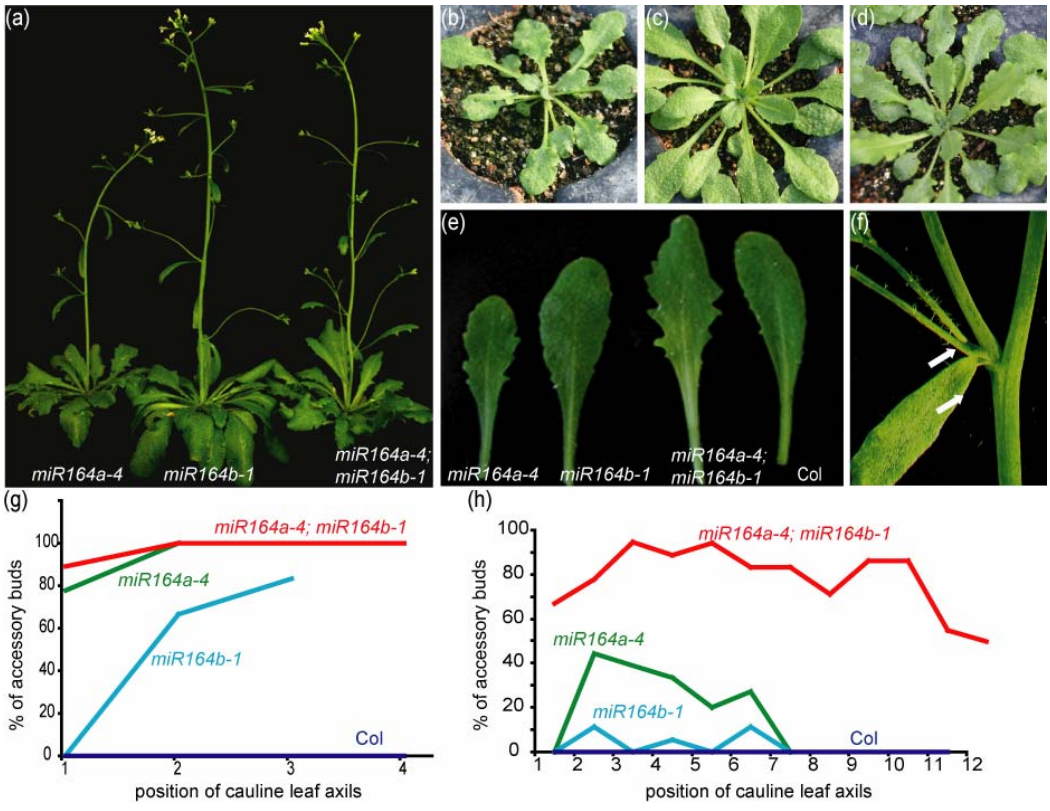


Fig. 3.1-6: Habitus and branching pattern in *mir164* mutants. (a) Comparison of growth habit of *mir164a-4*, *mir164b-1,* and *mir164a-4 mir164b-1* double mutants grown for 35d in short photoperiods and subsequently shifted to long days to induce flowering. (b)-(d) 30d old *mir164a-4* (b), *mir164b-1* (c), and *mir164a-4 mir164b-1* (d) seedlings grown in short days. (e) Comparison of leaf morphology of *mir164a-4*, *mir164b-1,* and *mir164a-4 mir164b-1* plants with Col wild-type. (f) Close-up of a shoot of a *mir164a-4 mir164b-1* plant grown for 35d in LD conditions, which developed two accessory side-shoots (arrows) from a single cauline leaf axil. (g) and (h) Graphic representation of accessory side-shoot formation in *mir164a-4, mir164b-1,* and *mir164a-4 mir164b-1* plants grown 42d in LD (g) and for 35d in short photoperiods and subsequently 14d in LD(h). n=20.

2004). During this study, the roles of two of these genes, *MIR164A* and *MIR164B,* in the development of axillary meristems were assayed by studying the loss of function alleles *mir164a-4* (Nikovics *et al.,* 2006), *mir164b-1* (Mallory *et al.,* 2004), and the double mutant *mir164a-4 mir164b-1*(Fig. 3.1-6a). As reported recently by Nikovics *et al.* (2006), the leaves of *mir164a-4* mutants were highly serrated. This phenotype was clearly visible early in development beginning from four weeks old seedling stage (Fig. 3.1-6b and 3.1-6e). *mir164b-1* displayed no severe alterations in leaf blade morphology when compared to the wild-type leaf (Fig. 3.1-6c and 3.1-6e). The severity of the serration phenotype seen in *mir164a-4* was increased in the *mir164a-4 mir164b-1* double mutants (Fig. 3.1-6d and 3.1-6e).

The shoot branching phenotypes of plants homozygous for the loss of function alleles *mir164a-4*, *mir164b-1* and *mir164a-4 mir164b-1* were characterised under long day and short day conditions (Fig. 3.1-6a, 3.1-6g and 3.1-6h).

Under short day conditions *mir164b-1* mutants showed a slight increase in accessory side-shoot formation from cauline leaf axils as compared to Col wild-type plants (Fig. 3.1-6h). *mir164a-4* mutants displayed a more pronounced increase in accessory side-shoot formation and this phenotype was highly accentuated in *mir164a-4 mir164b-1* double mutants (Fig. 3.1-6f and 3.1-6h). In the *mir164a-4 mir164b-1* double mutant almost all the cauline leaf axils harboured an accessory bud. Furthermore, the number of cauline leaves was reduced in *mir164a-4* mutants. Development of accessory side-shoots was also observed in *mir164a-4*, *mir164b-1,* and *mir164a-4 mir164b-1* under long day conditions (Fig. 3.1-6g). This tendency was more pronounced in late than in early cauline leaf axils and *mir164a-4* as well as the double mutant displayed a stronger increase than *mir164b-1*. Accessory side-shoot formation was not observed in the axils of rosette leaves in either day-length condition in any of the *mir164* mutants. These results demonstrated that, *MIR164A* and *MIR164B* redundantly regulate the number of side-shoots formed during reproductive development, with a greater contribution of *MIR164A* than *MIR164B* in this process. These results show that axillary meristem development is regulated by miR164.

3.1.5 Analysis of expression pattern of *LAS* in *cuc3-2*

Important indications about the function of a gene in an organism can be obtained either by studying the phenotype of the appropriate mutant or by the analysis of the pattern of its transcript accumulation. The expression pattern of the genes *CUC1, CUC2* and *CUC3-2* in *Arabidopsis* wild-type plants have been analysed in detail in previous studies. During embryogenesis, *CUC1, CUC2* and *CUC3* mark the boundaries of the cotyledons and the SAM with an overlapping expression pattern; whereas during post-embryonic development, expression of the *CUC* genes is seen in a wide variety of boundaries in the seedling and adult plant, generally separating two proliferating organs or a proliferating

organ or cell from its surrounding cells (Aida *et al.,* 1999; Ishida *et al.,* 2000; Takada *et al.,* 2001; Vroemen *et al.,* 2003). The expression of all the three *CUC* genes is also seen in the axillary region of leaves (Greb, 2003). *CUC1* transcript is seen in the axils of young leaf primordia from P0 to P6/7 in a domain that is about 3-5 cell layers deep, spanning the L1-L3 layers of the SAM, and extends one or two cell layers in the adaxial-abaxial dimension. *CUC1* expression is not detectable in the axils of P7/8 to P20/P21 primordia, but is again found from P21/P22 onwards, which indicates the onset of axillary meristem activity in these leaf axils. *CUC2* mRNA is found in a domain similar to the *CUC1* domain. Different from *CUC1*, *CUC2* transcript is detected in the axils of leaf primordia from P0 to P17. Vroemen *et al*. (2003) and Hibara *et al*. (2006) have shown that during vegetative development *CUC3* transcript also accumulates in a narrow domain in the axils of leaf primordia. Thus, *CUC1, CUC2 and CUC3* transcripts have been reported to accumulate in a band-shaped domain along the adaxial boundary of the leaf primordium. Greb (2003) reported that pattern of transcript accumulation of *CUC1* and *CUC2* in the axils of leaf primordia in a *las-4* mutant background did not deviate from that in the wild-type.

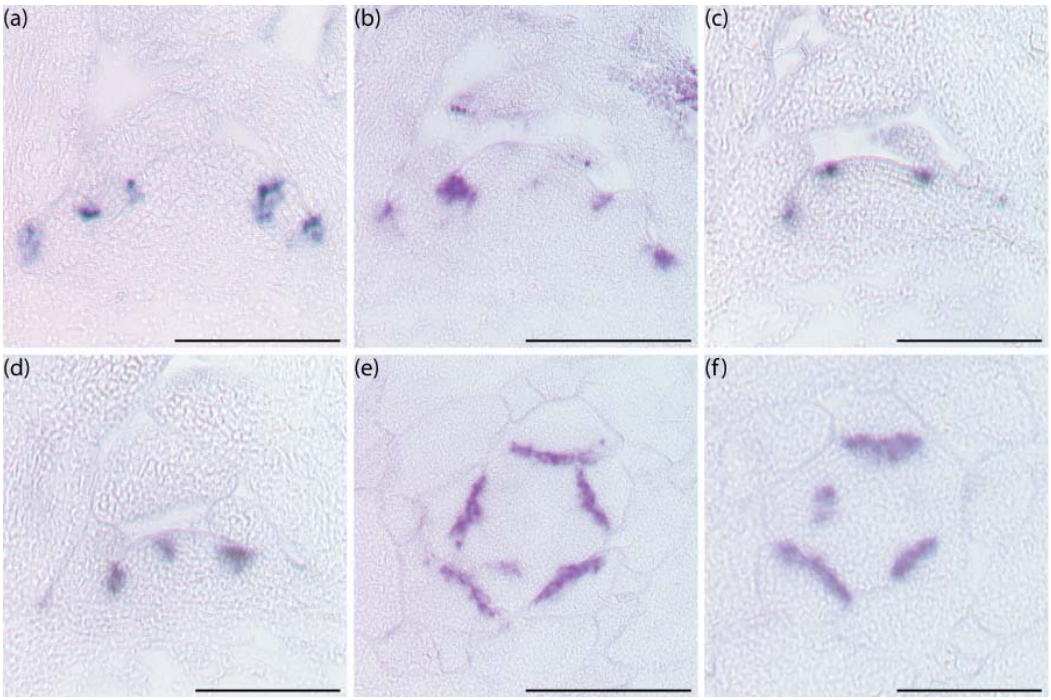


Fig. 3.1-7: Pattern of *LAS* transcript accumulation in the vegetative shoot apex of *cuc3-2*. Longitudinal (a to d) and transverse (e and f) sections through shoot apices of 30 day old Ws (a, e) and *cuc3-2* (b, c, d, f) plants grown under short day conditions were hybridized with a *LAS* antisense probe. (c) and (d) are sections of the same apex, (d) being closer to the centre of the apex than (c). Bar = 200 μm for (a) to (f).

Similarly, expression of *LAS* was also not altered in the apices of *cuc1* and *cuc2* mutants (Greb, 2003). *CUC1* and *CUC2* are known to function redundantly. Since the *cuc1* and *cuc2* mutants are not compromised in axillary meristem formation, and the *cuc1 cu2* double mutant is arrested in development, these *in situ* hybridisation results could not conclusively state the hierarchical order of *LAS* and *CUC* activities.

The *cuc3-2* mutant shows a defect in axillary meristem development in rosette leaf axils under all growth conditions, and in cauline leaf axils when grown in short days (Fig. 3.1-1b, 3.1-1d, 3.1-1f, 3.1-1g, 3.1-1i, 3.1-1k, 3.1-1l and 3.1-1m). Hence expression patterns of two genes known to regulate axillary meristem development, *LAS* and *RAX1*, were monitored by RNA in situ hybriddization experiments on apex sections of *cuc3-2* plants that were grown under short day conditions and fixed 30 days after sowing.

The wild-type expression of *LAS* in the vegetative apex has been described earlier (Greb, 2003; Greb *et al.,* 2003). *LAS* transcripts accumulate in the axils of leaf primordia and leaves from P1 to about P22 in a 3-5 cell layers deep domain covering L1 to L3 layers of the SAM (Fig. 3.1-7a). In transverse sections, *LAS* mRNA accumulates in a band-shaped domain at the adaxial side of the leaf primordium, marking the inner boundary of the primordium (Fig. 3.1-7e). In *cuc3-2* mutants, the expression domain of *LAS* remained unaltered when compared to that in the wild-type (Fig. 3.1-7). *LAS* expression was detected in the axils of leaf primordia as in wild-type (Fig. 3.1-7b, 3.1-7c and 3.1-7d). The expression pattern was restricted to a band shape domain at the adaxial side of the leaf primordium, as visualised in transverse sections (Fig. 3.1-7f).

3.1.6 Analysis of expression pattern of *RAX1* in *cuc3-2*

The *RAX1* gene function is needed for the formation of axillary meristems in the early phase of vegetative development (Keller *et al.,* 2006; Müller *et al.,* 2006). In wild-type plants, *RAX1* transcripts are detected transiently in the axils of leaf primordia from P0 to P10 or P11. This expression domain is three to five cell layers deep and extends across the L1, L2 and L3 layers of the SAM (Keller *et al.,* 2006; Müller *et al.,* 2006; Fig. 3.1-7a).

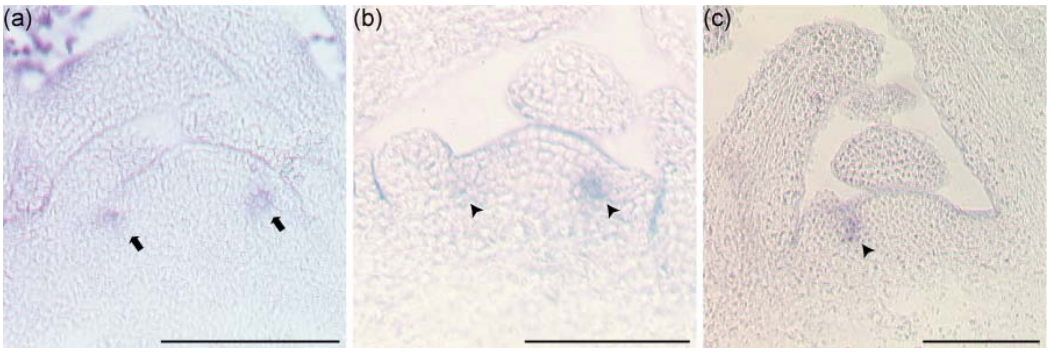


Fig. 3.1-8: Pattern of *RAX1* transcript accumulation in the vegetative shoot apex of *cuc3-2*. Longitudinal sections through shoot apices of 30 day old Ws (a) and *cuc3-2* (b, c,) plants grown under short day conditions were hybridized with a *RAX1* antisense probe. (b) and (c) are sections from two individual *cuc3-2* apices. Bar = 200 μm for (a) to (f).

In transverse sections, the *RAX1* expression is seen in a circular domain at the adaxial center of the boundary between the SAM and the leaf primordia, extending approximately three to five cell layers in the adaxial-abaxial and the tangential dimensions (Keller *et al.,* 2006; Müller *et al.,* 2006).

In *cuc3-2* mutants, no changes in the expression pattern of *RAX1* were observed when compared to that in the wild-type (Fig. 3.1-8). In longitudinal sections through the apex of *cuc3-2* mutants, faint expression of *RAX1* was found in a narrow domain extending downwards from L1-L2 at the border between the SAM and the two youngest primordia (Fig. 3.1-8b and 3.1-8c, arrowheads). The intensity and cellular distribution of the *RAX1* transcript was comparable to that observed in Ws wild-type apices (Fig. 3.1-8a, arrow).

3.2 ANALYSIS OF DEVELOPMENTAL SPECIFICITY OF *LAS* FUNCTION IN THE MERISTEM

LATERAL SUPPRESSOR (LAS) belongs to the plant specific GRAS family of putative transcription factors. The *Arabidopsis* GRAS family consists of 33 genes, and is named after its founding members *GIBBERELLIC ACID-INSENSITIVE (GAI), REPRESSOR of ga1-3 (RGA)* and *SCARECROW (SCR)*. GRAS proteins exhibit variable N-termini, but show considerable homology to each other in their respective C-termini containing a highly conserved VHIID motif flanked by leucine-rich domains, the PFYRE motif and a SAW motif (Pysh *et al.,* 1999; Bolle, C., 2003; Fig. 3.2-1). GRAS proteins play important roles in diverse processes such as signal transduction, root development and meristem maintenance.

The *LAS* gene plays an important role in the regulation of axillary meristem formation in *A.thaliana* (Greb *et al.,* 2003). *las* mutants are characterized by the failure to initiate axillary meristems during vegetative phase of development. In accordance with the mutant phenotype, *LAS* is expressed in the axils of all leaf primordia. *in situ* mRNA hybridisation experiments have revealed that, in the shoot apex, *LAS* transcripts accumulate in a band shaped domain on the adaxial side of the primordia P1 to P21. The expression domain extends 3-5 cell layers in apical-basal direction and has an adaxial-abaxial width of 1-2 cell layers 1-2 cell layers in adaxial-abaxial width. In the reproductive phase, *LAS* expression is detected in similar domains in axils of floral primordia and sepals. This precise expression pattern in the axils of developing leaves is very similar to the expression pattern of other genes, like the *CUP-SHAPED COTYLEDON1 (CUC1), CUC2, CUC3 and LATERAL ORGAN BOUNDARIES (LOB)*, which indicates that the axillary tissue has a specific identity. The characteristic expression pattern of *LAS* probably results from transcriptional regulation by the SAM and the flanking leaf primordia.

To identify more precisely the tissues that require *LAS* activity and to determine the time window in development during which *LAS* carries out its function, misexpression approaches were employed. Promoters driving specific patterns of expression in domains very close to or overlapping with the *LAS* expression domain in the shoot apex were fused directly to the *LAS* open reading frame. For these studies, the promoters of the genes *SHOOT MERISTEMLESS (STM), KNAT1* and *UNUSUAL FLORAL ORGANS (UFO)* were chosen to drive the expression of *LAS* in transgenic plants. These promoter swapping experiments were used to assess the effect of *LAS* misexpression on axillary meristem formation in the *las-4* mutant. The transgenic plants thus generated were tested for complementation of the *las-4* branching phenotype.

A subgroup of the GRAS family is the DELLA protein family. These proteins share a high degree of homology within their N-termini, including a conserved DELLA motif (Fig. 3.2-1). In *Arabidopsis*, this subgroup of GRAS proteins comprises five members: GAI, REPRESSOR OF *ga1-3* (RGA), RGA-Like1 (RGL1), RGL2 and RGL3. DELLA proteins act as negative regulators of gibberellic acid (GA) signaling in plants (Fleet and Sun, 2005). GA is known to be involved in a wide variety of developmental pathways in plants, such as embryonic development, seed germination, hypocotyl and stem elongation, leaf expansion, pollen development and flower initiation. GA down-regulates DELLA proteins through protein degradation via the ubiquitin-proteasome pathway (Sun and Gubler, 2004). In *Arabidopsis* this degradation is mediated via SLEEPY1, an F-Box protein, as a part of the E3 ubiquitin ligase SCF complex (McGinnis *et al.,* 2003). *LAS* lacks the DELLA domain, and thus does not belong to this subgroup of GRAS family genes.

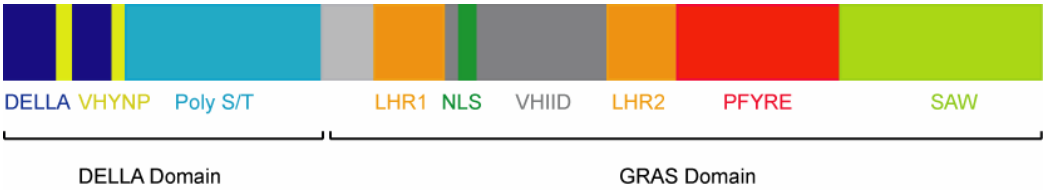


Fig. 3.2-1: The GRAS proteins and the DELLA subfamily. The C-terminal region is conserved among all GRAS family members. VHIID, PFYRE and SAW are the conserved motifs in the C-terminal region. The unique N-terminal "DELLA domain" of the DELLA proteins contains two highly conserved motifs (named DELLA and VHYNP) and a Poly S/T region. Poly S/T = polymeric Ser and Thr; LHR = Leu heptad repeat; NLS = nuclear localization signal. (Adapted from Pysh *et al.,* 1999 and Sun and Gubler, 2004).

In this study, a possible role of *LAS* as a local regulator of GA signaling in the axils of leaves was also examined. To this end, *LAS* promoter was used to drive the expression of the *gai-1* ORF (a mutant which is devoid of the DELLA domain) transgenically in a *las-4* mutant background to check for any complementation of the *las-4* branching phenotype. Furthermore, a direct role of GA in axillary meristem development was evaluated by conducting GA spraying experiments on wild-type and *las-4* mutant plants.

3.2.1 Spatial and temporal window of *LAS* function during axillary meristem development

3.2.1.1 Cloning of misexpression constructs using the Gateway system

In the vegetative shoot apex *LAS* transcript accumulates in the axils of leaf primordia. This boundary region is flanked by the SAM and the leaf primordium. In this study, promoter swapping experiments were performed in which *LAS* was expressed under promoters of genes whose expression patterns in the shoot apex overlap or adjoin with the *LAS* expression domain. For this purpose, promoters of the genes *STM, KNAT1* and *UFO* were fused to the *LAS* ORF. The *AtSTM*, *AtKNAT1* and *AtUFO* promoters were

amplified by PCR from Col genomic DNA using specific primers containing GATEWAY tails. The forward primers contain the AttB1 tail and reverse primers contain the AttB2 tail of the GATEWAY cassette.

STM is required for SAM initiation and maintenance (Barton and Poethig, 1993; Clark *et al.,* 1996). The *STM* gene encodes a homeodomain protein of the KNOTTED class I, first identified in maize as KNOTTED1 (KN1) (Hake and Freeling, 1986). STM is expressed in the SAM founder cells in the embryo (Long *et al.,* 1996; Long and Barton, 1998). The *STM* transcript remains expressed throughout the SAM during the entire life span of the plant and it is found in all types of shoot meristems: primary, axillary, and floral. Thus, *STM* can be used as a marker of SAM fate. However, *STM* expression is absent from all leaf primordia that arise from the SAM (Long *et al.,* 1996; Long and Barton, 2000; Fig.3.2-2d). *STM* (AT1G62360) is located on the lower arm of chromosome I. A 4.5 kb genomic sequence preceding the ATG of *STM* open reading frame (ORF) was cloned upstream of the *LAS* ORF to assemble a *STM::LAS* construct in pGW-nos vector (Fig. 3.2-2a). The expression pattern of this promoter has been previously studied (Long *et al.,* 1996) and is schematically illustrated in Fig. 3.2-2d. This construct was transformed into wild-type and *las-4* mutant background to look for possible complementation of *las-4* branching defect.

KNAT1 (*kn*otted-like from *A*rabidopsis *t*haliana) or *BREVIPEDICELLUS* (*BP*) is another putative transcription factor belonging to the class I KNOTTED-like homeodomain family (Lincoln *et al.,* 1994; Douglas *et al.,* 2002; Venglat *et al.,* 2002). *Arabidopsis* encodes four class I *KN1*-like homeobox (*KNOX*) genes, the most closely related to *KN1* being *KNAT1/BP* and *STM*. *KNAT1* is the *Arabidopsis* orthologue of *KN1*, although *STM* has closer functional similarity to *KN1* on the basis of similar null mutant phenotypes and expression patterns (Long *et al.,* 1996; Vollbrecht *et al.,* 2000). *KNAT1* is expressed in several different tissues during vegetative and reproductive phases of growth. In the vegetative shoot apex, *KNAT1* is expressed in the meristem and in a region just proximal to the meristem that extends to the base of several newly initiated leaf primordia (Fig. 3.2-2d).

The expression is more abundant in the peripheral and rib zones than in the central zone. The *KNAT1* mRNA is not detected in leaf primordia, even at very early stages of primordium formation. This is due to the selective downregulation of *KNAT1* expression as cells are recruited into organ primordia (Lincoln *et al.,* 1994; Reiser *et al.,* 2000; Fig. 3.2-1d). *KNAT1* confers a switch mechanism in the SAM which governs indeterminate to determinate development, and is also shown to be involved in the regulation of inflorescence architecture (Byrne *et al.,* 2002; Douglas *et al.,* 2002; Venglat *et al.,* 2002). *KNAT1* (AT4G08150) is located on the top of the lower arm chromosome IV. To study the effects of misexpression of *LAS* under the *KNAT1* promoter, a 1.48 kb promoter fragment of *KNAT1* was used. The pattern of expression driven by this promoter has been

previously reported by Lincoln *et al.* (1994), and is schematically represented in Fid. 3.2-2d. This *KNAT1* promoter was used to drive the expression of *LAS* gene in a pGW-nos vector (Fig. 3.2-2b). The construct was transformed into wilt-type as well as *las-4* mutant background.

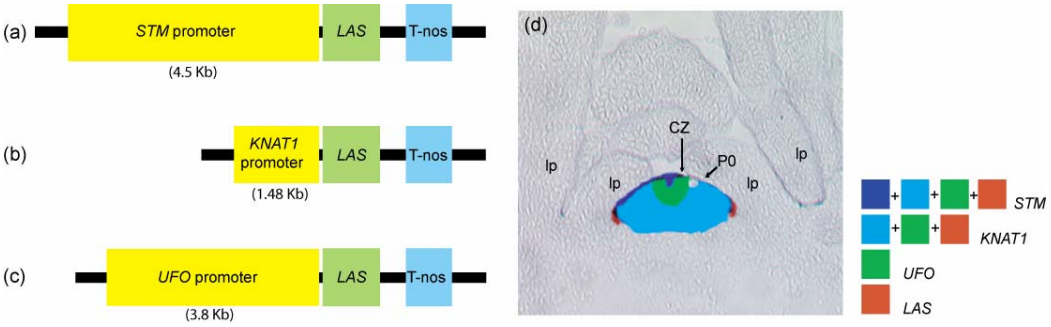


Fig. 3.2-2: Misexpression constructs to study the effects of ectopic expression of *LAS*. Schematic representations of *STM::LAS* (a), *KNAT1::LAS* (b), and *UFO::LAS* (c) constructs in pGW-nos vector, and the expression domains of *STM, KNAT1, UFO* and *LAS* in the shoot apex (d; adapted from expression pattern descriptions of Long *et al.,* 1996; Long and Barton, 1998; Lincoln *et al.,* 1994; Lee *et al.,* 1997) . The overlap in the expression domains of the genes is indicated by colour codes in (d). CZ= central zone, PZ= Peripheral zone, lp= leaf primordium, P0= incipient primordium.

UFO encodes an F-box-containing protein, and is involved in multiple processes in flower development including growth of floral primordia, specification of organ identity in the second and third whorl and the proper patterning of primordium initiation in the inner three whorls (Ingram *et al.,* 1995; Wilkinson and Haughn, 1995; Levin *et al.,* 1998). In agreement with its complex developmental role, the expression pattern of *UFO* in the flower is highly dynamic. In the floral meristem, *UFO* is expressed in the central dome early on, whereafter it becomes progressively restricted to the presumptive whorls 2 and 3 and, finally, to the base of the petals. In the vegetative meristem, *UFO* is expressed in a ring-like domain surrounding the central zone (Lee *et al.,* 1997; Samach *et al.,* 1999; Laufs *et al.,* 2003; Fig. 3.2-2d). *UFO* expression in the SAM is dependent on *STM* (Long and Barton, 1998). However its function in the vegetative apex is unclear. *UFO* (AT1G30950) is located on the upper arm of Chromosome I, proximal to the centromere. In this study, misexpression of *LAS* under the *UFO* promoter was carried out by fusing a 3.8 kb *UFO* promoter fragment to the *LAS* ORF in a pGW-nos vector (Fig. 3.2-2c). The expression pattern driven by this *UFO* promoter is schematically depicted in Fig. 3.2-2d according to the description in previously reported studies (Lee *et al.,* 1997; An *et al.,* 2004). This construct was transformed into *las-4* mutant plants to study the effects of ectopic expression of *LAS*.

3.2.1.2 Analysis of transgenic plants misexpressing *LAS* under the *STM* promoter

The pGW-nos vector containing the *STM::LAS* construct was transformed both into wild-type Col and *las-4* plants. In the wt background, these transgenic plants did not show any notable phenotypes different from the wild-type. *STM::LAS* transgenic plants in *las-4*

mutant background were analysed for complementation of the *las-4* branching defect and other additional phenotypes. These transgenic plants were characterised by a dark green colour (Fig. 3.2-3a). Rosette leaf axils of twenty plants each from thirteen T1 lines of *STM::LAS* in *las-4* were subjected to closer inspection under a stereomicroscope. Partial complementation of the *las-4* branching defect was observed in 8 lines, and the remaining 5 lines displayed little or no complementation.

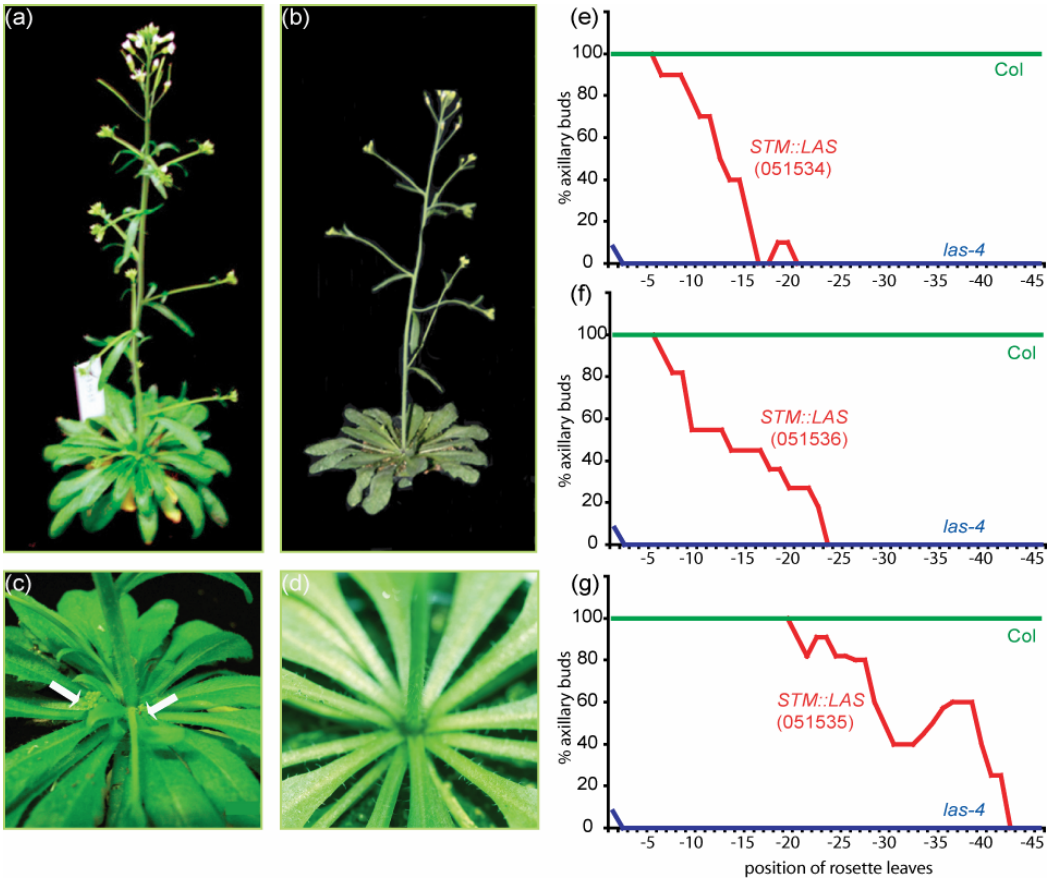


Fig. 3.2-3: Habitus and axillary shoot formation in *STM::LAS* transgenic plants. (a) Habitus of an *STM::LAS* transgenic plant in *las-4*. (b) Habitus of a *las-4* mutant. (c) Close-up of the rosette of an *STM::LAS* transgenic plant in *las-4*. Arrows point to emerging axillary shoots. (d) Close-up of the rosette of an *las-4* mutant showing empty leaf axils. (e)-(g) Graphical representation of axillary bud formation in three independent *STM::LAS* lines, 051534, 051536 and 051535, showing various degrees of partial complementation of the *las-4* branching phenotype (n=20). Plants were grown for 6 weeks in short days and then shifted to long days to induce flowering.

3.2.1.3 Analysis of transgenic plants misexpressing *LAS* under the *KNAT1* promoter

KNAT1::LAS construct was transformed both into wt Col and *las-4* mutant plants. No striking phenotypes were observed in the transformed Col plants. The efficiency of transformation of the *KNAT1::LAS* construct was very low in the *las-4* mutant

background, resulting in only three T1 lines. These lines were grown to maturity under short day conditions and characterized in detail.

KNAT1::LAS transgenic lines in *las-4* mutant background displayed a very characteristic growth habitus. They developed axillary shoots from most leaf axils. The axillary shoots arising from cauline leaf axils in these transgenic plants subtended an obtuse angle with the main axis when compared to those in *las-4* (Fig. 3.2-4a and 3.2-4b).

The tip of these axillary shoots pointed towards the ground rather than standing erect, giving the plants a broader habitus. Furthermore, whereas the rosette of *las-4* mutants showed almost a complete lack of axillary bud development, in the *KNAT1::LAS* transgenic lines a robust formation of axillary shoots was observed (Fig. 3.2-4c and 3.2-4d). These side-shoots flowered very early and were much shorter in length than in wild-type plants (Fig. 3.2-4c). The early flowering phenotype was a general characteristic of

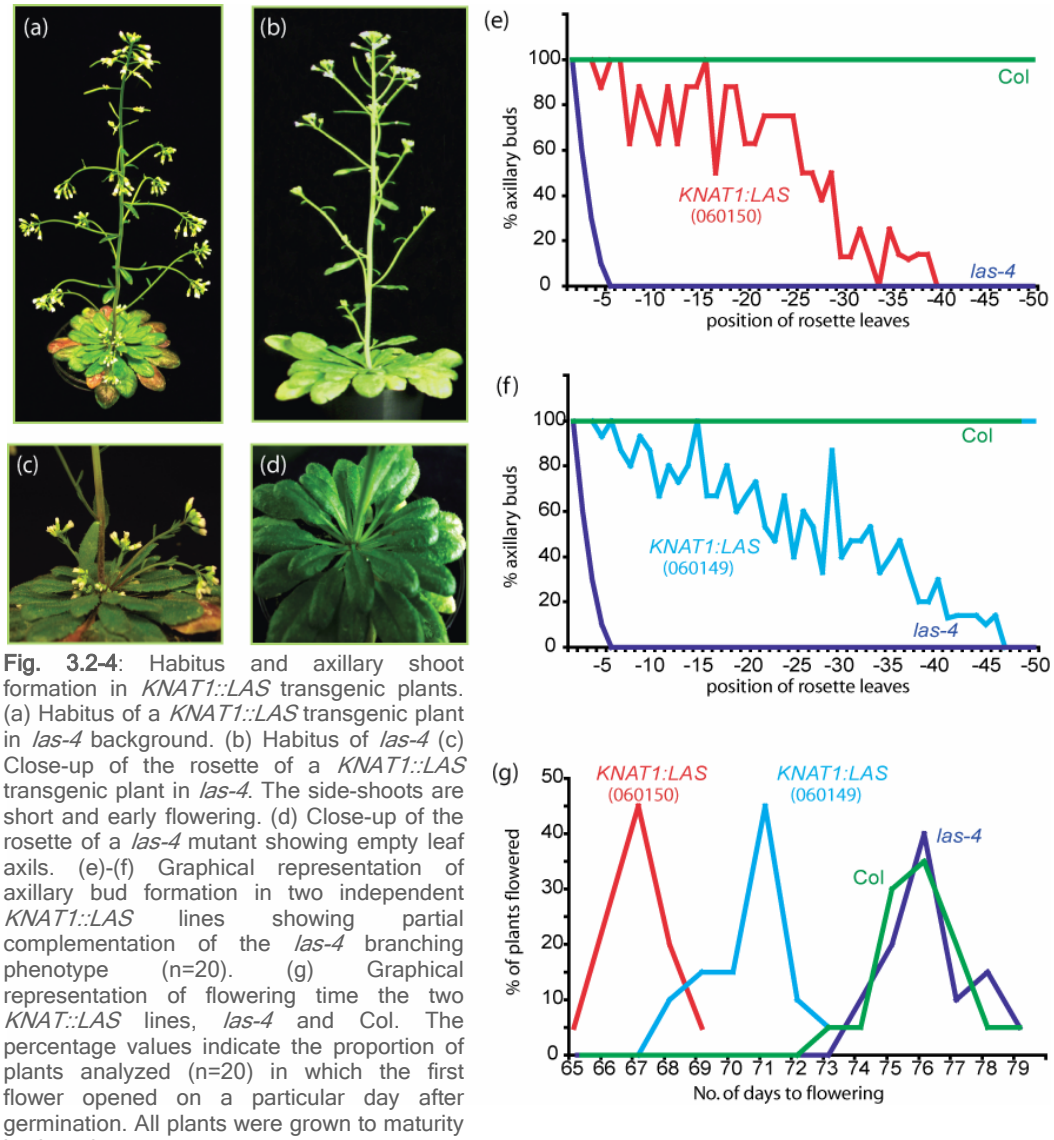


Fig. 3.2-4: Habitus and axillary shoot formation in *KNAT1::LAS* transgenic plants. (a) Habitus of a *KNAT1::LAS* transgenic plant in *las-4* background. (b) Habitus of *las-4* (c) Close-up of the rosette of a *KNAT1::LAS* transgenic plant in *las-4*. The side-shoots are short and early flowering. (d) Close-up of the rosette of a *las-4* mutant showing empty leaf axils. (e)-(f) Graphical representation of axillary bud formation in two independent *KNAT1::LAS* lines showing partial complementation of the *las-4* branching phenotype (n=20). (g) Graphical representation of flowering time the two *KNAT::LAS* lines, *las-4* and Col. The percentage values indicate the proportion of plants analyzed (n=20) in which the first flower opened on a particular day after germination. All plants were grown to maturity in short days.

KNAT1::LAS lines. Flowering time in two independent transgenic lines was monitored by blotting the days to appearance of the first petal against the percentage of plants that flowered for the first time on that given day. As illustrated in Fig. 3.2-3g, 060149 and 060150, the two *KNAT1::LAS* lines, had flowering time peaks at about 71 days and 67 days after germination, respectively, whereas most *las-4* and Col plants flowered at about 76 days after germination in short days. In accordance to this early transition to flowering, the two *KNAT1::LAS* lines produced less rosette leaves (44 and 39 rosette leaves on an average in the lines 060149 and 060150, respectively) than *las-4* (47 rosette leaves on an average).

The rosette leaf axils of twenty plants each from these two *KNAT1::LAS* lines, and of *las-4* and Col plants grown in parallel were further inspected using a stereomicroscope. All rosette leaf axils of Col harboured axillary buds. In *las-4*, the youngest four or five rosette leaf axils developed axillary buds, as is typical for this mutant when grown in short day. A high degree of complementation of the *las-4* phenotype was observed in both the *KNAT1::LAS* transgenic lines characterised (Fig. 3.2-4e and 3.2-4f). Formation of axillary buds was observed in most leaf axils during the middle and late phase of vegetative development. A low percentage of the older rosette leaf axils also supported the formation of axillary buds. Notably, no ectopic bud development was observed in cauline- or rosette leaf axils in the *KNAT1::LAS* transgenes, either in wt or in *las-4* mutant background.

3.2.1.4 Analysis of transgenic plants misexpressing *LAS* under the *UFO* promoter

Col as well as *las-4* plants were transformed with the *UFO::LAS* construct cloned into a pGW-nos vector. No characteristic phenotypes were observed in wild-type plants transformed with this construct. *las-4* mutants carrying the *UFO::LAS* construct were analysed for complementation of the *las-4* branching phenotype.

The phenotypes of eleven independent T1 lines of *UFO::LAS* transgenes in *las-4* background were analysed in detail. Leaf axils of 20 plants from each line were inspected using a stereomicroscope. Six of these lines displayed almost complete complementation of the *las-4* branching phenotype (Fig. 3.2-5b). Axillary bud formation was observed in the axils of almost all rosette leaves, except in a low proportion of plants where the oldest 3 to 4 rosette leaf axils were empty (Fig. 3.2-5h). Formation of axillary shoots in the cauline leaf axils in these transgenic lines did not deviate from that in *las-4* (Fig. 3.2-5b and 3.2-5h). The remaining five transgenic lines did not show any notable complementation of the branching defect (Fig. 3.2-5c). Axillary buds were formed only in a low number of plants late in vegetative development (Fig. 3.2-5f). Additionally, these plants displayed an

enhancement of the *las-4* phenotype, in that axillary buds failed to initiate in the axils of a large number of older cauline leaves (Fig. 3.2-5e and 3.2-5f). Moreover, the axils of a high proportion of younger cauline leaves supported the development of a flower instead of an axillary shoot (Fig. 3.2-5d and 3.2-5g). Once again, similar to the other two misexpression transgenes, accessory structures or ectopic shoot development was not observed in any *UFO::LAS* transgenic line.

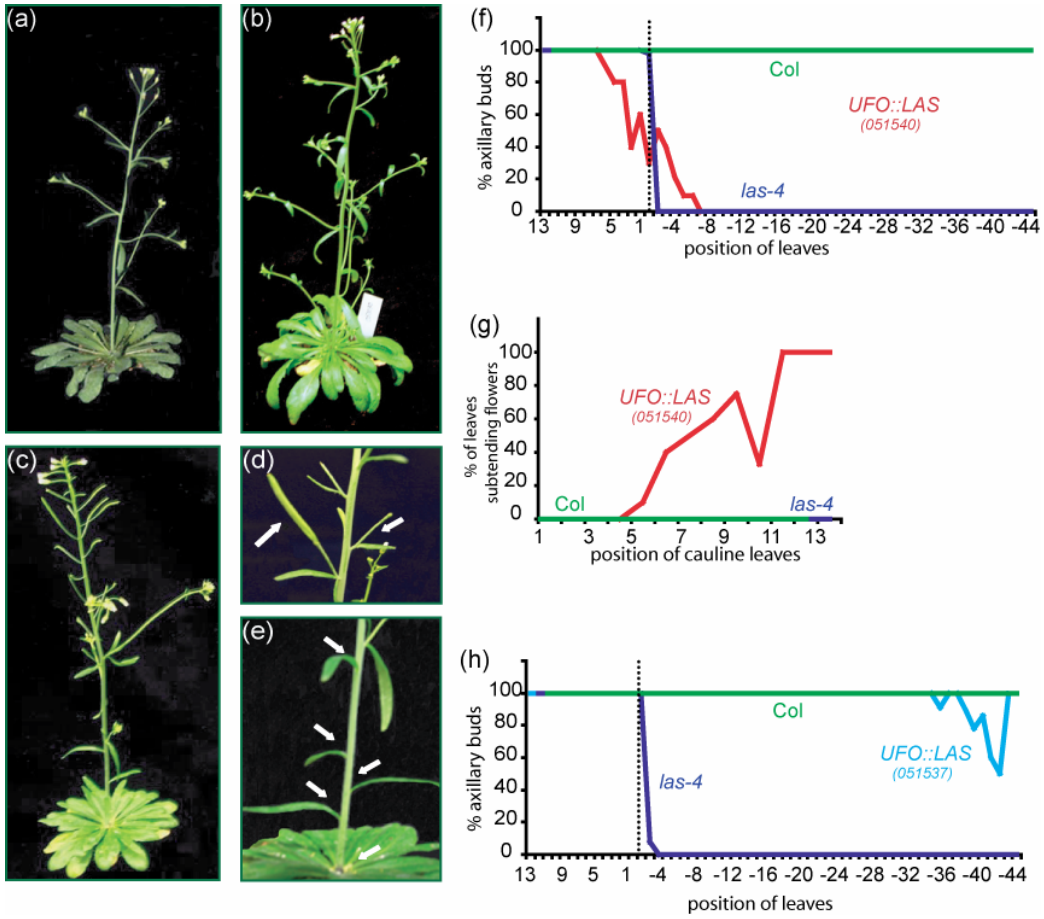


Fig. 3.2-5: Habitus and axillary shoot formation in *UFO::LAS* transgenic plants. (a) Habitus of a *las-4* plant. (b) Habitus of an *UFO::LAS* plant (051537) in *las-4* background that complements the *las-4* branching phenotype. (c) Habitus of an *UFO::LAS* plant (051540) in *las-4* background that does not complement the *las-4* branching phenotype. (d) Close-up of the non-complementing *UFO::LAS* plant (051540) showing flowers/siliques (arrows) subtended by cauline leaf axils. (e) Close-up of a non-complementing *UFO::LAS* plant (051540) displaying empty cauline and rosette leaf axils. (f) Graphical representation of axillary bud formation in leaf axils of *UFO::LAS* (051540). (n=20) (g) Graphical representation of proportion of cauline leaf axils subtending a flower instead of an axillary shoot in *UFO::LAS* (051540). (n=20). (h) Graphical representation of axillary bud formation in leaf axils of *UFO::LAS* (051537). (n=20). In (f) and (h), the dotted line represents the position of the youngest rosette leaf. To the right of the dotted line are positions of progressively older rosette leaves, and to the left are positions of progressively younger cauline leaves. Plants were grown for 6 weeks in short days and then shifted to long days to induce flowering.

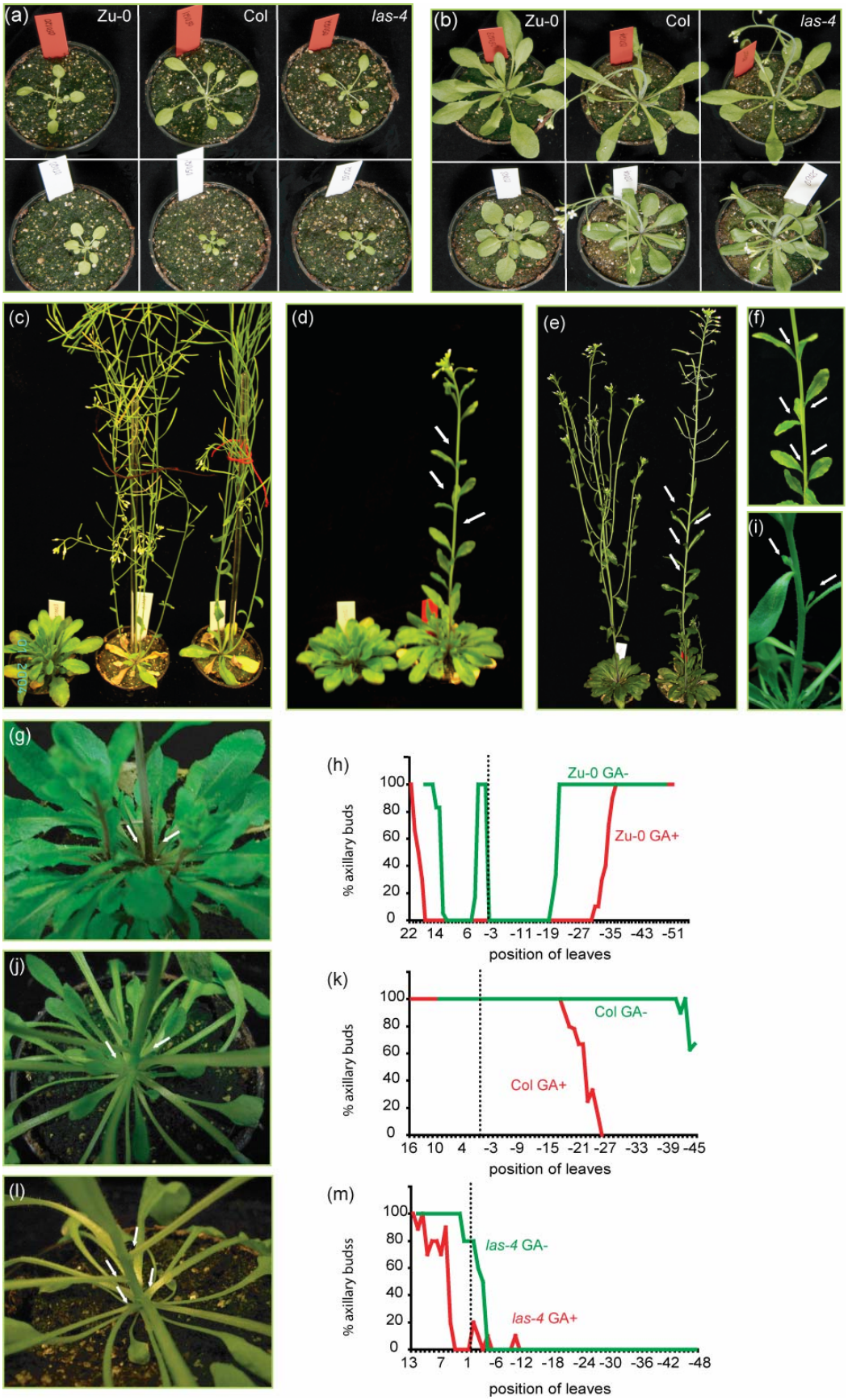
3.2.2 Gibberellic acid signalling and axillary meristem development

3.2.2.1 Analysis of effect of gibberellic acid on axillary meristem development

The plant hormone GA is known to regulate germination, growth and flowering. Application of GA accelerates germination of seeds and results in elongation of internodes and leaves, and early flowering in plants. To assay any potential role of GA in the development of axillary meristems, spraying experiments were carried out on wilt-type Zu-0 and Col ecotypes, and *las-4* mutant plants. Zu-0 is a late-flowering ecotype which is characterised by lack of axillary shoot formation during the late vegetative phase of development (Kalininia *et al.,* 2002; Fig. 3.2-6c, 3.2-6f, 3.2-6g, 3.2-6h). Monitoring of *STM* expression in Zu-0 has revealed that axillary meristems are initiated but arrested at an early stage of development (Kalininia *et al.,* 2002). In this study, Zu-0, Col, and *las-4* mutants in Col were sprayed with 0.1 mM GA_{4+7} every second day beginning from 7 days after germination. The experiment was carried out both in short day and long day growth conditions.

The results obtained from GA_{4+7} application experiments are illustrated in Fig. 3.2-6. Comparison of twenty days old short day grown plants with and without GA treatment demonstrated that plants treated with GA_{4+7} had longer leaf petioles and expanded leaf blade area (Fig. 3.2-6a). Similar results were obtained in plants grown for 20 days in long days, except that, in addition to increased leaf size, the GA_{4+7} sprayed Col and *las-4* plants displayed an early transition to flowering when compared to the corresponding control plants (Fig. 3.2-6b). However, no transition to the reproductive phase was observed in GA_{4+7} treated or control Zu-0 plants (Fig. 3.2-6b). When grown under long days, Zu-0 plants bolted 17 weeks after germination (Fig. 3.2-6d and 3.2-6e). However, GA_{4+7} treatment of Zu-0 plants led to an earlier transition to flowering at 13 weeks after germination (Fig. 3.2-6d).

As reported earlier, Zu-0 plants were also characterised by zones lacking axillary buds in the axils of late rosette leaves and early cauline leaves (Fig. 3.2-6d). These zones occur from the 4^{th} to the 10^{th} cauline leaf axils on the main bolt and in the axils of the twenty youngest rosette leaves (Fig. 3.2-6h). In GA_{4+7} treated Zu-0 plants, a substantial expansion of this zone was observed, with a large number of older cauline leaf axils remaining empty (Fig. 3.2-6d to 3.2-6f). This zone also expanded into the axils of upper rosette leaves (Fig. 3.2-6g). Closer inspection of the leaf axils of Zu-0 plants under a stereomicroscope revealed that in addition to the empty cauline leaf axils in the lower part of the main bolt (cauline leaf 4 to 10), the youngest 20 rosette leaf axils also failed to support the development of axillary buds (Fig. 3.2-6h).


(a)
Zu-0
Col
las-4
(b)
Zu-0
Col
las-4
(c)
(d)
(e)
(f)
(i)
(g)
(h)
% axillary buds
100
80
60
40
20
0
22 14 6 -3 -11 -19 -27 -35 -43 -51
position of leaves
Zu-0 GA-
Zu-0 GA+
(j)
(k)
% axillary buds
16 10 4 -3 -9 -15 -21 -27 -33 -39 -45
position of leaves
Col GA-
Col GA+
(l)
(m)
% axillary budss
13 7 1 -6 -12 -18 -24 -30 -36 -42 -48
position of leaves
las-4 GA-
las-4 GA+

However, in the corresponding GA_{4+7} treated plants the youngest 18 cauline leaf axils and about 30 youngest rosette leaf axils failed to initiate any axillary buds (Fig. 3.2-6h). Thus, application of GA_{4+7} resulted in aggravation of the branching defect in Zu-0 plants. Surprisingly, even though the application of GA_{4+7} lead to an accelerated transition to flowering, the number of rosette leaves formed remained the same. This indicates that the plastochron (i.e. the time period between initiation of two successive leaves) index is substantially reduced by treatment with GA_{4+7} in Zu-0.

The effect of application of GA4+7 on axillary meristem development was also investigated on Col wilt-type and *las-4* plants. In Col, axillary meristem development was not affected by GA4+7 treatment. Axillary shoots developed in the axils of all cauline and rosette leaves (Fig. 3.2-6i and 3.2-6j). However, as expected, application of GA4+7 caused an early transition to flowering, as indicated by the lower number of rosette leaves formed (Fig. 3.2-6k). Both in treated and non-treated Col plants, axillary bud formation seemed to be compromised in the axils of the oldest rosette leaves (Fig. 3.2-6k). However, rotting away of older rosette leaves rendered it difficult to assess the axils of these leaves, and this may account for the seeming lack of axillary buds in these leaf axils.

In *las-4* mutants, application of GA4+7 had a minor effect on axillary bud formation. In non-GA4+7 treated control plants, the axils of the uppermost 4-5 rosette leaves in most plants supported the formation of axillary buds. However, in the GA4+7 treated plants, only very few plants produced any axillary buds during vegetative development (Fig. 3.2-6l and 3.2-6m). Additionally, unlike in the control plants, axillary buds were absent in the axils of most of the older cauline leaves (Fig. 3.2-6l and 3.2-6m).

Fig. 3.2-6: Effects of GA4+7 spraying on Zu-0, Col and *las-4* plants. (a) 20 days old Zu-0 (left), Col (centre) and *las-4* (right) seedlings grown in short days, non treated (lower row with while labels) and treated with GA4+7 (upper row with red labels). (b) 20 days old Zu-0 (left), Col (centre) and *las-4* (right) seedlings grown in long days, non treated (lower row) and treated with GA4+7 (upper row). (c) 10 weeks old long day Zu-0 (left), Col (centre) and *las-4* (right) plants treated with GA4+7. (d) 14 weeks old long day GA4+7 non-treated (left) and treated (right) Zu-0 plants. (e) 18 weeks old GA4+7 treated (right) and non-treated (left) long day Zu-0 plants. (f)- (g) Close up of cauline (f) and rosette (g) leaf axils of 18 weeks old GA4+7 treated Zu-0 plants grown in long days. (h) Graphical representation of axillary bud formation in leaf axils of 18 weeks old GA4+7 non-treated and treated Zu-0 plants grown in long days. (i)-(j) Close up of cauline (i) and rosette (j) leaf axils of 6 week old Col plants grown in long days. (k) Graphical representation of axillary bud formation in leaf axils of 10 weeks old GA4+7 treated and non-treated Col plants grown in short days. (l) Close up of cauline and rosette leaf axils of 6 week old *las-4* plants grown in long days. (m) Graphical representation of axillary bud formation in leaf axils of 10 weeks old GA4+7 treated and non-treated *las-4* plants grown in short days. The arrows in (d)-(g) and (i) point at empty leaf axils, and in (i)-(j) indicate the presence of axillary buds. In (h), (k) and (m), n=20, the red and the green curves indicates axillary bud development in plants treated and not treated, respectively, with GA4+7.

3.2.2.2 Analysis of a role of *LAS* as a regulator of GA signalling during axillary meristem development

LAS is a member of the plant specific GRAS family of putative transcription factors. A subgroup of the GRAS family is the DELLA protein family, a class of GA signaling repressors (Fleet and Sun, 2005). A conserved and unique domain (named DELLA, after a conserved amino acid motif) is present near the N terminus of the DELLA proteins (Fig. 3.2-1). Although the DELLA proteins do not have a clearly identified DNA-binding domain, they may act as co-activators or co-repressors by interacting with other transcription factors that bind directly to the DNA sequence of GA-regulated genes. RGA and GAI were the first two DELLA proteins to be identified in *Arabidopsis*. The levels of both the RGA and GAI proteins have been shown to be reduced rapidly by GA treatment (Silverstone *et al.,* 2001). This downregulation of DELLA proteins by GA is brought about by protein degradation via the ubiquitin-proteasome pathway (McGinnis *et al.,* 2003; Sasaki *et al.,* 2003). Removal of repression of target genes of DELLA proteins by an increase in GA levels triggers cell differentiation and regulates various developmental processes, like germination, stem elongation and flowering.

LAS lacks the DELLA domain, and hence cannot be downregulated by GA. A tempting hypothesis would be that *LAS* aids the cells in the axillary region to retain their meristematic character by repressing GA signaling in these cells, thereby protecting them from further differentiation. To analyse a potential role of GA signalling being regulated by *LAS* during axillary meristem development, one of the DELLA domain genes, *GAI*, was chosen to be transgenically expressed, with and without its DELLA domain, under the *LAS* promoter in the *las-4* mutant background.

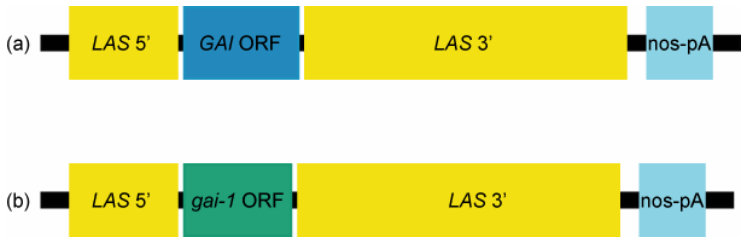


Fig. 3.2-7: Constructs to study the effects of expression of *GAI* and *GAI-Δ DELLA* under the *LAS* promoter. Schematic representations of *LAS::GAI* (a) and *LAS::GAI-Δ DELLA* (b) constructs in pGPTV-Bar and pGEM binary vectors, respectively.

Two constructs, *LAS::GAI* and *LAS::GAI-Δ DELLA*, were designed to drive the expression of *GAI* and *GAI-Δ DELLA*, respectively, under the *LAS* promoter. For constructing *LAS::GAI,* a 1.6 Kb GAI ORF amplified from genomic DNA was cloned into a pGPTV-Bar plant binary vector containing a 1.5 Kb 5' promoter and a 3.5 Kb 3' regulatory sequence of the *LAS* gene (Fig. 3.2-7a). In a previous study, transgenic expression of *LAS* by these 5' and 3' promoter regions have been shown to result in a complete complementation of

the *las-4* mutant phenotype (Eicker, 2006). The *LAS::GAI-Δ DELLA* construct was generated by amplifying the ORF of *gai-1* and cloning it between the 1.5 Kb 5' and 4.5 Kb 3' promoter sequences of *LAS* in a pGEM binary vector (Fig. 3.2-7b). The gain-of-function *gai-1* allele is GA-unresponsive and displays a dwarf phenotype. In *gai-1* a 51bp in-frame deletion in the *GAI* sequence results in the loss of 17 amino acids spanning the DELLA motif (Peng *et al.,* 1997), making it a constitutive repressor of GA the response.

These two constructs were transformed into *las-4* mutant plants, and the transgenic plants were then analysed for complementation of the *las-4* branching phenotype. Phenotypic analysis of T1 plants revealed that, whereas none of the 115 *LAS::GAI* transgenic plants complemented the *las-4* phenotype, about 65 of the 107 *LAS::GAI-Δ DELLA* transgenic plants complemented the *las-4* branching phenotype. These T1 plants were grown under long day growth conditions and were not subjected to a detailed analysis under a microscope. Further analysis of the T2 lines deriving from these plants is ongoing and necessary to draw clear conclusions.

3.3 GENETIC SCREEN TO IDENTIFY SUPPRESSORS OF *lateral suppressor-4*

An effective approach for determining the biological functions of genes is the identification of mutants with altered phenotypes. These mutants can be used as a tool to uncover the role of genes in various developmental and physiological processes. However, using mutants for developmental studies in *Arabidopsis* has a few distinct limitations. Several developmental processes in plants are regulated redundantly by multiple genes or similar genes belonging to a gene family. Thus, loss of function of a single gene may not yield a mutant phenotype as it might be adequately compensated by the function of redundant gene(s). Additionally, loss of function of an important gene may be lethal to the organism, thus rendering it impossible to study such null mutants.

However, once an interesting developmental mutant is identified, it can be used as a starting point for identification of additional genes that are involved in that particular developmental pathway. A second site mutagenesis screen, otherwise termed as a modifier screen, can be devised to identify enhancers or suppressors of the existing mutant phenotype. Such a screen has two advantages. First, new genes identified in the screen are often involved in the same biological process as the original mutant in the genetic background. Second, it helps to uncover genetic redundancy, if any, in the original mutant background.

In this work, the *las-4* mutant was used as a starting point in a suppressor screen to identify and isolate other loci involved in the process of axillary meristem formation. The *LAS* gene encodes a putative transcription factor belonging to the GRAS family involved in the formation of axillary meristems in *A.thaliana* (Greb *et al.,* 2003). Homozygous *las-4 max1-1* seeds were chemically mutagenised using ethyl methanesulphonate (EMS), and a population of M2 plants arising from this mutagenesis was analysed for suppression of the *las-4* phenotype. The *MAX1* gene encodes a member of the Cytochrome P450 family and has been shown to be involved the formation of a carotenoid-derived hormone which inhibits branching (Booker *et al.,* 2005), and the *max1-1* mutant is characterized by a bushy habitus (Stirnberg *et al.,* 2002). In an earlier study (Clarenz, 2004), the *las-4* mutation was found to be epistatic to *max1-1* mutation, the double mutant being characterized by a drastic reduction in side-shoot formation from rosette leaf axils. The logic behind screening for suppressors of the *las-4* phenotype in a *las-4 max1-1* double mutant background was to facilitate the easy scoring of suppressor phenotypes by identifying new mutations which would remove the epistatic effect of *las-4* over *max1-1*, thus resulting in suppressor candidates that ‘stand out’ with a bushy habit.

3.3.1 EMS mutagenesis and screening for suppressors of *las-4*

Approximately 5000 seeds of the homozygous *las-4 max1-1* double mutant were treated with 0.3% EMS for 8 hours. These mutagenised seeds were sown and 2000 M1 plants were harvested separately. The success of the mutagenesis was analysed in the M1 population by random sampling for plants showing discoloured/non-green clonal sectors on leaves indicating mutations in the components of photosynthesis apparatus. Approximately 2% of the M1 population showed such clonal sectors, indicating the success of the mutagenesis experiment. Since the M1 uncovers only dominant mutations, the screening for suppressors of *las-4* was carried out in an M2 population.

Sixteen plants each from 2000 separate M2 lines were screened. The seeds were first stratified at 4^{o}C for 36 hrs before being sown to ensure uniform germination. The plants were first grown for five weeks under short day conditions to facilitate development of a large number of rosette leaves. They were subsequently shifted to long days to induce bolting. When the bolts were about 10 cm long, the main bolt was cut off 1 cm from the top of the rosette. Two weeks after decapitation, plants were visually analysed for formation of axillary shoots in the rosette axils. The increase in axillary shoot formation in the putative suppressor candidates was measured against side-shoot formation in *las-4 max1-1*. Col and Ler plants were used as additional controls. Ler and Col-0 wilt-type plants grown for 80d in short day conditions displayed 100% side-shoot formation in rosette and cauline leaf axils, whereas *las-4 max1-1* plants were marked by a severe decrease in the number of side-shoots arising from rosette leaf axils (Fig. 3.3-1).

Plants that showed formation of five or more axillary buds were short listed as candidates. These candidates were first molecularly tested for the presence of the *las-4* deletion using a CAPS marker. The false positive candidates (wild-type contamination) were thus excluded from being selected as suppressor candidates. The remaining putative candidates were selfed and the M3 was further analysed for axillary bud formation. Whereas the *las-4 max1-1* mutant is characterized by a complete suppression of side-shoot formation in the rosette (Fig. 3.3-1), the introduction of certain new mutations removed this suppression and resulted in formation of side-shoots from the rosette axils. These candidates were designated as *suppressors of las-4* (*sol*) and were further characterized in detail. A total of 11 independent lines that showed a suppression of the *las-4* phenotype were identified. Three of these lines, which displayed a stable phenotype in several selfings, were selected for further detailed characterisation.

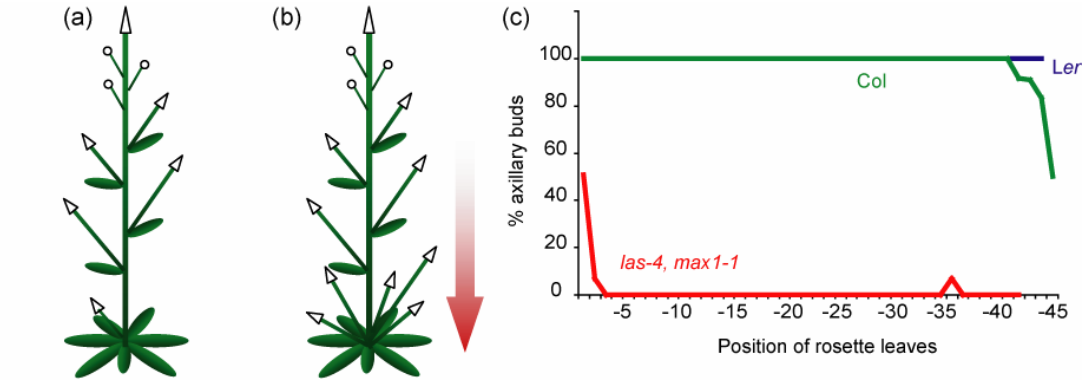


Fig. 3.3-1: Schematic and graphic comparison of the branching in *las-4 max1-1* and the *suppressors of las-4* identified in the screen. *las-4 max1-1* (a) is characterized by the absence of axillary shoots from the rosette. The *suppressors of las-4* (b) developed five or more axillary buds from the rosette axils. (c) Graphic representation of axillary bud formation in the rosette leaf axils of *las-4 max1-1*, Col and L*er* grown for 80 d in short day conditions. Genotypes are indicated next to the graphs. Position -1 corresponds to the uppermost rosette leaf, and position -45 corresponds to the oldest rosette leaf axil analysed. The percentage values indicate the proportion of plants analysed (n=30) that formed an axillary bud in a specific position along the shoot axis.

3.3.2 Characterisation of three *suppressor of las-4* lines

The three *suppressor of las-4* lines selected for further characterization were first analysed for their branching phenotype in the M3 generation. The suppression of the *las-4* phenotype in these plants was found to be stably inherited. Since EMS mutagenesis can generate multiple point mutations, the number of additional (new) mutations in the *sol* candidates was first analysed by backcrossing the suppressor candidates to *las-4*. If the suppressor phenotype is conferred by a recessive mutation in a single locus, it would result a 1:3 segregation of the suppressor phenotype in the F2 of the backcross. If two separate loci are mutated, the suppressor phenotype would segregate 1:15 in the F2. Furthermore, to test whether the new mutation confers a phenotype on it own, the *sol* lines were crossed to Col wild-type plants to remove the *las-4* mutation.

In addition, crossings were carried out to create mapping populations to later facilitate molecular mapping of the loci responsible for the *sol* phenotype. Since the new mutation was in the Col ecotype, the *sol* mutants were crossed to the Landsberg ecotype. The Landsberg parent used for creating mapping populations was either Landsberg *erecta* (L*er*) or L*er* carrying *las-4* mutation introgressed from Col background with three back crosses (Clarenz, 2004). Multiple DNA polymorphisms between the Col and L*er* ecotypes have been reported and documented. Extensive information about molecular markers is publicly accessible in the *Arabidopsis* data base (TAIR).

3.3.3 Phenotypic characterisation of *sol2*

sol2 identified from the screen segregated for the branching phenotype. Plants displaying the characteristic bushy *sol2* phenotype were selected and selfed for three generations to

establish the stability of the phenotype. For phenotypic analysis, *sol2* plants where grown to maturity under short day as well as long day conditions and compared to the *las-4 max1-1* and Col wt controls. In short days, the *sol2* plants were characterized by a dwarfish, bushy habitus with reduced apical dominance (Fig. 3.3-2a). When compared to the *las-4 max1-1* parents (Fig. 3.3-2c), *sol2* plants showed a significant increase in side-shoot formation from the rosette axils (Fig. 3.3-2d). During the early and middle phase of vegetative development, axillary buds developed in all leaf axils. Around 50-60% of leaf axils in the late vegetative phase supported the formation of an axillary bud (Fig. 3.3-2e). These buds grew out to side-shoots, which where almost as tall as the main shoot, giving the *sol2* plants a very bushy appearance (Fig. 3.3-2a). Under long day conditions, the bushy and dwarfish phenotype in *sol2* plants was strongly reduced. Formation of axillary buds in *sol2* grown first in short periods and then induced to bolt by shifting to long days was similar to those grown to maturity in short days. However, not all the axillary buds grew out to form side-shoots, thus giving the plant a less bushy appearance than when grown to maturity in short-day conditions (Fig. 3.3-2b and 3.3-2b). Although *sol2* plants grown to maturity under long day conditions displayed a reduction in the severity of the phenotype, they still exhibited more axillary buds than *las-4 max1-1* grown simultaneously under the same growth conditions. Axillary bud formation was not affected during reproductive development in *sol2* in any growth condition.

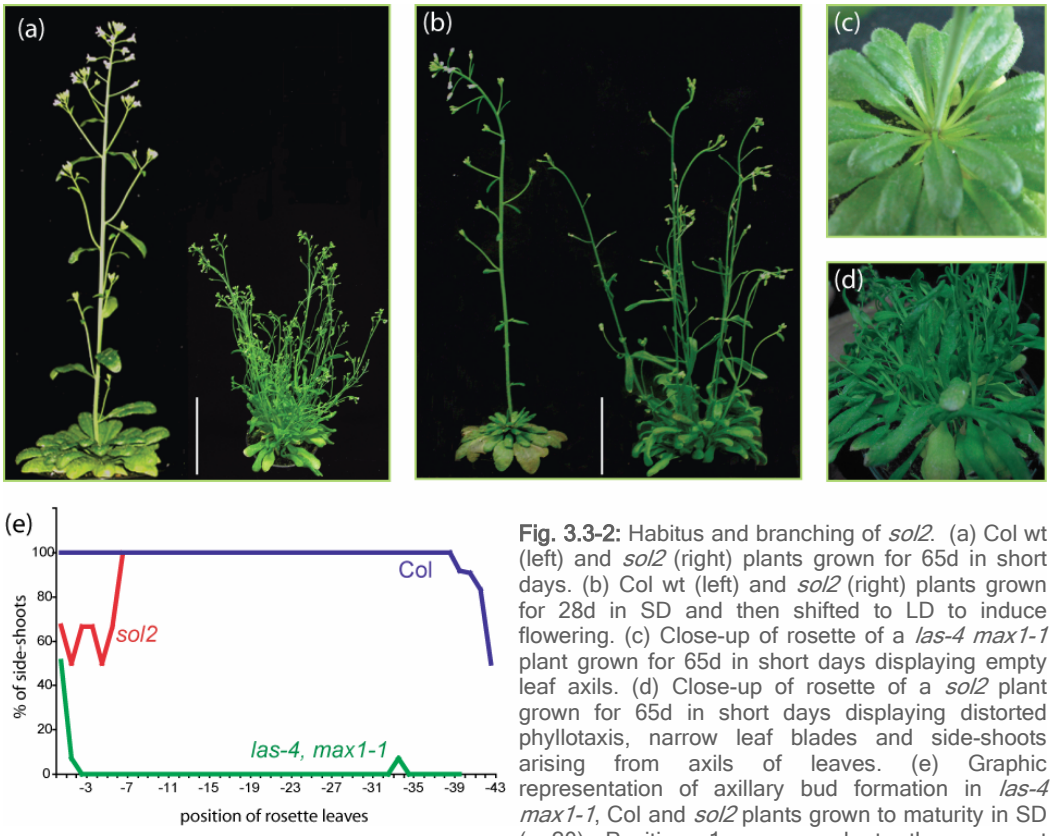


Fig. 3.3-2: Habitus and branching of *sol2*. (a) Col wt (left) and *sol2* (right) plants grown for 65d in short days. (b) Col wt (left) and *sol2* (right) plants grown for 28d in SD and then shifted to LD to induce flowering. (c) Close-up of rosette of a *las-4 max1-1* plant grown for 65d in short days displaying empty leaf axils. (d) Close-up of rosette of a *sol2* plant grown for 65d in short days displaying distorted phyllotaxis, narrow leaf blades and side-shoots arising from axils of leaves. (e) Graphic representation of axillary bud formation in *las-4 max1-1*, Col and *sol2* plants grown to maturity in SD (n=20). Position -1 corresponds to the youngest rosette leaf, and position -45 corresponds to the oldest rosette leaf axil analysed.

The *sol2* mutation had pleiotropic effects. Other than the dwarfish habitus and bushy outgrowth, *sol2* plants were characterized by distorted leaf morphology and floral organ fusions. In *sol2* flowers, sepals were fused to each other, thus encasing the floral organs permanently within the flower bud (Fig. 3.3-3a).

Dissection of flower buds revealed that petals were either absent or rudimentary in structure (Fig. 3.3-3b). The *sol2* flowers usually failed to open and were smaller in size compared to the *las-4 max1-1* flowers, due to the lack of petal development (Fig. 3.3-3b). The inflorescence morphology of *sol2* was also altered with fewer flowers per inflorescence than in *las-4 max1-1* (Fig. 3.3-3c and 3.3-3d). The siliques in *sol2* were small and displayed delayed dehiscence (Fig. 3.3-3f and 3.3-3g). The number of mature seeds formed per silique was also reduced when compared to the *las-4 max1-1* parent. The fused flower and reduced seed fertility phenotype made it very difficult to use *sol2* homozygotes for crossing. Often, crossing had to be performed several times before being able to obtain seeds. *sol2* plants had smaller and narrower leaves with dark green colour (Fig. 3.3-3e). The surface of leaves was slightly wavy, with a thick waxy coating, indicating defective development of epidermis. Slight fusions in leaf margins were also observed.

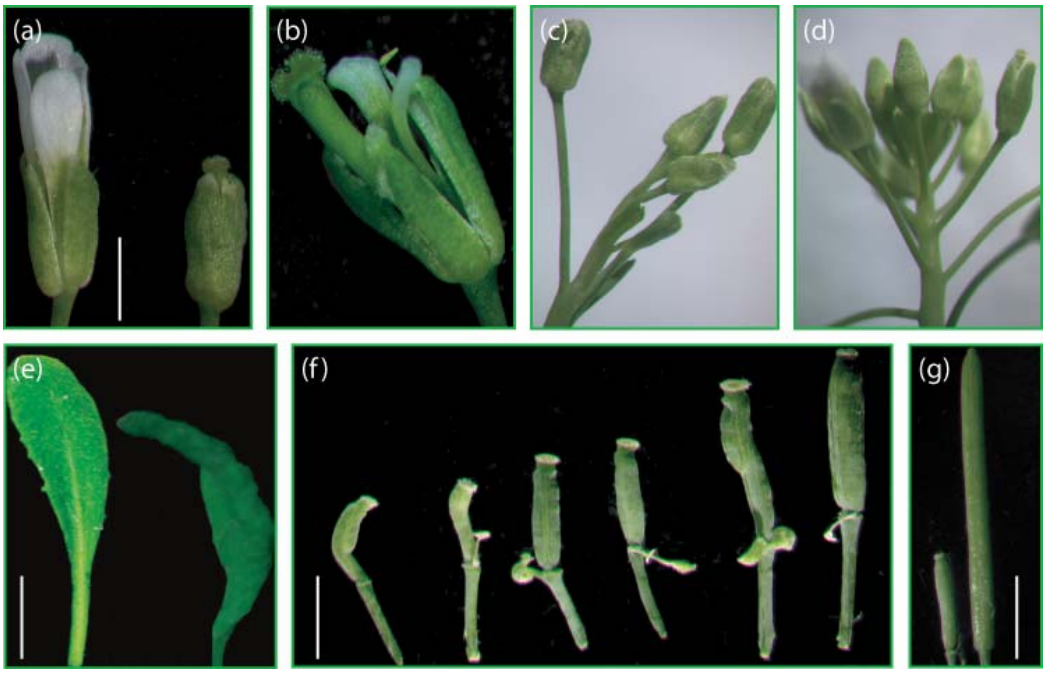


Fig. 3.3-3: Floral and leaf phenotypes of *sol2*. (a) Comparison of flowers of the similar developmental stage in *las-4 max1-1* (left) and *sol2* (right). Bar = 1mm. (b) A late *sol2* flower bud dissected open to show rudimentary petal formation. (c) Inflorescence branching in *sol2*. (d) Inflorescence of *las-4 max1-1*. (e) Comparison of rosette leaves of *las-4 max1-1* (left) with those of *sol2* (right). Bar = 1cm. (f) A range of smallest to largest siliques in *sol2*. Bar = 2mm. (g) Comparison of siliques of similar stage in *sol2* (left) and *las-4 max1-1* (right). Bar = 5mm.

The floral organ fusions and the defective leaf morphology of *sol2* mutants were reminiscent of those in other *Arabidopsis* mutants like *fiddlehead (fdh), lacerata (lcr)*, *hothead (hth)* and *bodyguard (bdg)*. *FIDDLEHEAD* is involved in the synthesis of long-chain lipids found in the cuticle, and is similar to a family of genes encoding β-ketoacyl-CoA synthases and chalcone synthases (Pruitt *et al.,* 1999). The *LACERATA* gene encodes a cytochrome P450 monooxygenase that catalyzes hydroxylation of fatty acids (Wellesen *et al*, 2001). *HOTHEAD* is required for cellular interaction in epidermal layer and it codes for an enzyme related to FAD-containing oxidoreductases (Krolikowski *et al.,* 2003). *BODYGUARD* also regulates epidermal development and codes for a protein belonging to α/β- hydrolase superfamily (Kurdyukov *et al.,* 2006). Mutations in all these genes resulted in defective development of the epidermal layer, in some cases resulting in severe fusions in floral organs and leaves. Additionally, the *lcr* mutant is also characterized by a bushy habit. However, sequencing of genomic DNA revealed that *sol2* contained the wild-type allele of *FDH, LCR, HTH* and *BDG* genes. In addition to that, analysis of double mutants of *sol2* with all the above mentioned mutants disclosed that *sol2* was not allelic to any of them (S.Yephremov, personal communication).

Cross	Total no. of F2 plants analysed	No. of plants with *sol2* phenotype	No. of plants with *las-4* phenotype	No. of plants with *max1-1* phenotype	No. of plants with wt phenotype
Sol2 X *las-4*	54	12	42	-	-
Expected	54	13.5	40.5	-	-
sol2 X Col	108	6	24	18	60
Expected	108	6.75	20.25	15.1	65.8

Table 3.3-1: Analysis of segregation in an F2 population arising from backcrossing and out-crossing of *sol2* to *las-4* and Col, respectively. Plants were grown for 28d in SD and then shifted to LD to induce flowering. Phenotypic classification was carried out visually. Genotypes of the different classes were confirmed using molecular markers.

In order to remove any possible additional mutations in the background, *sol2* was back-crossed to *las-4*. Out of 54 plants analysed in the F2 generation of this backcross, 12 plants displayed the bushy *sol2* phenotype and the rest were characterised by the *las-4* phenotype (Table 3.3-1). This corresponds to a 3:1 segregation (expected numbers: 40.5:13.5), thus indicating that *sol2* phenotype probably results from a mutation in one additional locus in the *las-4* background. This segregation also suggested that the *sol2* phenotype was not dependent on the additional *max1-1* mutation in the background. *sol2* was also crossed to Col wild-type in order to determine if the new mutation had any phenotypes on its own. 108 F2 plants arising from this out-crossing were analysed for their phenotypes. 57 of these plants were wild-type in their branching pattern; 25 plants were characterized by the *las-4* phenotype and 20 by the bushy *max1-1* phenotype. Only

six plants displayed a *sol2* phenotype (Table 3.3-1). This segregation suggested that the *sol2* phenotype was dependent on *las-4*, but independent of *max1-1*.

3.3.4 Phenotypic characterisation of *sol6*

sol6 plants identified in the suppressor screening were selfed for three generations to validate their phenotype. In short day growing conditions, *sol6* displayed a robust outgrowth of side-shoots from the uppermost rosette leaf axils (Fig. 3.3-4a and 3.3-4c) when compared to the *las-4 max1-1* parent. Closer inspection of the rosette under a stereomicroscope revealed that other than a high proportion of side-shoots developing from the youngest leaf axils, the leaves formed during the middle phase of vegetative development in *sol6* also supported varying proportion of axillary bud formation (Fig. 3.3-4c). Furthermore, *sol6* was characterized by a dwarf habitus, smaller rosette leaves and more robust axillary shoots when compared to *las-4 max1-1*(Fig. 3.3-4a). These phenotypes almost disappeared in plants grown either to maturity in long days or grown first under short photoperiods and then shifted to long days to induce flowering (Fig. 3.3-4b). However, in both these growing conditions axillary buds were produced from rosette leaf axils comparable to when these plants were grown to maturity in short days. No defects were observed in the formation of axillary buds from cauline leaf axils in any of these growth conditions.

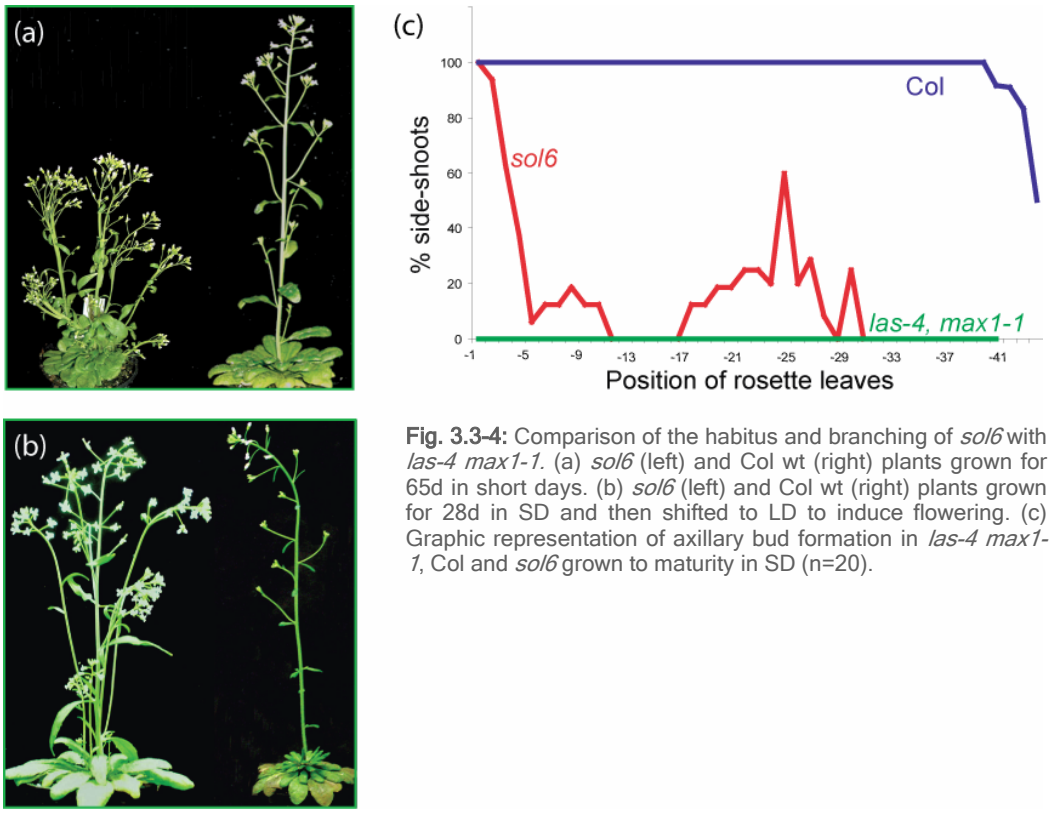


Fig. 3.3-4: Comparison of the habitus and branching of *sol6* with *las-4 max1-1*. (a) *sol6* (left) and Col wt (right) plants grown for 65d in short days. (b) *sol6* (left) and Col wt (right) plants grown for 28d in SD and then shifted to LD to induce flowering. (c) Graphic representation of axillary bud formation in *las-4 max1-1*, Col and *sol6* grown to maturity in SD (n=20).

sol6 was back-crossed to *las-4* and segregation of phenotypes in the F2 was analysed. Of 54 plants analysed, 11 plants displayed the *sol6* phenotype and the remaining 43 were characterised by the *las-4* phenotype. This corresponds to a 3:1 segregation (expected numbers: 40.5:13.5), and suggests that *sol6* phenotype arises from a mutation in one additional locus in the *las-4 max1-1* background.

Total no. of F2 plants analysed	No. of plants with *sol6* phenotype	No. of plants with *las-4* phenotype	No. of plants with *max1-1* phenotype	No. of plants with wt phenotype
200	3	42	33	122
Expected	12.5	37.5	37.5	112.5

Table 3.3-2: Analysis of segregation in an F2 mapping population arising from crossing of *sol6* to L*er*. Plants were grown for 28d in SD and then shifted to LD to induce flowering. Phenotypic analysis was performed visually. Genotypes of classes showing *las-4* and *max1-1* phenotypes were confirmed using molecular markers.

sol6 was crossed to L*er* to generate an F2 rough mapping population. *sol6* was expected to segregate 1 in 16 in this population. However, of the 200 F2 plants screened, only 3 plants, as opposed to the expected 12, displayed the *sol6* phenotype (Table 3.3-2). This suggested that the L*er* background might have a modifier that obscures the *sol6* phenotype.

3.3.5 Phenotypic characterisation of *sol7*

Another mutant identified from the screening which displayed a strong suppression of the *las-4 max1-1* phenotype was *sol7*. It was characterized by a very bushy phenotype whose intensity was dependent on day-length conditions. *sol7* plants grown continuously in short days produced a large number of side-shoots compared to *las-4 max1-1* (Fig. 3.3-5a). Analysis of rosette leaf axils of *sol7* using a stereomicroscope revealed that axillary buds were formed in a high proportion in the axils of rosette leaves during late vegetative development (Fig. 3.3-5c). However, almost no axillary buds were detected in the axils of early rosette leaves (Fig. 3.3-5c). In *sol7* plants grown for 28d in short days and then subsequently induced to flower in long days, the pattern of axillary bud formation was very similar to that observed in *sol7* grown to maturity in short days (Fig. 3.3-5c and 3.3-5d). However, when grown to maturity under long day conditions, *sol7* does not show an increase in axillary bud formation when compared to the *las-4 max1-1* parent (Fig. 3.3-5e). This suggests that the *sol7* phenotype is dependent on day length. Short photoperiods for the first few weeks are mandatory to obtain the *sol7* bushy habit. All cauline leaf axils of *sol7* plants support the formation of axillary buds, irrespective of growing and day-length conditions.

In addition to the bushy habit, *sol7* plants were paler green in colour. Another notable feature of *sol7* plants was their reduced flowering time. *sol7* flowered much earlier than

the *las-4 max1-1* and Col wt plants. Flowering time was monitored by growing the plants to maturity in short day conditions, and counting the number of rosette leaves formed as a measure of the duration of the vegetative phase in the plants. *sol7* plants formed around 19-22 rosette leaves before transition to reproductive phase. This demonstrated that *sol7* was very early flowering when compared to the *las-4 max1-1* or Col plants, which formed between 34-40 and 30-43 rosette leaves, respectively (Fig. 3.3-5f).

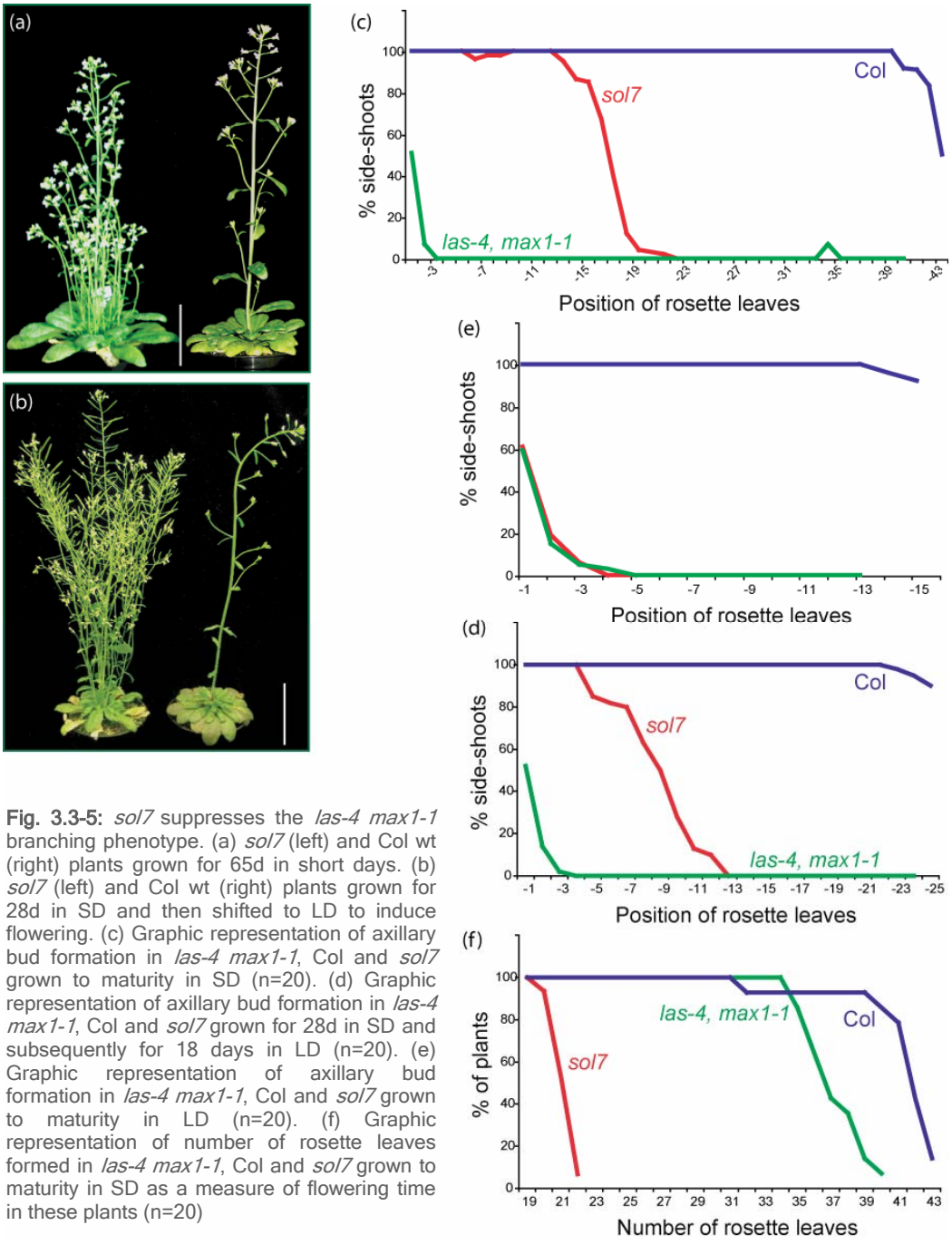


Fig. 3.3-5: *sol7* suppresses the *las-4 max1-1* branching phenotype. (a) *sol7* (left) and Col wt (right) plants grown for 65d in short days. (b) *sol7* (left) and Col wt (right) plants grown for 28d in SD and then shifted to LD to induce flowering. (c) Graphic representation of axillary bud formation in *las-4 max1-1*, Col and *sol7* grown to maturity in SD (n=20). (d) Graphic representation of axillary bud formation in *las-4 max1-1*, Col and *sol7* grown for 28d in SD and subsequently for 18 days in LD (n=20). (e) Graphic representation of axillary bud formation in *las-4 max1-1*, Col and *sol7* grown to maturity in LD (n=20). (f) Graphic representation of number of rosette leaves formed in *las-4 max1-1*, Col and *sol7* grown to maturity in SD as a measure of flowering time in these plants (n=20)

sol7 was backcrossed to *las-4* to remove any additional mutations in the background. Eleven of the 54 F2 plants analysed displayed the characteristic *sol7* phenotype. These plants were also early flowering. The remaining 43 plants in the F2 population were characterised by the *las-4* phenotype (Table 3.3-3). This fits with an expected 3:1 segregation (expected numbers: 40.5:13.5), and suggested that *sol7* phenotype resulted from a mutation in one additional locus in the *las-4* background and is independent of the *max1-1* mutation. To evaluate if *sol7* had any independent phenotype of its own, it was also out-crossed to Col wt. Out of the 108 F2 plants analysed, 8 plants displayed a *sol7* phenotype (Table 3.3-3). The remaining plants could be classified phenotypically as follows: 28 *las-4*, 18 *max1-1* and 54 Col wt. This segregation suggested that the *sol7* phenotype was dependent on *las-4*.

Cross	Total no. of F2 plants analysed	No. of plants with *sol7* phenotype	No. of plants with *las-4* phenotype	No. of plants with *max1-1* phenotype	No. of plants with wt phenotype
sol7 X *las-4*	54	11	43	-	-
Expected	54	13.5	40.5	-	-
sol7 X Col	108	8	28	18	54
Expected	108	6.75	20.25	20.25	60.75

Table 3.3-3: Analysis of segregation in an F2 population arising from backcrossing and outcrossing of *sol7* to *las-4* and Col, respectively. Plants were grown for 28d in SD and then shifted to LD to induce flowering. Phenotypic classification was carried out visually. Genotypes of classes showing *las-4* and *max1-1* phenotypes were confirmed using molecular markers.

3.3.6 Complementation analysis for allelism among *sol2, sol6* and *sol7*

To unravel whether the *sol2, sol6* and *sol7* phenotypes resulted from independent mutations in the same gene or in different genes, complementation tests for functional allelism were carried out. The three different *sol* lines described above were crossed to each other, and the F1 arising from each of the three crossings were grown for 35d in short day conditions and subsequently shifted to long days to induce bolting.

Fifteen F1 plants from each of the three crossings were analysed under a stereomicroscope for presence or absence of axillary buds in the rosette leaf axils. If the different *sol* mutations are allelic, one would expect a *sol* phenotype in the F1. However, all the F1 lines were characterized by the *las-4* branching phenotype. As illustrated graphically in Fig. 3.3-6, the F1 plants of all the three crossings developed axillary buds during late vegetative phase of development, comparable to the axillary bud formation pattern in *las-4 max1-1* plants.

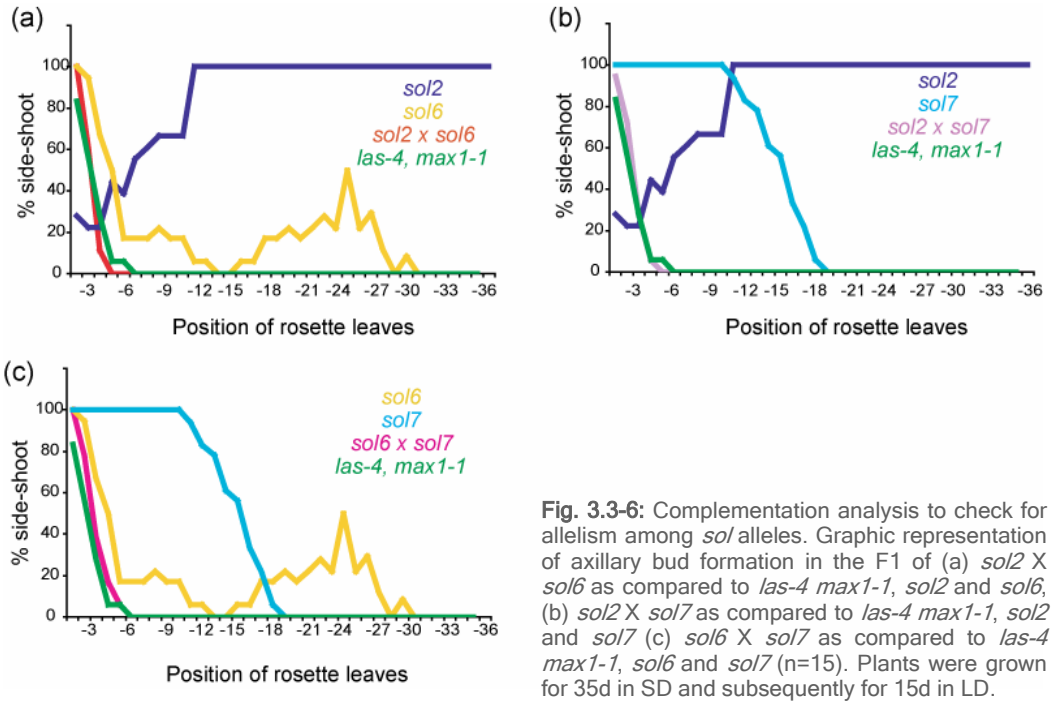


Fig. 3.3-6: Complementation analysis to check for allelism among *sol* alleles. Graphic representation of axillary bud formation in the F1 of (a) *sol2* X *sol6* as compared to *las-4 max1-1*, *sol2* and *sol6*, (b) *sol2* X *sol7* as compared to *las-4 max1-1*, *sol2* and *sol7* (c) *sol6* X *sol7* as compared to *las-4 max1-1*, *sol6* and *sol7* (n=15). Plants were grown for 35d in SD and subsequently for 15d in LD.

These results demonstrated that the *sol2, sol6* and *sol7* are independent mutations in different genes.

3.3.7 Molecular mapping of *sol2* and *sol7*

Since the *sol* mutations were induced by EMS, which typically causes single base pair exchanges, it was possible to pursue positional mapping of these loci. From among the three *sol*s detailed in this chapter, *sol2* and *sol7* were selected for molecular mapping based on their distinct suppressor phenotypes and clear segregation in backcross- and outcross analyzes. *sol6*, as described earlier, exhibited an anomalous segregation in the F2 when crossed to Ler, and hence was excluded from further mapping analysis. A detailed description of the generation of mapping populations and the evaluation of polymorphic PCR-based SSLP markers in this mapping population is provided in this section.

3.3.8 Generation of mapping populations

As described earlier, the *sol* lines arose from an EMS mutagenesis of seeds obtained from *las-4 max1-1* homozygous plants in Col ecotype. In order to generate a mapping population and facilitate positional mapping, it was essential to cross *sol2* and *sol7* to a different genetic background exhibiting sufficient molecular polymorphism to Col. To this end, L*er* was chosen as the crossing partner. An M4 *sol2* plant was selected and crossed to a L*er* plant. The F1 plants were grown and selfed in long days, and exhibited a wild-type branching phenotype. Since *sol2* phenotype is dependent on the *las-4* mutation in the background, it was expected to segregate 1 in 16 in the F2 of the above cross.

In a second crossing, an M3 *sol7* plant was selected and crossed to a L*er* plant carrying the *las-4* mutation. This L*er* parent was obtained from a third backcross of the original *las-4* mutant in Col background to L*er* (Clarenz, 2004). The F1 plants were grown and selfed in long days. These plants displayed the *las-4* branching phenotype, as they remained homozygous for the *las-4* mutation. Consequently, in the F2 of such a cross, *las-4* would not segregate anymore, and *sol7* phenotype was thus expected to display a segregation ratio of 1 in 4.

3.3.9 Analysis of *sol2* mapping population

F2 seeds arising from the crossing of *sol2* to L*er* were sown and grown to maturity under short photoperiods. A population of 480 F2 plants was visually analysed for segregation of *sol2* plants. The *sol2* phenotype was slightly modified in the L*er* background. In the L*er* background, the dwarf habitus and the leaf phenotype of *sol2* was clearly reduced. Hence, it was difficult to distinguish *sol2* from plants segregating for the *max1-1* mutation. Since visual identification of *sol2* in this mapping population was difficult and did not conform to the expected numbers, an alternative analysis using molecular markers was employed. DNA was extracted from all the 480 plants, and analysed molecularly for segregation of the *las-4* homozygotes using PCR markers. The primers were designed to produce a 244 bp *las-4* mutant product and a 471 bp wild-type product, which could be easily distinguished by 4% agarose gel electrophoresis. The heterozygotes displayed both the bands. An example of such an agarose gel is illustrated in Figure 3.3-7.

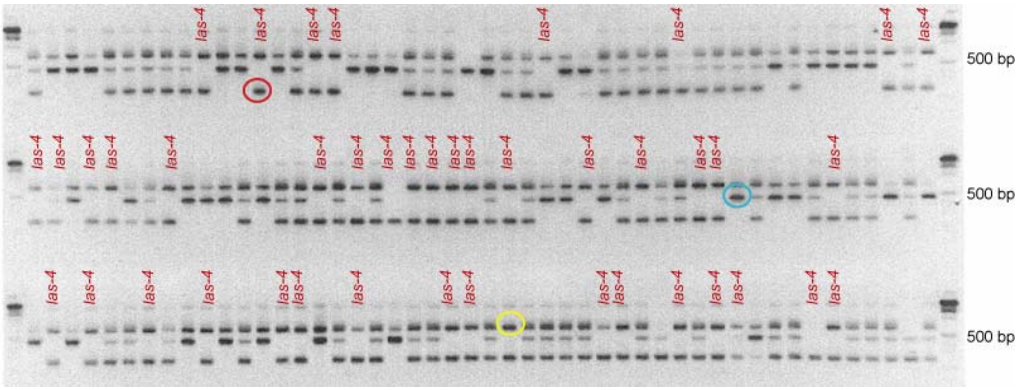


Fig. 3.3-7: Molecular analysis of segregation of *las-4* in *sol2* x L*er* F2 mapping population using PCR-based markers. Two pairs of primers, namely AtLs2599F, AtLs3070R, AtLs2349F and AtLs2593muR, were used for this analysis. The primer pair AtLs2599F and AtLs3070R produced a 471 bp product (indicated, for instance, by the blue circle) for wild-type. The primer pair AtLs2349F and AtLs2593muR produced a 244 bp product (indicated, for instance, by the red circle) specific for *las-4* mutation. Presence of both the bands indicates heterozygosity. The primers AtLs2349F and AtLs3070R could produce a 721 bp ubiquitous band (indicated, for instance, by the yellow circle).

As expected, one quarter of the plants in the mapping population were homozygous for the *las-4* mutation (Table 3.3-4). Of the 125 plants that were found to be homozygous for the *las-4* mutation, one quarter was expected to have *sol2* phenotype. Phenotypic examination of these homozygous *las-4* mutants revealed that 27 plants were *sol2* in

phenotype with axillary buds arising from a large number of leaf axils. DNA extracted from 24 of these homozygous plants was used for a rough molecular mapping of *sol2*

Cross	Total no. of F2 plants analysed	No. of plants homozygous for *las-4*	No. of plants heterozygous for *las-4*	No. of plants wt for *LAS*
sol2 X L*er*	480	125	249	106
Expected	480	120	240	120

Table 3.3-4: Molecular analysis of segregation in an F2 population arising from crossing of *sol2* to L*er*. Plants were grown for 70d in SD. DNA was extracted from all 480 plants, and molecularly analysed using PCR markers polymorphic for the *las-4* mutation.

3.3.10 Molecular mapping of *sol2*

PCR-based *simple sequence length polymorphism* (SSLP) markers were used for molecular mapping of *sol2*. A set of 22 SSLP markers distributed evenly over the *A.thaliana* genome was used for this purpose (Lukowitz *et al.,* 2000). Fig. 3.3-8 illustrates the positions of these SSLP markers on the five chromosomes.

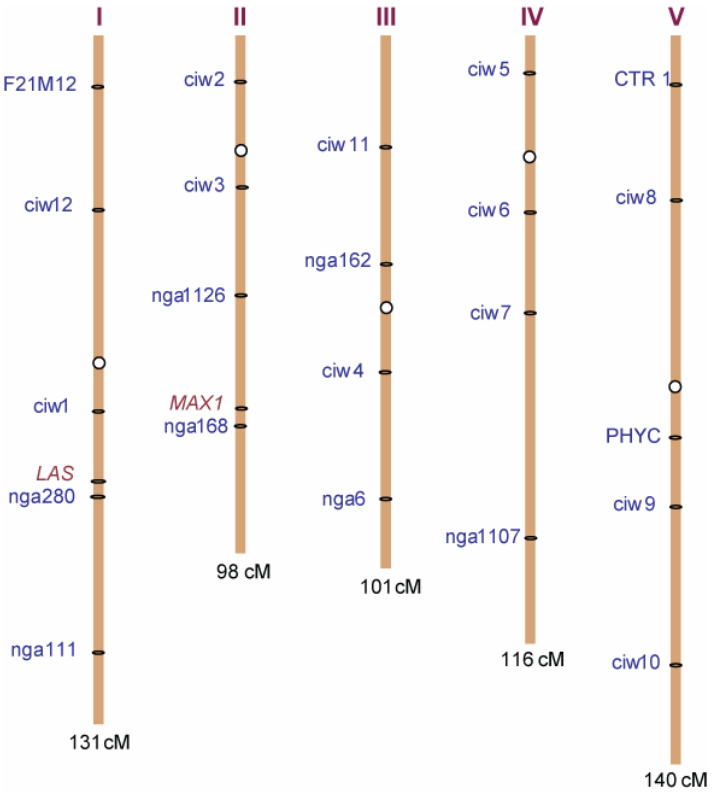


Fig. 3.3-8: Schematic representation of the SSLP marker positions on the five chromosomes of *A. thaliana.* Each of the five chromosomes is depicted as a bar, with their respective lengths given in centi-Morgan. The centromeric region of each chromosome is represented by the black circle. The position of each of the twenty two SSLP markers is indicated. The positions of *LAS* and *MAX1* on chromosome 1 and chromosome 2, respectively, are also marked.

SSLPs are co-dominant markers, and therefore both chromosomes of the diploid pair can be genotyped and maximum amount of information can be gathered. These markers are PCR-based and can be easily analysed on agarose gels, thus making them relatively inexpensive as well as easy to use. The set of 22 SSLP markers used were polymorphic for Col and L*er* ecotypes, and information about these polymorphisms could be obtained

from The Arabidopsis Information Resource (TAIR). The Col and L*er* PCR products differed in sizes varying from 10 to 100 bp, and these size differences could be distinctly resolved on a 4% agarose gel by electrophoresis.

Since the *sol2* mutation was in a Col background, and was crossed to L*er* to create the F2 mapping population, a mixture of genetic material from the two different backgrounds was expected. The co-dominant SSLP markers would therefore, under normal conditions, segregate 1:2:1 for the Col and L*er* alleles at each locus. However, since only plants homozygous for the *sol2* allele were selected for mapping, one would expect that the markers would segregate predominantly for the Col allele in the vicinity of the new mutation, and the closer the marker is to the new mutation, the higher would be the percentage of the Col polymorphic marker segregation. A good control for this was the *las-4* mutation in the *sol2* background. Since all *sol2* plants are also homozygous for the *las-4* mutation, a 100% co-segregation for the Col allele was expected at the *las-4* locus.

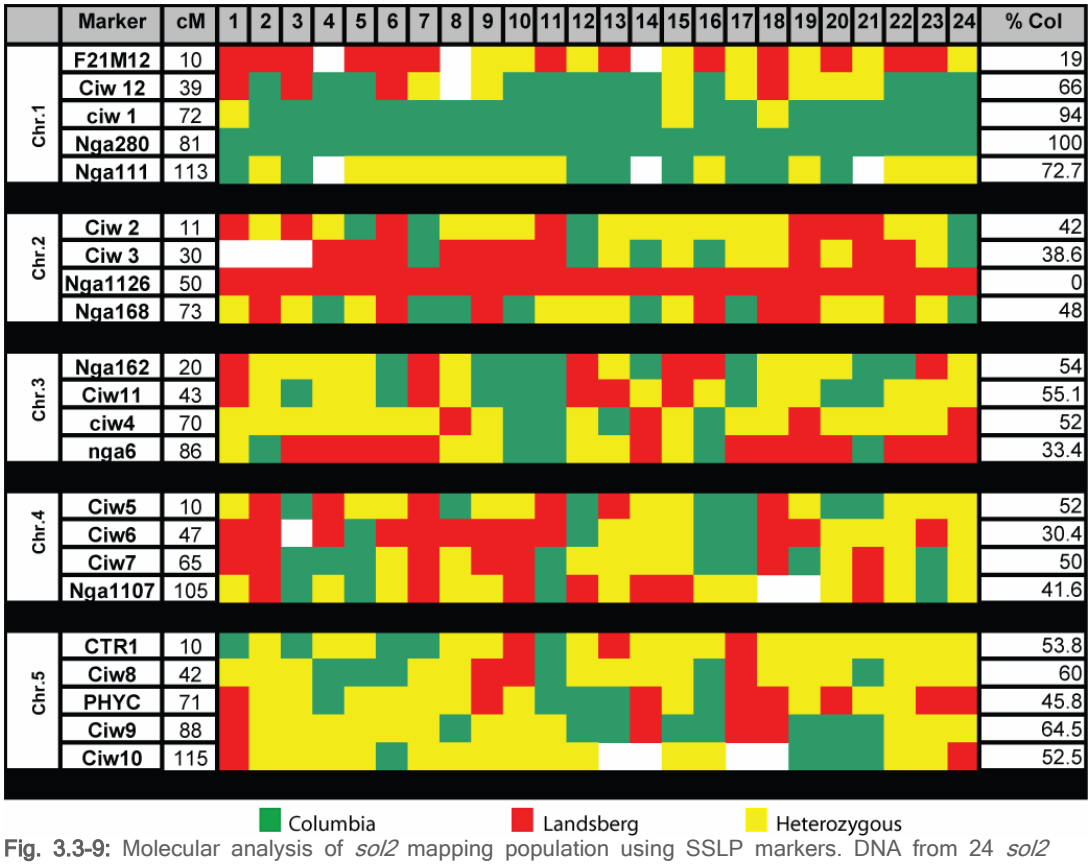


Fig. 3.3-9: Molecular analysis of *sol2* mapping population using SSLP markers. DNA from 24 *sol2* homozygous plants was analysed with a set of 22 SSLP markers polymorphic for Col and L*er*. Col alleles are scored in green, L*er* alleles in red and heterozygotes in yellow. White boxes represent cases were scoring was ambiguous or where no score could be obtained. Percentage segregation for Col chromosome is denoted in the last column.

The segregation of the selected SSLP markers in the *sol2* mapping population is summarised in Fig. 3.3-9. As expected, the Nga280 marker, which lies closest to the *las-4* locus, co-segregated 100% for the Col allele. On chromosomes 3, 4 and 5, the markers

showed an approximate 1:2:1 segregation for Col, heterozygous and L*er* alleles. Since none of these markers displayed a clear co-segregation for the Col allele, the occurrence of the *sol2* locus on these three chromosomes could be ruled out. Similarly, most of the marker on chromosome 1 and chromosome 2, displayed an approximate 50% segregation for Col and L*er* alleles. However, surprisingly, markers F21M12 (chromosome 1) and Nga1126 (chromosome 2) displayed very strong co-segregation (81% and 100%, respectively) for the L*er* allele. Since the *sol2* mutation is in Col background, these co-segregations can only be explained by the presence of some quantitative trait loci (QTLs) in these regions of the L*er* genome that could suppress the *las-4* branching phenotype.

The high percentage for Col segregation at Ciw1 (94%) and Nga111 (73%), the two markers flanking Nga280 could be explained on the basis of their proximity to the *las-4* locus. However, since Nga111 is more than 30cM from the *las-4* locus, its surrounding was identified as a potential region for the location of the *sol2* locus. Further molecular markers needed to be applied to investigate this region and to get closer to the *sol2* locus. A bigger mapping population segregating for about a 100 *sol2* plants was also necessary to be able to fine map the locus. Hence, efforts were concentrated on developing a second mapping population with a better segregation ratio for *sol2*. To create such a mapping population, *sol2* was crossed to *las-4* introgressed into L*er* from Col by three backcrosses. In the F2 of this population, *sol2* would segregate 1 in 4. Such a population is now ready to be used for fine mapping the *sol2* locus.

3.3.11 Analysis of *sol7* mapping population

F2 seeds arising from the crossing of *sol7* to *las-4* introgressed into L*er* were sown and grown to maturity under short photoperiods. Since the *las-4* locus remains homozygous in this population, *sol7* was expected to segregate in the ratio of 1 in 4. Of 160 plants analysed in this F2 population, 41 plants could be identified as *sol7*. The phenotype of *sol7* was slightly altered in the L*er* background, the plants appearing less bushy and more erect. DNA extracted from these *sol7* plants was used for a rough molecular mapping using of the *sol7* locus.

Cross	Total no. of F2 plants analysed	No. of plants with *sol7* phenotype	No. of plants with *las-4* phenotype
sol7 X *las-4*/ L*er*	160	41	119
Expected	160	40	120

Table 3.3-5: Analysis of segregation in an F2 population of *sol2* crossed to *las-4* in L*er*. Plants were grown for 70d in SD. Phenotypic classification was carried out visually.

3.3.12 Molecular mapping of *sol7*

The same set of SSLP markers as described in section 3.3.10 was employed for rough mapping of the *sol7* locus. *sol7*, *las-4* introgressed into L*er*, Col wt, L*er* wt and Enkheim-2 (En-2) wt DNA were used as controls to check for polymorphism of the SSLP markers used. The *max1-1* mutation was introgressed into Col from the En-2 accession with seven backcrosses (Stirnberg *et al.,* 2002). An illustration of the segregation of these PCR-based molecular markers in the *sol7* mapping population is provided in Fig. 3.3-10.

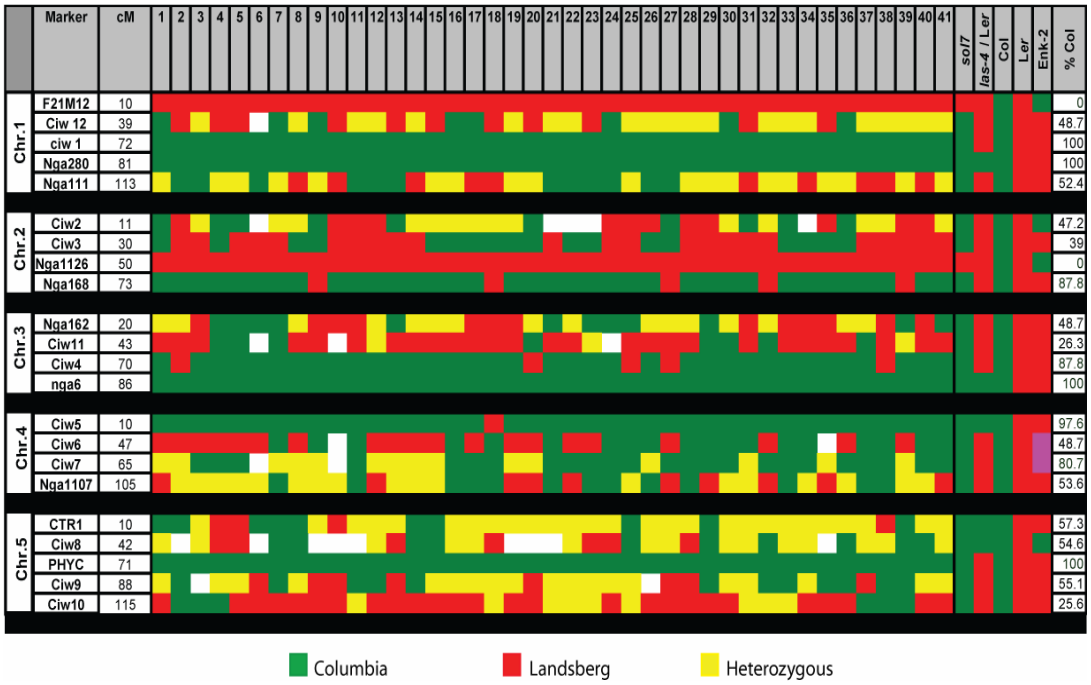


Fig. 3.3-10: Molecular analysis of *sol7* mapping population using SSLP markers. DNA from 41 homozygous *sol7* plants were analysed with a set of 22 SSLP markers polymorphic for Col and L*er*. Five controls included are: *sol7*, *las-4* introgressed into L*er*, Col wt, L*er* wt and En-2 wt. Col alleles are scored in green, L*er* alleles in red and heterozygote in yellow. Pink boxes represent polymorphic En-2 alleles. White boxes represent cases were scoring was ambiguous or where no score could be obtained. Percentage segregation for Col chromosome is denoted in the last column.

On chromosome 1, marker Nga280 co-segregated 100% for the Col allele as expected, since both parents are homozygous for the *las-4* mutation in a Col background in this region. Additionally, *las-4* introgressed into L*er* also retains Col background at the end of chromosome 3 and the beginning of chromosome 4, which explains the lack of polymorphism between the two parents for markers Nga6 and Ciw5, and the resulting 100% co-segregation for the Col allele. Interestingly, the *sol7* parent contains the L*er* polymorphic allele for the markers F21M12 and Nga1126 on chromosome 1 and chromosome 2, respectively. Not surprisingly, thus, these two markers co-segregate 100% for the L*er* allele in the mapping population. Since *sol7* contains the *max1-1* mutation that was introgressed from En-2 background, any potential non-polymorphism for the En-2 allele with the L*er* allele of these two markers was tested. However, the En-2 alleles of these markers were similar to their Col alleles, and exhibited clear

polymorphism with the L*er* alleles. Thus, the appearance of L*er* backgound at these two markers in *sol7* could not be resolved. The segregation of F21M12 and Nga1126 in the *sol2* mapping population had also followed a similar pattern as in *sol7*.

In addition to those mentioned above, one marker on each of the five chromosomes showed a high percentage of segregation for the Col allele. These were: Ciw1 (chromosome1, 100%), Nga168 (chromosome 2, 88%), Ciw4 (chromosome 3, 88%), Ciw7 (chromosome 4, 81%) and PHYC (Chromosome 5, 100%). The high Col segregation in Ciw1 may be on account of linkage with the *las-4* mutation in the vicinity. The remaining multiple regions of high Col background and the low frequency of heterozygous plants are not unequivocally explainable and suggested a flaw in the mapping population and/or the markers used. Heterozygous plants from the F1 of the mapping population need to be included as controls for the markers.

Two additional mapping populations for *sol7* were created. The first is an F1BC1 back cross population created by crossing the F1 of a *sol7* plant crossed to *las-4* introgressed into L*er* back to the original *sol7* mutant. This population segregates 1:1 for the *sol7* phenotype. The second population is an F2 of *sol7* crossed to *las-11*, a new allele of *las* in L*er*. Both these populations are ready to be analysed and rough-mapped.

4 DISCUSSION

4.1 *CUP-SHAPED COTYLEDON* GENES AND miRNA164 REGULATE THE FORMATION OF AXILLARY MERISTEMS

The *Arabidopsis CUC* genes are expressed in a variety of boundary regions between organs as well as between the SAM and developing organs (Vroemen *et al.,* 2003; Greb, 2003; Keller *et al.,* 2006; Hibara *et al.,* 2006). The characteristic expression pattern of these genes in the axils of developing leaves suggests a role of *CUC* genes in axillary meristem development. Mutations in the corresponding homologous *CUPULIFORMIS* gene from *Antirrhinum* lead to the development of misformed leaf axils that do not support the formation of lateral buds (Weir *et al.,* 2003). This also suggests a role for *CUC1/CUC2* in axillary meristem initiation.

4.1.1 *CUC* GENES ARE REDUNDANTLY REQUIRED FOR AXILLARY MERISTEM FORMATION

Axillary meristems develop from the boundary regions between leaf primordia and the inner part of the shoot apex. Since the transcripts of the three *Arabidopsis CUC* genes, *CUC1, CUC2* and *CUC3* accumulate in this region, a possible role of these genes in axillary meristem formation was investigated during this study. *cuc1-1* and *cuc2-1* single mutants did not display any defects in axillary meristem development. Phenotypic analysis demonstrated that *cuc3-2* plants do not develop axillary buds in rosette leaf axils in the early and middle phase of vegetative development. Axillary bud formation was compromised to a lesser extent towards the top of the rosette and in the reproductive phase. The intensity of this phenotype also depended on the day-length conditions. In long days, absence of axillary buds in *cuc3-2* plants was restricted mainly to younger rosette leaf axils. However, in *cuc3-2* plants grown under short day conditions, in addition to absence of axillary bud formation in the axils of rosette leaves during the middle and late vegetative phase, axillary bud formation was also lacking in the cauline leaf axils. SEM analysis demonstrated that empty leaf axils did not contain any morphological structures that could be traced back to the activity of an axillary meristem. These observations suggest that *CUC3* activity is required in the early and middle phase of vegetative development to initiate axillary meristems in a day-length dependent manner.

The block of axillary bud formation in *cuc3-2* mutants was not absolute as some axillary buds were formed, mainly during the reproductive phase. On the other hand *cuc1-1* and *cuc2-1* mutants are not compromised in axillary bud formation. Likewise, wild type plants overexpressing miR164 also did not show any defects in axillary meristem development. Post-embryonic shoots regenerated from *cuc1, cuc2* calli also display wild type axillary shoot formation (Hibara *et al.,* 2006). To test for a possible redundant involvement of

CUC1 and *CUC2* in the process of axillary meristem initiation, the consequences of misexpression of *MIR164A* and *MIR164B* under the control of the *CaMV35S* promoter in a *cuc3-2* background was studied. A subgroup of plants arising from such a population was characterised by the formation of cup-shaped cotyledons, thereby indicating a strong reduction in *CUC1* and/or *CUC2* activity in these plants. The presence of the T-DNA overexpressing MIR164A was molecularly confirmed. In most cases, these cup-shaped seedlings initiated a new meristem in the hypocotyl region below the base of the fused cotyledons. The rosette and the main shoot arising from this new SAM characteristically displayed an almost complete block in axillary bud formation. Taken together, these results demonstrate that, in addition to *CUC3*, *CUC1* and *CUC2* also play a role in the regulation of axillary meristem initiation.

CUC1 and *CUC2* are post-transcriptionally regulated by a microRNA, miR164 (Laufs *et al.,* 2004; Mallory *et al.,* 2004). The role of these two genes in lateral branch formation was further studied by analysing the shoot branching pattern of a transgenic line containing miR164-resistant variants of *CUC1* and *CUC2.* In *CUC2g-m4*, silent mutations are introduced into the miRNA-binding site in the *CUC2* gene, thus making it resistant to miR164 regulation (Nikovics *et al*. 2006). These plants developed accessory buds in the axils of late rosette leaves and early cauline leaves, showing that increased *CUC2* activity leads to additional axillary meristems. Similar formation of accessory buds was observed in the leaf axils of *5mCUC1*, a miR164-resistant allele of *CUC1*. In *5mCUC1* transgenic plants, accessory buds were formed in most of the older cauline leaf axils and in the axils of rosette leaves formed during middle-phase of vegetative development. Altogether, these data indicate that *CUC1* and *CUC2* activity is not only required for axillary meristem formation but is also rate limiting during normal development and that additional axillary meristems form in response to an increase in *CUC1* and *CUC2* activity. Also, the differences in the pattern of accessory bud formation in rosette leaves of *5mCUC1* and *CUC2g-m4* suggests *CUC1* and *CUC2* function in the control of axillary meristem formation along different zones on the main shoot.

4.1.2 miR164 CONTROLS THE NUMBER OF BUDS PER LEAF AXIL

Schwab *et al*. (2005) have shown that six NAC-domain transcription factors contain binding sites for miR164, which is encoded by the genes *MIR164A, MIR164B,* and *MIR164C*. Among those NAC-domain genes regulated by miR164 are *CUC1* and *CUC2,* but not *CUC3*. In this study, phenotypic analysis of *mir164a-4* and *mir164b-1* (Mallory *et al.,* 2004; Nikovics *et al.,* 2006) showed that these loss-of-function mutants develop accessory buds in the axils of cauline leaves. This phenotype is strongly enhanced in the *mir164a-4 mir164b-1* double mutant, in which almost every cauline leaf axil developes an accessory bud. However, different from the accessory bud development observed in miRNA-resistant *CUC*1 and *CUC2* plants, *5mCUC* and *CUC2g-m4* respectively, where

accessory bud developed in axils of rosette as well as cauline leaves, in the *mir164a* and *mir164b* mutants the formation of accessory buds occurred only in cauline leaf axils. This difference in the mutant phenotypes indicates that the third gene encoding miR164, *MIR164C*, may have a role in regulating *CUC1* and *CUC2* transcript accumulation in the axils of the rosette leaves. Alternatively, this observation may be explained by the different genetic backgrounds of the respective lines. *mir164* mutants are in Columbia background and the *CUC2g-m4* plants have a Ws background, which shows a stronger tendency to produce accessory side shoots.

In situ hybridization of miR164 in *Nicotiana benthamiana* reveals specific temporal and spatial expression patterns in developing flowers, ovules, ovary, pollen sacs and anthers (Valoczi *et al.,* 2006). miR164 also accumulates to high levels in vascular bundles, procambium and meristems. However, the precise cellular expression domain of *Arabidopsis* miR164 in the shoot apex is not yet known. This study shows that miR164 is required to control the transcript levels of *CUC1* and *CUC2* in leaf axils. Loss of miR164 activity may lead to a broader domain of *CUC1* and *CUC2* transcript accumulation resulting in the formation of more than one axillary bud. Alternatively, miR164 may be needed to restrict the developmental time window of *CUC1* and *CUC2* mRNA accumulation.

4.1.3 *CUC* GENES ARE EARLY REGULATORS OF AXILLARY MERISTEM FATE

The *cuc3-2* branching pattern is similar to the patterns of shoot branching observed in the *Arabidopsis las-4* (Greb *et al.,* 2003) and *rax1-3* mutants (Müller *et al.,* 2006; Keller *et al.,* 2006). In *rax1-3* mutants, axillary meristem formation is impaired in the early phase of vegetative development and restored when the plant matures. *las-4* mutants usually do not develop axillary buds in the vegetative phase, but depending on growth conditions and environmental factors, axillary shoots are seen in the axils of the topmost rosette leaves at a low frequency. In *cuc3-2* mutants, axillary bud formation is severely compromised during early vegetative development and moderately during late vegetative and early reproductive phases of development. Transcripts of the *CUC3, RAX1,* and *LAS* genes accumulate in overlapping domains in the axils of developing leaf primordia (Greb *et al.,* 2003; Vroemen *et al.,* 2003; Müller *et al.,* 2006; Keller *et al.,* 2006). Taken together, the overlap in the mutant phenotypes and the patterns of transcript accumulation of these genes raise the possibility that *CUC3*, *RAX1*, and *LAS* interact to maintain the competence for axillary meristem formation in the early phase of vegetative development.

CUC1 and *CUC2* expression in a *las-4* mutant background remains unaltered when compared to their wild type expression pattern (Greb, 2003). Conversely, expression of *LAS* in *cuc1* and *cuc2* apices also mirrors the wild type expression pattern of *LAS* (Greb, 2003). However, *cuc1* and *cuc2* single mutants are not defective in axillary meristem

formation. In a recent study, *LAS* expression in the intercotyledon region was shown to be specifically eliminated in the *cuc1 cuc2* double mutant embryo (Hibara *et al.,* 2006). During this study, the RNA *in situ* hybridisation pattern of *LAS* in *cuc3-2* mutant, which is compromised in axillary meristem formation, was monitored. *LAS* expression pattern is not altered in *cuc3-2* apices. Also, double mutant analyses have revealed that *las-101* enhances the loss of tertiary shoot formation in *cuc2* and *cuc3* mutants (Hibara *et al.,* 2006). Taken together, these expression pattern studies and double mutant analyses indicate that *CUC3* might either be a regulator of *LAS* or act independently in a separate pathway to control axillary meristem development.

RNA *in situ* hybridisation pattern of *CUC2* in *rax1-2* reveals absence of *CUC2* transcripts in the domain overlapping RAX expression in the apex, thus indicating that *RAX1* regulation of axillary meristem initiation is mediated through *CUC2* (Keller *et al.,* 2006). In this work, the wild type expression pattern of *RAX1* was found to be unaltered in *cuc3-2* mutant apices. Double mutant analysis in tomato and in *Arabidopsis* have revealed that *LAS* and the *RAX* genes are involved in two different pathways regulating axillary meristem initiation (Schmitz *et al.,* 2002; Müller *et al.,* 2006). It remains to be conclusively tested whether or not *CUC3* plays a role in either of these two pathways or if it is involved in a third yet unknown pathway regulating axillary meristem initiation.

4.2 *LAS* MAINTAINS MERISTEMATIC POTENTIAL OF AXILLARY CELLS

The phenotypes of *las* mutants and the leaf axil specific expression pattern of *LAS* transcript have established it as a primary regulator of axillary meristem development (Greb *et al.,* 2003). However, axillary meristems are initiated only in the middle of the band-shape *LAS* expression domain at the boundary between SAM and the newly formed leaf primordia, suggesting that additional positional cues are required to specify the exact position of axillary meristem initiation. The specific spatial and temporal function of *LAS* was investigated by misexpressing it transgenically from the promoters of other meristem-specific genes that show an adjoining or overlapping expression domain with *LAS*.

4.2.1 Misexpression of *LAS* in the SAM does not produce ectopic meristems

In this study, *LAS* was ectopically expressed from the promoters of *STM, KNAT1* and *UFO*. The expression patterns of these genes overlap with that of *LAS*, although they are expressed in broader domains in the SAM (Lincoln *et al.,* 1994; Long *et al.,* 1996; Lee *et al.,* 1997; Long and Barton, 1998; Reiser *et al.,* 2000; Samach *et al.,* 1999; Laufs *et al.,* 2003).

In *las-4* mutants, the characteristic focused *STM* expression in the axils of older leaf primordia is found to be missing indicating the absence of axillary meristem initiation (Greb *et al.,* 2003). Misexpression of *LAS* from the *STM* promoter in a *las-4* mutant background partially complements the *las-4* branching defect. *las-4* plants harbouring an *STM::LAS* transgene produced axillary buds in most of the rosette leaf axils formed during the middle and late phase of vegetative development. Notably, the broadening of the *LAS* expression domain under the *STM* promoter did not result in formation of ectopic meristems. The *KNAT1::LAS* transgenes in *las-4* background complemented the *las-4* phenotype more strongly than the *STM::LAS* transgenes. In addition to most of the rosette leaf axils produced during late and middle vegetative development, a small proportion of the early rosette leaf axils also supported the formation of lateral buds. Furthermore, *KNAT1::LAS* plants flowered earlier than the *las-4* controls. Again, ectopic meristem formation was not observed in these transgenic plants. The *las-4* plants harbouring a *UFO::LAS* transgene displayed two different sets of phenotypes. One set of these transgenic lines displayed an almost complete complementation of the *las-4* branching phenotype. The other set of *UFO::LAS* transgenic lines did not only lack complementation, but also enhanced the *las-4* branching defect by eliminating axillary bud formation in most cauline leaf axils. The non-complementation and enhancer effects

in these transgenic lines may have been the result of silencing brought about by the *UFO::LAS* misexpression T-DNA. In the younger cauline leaf axils of these transgenic lines, wherever axillary meristems did initiate, they supported the formation of a flower rather than an axillary shoot. This observation can be vindicated by the role of *UFO* as a regulator of floral primordia development. However, most significantly, ectopic expression of *LAS* from the *UFO* promoter did not lead to the formation of any ectopic meristems.

Taken together, these results reconfirm the role of *LAS* as an early regulator of axillary meristem development. *LAS* function maintains the meristematic potential in the cells in which it is expressed. The lack of ectopic axillary meristem formation in the domains were *LAS* is misexpressed can thus be explained by the absence of additional downstream regulators and events which are required to spatially and temporally define the morphological initiation of an axillary meristem. Hence the main mechanism of *LAS* functions seems to be to protect the leaf axil from full differentiation, thus retaining its meristematic potential.

4.2.2 GA INHIBITS FORMATION OF AXILLARY MERISTEMS

Bioactive gibberellins (GAs) are plant hormones that promote cell differentiation and thus control various developmental processes throughout the life cycle of plants. Several positive and negative regulators of GA signaling have been identified in *Arabidopsis* (Sun and Gubler, 2004). GA biosynthesis and response mutants are often characterized by dwarf habitus, dark green colour, delayed flowering and defects in germination (Thomas and Sun, 2004; Peng and Harberd, 2002; Olszewski *et al.,* 2002). Frequently, GA response mutants cannot be recovered by GA spraying. On the other hand, loss of function mutants of negative regulators of GA signaling have increased GA levels and display tall and slender habitus (Richards *et al.,* 2001).

To analyse the role of GA in axillary meristem formation, spraying experiments were conducted on wild-type and *las-4* mutant plants to provide them with exogenous GA. Spraying Col wild-type plants with GA does not lead to any alteration in axillary meristem development. However, the late flowering Zu-0 ecotype, which fails to initiate axillary meristems in certain zones along the vegetative and the flowering shoot, displays an enhanced axillary meristem development defect upon application of GA. Most leaves of GA treated Zu-0 plants produced late during vegetative development and early in the reproductive phase fail to initiate axillary buds, suggesting that GA negatively regulates the formation of axillary meristems. Although the GA-treated Zu-0 plants display an accelerated transition to flowering when compared to non-treated Zu-0 plants, the number of leaves produced is comparable in GA treated and non-treated plants. This observation indicates that application of GA increases the rate of leaf initiation and development.

Application of GA on *las-4* mutants, likewise, resulted in a slight enhancement of the *las-4* branching defect. In addition to a complete loss of axillary bud formation in the axils of rosette leaves, older cauline leaf axils of GA-treated *las-4* plants also remained devoid of axillary buds. Collectively, these results suggest that GA negatively regulates axillary meristem initiation and that *LAS* function might be required to protect the leaf axils from GA signalling. It will be interesting to study whether *LAS* expression in Zu-0 is differentially regulated in those zones along the main shoot where axillary meristems fail to initiate.

4.2.3 Is *LAS* a regulator of GA signalling in leaf axils?

The results of the GA spraying experiments indicate a role for *LAS* as a negative regulator of GA signalling in the leaf axils. This hypothesis is substantiated by the fact that the GRAS family of putative transcription factors, of which *LAS* is a member, also comprises the DELLA sub-family of GA repressors, such as *GAI* and *RGA*. DELLA proteins negatively regulate GA signalling by transcriptionally repressing genes involved in various developmental pathways, like germination, stem elongation, flowering time and flower development (Richards *et al.,* 2001; Olszewski *et al.,* 2002; Lee *et al.,* 2002; Tyler *et al.,* 2004; Cheng *et al.,* 2004). This repression is removed by GA induced degradation of DELLA proteins via the ubiquitin-proteasome pathway mediated by F-Box proteins (McGinnis *et al.,* 2003; Sasaki *et al.,* 2003; Sun and Gubler, 2004).

Since *LAS* is devoid of a DELLA domain, its potential repression of GA signaling in the axils of leaves can not be overcome by GA-induced ubiquitin-mediated degradation. To test this hypothesis, *GAI,* both with and without its DELLA domain, was expressed transgenically from the *LAS* promoter in an *las-4* mutant background, and the transgenic plants were subsequently analysed for complementation of the *las-4* phenotype. Analysis of the T1 transgenic plants have revealed that, whereas *LAS::GAI* plants failed to complement the *las-4* phenotype, many *LAS::GAI-ΔDELLA* plants complement the *las-4* branching defect. Removal of the DELLA domain in the *GAI-Δ DELLA* transgene prevents it from being subjected to degradation via the ubiquitin-proteasome pathway by increase in GA levels.

A possible mechanism of *LAS* function in regulating axillary meristem fate can be elucidated from comparing the function of the *LAS* gene to the *GAI-Δ DELLA* transgene. This would suggest that *LAS* acts to prevent gibberellin induced differentiation of tissue with meristematic capacity. Additional support for this hypothesis can be derived from the fact that GA levels are found to be substantially increased in the tomato *lateral suppressor (ls)* mutant (Schumacher *et al.,* 1999). Furthermore, the function of suppression of GA signaling in the axillary meristem has been previously speculated for the *LAX* gene in rice. The *lax* mutants are characterised by a complete absence of

branching during vegetative and reproductive development. *LAX* encodes a bHLH transcription factor and is expressed in a narrow band of cells between the SAM and the developing axillary meristem. It is speculated to non-cell autonomously prevent an inhibitory signal, possibly GA, from affecting the axillary meristem (Komatsu *et al.*, 2003a). This speculation is based on the observation that plants overexpressing *LAX* from an ACTIN promoter show phenotypes similar to gibberellin deficient plants, including dwarfing, sterility and dark green colouration.

However, further characterisation of complementation of the *las-4* branching defect in *LAS::GAI-Δ DELLA* T2 lines needs to be performed to conclusively prove a role of *LAS* in regulating axillary meristem development by antagonising GA signalling in leaf axils.

4.3 SUPPRESSOR SCREEN REVEALS NOVEL LOCI INVOLVED IN REGULATION OF AXILLARY MERISTEM DEVELOPMENT

Modifier screens help to unravel enhancers and suppressors of known mutant phenotypes. Enhancers of a mutant phenotype usually result from mutations in genes acting in a parallel or redundant pathway. On the other hand, suppressors of a mutant phenotype are mostly a result of mutations in other genes that act as in the same pathway. During this work, screening of a mutagenised population of 32,000 M2 plants for suppression of the *las-4* phenotype led to the identification of eleven suppressor lines. These lines were characterised by an increase in axillary shoot formation from the rosette leaf axils when compared to the *las-4 max1-1* parent, and hence were designated as *suppressor of las-4 (sol)*. Three of these *sol* lines, *sol2, sol6* and *sol7,* were further characterised in detail.

4.3.1 *sol2, sol6* AND *sol7* SUPPRESS THE *las-4 max1-1* BRANCHING PHENOTYPE

Complementation analysis for functional allelism revealed that *sol2, sol6* and *sol7* resulted from independent mutations in different genes. These three lines suppressed the *las-4 max1-1* branching phenotype to different extents. In *sol2* plants, almost a complete restoration of axillary shoot formation was observed in the early and middle phase of vegetative development. Additionally, *sol2* plants were characterised by a dwarfish, bushy habitus, and displayed fusions of floral organs and leaves. These fusion phenotypes are reminiscent of several mutants defective in development of the epidermis, such as *fiddlehead, lacerata, hothead* and *bodyguard* (Pruitt *et al.,* 1999; Wellesen *et al*, 2001; Krolikowski *et al.,* 2003; Kurdyukov *et al.,* 2006). However, allelism of *sol2* with any of the above mutants could be ruled out by double mutant analysis and sequencing of the *FDH, LCR, HTH* and *BDG* genes in *sol2*.

Suppression of the *las-4 max1-1* branching defect was observed during the middle and late phase of vegetative development in *sol6* plant. *sol7* plants also formed side-shoots in the axils of the middle and late rosette leaves. However, suppression of the *las-4 max1-1* branching defect was stronger in *sol7* compared to *sol6*. Furthermore, *sol7* plants flowered earlier than the *las-4 max1-1* parent, and had a characteristic pale green colour.

Analyses of back crosses of the three *sol* lines to *las-4* showed that the *sol2, sol6* and *sol7* phenotypes resulted from single recessive mutations and were independent of the *max1-1* mutation. Out-crossing of *sol2* and *sol7* to Col wt revealed that the *sol2* and *sol7* phenotypes were dependent on *las-4* mutation. Hence, these plants would not have been

identified in a mutagenesis screen with wild-type plants, thus demonstrating the advantage of modifier screens.

4.3.2 Severity of the *sol2, sol6* and *sol7* phenotype is day length dependent

Although suppression of the *las-4 max1-1* branching defect was observed in the three *sol* lines both in short day and long day conditions, the extent of this suppression was day-length dependent. *sol* plants grown in short photoperiods displayed a stronger suppression of *las-4 max1-1* phenotype than those grown under long day conditions. The influence of environmental conditions on the severity of branching defects in mutants has been reported by Müller *et al*. (2006), who showed that the *RAX* genes control branching along different zones of the main bolt in *Arabidopsis* in a day-length dependent manner. When grown under long day conditions, the defects in axillary meristem formation in *rax1-3, rax2-1,* and *rax3-1*, as well as the double and triple mutants were highly reduced or no longer detectable. Similarly, branching patterns in *Arabidopsis* have also been shown to be influenced by vernalisation. The ecotype Zu-0, which displays zones without axillary branches along its primary axis, overcomes its late flowering phenotype as well as the branching defect when subjected to a period of vernalisation (Kalinina *et al.,* 2002). These observations suggest the presence of alternate pathways that are activated by photoperiod and vernalisation.

4.3.3 Rough molecular mapping of *sol2* and *sol7*

Since *sol2* and *sol7* mutations were identified in a Col background, polymorphic mapping populations were created by crossing them into a L*er* background. A set of twenty two simple sequence length polymorphism (SSLP) markers distributed over the genome were used for rough mapping these two loci. These are PCR-based co-dominant molecular markers generated on the basis of naturally occurring polymorphisms between different ecotypes that occur due to variations in the number of tandemly repeated short nucleotide motifs. These markers have been used to construct linkage maps in *Arabidopsis* (Lukowitz *et al.,* 2000). In a polymorphic mapping population, the vicinity of the new mutation would be tightly linked to the original background in which the mutation occurred, in this case, Col. Hence, the SSLP markers in the vicinity of the new mutation would co-segregate with the Col allele.

The F2 population arising from the cross of *sol2* to L*er* was used for rough molecular mapping of the *sol2* locus. A high percentage of co-segregation for the Columbia allele was found on chromosome 1 at markers ciw1, Nga280 and Nga111. The high Columbia co-segregation at markers ciw1 and Nga280 is expected due to the presence of the *las-4* mutation in their vicinity. The Nga111 marker is, however, more than 30cM from the *las-4*

locus. Hence its chromosomal surrounding is a potential region for the location of the *sol2* locus, and will be further investigated with more markers.

Rough mapping of *sol7* was carried out in a F2 population arising from a cross of *sol7* to L*er* carrying the *las-4* mutation introgressed from Col by three back-crosses. Marker analysis revealed that this *las-4*/L*er* parent still retains Col genome at the bottom of chromosome 3 and top of chromosomes 4 and 5, in addition to the expected region on chromosome 1 which carries the introgressed *las-4* mutation. Surprisingly, the *sol7* mutant is polymorphic for L*er* alleles of the markers F21M12 and Nga1126. The possibility of remnants of the En-2 background at these positions, arising from the *max1-1* mutation present in *sol7,* was ruled out by testing for polymorphism between the L*er* and En-2 alleles of these markers. The original *max1-1* mutant which was crossed to *las-4* to create the mutagenesis population to screen for *sol*s can be tested for polymorphism at the markers F21M12 and Nga1126, to unravel the origin of L*er* alleles at these loci. The analysis of this *sol7* mapping population indicates the regions around markers PHYC (chromosome 5) and Nga168 (chromosome 2), which show 100% and 87.8% co-segregation, respectively, for the Col alleles, as the potentially interesting areas to be further investigated with more molecular markers. Two additional *sol7* mapping populations (an F2 and an F1BC1) now available may further aid this process.

5 ABSTRACT

Aerial architecture and reproductive success in higher plants is determined by the formation of secondary axes of growth which are formed by axillary meristems initiated post-embryonically in the axils of leaves. Among the genetic modulators of axillary meristem fate in *Arabidopsis* is *LATERAL SUPPRESSOR*, a putative transcription factor belonging to the GRAS family, which specifically regulates the initiation of axillary meristems during the vegetative phase of development. The aim of this work was to study the mechanism of *LAS* function in the meristem and to identify new regulators of axillary meristem initiation in *Arabidopsis*.

To study the spatio-temporal specification of its function, *LAS* was misexpressed from promoters of meristematic genes possessing adjoining or overlapping expression domains in the SAM. Analysis of *STM::LAS, KNAT1::LAS* and *UFO::LAS* transgenic plants in *las-4* background revealed partial to complete complementation of the *las-4* branching phenotype, but did not lead to the formation of ectopic meristems. These results imply a function for *LAS* in maintaining the meristematic potential in axillary cells which can later initiate axillary meristems upon activation by other developmental cues. A potential mechanism of *LAS* function in axillary meristems was investigated by GA spraying experiments and complementation analysis of *LAS::GAI* and *LAS::GAI ΔDELLA* transgenic plants in *las-4* mutant background. Preliminary results indicate a role for *LAS* as a regulator of GA signaling in axillary meristems.

To identify new regulators of axillary meristem development, two approaches were employed. Firstly, an EMS mutagenesis screen was carried out to identify supperssors of the *las-4 max1-1* phenotype. Characterisation of three *suppressor of las-4* (*sol*) candidates, *sol2, sol6* and *sol7*, revealed three novel loci that regulate axillary meristem development. *sol2, sol6* and *sol7* complemented the branching defect in *las-4 max1-1* to different degrees and were found to be non-allelic to each other. Their phenotypes were dependent on the *las-4* mutation. Molecular mapping of two of these loci is underway. Secondly, the NAC domain transcription factors *CUP-SHAPED COTYLEDON1*, *CUC2* and *CUC3*, exhibiting a characteristic expression pattern in the axils of leaf primordia, were investigated for potential roles in the development of axillary meristems. Investigation of loss-of-function mutants of these genes revealed that *cuc3-2* is impaired in axillary bud formation, and that the severity of this phenotype is day length dependent. Transcripts of the other two *CUC* genes, *CUC1* and *CUC2*, are targeted for degradation by miR164. Overexpression of *MIR164A* or *MIR164B* in the *cuc3-2* mutant caused an almost complete block in axillary bud development. Conversely, plants harbouring miR164-resistant alleles of *CUC1* and *CUC2* developed accessory buds in rosette and

cauline leaf axils, revealing redundant functions of *CUC1* and *CUC2* in axillary meristem development. Development of accessory buds was also observed in *mir164* mutants. Thus, the role of *CUC* genes and miR164 in regulation of axillary meristem development was unveiled in this study.

6 ZUSAMMENFASSUNG

Die Architektur und damit auch der reproduktive Erfolg höherer Pflanzen hängt in hohem Maße von der Bildung sekundärer Wachstumsachsen ab. Diese werden von Lateralmeristemen gebildet, die während der postembrionalen Entwicklung in den Blattachseln initiiert werden. Das *LATERAL SUPPRESSOR (LAS)*-Gen ist einer der Faktoren, die die Achselmeristemanlage genetisch kontrollieren. *LAS* gehört der GRAS-Familie von putativen Transkriptionsfaktoren an und reguliert die Initiation von Lateralmeristemen während der vegetativen Wachstumsphase. Ziel dieser Arbeit war es, den Mechanismus dieser Regulation zu untersuchen und neue Regulatoren der Achselmeristementwicklung zu identifizieren.

Um die räumliche und zeitliche Spezifität der *LAS*-Funktion zu untersuchen, wurde dieses Gen unter den Promotoren verschiedener meristematischer Gene, mit angrenzenden oder überlappenden Expressionsdomänen, fehlexprimiert. In den Analysen von transgenen *STM::LAS-, KNAT1::LAS*- und *UFO::LAS*-Pflanzen im *las-4*-Hintergrund zeigte sich eine partielle oder vollständige Komplementation der *las*-Verzweigungsdefekts, nicht jedoch die Bildung ektopischer Meristeme. Diese Ergebnisse deuten darauf hin, dass *LAS* ein Rolle in der Erhaltung des meristematischen Potentials von Achselzellen spielt, welche später durch entwicklungsspezifische Signale aktiviert, Lateralmeristeme anlegen können. Eine potentielle Rolle von *LAS* im GA-Signalweg wurde durch GA-Sprühexperimente und Analyse von transgenen *LAS::GAI*- und *LAS::GAI ΔDELLA*-Pflanzen im *las-4*-Hintergrund untersucht. Vorläufige Ergebnisse deuten auf eine Rolle von *LAS* als Regulator im GA-Signalweg hin, der die Lateralmeristemanlage beeinflußt.

Um neue Regulatoren der Achselmeristementwicklung zu identifizieren, wurden zwei Ansätze gewählt. Einerseits wurde ein EMS-Mutagenesescreen durchgeführt, um Mutanten zu identifizieren, die den *las-4 max1-1*-Phänotyp komplementieren. Auf diese Weise wurden drei Kandidatenlinien identifiziert, die *suppressor of las-4 2 (sol2), sol6* und *sol7* genannt wurden. Bei der Charakterisierung zeigte sich, dass es sich um nicht-allelische Loci handelt, die die Lateralmeristementwicklung beeinflussen und in der Lage sind, den *las-4 max1-1*-Phänotyp zu einem gewissen Grad komplementieren, wobei der Phänotyp vollständig von der *las-4*-Mutation abhängig ist. Die molekulare Kartierung dieser Loci ist in Arbeit.

In einem weiteren Ansatz wurde untersucht, ob die Transkriptionsfaktoren *CUP-SHAPED COTYLEDON1 (CUC1), CUC2* und *CUC3* eine Rolle bei der Lateralmeristemanlage spielen, da sie eine charakteristische Expressionsdomäne in den Achseln von Blattprimordien besitzen. Die Analyse von Verlustmutanten zeigte, dass in *cuc3-2*-

Pflanzen die Achselknospenbildung gestört ist, wobei das Ausmaß tageslängenabhängig ist. Die Transkripte von *CUC1* und *CUC2* werden durch die mikroRNA164 (miR164) abgebaut. Die Überexpression von *MIR164A* oder *MIR164B* im *cuc3-2*-Hintergrund führte zu einem annähernd vollständigen Ausfall der Achselknospenbildung. Im Gegensatz dazu entwickelten transgene Pflanzen mit miR164-resistenen Allelen von *CUC1* und *CUC2* akzessorische Knospen in Rosetten- und Stengelblattachseln, was auf eine redundante Funktion von *CUC1* und *CUC2* in der Lateralmeristementwicklung hinweist. Dementsprechend wurden auch akzessorische Seitentriebe in *mir164*-Mutanten beobachtet. Folglich konnte in dieser Arbeit gezeigt werden, dass die *CUC*-Gene und miR164 eine Funktion haben bei der Regulation der Achselmeristementwicklung.

7 GLOSSARY

AG	*AGAMOUS*
AS1	*ASYMMETRIC LEAVES*
1	
AP1	*APETALA1*
AP2	*APETALA2*
AP3	*APETALA3*
AXR1	*AUXIN RESISTANT1*
Bp	*Base pair*
BP	*BREVIPEDICELLUS*
Bl	*Blind*
BUS	*BUSHY*
CAF	*CARPEL FACTORY*
CAPS	*cleaved-amplified-polymorphic-sequence*
CLV	*CLAVATA*
Col	*Columbia*
CUC	*CUPSHAPED COTYLEDON*
CUP	*CUPULIFORMIS*
DNA	*Deoxyribonucleic acid*
eol	*enhancer of las-4*
En-2	*Enkheim-2*
GA	*Gibberellic acid*
GAI	*GIBERRELIC ACID INSENSITIVE*
HAM	*HAIRY MERISTEM*
IFL	*INTERFASCICULAR FIBRELESS*
Kb	*Kilo bases*
KN1	KNOTTED1 *from maize*
KNAT	KNOTTED-*Like from* Arabidopsis thaliana
KNOX	*Knotted1-like Homeobox-Gene*
LAS	*LATERAL SUPPRESSOR from Arabidopsis*
Ler	*Landsberg erecta*
LFY	LEAFY
LOB	LATERAL ORGAN BOUNDARY
Ls	Lateral suppressor from Tomato
MAX	MORE AXILLARY GROWTH
Mb	Mega bases
MOC1	MONOCULM1
mRNA	messenger RNA
miRNA	micro RNA
ON	over night
PCR	Polymerase Chain Reaction
PNH	PINHEAD
RAM	root apical meristem
RAX1	REGULATOR OF AXILLARY MERISTEM 1
REV	REVOLUTA
RGA	REPRESSOR OF GA1-3
RNA	Ribonucleic acid
RT	Room temperature
SAM	Shoot Apical Meristem
SEM	Scanning Electron Microscopy
SCR	SCARECROW
SHR	SHORTROOT
sol	suppressor of las-4
SPY	SPINDLY
SPS	SUPERSHOOT
STM	SHOOT MERISTEMLESS
SSLP	simple-sequence-length-polymorphisms
UFO	UNUSUAL FLORAL ORGANS
Ws	Wassilewskija
WUS	WUSCHEL
ZLL	ZWILLE
Zu-0	Zürich-0

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9 ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Priv. Doz. Dr. Klaus Theres betreut worden.

Ein Teil diser Arbeit wurde zur Veröffentlichung bei der Fachzeitschrift "The Plant Journal" eingereicht:

Raman, S., Greb, T., Peaucelle, A., Laufs, P. and Theres, K. (2006). *CUP-SHAPED COTYLEDON* genes and miR164 regulate the formation of axillary meristems in *Arabidopsis thaliana.*

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11 ACKNOWLEDGEMENTS

The completion of this work was made possible by support and assistance from many people, to whom I wish to express my earnest gratitude.

Thanks to Prof. Klaus Theres for the supervision of this dissertation, timely advice and valuable discussions.

Thanks to Prof. Maarten Koornneef for helpful discussions.

Thanks to Prof. Patrick Laufs and Dr. Sascha Yephremov for fruitful collaborations on parts of this work.

Thanks to Bodo Raatz, Dr. Gregor Schmitz, Dr. Jagreet Kaur, Bernhard Busch and Dr. Ralf Petri for critical reading and suggestions during the manuscription of this work.

Thanks to all my colleagues for a dynamic and pleasant working atmosphere, helpful discussions and amiable banter.

Thanks to Ursula Pfordt, Alexandra Kalda and Ute Tatler for excellent technical assistance in the laboratory.

Thanks to Rolf-Dieter Hirtz for help with scanning electron microscopy.

Thanks to Prof. Wolgang Werr, Prof. Martin Hülskamp and Dr. John Chandler for constituting my thesis committee.

Thanks to some remarkable people whose company and certitude kept me anchored: Thomas Seher, Jagreet Kaur, Inge Kappen, Marcella Santaella, Imke Drews, Revathy Uthaiah, Jiji Thomas and Jürgen Porath, for camaraderie, cheer and counsel; my home-away-from-home Christa and Dieter Metzinger, for lovely food and memorable Christmases; my beloved mother, my sisters Sneha and Sapna, and brother-in-law Padma, for their incessant love, encouragement and prayers across the miles; and Sacha, for his endless patience, understanding and care.