# On the Role of CLE40

# A Peptide with Antagonistic Functions in *Arabidopsis thaliana* Shoot Meristem Development

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# 1 Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Ferner erkläre ich, dass ich die Dissertation nur in diesem und keinem weiteren Promotionsvorhaben eingereicht habe und diesem kein gescheitertes Promotionsvorhaben vorausgegangen ist.

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“Captain, the most elementary and valuable statement in science,

the beginning of wisdom, is 'I do not know.'”

(*Star Trek TNG: Where Silence Has Lease,* 1988 by W. Kolbe)

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## 4.1 Tables



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# 5 Abbreviations

| | |
|---|---|
| ACR4 | ARABIDOPSIS CRINKLY 4 |
| *acr4-2* | mutant allele of *ARABIDOPSIS CRINKLY 4* |
| AG | AGAMOUS |
| BAM1 | BARELY ANY MERISTEM 1 |
| *bam1-3* | mutant allele of *BARELY ANY MERISTEM 1* |
| bp | base pairs |
| ° C | degree Celsius |
| *CaMV35S* | constitutive promoter of the *Cauliflower mosaic virus* |
| CNA | CORONA |
| CLE40 | CLAVATA3 EMBRYO SURROUNDING REGION RELATED 40 |
| *cle40-2* | mutant allele of *CLAVATA3 EMBRYO SURROUNDING REGION RELATED 40* |
| CLV1 | CLAVATA1 |
| *clv1-11* | mutant allele of *CLAVATA1* |
| *clv1-20* | mutant allele of *CLAVATA1* |
| CLV2 | CLAVATA2 |
| *clv2-101* | mutant allele of *CLAVATA2* |
| CLV3 | CLAVATA3 |
| *clv3-9* | mutant allele of *CLAVATA3* |
| cm | centimetre |
| cn | carpel number |
| Col-0 | Columbia-0 (ecotype) |
| CZ | central zone |
| dag | day(s) after germination |
| DNA | deoxyribonucleic acid |
| *E.coli* | *Escherichia coli* |
| EPF2 | EPIDERMAL PATTERNING FACTOR 2 |
| EPFL9 | EPIDERMAL PATTERNING FACTOR LIKE 9 |
| ER | ERECTA |
| *er-105* | mutant allele of *ERECTA* |
| ESR | EMBRYO SURROUNDING REGION |
| EtOH | ethanol |

| | |
|---|---|
| FD *Ase*I | Fast digest *Ase*I (restriction enzyme from *Aquaspirillum serpens*) |
| GFP | GREEN FLUORESCENT PROTEIN |
| g | gram |
| HCl | hydrochloric acid |
| H2B | HISTON 2B |
| kb | kilo bases |
| KOH | potassium hydroxide |
| KNU | KNUCKLES |
| l | litre |
| LFY | LEAFY |
| LOG4 | LONELY GUY 4 |
| LRR | leucin-rich repeat |
| L1 | layer 1 |
| L2 | layer 2 |
| L3 | layer 3 |
| MAPK | mitogen-activated protein kinase |
| min | minute |
| MS | Murashige and Skoog growth medium |
| µm | micrometre |
| mM | millimolar |
| OC | organizing centre |
| µg | microgram |
| *Pac*I | restriction enzyme from *Pseudomonas alcaligenes* |
| PI | propidium iodide |
| RAM | root apical meristem |
| SAM | shoot apical meristem |
| sec | second |
| SEM | scanning electron microscope |
| TA cells | transit amplifying cells |
| TDIF | TRACHERY ELEMENT DIFFERENTIATION INHIBITORY FACTOR |
| T-DNA | transfer DNA |
| TDR | TDIF RECEPTOR |
| PCR | polymerase chain reaction |
| PHB | PHABULOSA |

| | |
|---|---|
| PHV | PHAVOLUTA |
| PIN1 | PIN-FORMED 1 |
| PXY | PHLOEM INTERCALATED WITH XYLEM |
| PZ | peripheral zone |
| RLK | receptor-like kinase |
| RPK2 | RECEPTOR-LIKE PROTEIN KINASE 2 |
| v/v | volume per volume |
| WOX4 | WUSCHEL HOMEOBOX 4 |
| WOX5 | WUSCHEL HOMEOBOX 5 |
| WUS | WUSCHEL |
| w/v | weight per volume |
| YFP | YELLOW FLUORESCENT PROTEIN |
| % | per cent |
| ~ | approximately |

# 6 Abstract

The continuous *de novo* formation of organs throughout a plant's life depends on stem cells that reside in stem cell niches, which are called meristems. All above-ground tissue is generated by the shoot apical meristem. The control of stem cell homeostasis, the basis of meristem maintenance and simultaneous organogenesis, is facilitated by cell-to-cell communication that involves the interaction of small secreted peptides and receptor-like kinases (RLKs). Meristem homeostasis in the shoot is controlled by a feedback regulatory loop, in which the homeodomain transcription factor WUSCHEL (WUS), which promotes stem cell fate, is restricted by CLAVATA 3, a small secreted peptide expressed in stem cells, via two parallel pathways including the RLK CLAVATA1 (CLV1), the kinase CORYNE (CRN), and the receptor-like protein CLAVATA 2 (CLV2).

This work provides evidence that CLAVATA3/ EMBRYO SURROUNDING REGION RELATED 40 (CLE40), the closest homologue of CLV3, functions in meristem size control in a pathway that acts antagonistically to the CLV pathway, promoting meristem size. Besides this, under heat stress conditions, CLE40 can also act in parallel to CLV3 in the CLV pathway to restrict meristem size, and consequently fulfils antagonistic functions in response to changing environmental influences.

Like in the root, where CLE40 function involves the RLK ARABIDOPSIS CRINKLY 4 (ACR4) to restrict columella stem cell fate, CLE40 signalling in the shoot involves ACR4. Besides this, CLE40 signalling depends on the stem cell restricting function of the RLK BARELY ANY MERISTEM 1 (BAM1), which was previously described to function in the CLV pathway upon loss of CLV1 function.

In summary, this work characterizes the function of CLE40 in the control of meristem size throughout shoot development. CLE40 is part of a complex network of antagonistic pathways that function together to ensure robustness and plasticity of meristem maintenance in response to changing environmental influences.

# 7 Introduction

## 7.1 Plant growth and development depends on meristems

In adaptation to a sessile life, plants generate their organs postembryonically, in response to the environmental influences surrounding them. This continuous formation of new organs is a fundamental difference between plants and animals, the latter generate their organs during their embryonic development.

Plants facilitate the generation of new organs throughout their whole life by stem cells, pools of undifferentiated cells, which can, on the one hand, differentiate to form new organs, and on the other, simultaneously sustain a sufficient stem cell pool (Hall and Watt, 1989; Stahl and Simon, 2005).

### 7.1.1 Plant meristems

Plant stem cells reside in meristems. All above ground tissues originate from the shoot apical meristem (SAM), whereas the root originates from the root apical meristem (RAM) (Stahl and Simon, 2005).

During vegetative growth, the vegetative SAM gives rise to leaves, whereas after floral transition, the inflorescence meristem generates side shoots and flowers, the latter consisting of floral organs that arise from floral meristems (Hempel and Feldman, 1994) (Figure 1). To clearly discriminate between the different meristem types of the shoot, hereinafter the term SAM is used exclusively for the vegetative SAM. The shoot apical meristem of the inflorescence is termed inflorescence meristem.

#### 7.1.1.1 The structure of shoot meristems

Plant shoot meristems can be divided into different layers and zones (Figure 2).

All cells of the outermost layers L1 and L2 divide anticlinally, and thus form two distinct layers, which together are termed tunica. Cells of the L3, which is also called corpus, divide in all directions, thus the L3 is actually not composed of a single-cell layer (Satina *et al.*, 1940; Schmidt, 1924; Stahl and Simon, 2005).

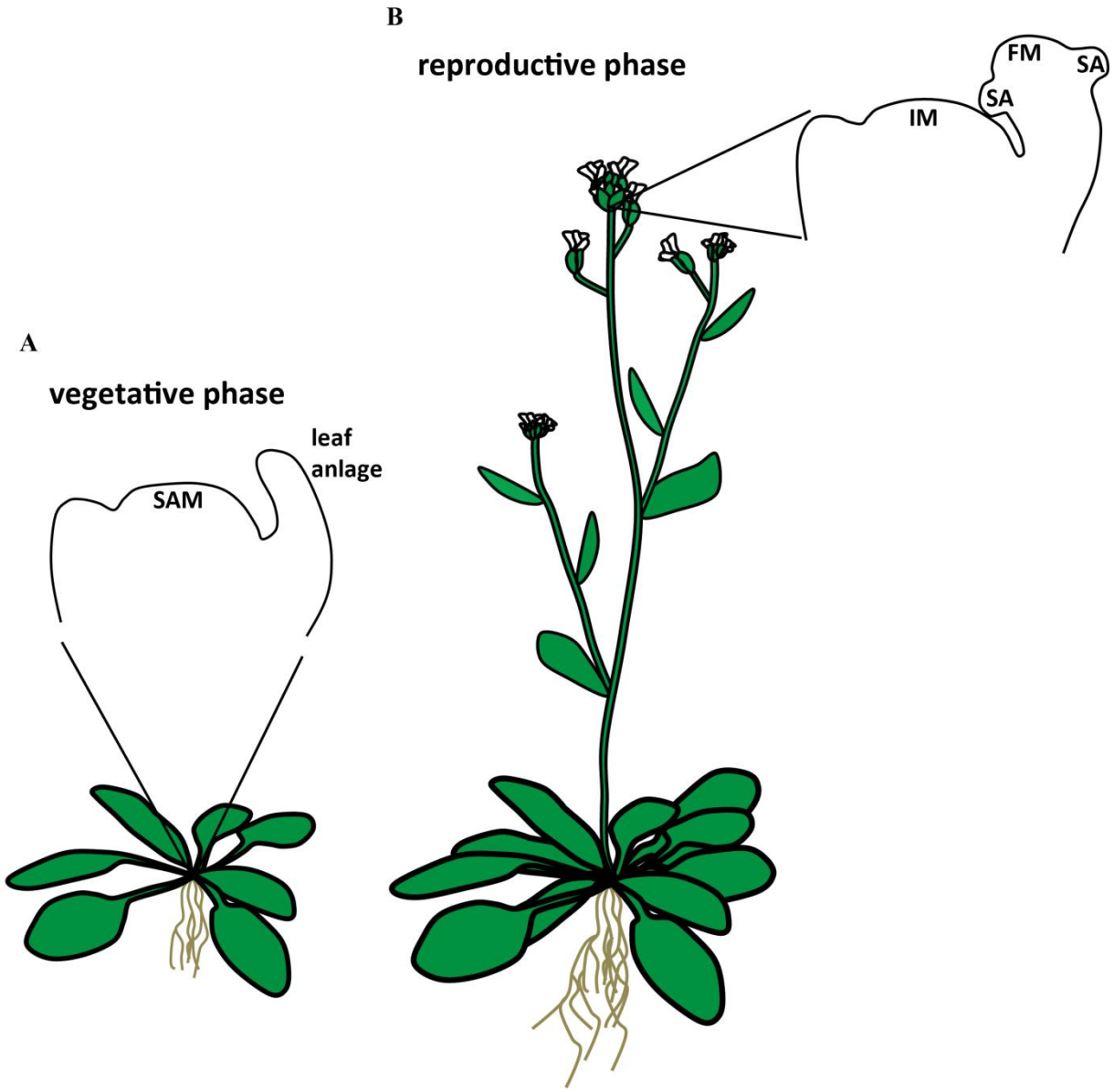



**Figure 1: *Arabidopsis thaliana* shoot meristems**

**A: overview of an *Arabidopsis* plant during vegetative growth; the vegetative shoot apical meristem (SAM) with its leaf anlagen is highlighted. B: During the reproductive phase, the inflorescence meristem (IM) forms flowers at its flanks that grow out of floral meristems (FM). The first organs to be generated at the flanks of the FM are the sepals, SA = sepal anlagen**

Cells within one layer have a clonal origin, but their final fate depends on their position, not on their linage. Consequently, cells that occasionally divide into other layers adapt to the fate of the new layer, causing no detectable abnormabilites (Furner and Pumfrey, 1992; Jenik and Irish, 2000; Lanza, 2004; Stahl and Simon, 2005).

The L1 generates epidermal tissues, while the L2 generates subepidermal tissues and gametes, and the majority of a plant's ground tissue and the vasculature originate from the L3 (Jenik and Irish, 2000; Stahl and Simon, 2005; Lanza, 2004).

Besides the different layers of the shoot meristem, different zones were assigned that harbour typical cell types (Figure 2) (Stahl and Simon, 2005). Stem cells reside in the central zone (CZ). They divide to form transit amplifying cells (TA cells) that differentiate in the peripheral zone (PZ) to form organ anlagen or primordia, which grow out to generate organs at the flanks of the meristem (Hall and Watt, 1989). Although all cells of the meristem have the potential to divide, cell divisions occur less frequently in the CZ than in the periphery and one function of the TA cells is the generation of many daughter cell from few stem cells (Clowes, 1958; Reddy *et al.*, 2004; Hall and Watt, 1989).

The rib meristem is located beneath the CZ and generates the plant's ground tissue (Figure 2).

Another classification of cell groups was done according to specific gene expression. The group of cells expressing the homeodomain transcription factor *WUS*, which promotes stem cell fate, is called the organizing centre (OC) (Figure 2) (Laux *et al.*, 1996; Mayer *et al.*, 1998; Stahl and Simon, 2005).

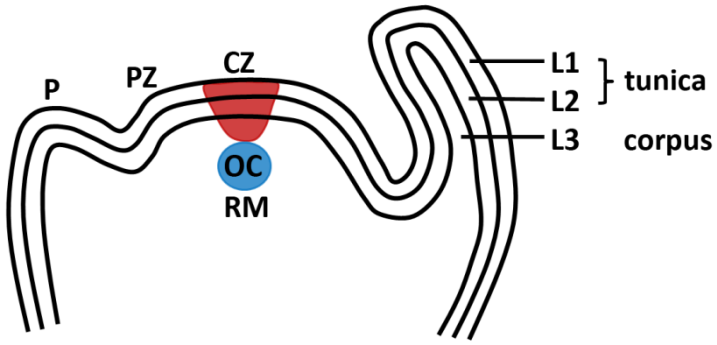



**Figure 2: Schematic illustration of a shoot meristem**

**CZ (red area): central zone with stem cells; PZ: peripheral zone with TA cells; P: primordium; OC (blue area): organizing centre; RM: rib meristem; L1-3: layer 1-3; L1 and L2 = tunica; L3 = corpus**

#### 7.1.1.2 Differences between shoot meristems

Basically, the vegetative SAM, the inflorescence meristem, and the floral meristem are homologous structures, which share regulatory mechanisms and exhibit only few differences (Clark, 1997; Endrizzi *et al.*, 1996; Laux *et al.*, 1996). The vegetative meristem forms leaves at its flanks, while after floral transition, the inflorescence meristem generates secondary

inflorescences and flowers, the organs of which grow out of floral meristems (Hempel and Feldman, 1994). One fundamental difference between the vegetative SAM and the inflorescence meristem on the one hand, and the floral meristem on the other hand, is the termination of the meristem in the latter upon formation of floral organs. This termination of the floral meristem is preceded by a down-regulation of the stem cell promoting homeodomain transcription factor WUS (Mayer *et al.*, 1998). In floral meristem, WUS together with the floral identity protein LEAFY (LFY) activates the transcription of the homeotic gene *AGAMOUS* (*AG*) (Schultz and Haughn, 1991; Bowman *et al.*, 1989; Busch *et al.*, 1999; Lohmann *et al.*, 2001; Drews *et al.*, 1991). AG activates the transcription of *KNUCKLES* (*KNU*) that encodes a transcriptional repressor, which represses *WUS* expression, thus facilitating the termination of the floral meristem (Sun *et al.*, 2009; Payne *et al.*, 2004; Mayer *et al.*, 1998).

In the SAM and in the inflorescence meristem, this down-regulation of *WUS* does not occur (Mayer *et al.*, 1998).

## 7.2 Cell-to-cell communication is crucial for meristem maintenance and organogenesis

Upon division of shoot stem cells, there are three possible outcomes. A stem cell can generate either two new stem cells or two new TA cells, or it can give rise to one new stem cell and one TA cell. An exact control of the balance between newly formed TA cells and newly formed stem cells is crucial for meristem maintenance and organ development throughout a plant's life. If the balance is shifted towards the production of TA cells, the meristem might be consumed. On the other hand, if the balance is shifted towards the production of stem cells, organ formation might be impaired.

The balance between organogenesis and stem cell maintenance is controlled by signals from neighbouring cells or niche cells. The communication among cells involves small secreted peptides acting as ligands for receptor-like kinases (Stahl and Simon, 2005).

Because plants as sessile organisms are exposed to changing environmental influences, control of plant growth by cell-to-cell signalling cannot be rigid, but has to include the possibility of adaptation in response to environmental effects.

### 7.2.1 Cell-to-cell communication is facilitated by the interaction of small secreted peptides and receptor-like kinases

Cell-to-cell communication involving small secreted peptides and receptor-like kinases is crucial for plant development (Tax and Kemmerling, 2012).

A large number of peptide gene families was found in plant genomes, but their function remains largely unknown (Hanada *et al.*, 2013). One quite well studied peptide family is the CLE-family, which also includes the stem cell restricting peptide CLV3.

#### 7.2.1.1 The CLE-family

The CLE-family (CLAVATA3/EMBRYO SURROUNDING REGION RELATED) was named after its first two members CLV3 from *Arabidopsis thaliana* and ESR from *Zea mays*. However, CLE peptides are not restricted to those species, but genes encoding CLE peptides have been found in many plant species. In *Oryza sativa*, the CLV3 orthologue FLORAL ORGAN NUMBER 4 (FON4) was identified. In tomato (*Solanum lycopersicum*), 15 *CLE* genes were found and named *SlCLE1-15*. Even in the moss *Physcomitrella patens*, CLE peptides were found (Chu *et al.*, 2006; Zhang *et al.*, 2014; Miwa *et al.*, 2014).

All *CLE* genes encode a predicted N-terminal signal peptide, which guides the peptide to the extracellular space, a process which is necessary for proper CLE peptide function, and a conserved region of 14 amino acids located at the C-terminus (Cock and McCormick, 2001; Rojo, 2002; Meng *et al.*, 2010). Outside of this conserved region, no sequence similarity could be identified. The conserved region encodes a motif that was named CLE motif and was described to be crucial for the function of CLE peptides (Figure 3) (Cock and McCormick, 2001). Consequently, point mutations affecting the CLE motif of CLV3 were described to cause hypomorphic phenotypes (Fletcher *et al.*, 1999). *In vitro* application of synthetic peptides corresponding to the CLE motif of either CLV3, CLE19, or CLE40 phenocopied the respective over-expression phenotypes (Fiers *et al.*, 2005). Besides this, deletion of the region encoding the CLE motif abolished the function of CLV3 (Fiers *et al.*, 2006).

Although the sequence outside the CLE motif is not characterized by any sequence similarities among CLE peptides, and the CLE motif was first suggested to be functionally independent of the flanking sequences, later studies suggested that the non-conserved region might contribute to functional specificity of CLE peptides *in vivo* by affecting peptide processing (Cock and McCormick, 2001; Fiers *et al.*, 2006; Meng *et al.*, 2010).

CLE peptides are expressed in diverse tissues and are associated with functions in meristem maintenance (Sharma *et al.*, 2003). In the shoot, CLV3 is known to be expressed in stem cells, functioning as a repressor of stem cell fate (Fletcher *et al.*, 1999; Clark *et al.*, 1995). In the vasculature, the CLE peptide TRACHERY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) promotes the proliferation of stem cells in the procambium, and in the root, CLE40 was found to function as a negative regulator of columella stem cell fate (Ito *et al.*, 2006; Hirakawa *et al.*, 2010; Stahl *et al.*, 2009).

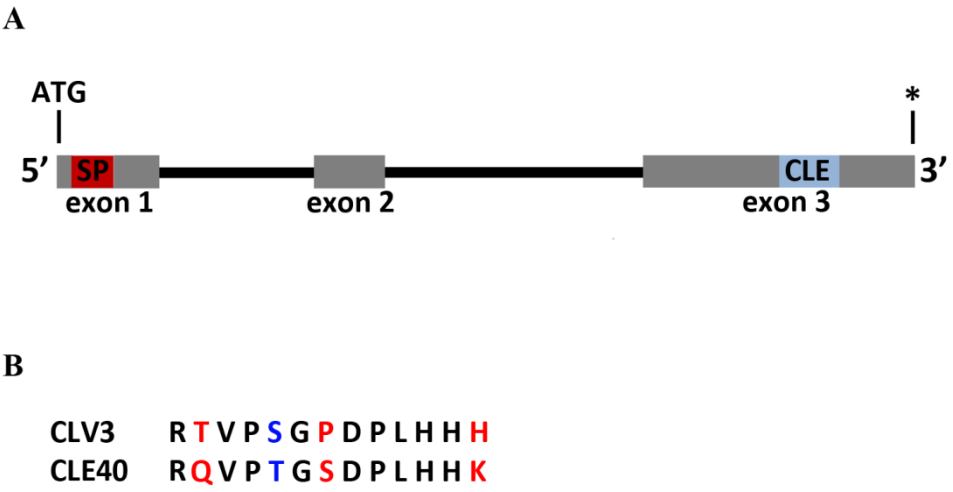



**Figure 3: Schematic illustration of the gene structure of *CLV3* and protein sequence alignment of CLE40 and CLV3 CLE motif**

**A: CLV3 has three exons (grey boxes) and two introns (black lines). SP (red area): region encoding the N-terminal signal peptide; CLE (blue area): region encoding the CLE motif; asterisk marks the position of the stop codon; The position of the start codon is indicated by ATG. B: Protein sequence alignment of the CLE motif of CLV3 and CLE40. Black: identical amino acids, blue: very highly conserved amino acids, red: not identical/ not highly conserved amino acids.**

#### 7.2.1.2 Receptor-like kinases

In *Arabidopsis,* about 600 receptor-like kinases form a monophyletic gene family, which is related to animal receptor kinases (Shiu and Bleecker, 2001).

RLKs have an extracellular domain, which can vary in structure, a single membrane spanning region, and a protein kinase domain that is located towards the cytoplasm. In contrast to animal receptor kinases, most of which are tyrosine kinases, plant RLKs are serine-threonine kinases (Walker, 1994).

Leucin-rich repeat RLKs (LRR RLKs) represent one of the largest subgroups of RLKs in *Arabidopsis*, containing ~235 members. This subgroup is characterized by the presence of an

extracellular leucin-rich repeat domain (Lehti-Shiu *et al.*, 2009). Some members of this group, like CLV1 or its close homologues BAM1, BAM2, and BAM3, as well as RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) function in the control of meristem homeostasis (Clark *et al.*, 1993; DeYoung *et al.*, 2006; DeYoung and Clark, 2008; Mizuno *et al.*, 2007; Kinoshita *et al.*, 2010). The RLK ACR4, which controls meristem homeostasis in the root, is classified into the group of RLKs that have a crinkly domain (Stahl *et al.*, 2009; Gifford *et al.*, 2003).

Some receptor-like proteins, like CLV2, lack the intracellular kinase domain and some receptor kinases, like CRN, have an extremely short extracellular domain (Jeong *et al.*, 1999; Müller *et al.*, 2008). CLV2 and CRN fulfil their function in the control of meristem homeostasis by interacting via their trans-membrane domains (Müller *et al.*, 2008; Bleckmann *et al.*, 2010). Generally, complex formation of receptor kinases is a common feature in plant signalling, controlling different developmental processes (Tax and Kemmerling, 2012).

### 7.2.2 The CLV pathway controls meristem maintenance in the shoot apex

The balance of organogenesis and meristem maintenance in the shoot apex is facilitated by the CLV pathway (Figure 4) (Brand, 2000; Schoof *et al.*, 2000). The small secreted peptide CLV3 is expressed in stem cells, from where it is secreted to underlying layers where it acts via the receptor-like kinase CLV1 and in parallel via the receptor-like protein CLV2 and the kinase CRN to repress the function of the homeodomain transcription factor WUS. (Rojo, 2002; Fletcher *et al.*, 1999; Clark *et al.*, 1997; Jeong *et al.*, 1999; Müller *et al.*, 2008; Brand, 2000; Schoof *et al.*, 2000; Laux *et al.*, 1996; Mayer *et al.*, 1998). *WUS* is expressed in the cells of the OC (Mayer *et al.*, 1998). It moves towards the apex via plasmodesmata to the cells of the CZ, where it promotes stem cell fate and restricts its own expression level by the activation of its repressor *CLV3* (Yadav *et al.*, 2011; Daum *et al.*, 2014). The activation of *CLV3* via WUS is mediated by direct binding of WUS to the *CLV3* promoter (Yadav *et al.*, 2011). Thus, a feedback loop is established that controls the balance of organogenesis and meristem maintenance (Brand, 2000; Schoof *et al.*, 2000).

Loss of the repressive function of CLV3 results in an over-proliferation of stem cells and eventually in the formation of additional organs, e.g. additional carpels within one flower (Clark *et al.*, 1995). On the other hand, over-expression of *CLV3* results in consumption of the shoot meristem, due to excessive repression *WUS*. Loss-of-function mutants of *WUS* phenotypically mimic *CLV3* over-expression phenotypes, whereas the over-expression phenotype of *WUS* mimics *clv3* null mutant phenotypes (Brand, 2000; Schoof *et al.*, 2000;

Laux *et al.*, 1996). The effects described for loss of the receptors of CLV3 are additive, because of their parallel function (Müller *et al.*, 2008). Loss of CLV1 function results in the same effects as loss of CLV3 function, but to a weaker degree (Clark *et al.*, 1995). Loss of either CLV2 or CRN function has the same effect as loss of CLV1. CLV2 and CRN were described to function together and loss of the function of one component abolishes the function of the whole complex, due to lack of proper localization of the CLV-CRN receptor complex at the plasma membrane (Müller *et al.*, 2008; Bleckmann *et al.*, 2010). Consequently, *clv1 clv2* double mutants phenotypically resemble *clv3* mutants (Müller *et al.*, 2008). *CLV1* expression was found in cells of the OC beneath the stem cell niche (Clark *et al.*, 1997). In contrast to the expression of *CLV1*, *CRN* expression is rather broad, spanning the whole meristematic region of the inflorescence and the floral meristem (Müller *et al.*, 2008; Bleckmann, 2010). *CLV2* was found in all layers of the CZ (Bleckmann, 2010) (Figure 4). The expression of the CLV pathway components starts during embryogenesis; *WUS* mRNA was detected in the 16 cell embryo at the earliest (Clark *et al.*, 1997; Bleckmann, 2010; Müller *et al.*, 2008; Brand, 2000; Mayer *et al.*, 1998).

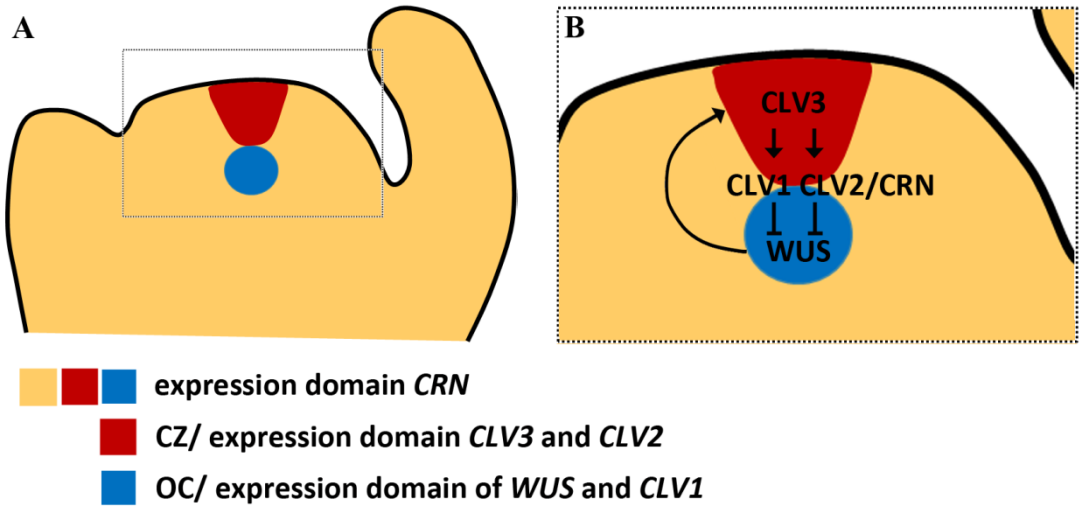



**Figure 4: The CLV pathway and expression domains of its components**

**A: Shoot meristem showing expression domains of CLV pathway components. Orange: expression domain of *CRN*, red: expression domain of *CLV3* and *CLV2*, blue: expression domain of *WUS* and *CLV1*. Dotted box shows region that is highlighted in B; B: *CLV3* is expressed in stem cells/ CZ and is perceived via CLV1 and in parallel CLV2 and CRN to restrict *WUS* in the OC. WUS in turn promotes stem cell fate, establishing a feedback loop. Arrows show perception of CLV3 by its receptors and positive effect of WUS on stem cells, bars show repression of *WUS*.**

#### 7.2.2.1 RPK2, BAM1, BAM2, and BAM3 are involved in the CLV pathway

Besides CLV1, CLV2, and CRN, which were the earliest receptors to be described to be functioning in CLV3 signalling, there are further receptors involved in the transmission of the CLV3 signal.

One of them is RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) also described as TOADSTOOL 2 (TOAD2) (Kinoshita *et al.*, 2010; Mizuno *et al.*, 2007; Nodine *et al.*, 2007). RPK2 was found in a screen for mutants resistant to treatment with exogenously applied CLV3 dodecapeptide. Treatment with this synthetic peptide was previously described to induce shoot phenotypes that mimic phenotypes caused by endogenous over-expression of CLV3 followed by *WUS* depression through CLV1 and CLV2 (Kondo *et al.*, 2006; Kinoshita *et al.*, 2010).

In the shoot, *RPK2* mRNA was detected in the SAM, the inflorescence meristem and floral meristems (Mizuno *et al.*, 2007; Kinoshita *et al.*, 2010).

Loss-of-function mutants of *RPK2* exhibit *clv*-mutant phenotypes in the SAM, but not in the inflorescence meristem. Floral organs were shown to be increased in number, as they are in *clv* mutants (Kinoshita *et al.*, 2010; Clark *et al.*, 1995). Overall, the phenotypes observed for *rpk2* mutants were weaker than those observed for *clv3* mutants. Over-expression of *RPK2* phenocopied plants over-expressing *CLV3* and *wus* mutants, leading to the conclusion that RPK2 functions in the CLV3-WUS feedback loop. However, an interaction of RPK2 with CLV1, CLV2, or CRN could not be detected. Thus, RPK2 is thought to act in an independent third pathway transmitting the CLV3 signal (Kinoshita *et al.*, 2010). A recent publication describes that RPK2 exhibits no direct binding to CLV3 peptide, thus the authors conclude that RPK2 is not involved in direct ligand interaction, but rather functions as co-receptor (Shinohara and Matsubayashi, 2015).

Besides RPK2, BAM1 and its homologues BAM2 and BAM3, the three of which are closely related to CLV1, were also described to function in the CLV pathway. Interestingly BAM1, BAM2, and BAM3 were originally described to positively regulate meristem size. Loss-of-function single mutants of the three *BAM* genes do not display any mutant phenotype. But, all double mutant combinations and the triple mutant exhibit a decrease in the size of the SAM, the inflorescence meristem, and the floral meristem, which led to the name BARELY ANY MERISTEM. In some cases the decrease in meristem size was so drastic, that meristems were terminated (DeYoung *et al.*, 2006).

However, mutant combinations of *BAM1* or *BAM2* with *CLV1* were described to increase the *clv1* single mutant phenotype, which is characterized by an enlarged meristem, due to the

partial loss of *WUS* repression. Thus, BAM1 and BAM2 can also function as repressors of meristem size (DeYoung and Clark, 2008; Durbak and Tax, 2011; Clark *et al.*, 1995; Brand, 2000; Schoof *et al.*, 2000).

A recent publication describes that in wild type shoots, *BAM1* is present exclusively in the L1 of the meristem, while *BAM3* is restricted to vascular strands below developing primordia. The study excluded *BAM2* from expression analyses, because of the very high similarity to *BAM1*, which was analysed instead (Nimchuk *et al.*, 2015; DeYoung *et al.*, 2006). Upon loss of CLV1 function, *BAM1* and *BAM3* are ectopically expressed in the cells of the rib meristem, where in wild type plants *CLV1* expression is detected. This ectopic expression is thought to facilitate the contribution of BAM1 and BAM3 to the CLV pathway upon loss of CLV1 function (Nimchuk *et al.*, 2015).

#### 7.2.2.2 Complex formation in the CLV pathway

As previously mentioned, receptor complex formation is a common feature of plant signalling pathways (Tax and Kemmerling, 2012).

CRN and CLV2 have first been hypothesized to form hetero-dimers, because the extracellular domain of CRN is very short and CLV2 lacks an intracellular kinase (Müller *et al.*, 2008; Jeong *et al.*, 1999). Thus, hetero-dimerization was thought to facilitate signal transduction (Müller *et al.*, 2008). A later study described that hetero-dimerization of CRN and CLV2 is necessary even for proper localization of the CLV2-CRN complex to the plasma membrane. If the respective interaction partner is lacking, CRN or CLV2 were described to localize to the endoplasmic reticulum instead. In the same study, CLV1 and CRN were found to form homomers, the latter of which are formed at the endoplasmic reticulum, prior to the transport of the CRN-CRN complex to the plasma membrane (Bleckmann *et al.*, 2010).

In a recent study, the authors could identify ligand-dependent receptor complex formation *in vivo*. According to this study, in the absence of CLV3, CLV1 preferentially forms homomers, whereas CLV2 and CRN form heteromers. Few CLV1-CLV2-CRN multimers were detectable. However, in the presence of CLV3, CLV1-CLV2-CRN multimers were detectable forming clusters in plasma membrane subdomains.

The authors conclude that either, CLV1 homomers, CLV2-CRN heteromers, and the multimeric complex consisting of CLV1-CLV2-CRN can all contribute to the CLV pathway, or CLV1 homomers and CLV2-CRN heteromers contribute to the CLV pathway, whereas the multimeric complex could be inactive and its formation could provide a possibility of

buffering against high concentrations of CLV3. Both models are in agreement with the previously described model of the CLV pathway, which was developed on the basis of genetic data, and in which CLV1 and CLV2-CRN are described to act in parallel to transmit the CLV3 signal (Somssich *et al.*, 2015; Müller *et al.*, 2008) (Figure 4).

### 7.2.3 CLE40

The closest homologue of *CLV3* is *CLE40*, which was found in a search for sequences with similarity to *CLV3*. Both genes consist of three exons and two introns, which are similar in their size between the two genes, and their peptides exhibit very high similarity within the CLE motif (Hobe *et al.*, 2003; Sharma *et al.*, 2003) (Figure 3 B).
In contrast to *CLV3* that is restricted in its expression to the stem cell region, *CLE40* mRNA was detected in the whole plant at a low level (Fletcher *et al.*, 1999; Hobe *et al.*, 2003). *CLE40* mRNA was detected at the earliest in the globular stage of the developing embryo (Stahl *et al.*, 2009).

#### 7.2.3.1 In the shoot, no function of CLE40 was described

Because of the high similarity between CLV3 and CLE40, previous studies focused on a potential function of CLE40 in the restriction of stem cell fate in the shoot. In fact, *CLE40* over-expression under the control of the *CaMV35S* promoter resembles over-expression of *CLV3*, upon which plant meristems are terminated due to the massive repression of *WUS* (Brand, 2000; Schoof *et al.*, 2000). Besides this, expression of *CLE40* under the control of the *CLV3* promoter is sufficient to restore the wild-type phenotype in *clv3-2* mutants. Thus, CLE40 actually has the potential to function in stem cell restriction in the shoot. However, *cle40 clv3* double mutants were found to phenotypically resemble *clv3* single mutants, thus in previous publications, it was concluded that CLE40 has only a minor if any role in activation of the CLV signalling pathway in the shoot of wild type plants (Hobe *et al.*, 2003).

#### 7.2.3.2 In the root, CLE40 restricts columella stem cell fate

Later studies focused on the function of CLE40 in the root. *CLE40* expression in the root was detected in the vasculature and in columella cells in the root tip. CLE40 was described to act

via its potential receptor ACR4 and CLV1 to restrict the *WUS* homologue *WUSCHEL HOMEOBOX 5* (*WOX5*), which promotes columella stem cell fate. Thus in the root, CLE40 functions as a negative regulator of columella stem cell fate (Stahl *et al.*, 2009; Stahl *et al.*, 2013).

# 8 Terms of Reference

## 8.1 Starting situation

The small secreted peptide CLE40 was previously described to function in the root regulating columella stem cell fate. In the shoot, *CLE40* was described to be expressed broadly at a low level, but no clear shoot specific function was described so far.

Meristem homeostasis in the shoot is achieved by the CLV pathway and CLE40 has the potential to substitute for CLV3 function in the CLV pathway. However, previous studies could not identify any functional relevance of this potential.

Different publications deal with the question how the shoot meristem can compensate high concentrations of CLV3. It was hypothesized that the receptors of the CLV pathway might be inactivated due to CLV3 induced complex formation. CLV3 was shown to induce multimeric complexes of CLV1-CLV2-CRN. This is of interest, because once the level of CLV3 is too high, *WUS* expression is lost and meristems are terminated.

## 8.2 Problem

Although the question how plants deal with excess CLV3 was previously discussed, the question of how a plant could react to quickly dropping levels of *CLV3* expression remains to be investigated in more detail. In principle, reductions in CLV3 lead to less repression of *WUS* and thus to more stem cells, which consequently in total produce higher amounts of CLV3. However, this increase in total CLV3 amount is based on the derepression of *WUS* transcription that is followed by WUS movement and eventually stem cell division. The binding of CLV3 to CLV1 was described, but it is not known how many downstream targets are addressed by CLV3 signalling before eventually *WUS* is repressed. The question remains, if plants have evolved strategies to react faster to decreasing *CLV3* expression levels, in case of sudden fluctuations of *CLV3* to ensure robustness of meristem maintenance and organogenesis.

## 8.3 Hypotheses

*CLE40* is expressed in the shoot and has the potential to substitute for CLV3 function. Thus, CLE40 might function as buffer for CLV3. Quick changes in *CLV3* expression might be compensated by the function of CLE40. To fulfil this function of a buffer, CLE40 might act via the same receptors that act in the CLV pathway. Assuming that CLE40 fulfils the function of a buffer that is only needed under specific conditions, which lead to decreasing *CLV3* levels, could explain why previous experiments could not identify a shoot specific function of CLE40.

## 8.4 Experiments

To address the question if CLE40 fulfils the function of a buffer in shoot meristem homeostasis, genetic analyses were performed in isogenic backgrounds. Previous studies were performed in mixed backgrounds that could have influenced the outcome of the experiments.
The receptors of the CLV pathway and *CLE40* were analysed in detail concerning their expression patterns. Previous descriptions of their expression are based on *in-situ* hybridization results and high resolution confocal microscopy was expected to give deeper insights into the exact expression patterns. This detailed understanding of expression domains is crucial to answer the question if a shoot specific function of CLE40 includes the same receptors that function in the CLV pathway.
The aim of the thesis was to characterize the function of CLE40 in the shoot in detail and to integrate CLE40 into the network of factors controlling shoot meristem homeostasis.

# 9 Results

## 9.1 *CLE40* is broadly expressed in seedlings while its homologue *CLV3* is restricted to stem cells

To gain insight into the exact expression pattern of *CLE40,* transgenic Col-0 plants expressing *CLE40::Venus-H2B* (Wink, 2013) were analysed concerning shoot specific expression. To compare the expression of *CLE40* with that of its close homologue *CLV3* (Hobe *et al.*, 2003), a *CLV3::H2B-YFP* line was used.

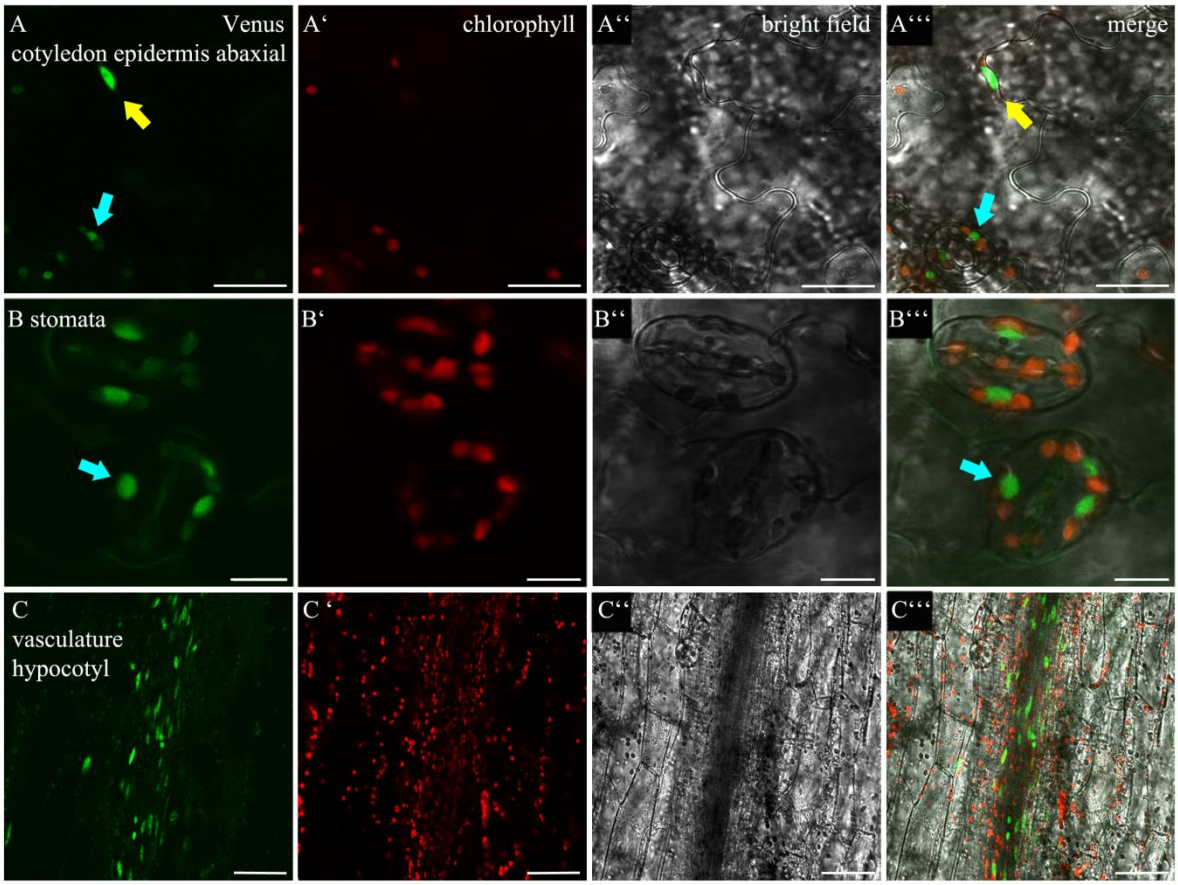



**Figure 5: *CLE40::Venus-H2B* in seedlings 4 dag**

**Expression of *CLE40::Venus-H2B* in abaxial epidermal cotyledon cells (A-A’’’), guard cells of stomata (B-B’’’), and hypocotyl vasculature (C-C’’’). A-C: Venus-H2B; A’-C’: chlorophyll autofluorescence; A’’-C’’: bright field; A’’’-C’’’: merge; Yellow arrows mark expression in the nucleus of an epidermal cell and light blue arrows mark expression in stomata nuclei (A, A’’’, B, B’’’). Scale bars represent 50 µm in A-A’’’, and C-C’’’ and 10 µm in B-B’’’.**

In agreement with previous publications, *CLE40* was broadly expressed in the shoot, while *CLV3* was restricted to stem cells (Sharma *et al.*, 2003; Hobe *et al.*, 2003; Fletcher *et al.*, 1999).

In 4 day old seedlings, *CLE40* expression was detected in the L1 of cotyledon cells, including guard cells of stomata (Figure 5 A-A''', B-B'''). *CLE40* expression was also found along the vasculature (Figure 5 C-C'''). However, the broad expression of *CLE40* hindered a clear localization of the SAM at this developmental stage.

In contrast to *CLE40*, the expression of *CLV3* was restricted to the stem cell region, in seedlings as well as after floral transition (Figure 10 A-A'', B-B''').

Taken together, despite the high similarity in the structure of *CLE40* and *CLV3*, they differ quite strongly in their expression patterns and this difference might correlate with functions in different developmental processes.

## 9.2 *CLE40* is expressed in the L1 and L3 of the inflorescence and the floral meristem

We asked if *CLE40* expression could also be detected in later developmental stages, like the expression of *CLV3,* which can be detected in inflorescence and floral meristems (Fletcher *et al.*, 1999). Thus, inflorescence and floral meristems of *CLE40::Venus-H2B* were analysed and compared to meristems of *CLV3::H2B-YFP*.

Microscopy of 57 plants revealed that *CLE40* is expressed in both the inflorescence and the floral meristem (Figure 6). In the inflorescence meristem, *CLE40* was detected in the L1 and in the L3, but in most plants analysed it was absent from the L2 (Figure 6 c). In about 9 % of the samples (5 of 57), *CLE40::Venus-H2B* expression was detected in the L2, but the L2 expression was always weaker than the expression in the L1 and L3 and in some meristems, it did not cover the whole L2 (Supplemental Figure 1). We noted that in the inflorescence meristem, cells of the L1 differed in *CLE40* expression strength (Figure 6 A'', A'''). L1 expression was decreased or lost in developing flower primordia (Figure 6 C'', C'''), which is reminiscent of an inverse auxin-signalling pattern (Vernoux *et al.*, 2011). Interestingly, in later stages of flower development, L1 expression could be detected again. In developing flowers, *CLE40* was found in the L1 and the L3 of the floral meristem, as well as in the anlagen of sepals (Figure 6 D'', D'''). In the L2 of the floral meristem, *CLE40* was absent in the majority of the plants, like in the L2 of the inflorescence meristem (Figure 6 c, D'', D''').

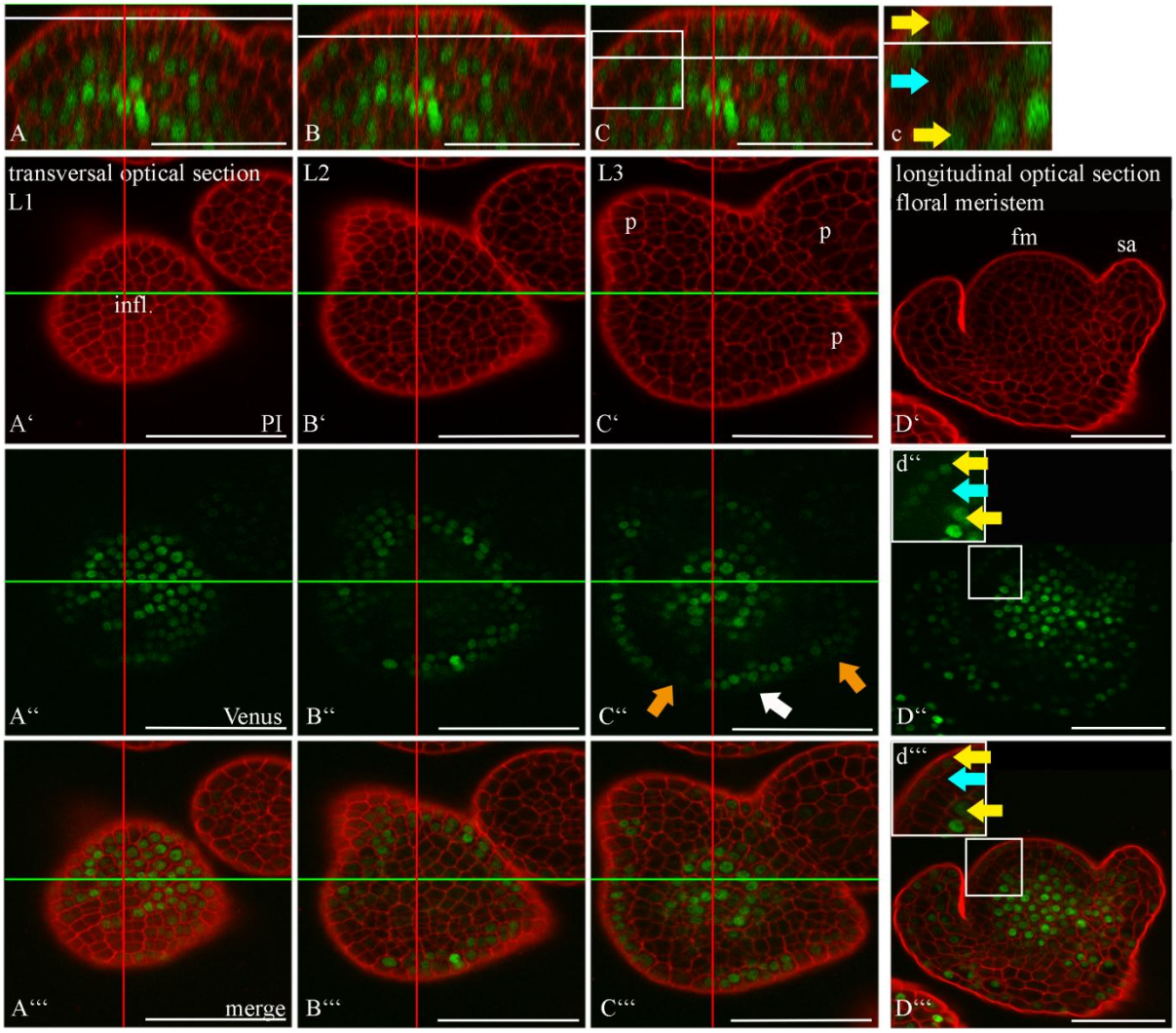



**Figure 6: Expression of *CLE40::Venus-H2B* in an inflorescence and a floral meristem**

**Transversal optical sections of the L1 (A'-A'''), L2 (B'-B'''), and L3 (C'-C''') of an inflorescence meristem (infl) and longitudinal optical sections of a floral meristem (fm) (D'-D''') showing PI staining (A'-D'), Venus-H2B expression (A''-D''), and merged pictures (A'''-D'''). Pictures in A-C show reconstructed longitudinal optical sections, each containing a white line that marks the plane of the respective transversal optical section. Red and green lines in A-C, A'-C', A''-C'', and A'''-C''' show the plane that was used to generate longitudinal optical sections. Those generated in the "green plane" are shown in A-C. Orange arrows point at positions of young developing primordia (p) in C'' that correlate with a decrease in expression strength compared to the adjacent region (white arrow). c, d'' and d''' show magnifications of areas surrounded by white boxes in C, D'' and D''' respectively. Light blue arrows point at regions of no expression in L2 cells of a floral and an inflorescence meristem. Yellow arrows mark expression in L1 and L3 cells (c, d''-d'''). The green channel was digitally enhanced in d''. Scale bars represent 50 µm.**

Overall, the L1-L3 pattern could be detected in about 77 % of the plants analysed (44 of 57). 8 of 57 plants showed expression that was either too weak or too patchy to allow a characterization of the expression pattern.

In contrast to this, *CLV3* expression was restricted to the stem cells of the floral and the inflorescence meristem and covered each of the three meristematic layers (Figure 10 A-A'').

Taken together, like in the vegetative SAM, the *CLE40* expression pattern in the floral and the inflorescence meristem is much broader than that of *CLV3*. In contrast to *CLV3* expression, *CLE40* is lacking in the L2 and its expression strength changes during different stages of organ development.

## 9.3 *CLV1* and *CLE40* have largely overlapping expression domains

To analyze whether CLE40, like CLV3, has the potential to act through the receptors of the CLV pathway, we compared the *CLE40* expression pattern to that of *CLV1*, *CLV2*, and *CRN*, with the aim to reveal the degree of overlap in expression.

For analysis of *CLV1*, we used a *CLV1::CLV1-2xGFP* line in the *clv1-11* mutant background, in which the mutant *clv1* phenotype was complemented (Nimchuk *et al.*, 2011). This line was previously described to show localization of CLV1 in the plasma membrane of the rib meristem, consistent with previous analyses of *CLV1* mRNA localization (Nimchuk *et al.*, 2011; Clark *et al.*, 1997). Occasionally, weak expression was observed in the L1 and the authors concluded that, while *CLV1* expression strength is highest in the central zone, some *CLV1* expression exists throughout the meristem (Nimchuk *et al.*, 2015; Nimchuk *et al.*, 2011).

In agreement with these descriptions, we found *CLV1::CLV1-2xGFP* in the plasma membrane of the L3 of the inflorescence meristem as well as in the L3 of developing flower primordia (Figure 7 C-C'''). However, in contrast to the previous descriptions of *CLV1* expression, we detected strong expression of *CLV1* in the L1 of all plants analysed (Figure 7 A, A''', C', C'''). In the L2, *CLV1* expression was absent (Figure 7 B, B''', C', C'''), resembling the expression pattern of *CLE40* (Figure 6).

Interestingly within the L1, we observed the same pattern that we also found for *CLE40* expression. Like *CLE40* expression, the expression of *CLV1* was decreased in regions where

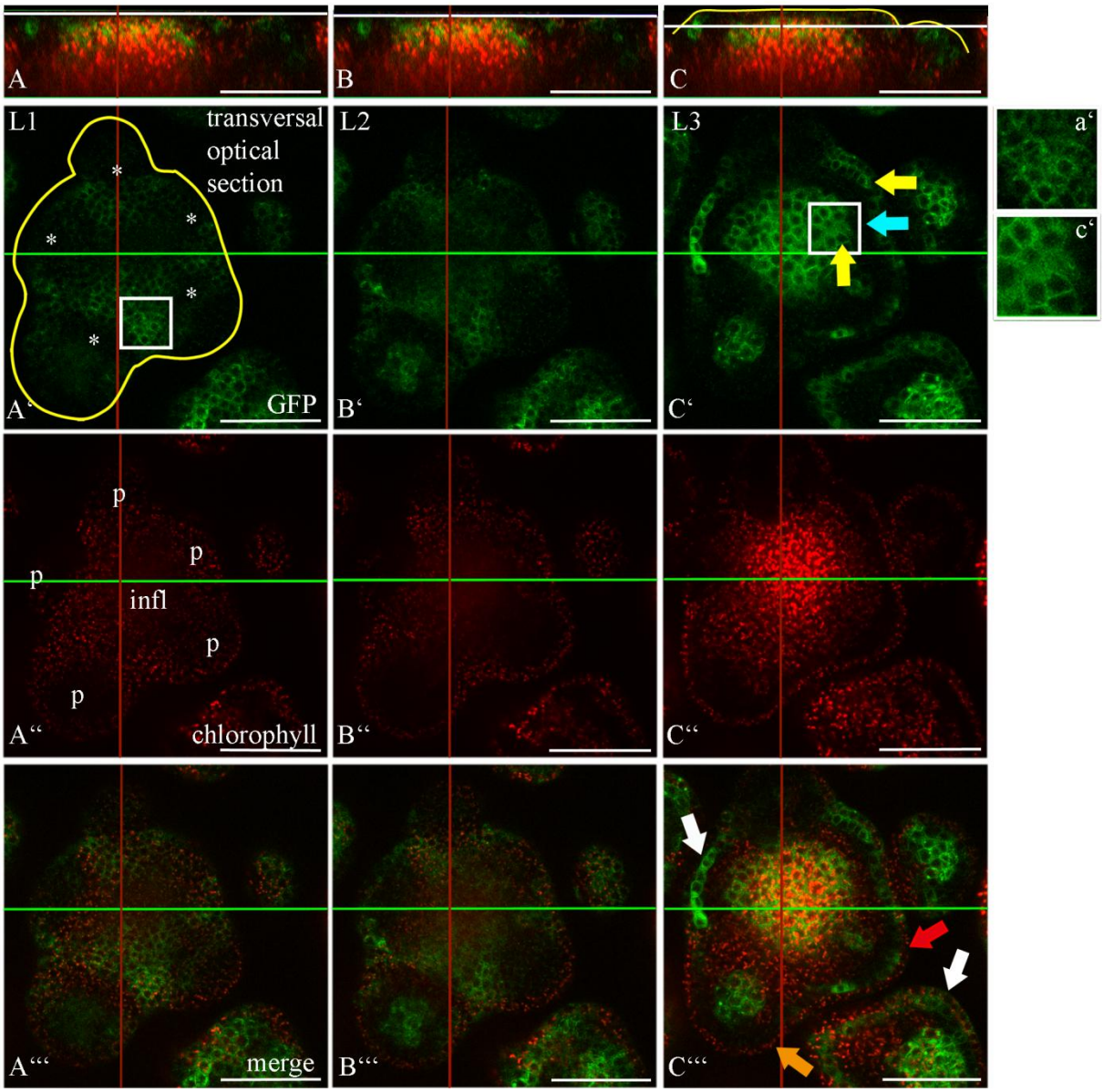



**Figure 7: Expression of *CLV1::CLV1-2xGFP* in an inflorescence and a floral meristem of a *clv1-11* mutant**

**Transversal optical sections of the L1 (A'-A'''), L2 (B'-B'''), and L3 (C'-C''') of an inflorescence meristem and reconstructed longitudinal optical sections (A-C) show expression of *CLV1::CLV1-2xGFP* (A'-C'), chlorophyll autofluorescence (A''-C''), and merged pictures (A-C, A'''-C'''). White lines in A-C mark the plane of the respective transversal optical section. Green and red lines show planes that were used to generate reconstructed longitudinal optical sections; those generated in the "green plane" are shown in A-C. Yellow lines in A' and C indicate the outer limits of the meristem and its adjacent primordia. Note that the meristem is flattened due to the pressure of the cover slide. a' and c' show magnifications of areas surrounded by white boxes in A' and C'. A'': infl = inflorescence meristem, p = primordium; A': Asterisks mark the position of lower GFP signal intensity compared to adjacent regions. C': Yellow arrows mark expression of *CLV1::CLV1-2xGFP* in the L1 and the L3 and light blue arrow points at the L2 that lacks a GFP signal. C''': White arrows mark L1 expression in the periphery of the inflorescence meristem and in the L1 of a young developing flower; orange arrow marks no L1 expression in a developing primordium; red arrow points at L1 of developing primordium that partially lacks the GFP signal. Scale bars represent 50 µm.**

primordia were formed (Figure 7 A', A'', C', C''', Supplemental Figure 2).

In contrast to primordia, in young developing flowers of *CLV1::CLV1-2xGFP* the GFP signal was restored, comparable to the restored Venus signal in developing flowers of *CLE40::Venus-H2B* (Figure 7 C''', Figure 6 D'-D''').

We concluded from these findings that, because *CLE40* and *CLV1* have overlapping expression patterns, CLE40 has the potential to function via CLV1 in the shoot. The fact that the expression domain of *CLV1* is much broader than the published expression domain of the CLV pathway target *WUS*, led to the conclusion, that CLV1 has the potential to be involved in further signalling processes in the shoot than only the CLV pathway (Mayer *et al.*, 1998).

## 9.4 *CRN* is expressed in all three layers of the inflorescence and the floral meristem, but *CLV2* is restricted to the L1 and the L3.

For the analysis of *CLV2* and *CRN* expression we chose a double marker line expressing *CLV2::Venus-H2B* and *CRN::mCherry-H2B* (Bleckmann, 2010).

In inflorescence meristems as well as in floral meristems, we found *CRN* broadly expressed in all three layers (Figure 8 A-C, A''-D'', A'''-D''', E). This result is consistent with previous analyses of *CRN* mRNA localization and previous analyses of another line expressing this construct (Müller *et al.*, 2008; Bleckmann, 2010).

*CLV2* expression was detected in the L1 and the L3 of inflorescence and floral meristems, but in both types of meristems it was lacking in the L2 (Figure 8 A-C, A'-D', A'''-D''', and E), reminiscent of the *CLE40* and the *CLV1* expression (Figure 6, Figure 7). These findings are consistent with previous descriptions of another *CLV2* reporter line that contained the same promoter. Though with that line, no sufficiently clear resolution could be obtained, which would have allowed to exclude *CLV2* from the L2 (Bleckmann, 2010).

Interestingly, for *CLV2* we could also detect regions of decreased expression correlating with the formation of primordia (Figure 8 B' and B'''), something we did not observe for *CRN*. However, in contrast to *CLV1* expression that was negatively correlated with the formation of young primordia, for *CLV2* this decrease in expression could only be found at later stages of primordia development. We concluded that either high stability of H2B hinders degradation of the fluorophore, or the effect occurs at later stages.

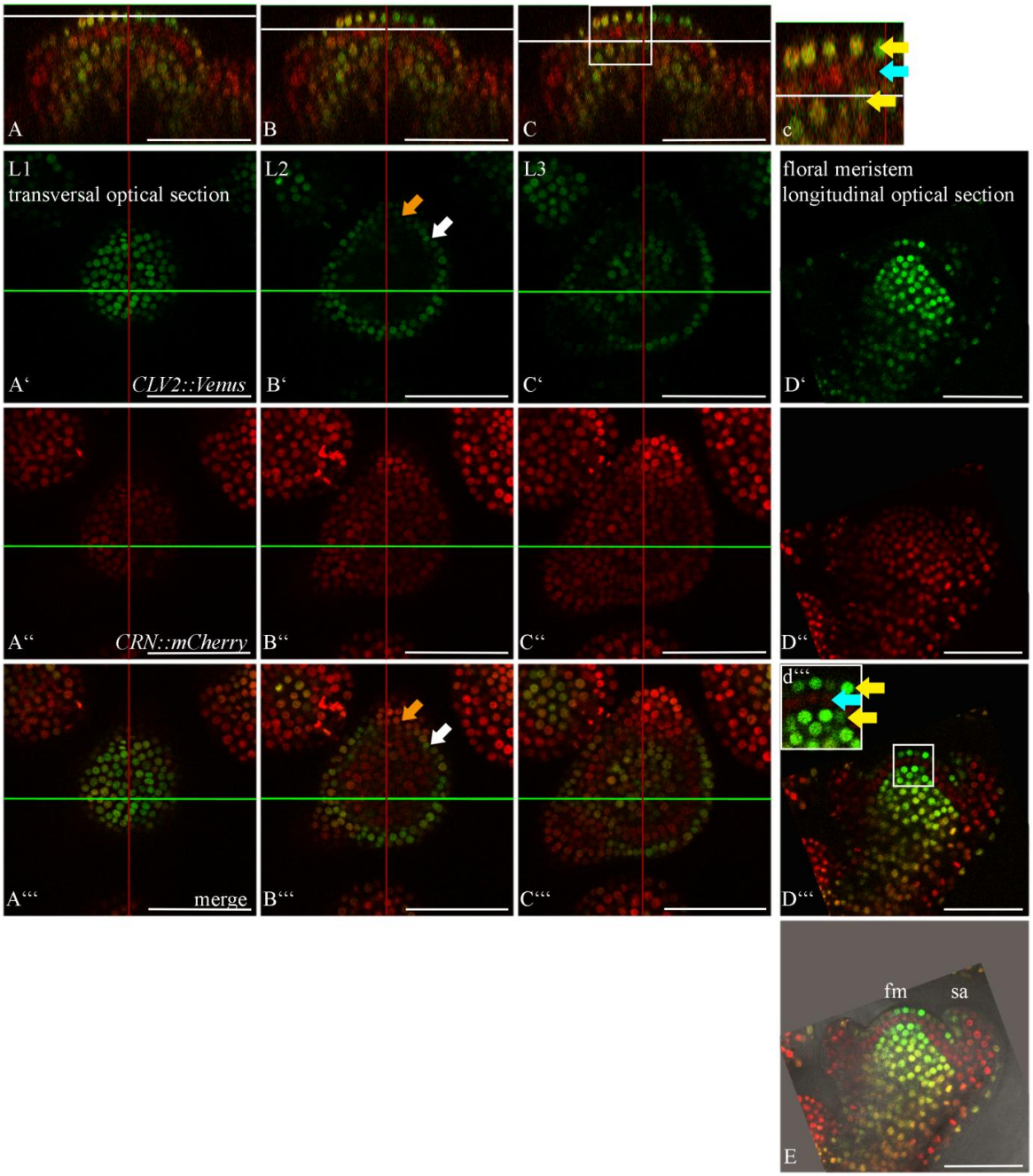



**Figure 8: Expression of *CLV2::Venus-H2B* and *CRN::mCherry-H2B* in an inflorescence and a floral meristem**

**Transversal optical sections of the L1 (A'-A'''), L2 (B'-B'''), and L3 (C'-C''') of an inflorescence meristem and longitudinal optical sections of a floral meristem (fm) with developing sepal anlagen (sa) (D'-D''', E) show expression of *CLV2::Venus-H2B* (A'-D'), *CRN::mCherry-H2B* (A''-D''), and merged pictures (A'''-D'''). Pictures in A-C show reconstructed longitudinal optical sections, each containing a white line that marks the plane of the respective transversal optical section. Red and green lines in A-C, A'-C', A''-C'', and A'''-C''' show planes that were used to generate reconstructions of longitudinal optical sections; those generated in the "green plane" are shown in A-C. E: like D''' plus bright field; c and d''': magnifications of areas surrounded by white boxes in C and D''' respectively; Yellow arrows mark *CLV2::Venus-H2B* expression in L1 and L3 cells and light blue arrows mark absence of *CLV2::Venus-H2B* expression in the L2 but presence of *CRN::mCherry-H2B* expression. Orange arrows mark regions of no *CLV2::Venus-H2B* in the L1 of a developing primordium and white arrows point at *CLV2* expression in the L1 of adjacent cells. Scale bars represent 50 µm.**

Taken together, we found that just like *CLV1*, *CLV2* and *CRN* are broadly expressed in the inflorescence and the floral meristem and that their expression largely overlaps with that of *CLE40*. Thus CLE40 has the potential to function via the same receptors that are involved in the transmission of the CLV3 signal (Clark *et al.*, 1995; Clark *et al.*, 1997; Jeong *et al.*, 1999; Müller *et al.*, 2008). Besides this, the broad expression domains of *CLV2* and *CRN* as compared to their target *WUS* indicate that CLV2 and CRN could have the potential to function in further developmental processes in the shoot than only the CLV pathway (Mayer *et al.*, 1998).

## 9.5 Loss of CLV3 results in ectopic expression of *CLE40* in the L2

We found that in the shoot, CLE40 has the potential to function via the same receptors that are involved in signal transmission of its close homologue CLV3 (Clark *et al.*, 1995; Clark *et al.*, 1997; Jeong *et al.*, 1999; Müller *et al.*, 2008; Hobe *et al.*, 2003). Previous publications reported that CLE40 can substitute for CLV3 in *clv3* mutants, if expressed under the control of the *CLV3* promoter (Hobe *et al.*, 2003).

Based on these findings, we asked if CLE40 could function as a buffer that would substitute for CLV3 in case of fluctuating expression levels and if it was transcriptionally regulated by CLV3 to control this potential substitution. To answer this question, we crossed *clv3-9* null mutants (Supplemental Figure 3) with Col-0 plants expressing *CLE40::Venus-H2B*. If *CLE40* is transcriptionally regulated by CLV3, we would expect that its expression might become up-regulated in the stem cell region in *clv3* mutants, where *CLV3* is expressed in wild type plants to buffer for loss of CLV3, because *CLV3* expression is stronger than *CLE40* expression in wild type (Hobe *et al.*, 2003) (Figure 10). However, we could not identify a clear upregulation of *CLE40* in the stem cell domain, either in inflorescence meristems, or in floral meristems (Figure 9). But in contrast to wild type plants, of which 91 % did not show *CLE40* expression in the L2, we found *CLE40* in the L2 of all *clv3* mutant inflorescence and floral meristems (Supplemental Figure 1, Figure 6 B'', Figure 9 a'', B''), which could be indicative of a general up-regulation of *CLE40* expression in the meristematic region. However, we cannot clearly state if there is in fact an up-regulation of *CLE40* expression in the whole meristematic region and not only in the L2, because there is some variability in expression strength of individual plants, which might cover slight differences between the wild type and *clv3* mutants.

Looking at the expression pattern of *CLE40* in the L1 of *clv3* mutants, we found that at positions of primordia formation, *CLE40* expression strength was reduced compared to the rest of the meristem; comparable to the differences in expression strength that we saw in wild type plants expressing *CLE40::Venus-H2B* (Figure 6 C'', Figure 9 A, a'').

We concluded from these findings that upon loss of CLV3, there is an up-regulation of *CLE40* in the L2, thus CLE40 might function as a buffer for CLV3.

The L1 specific expression of *CLE40* is not impaired in *clv3* mutants and its regulation appears to be present like in wild type plants. Besides this, the expression of *CLE40* is much broader than that of *CLV3*. Consequently, although CLE40 was previously described to have the potential to substitute for loss of CLV3 (Hobe et al., 2003), we assume that it also could have additional functions, in regions where *CLV3* is not expressed.

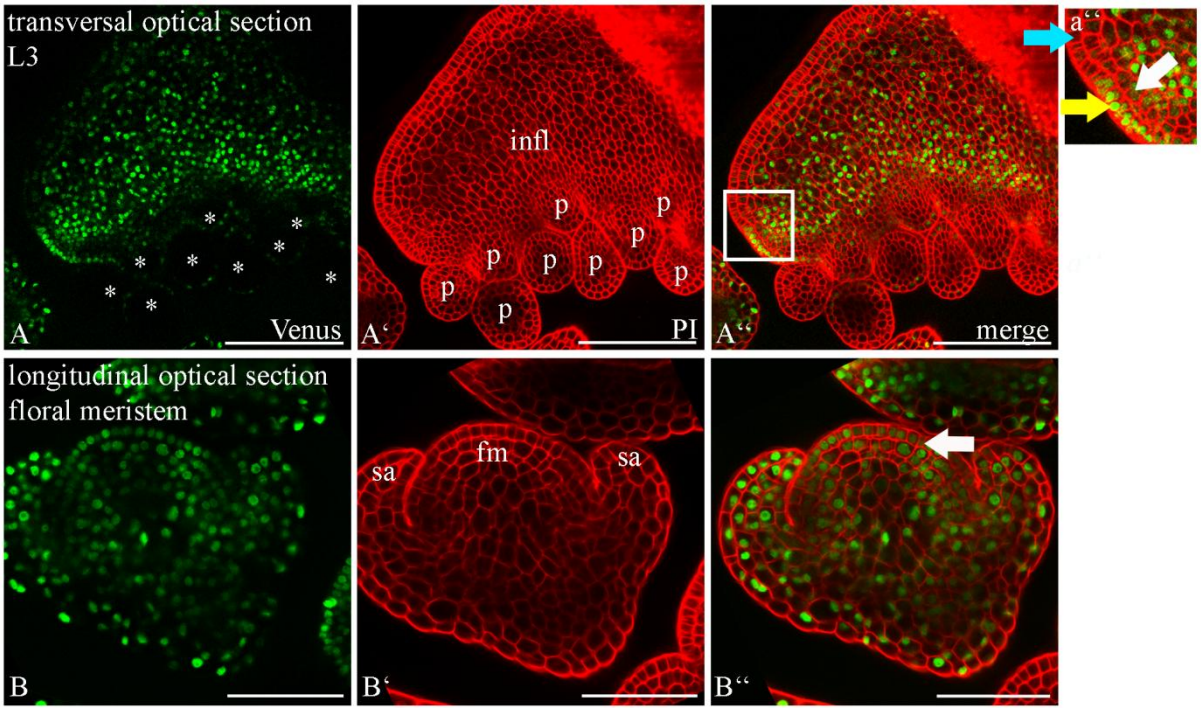



**Figure 9: Expression of *CLE40::Venus-H2B* in *clv3* mutants**

**Transversal optical sections of the L3 of an inflorescence meristem (infl) of *clv3* expressing *CLE40::Venus-H2B* show Venus-H2B (A), PI staining (A'), and merged pictures (A''). The meristem is strongly enlarged due to loss of the repressive function of CLV3. Longitudinal optical sections of a floral meristem (fm) of *clv3* with sepal anlagen (sa) show Venus-H2B (B), PI staining (B'), and merged pictures (B''). a'' shows a magnification of the area surrounded by a white box in A''. Yellow arrows mark expression in L1 cells of the inflorescence meristem (a''). Light blue arrow points at a region of no expression in L1 cells of the inflorescence meristem. White arrows mark expression in L2 cells of an inflorescence meristem (a'') and a floral meristem (B''). p = primordia; Asterisks in A mark regions of low expression that correlate with the location of primordia. Scale bars represent 100 µm in A-A'' and 50 µm in B-B''.**

## 9.6 Loss of CLE40 does not lead to ectopic *CLV3* expression in seedlings

From our findings that *CLE40* is broadly expressed outside of the stem cell domain and that it seems to be regulated in a different way than *CLV3*, we hypothesized that CLE40 might have additional functions in the shoot besides buffering of CLV3 fluctuations.

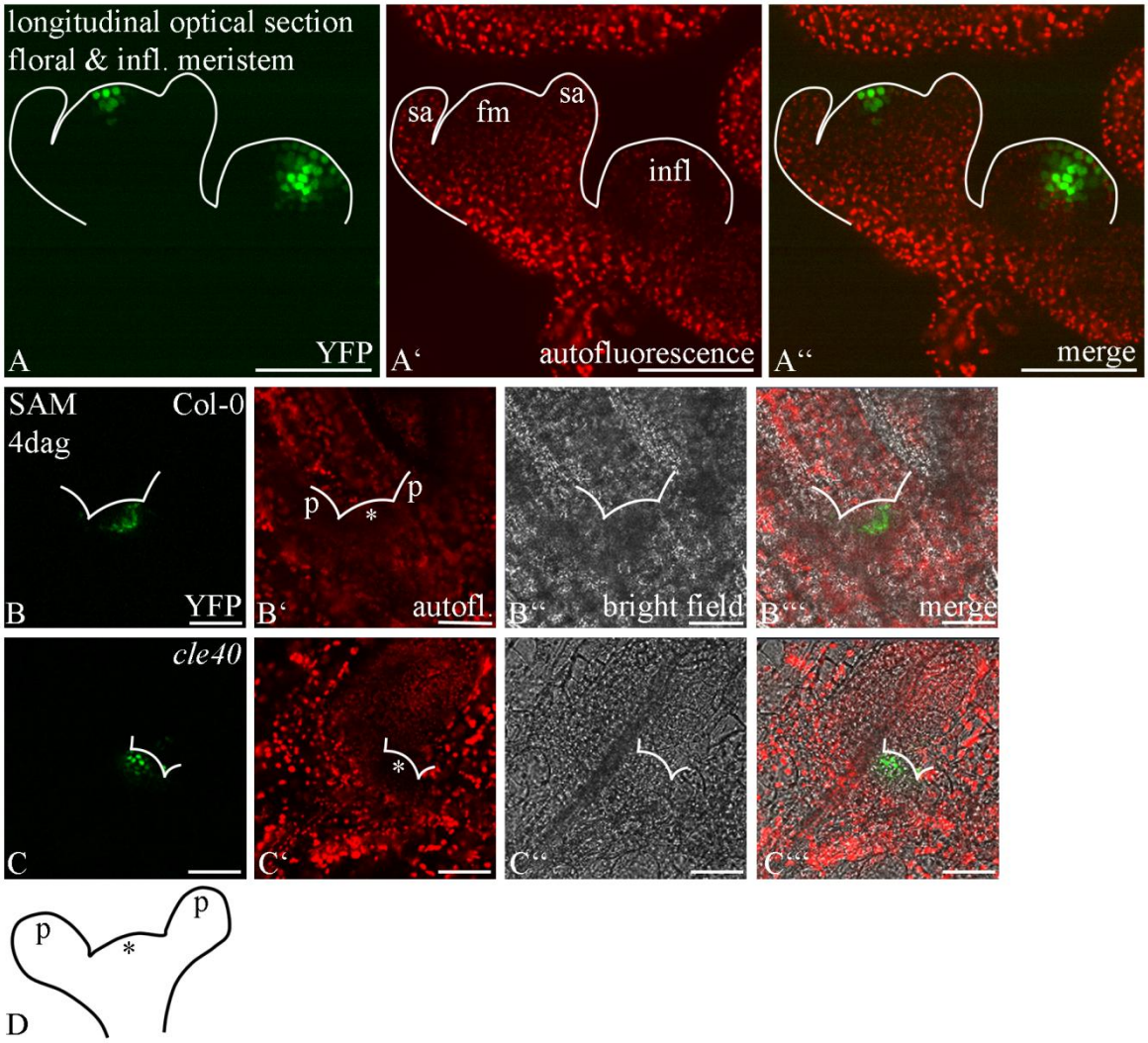



**Figure 10: Expression of *CLV3::H2B-YFP* in a Col-0 and a *cle40* SAM and in a floral and an inflorescence meristem of Col-0**

**A: Longitudinal optical sections of a floral meristem (fm) and an inflorescence meristem (infl) show H2B-YFP (A), chlorophyll autofluorescence (A'), and merged pictures (A''). sa = sepal anlagen (A'); White lines in A-A'' surround the L1 of the inflorescence meristem and the floral meristem including the sepal anlagen. B and C: Longitudinal optical sections of Col-0 (B-B''') and *cle40* (C-C''') seedlings 4 dag show H2B-YFP (B, C), chlorophyll autofluorescence ('), bright field (''), and merged pictures ('''). White lines in B-B''' and C-C''' mark the outer limits of the SAM (*) and the adjacent primordia (p). D: schematic illustration of the meristematic region in a seedling with the SAM (*) and leaf primordia (p); Scale bars represent 50 µm.**

*cle40* mutant shoots were previously described as aphenotypic (Hobe *et al.*, 2003). We hypothesized that if CLE40 had a function in the shoot, the lack of a strong mutant phenotype might be due to a compensation of its loss by CLV3. If CLV3 could compensate for loss of CLE40 in *cle40* mutants, it would be possible that the expression of *CLV3* would expand to regions where *CLE40* is expressed in wild type plants; there it could fulfil potential wild type functions of CLE40. To test this hypothesis, *cle40-2* null mutants (Stahl *et al.*, 2009) were crossed with Col-0 plants expressing *CLV3::H2B-YFP* and the expression of *CLV3* in homozygous F3 mutants was analysed.

Consistent with previous publications, *cle40-2* did not display an obvious shoot phenotype (Hobe *et al.*, 2003) (Figure 18 A, B).

However, in *cle40* mutant seedlings *CLV3* expression was not altered compared to Col-0 seedlings (Figure 10 B-B’’’, C-C’’’). In both cases, expression of *CLV3* was restricted to the stem cells. We concluded that upon loss of CLE40, there is no substitution of its potential function by CLV3, at least not beyond the stem cell region, where the expression domain of *CLE40* overlaps with that of *CLV3*.

## 9.7 CLE40 is a positive regulator of shoot apical meristem size

We found that, based on its expression domain and its potential regulation, CLE40 could have additional functions besides buffering for CLV3 fluctuations, and that the lack of an obvious *cle40* mutant shoot phenotype cannot be explained by ectopic *CLV3* expression in the *CLE40* expression domain.

However, *CLE40* and *CLV3* display an overlap in their expression in the stem cells (Figure 6, Figure 10). Thus, we carried out genetic analyses concentrating on meristem specific phenotypes, to answer the question if the two peptides have shared functions, e.g. regulation of meristem size within the CLV pathway.

For the genetic analyses, we chose *cle40-2* and *clv3-9* mutants. Both alleles are null mutants, because the conserved CLE motif is lacking due to a stop codon upstream of the region coding for this motif (Stahl *et al.*, 2009) (Supplemental Figure 3). Seedlings of homozygous plants were grown on MS plates for 4 days and SAM size was identified by measuring the distance between the two youngest primordia. In comparison to Col-0 wild type seedlings, *clv3* mutants exhibited a massive increase in SAM size (Figure 11 A-D), consistent with the described function of CLV3 as a repressor of meristem size (Fletcher *et al.*, 1999). In contrast,

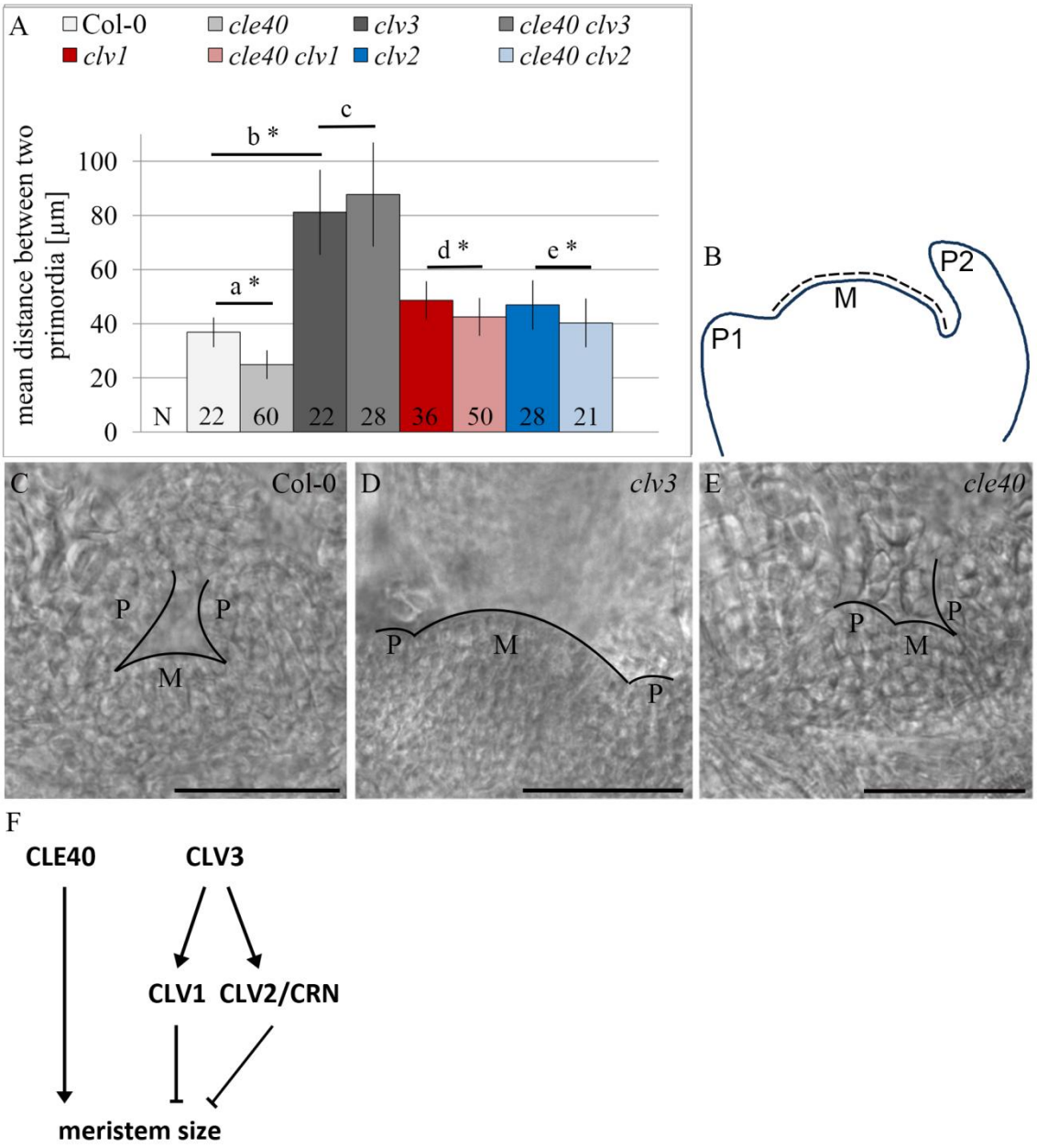



**Figure 11: CLE40 and CLV3 have antagonistic functions in the SAM.**

**A: Mean distance between two primordia 4 dag. Error bars represent standard deviation. Asterisks mark statistically significant differences. p-value (a) = $1.3 \times 10^{-14}$, p-value (b) = $2 \times 10^{-16}$, p-value (c) = 0.2, p-value (d) = 0.0001, p-value (e) = 0.01; N = number of seedlings; B: Schematic illustration of a SAM with the youngest (P1) and second youngest (P2) primordia and the meristem (M). Interrupted line represents the distance between the two youngest primordia, which was measured in A. C-E: SAM pictures of Col-0, *clv3*, and *cle40* respectively. Black lines mark the surface of the SAM and the youngest visible primordia. Scale bars represent 50 µm. F: Model of the signalling pathway depicting the function of CLE40 as an antagonist of the CLV pathway. Arrows show positive effect/activation of pathway and bars show repression.**

*cle40* mutants revealed a significant reduction in SAM size (Figure 11 A, C, E); indicating that CLE40 has a meristem size-promoting function during vegetative growth and that CLV3 and CLE40 are two peptides with antagonistic functions in meristem size control (Figure 11 F).

If CLV3 and CLE40 act in antagonistic pathways, one would expect that the *cle40* mutation would suppress phenotypic effects caused by mutations in the components of the CLV pathway. To test this hypothesis, *cle40* was crossed with *clv3,* with *clv1-20*, which is characterized by a reduced mRNA level (Durbak and Tax, 2011), and with the null mutant *clv2-101* (Kinoshita *et al.*, 2010). The receptor mutants *clv1* and *clv2* exhibited an increase in SAM size (Clark *et al.*, 1993; Clark *et al.*, 1997; Jeong *et al.*, 1999; Kayes and Clark, 1998), though less severe than that observed in *clv3*, due to the parallel function of the two receptors (Müller *et al.*, 2008) (Figure 11 A). Loss of CLE40 in either the *clv1* or the *clv2* mutant background caused a significant suppression of the respective *clv* phenotype, consistent with the hypothesis that CLE40 acts antagonistically to the CLV pathway (Figure 11 A). The receptor kinase CRN (Müller *et al.*, 2008) was not included into these analyses, because it was described to function together with CLV2 in the CLV pathway, and loss of either CRN or CLV2 abolishes the function of the whole CRN-CLV2 complex, due to loss of correct localization of the complex to the plasma membrane (Bleckmann *et al.*, 2010).

In *clv3 cle40* double mutants, suppression effects like those seen in *clv1 cle40* and *clv2 cle40* could not be detected (Figure 11 A). There was no significant difference in SAM size between *clv3 cle40* and *clv3*. As *clv3* has a very strong phenotype, it is possible that mild phenotypic changes due to loss of CLE40 cannot be detected in *clv3*. Thus, it was concluded that the CLV pathway has a stronger effect than the CLE40 pathway.

Taken together, the reduction of SAM size in *cle40* mutants and the suppression of the *clv1* and *clv2* phenotype by *cle40* indicate that CLE40 is a positive regulator of SAM size acting antagonistically to the CLV pathway (Figure 11 F).

## 9.8 CLE40 regulates the number, but not the size of meristematic cells

In *cle40* mutants, SAM size is decreased, but the pictures used to measure SAM size do not allow for conclusion on whether this reduction is due to a decrease in cell number or cell size. To answer this question, four day old seedlings of Col-0 and *cle40* were stained with propidium iodide to visualize cell walls, and to allow counting of individual cells. The cell

size was determined by measuring the cell diameter in the radial axis of the L1 (Figure 12). This analysis revealed no difference in the size of Col-0 and *cle40* SAM cells. Mean cell diameter was 7.0 µm and 6.8 µm for Col-0 and *cle40* respectively (Figure 12 C). Because SAM cell size does not differ between the two genotypes, the difference in meristem size must be due to a reduced cell number in meristems of *cle40* mutants.

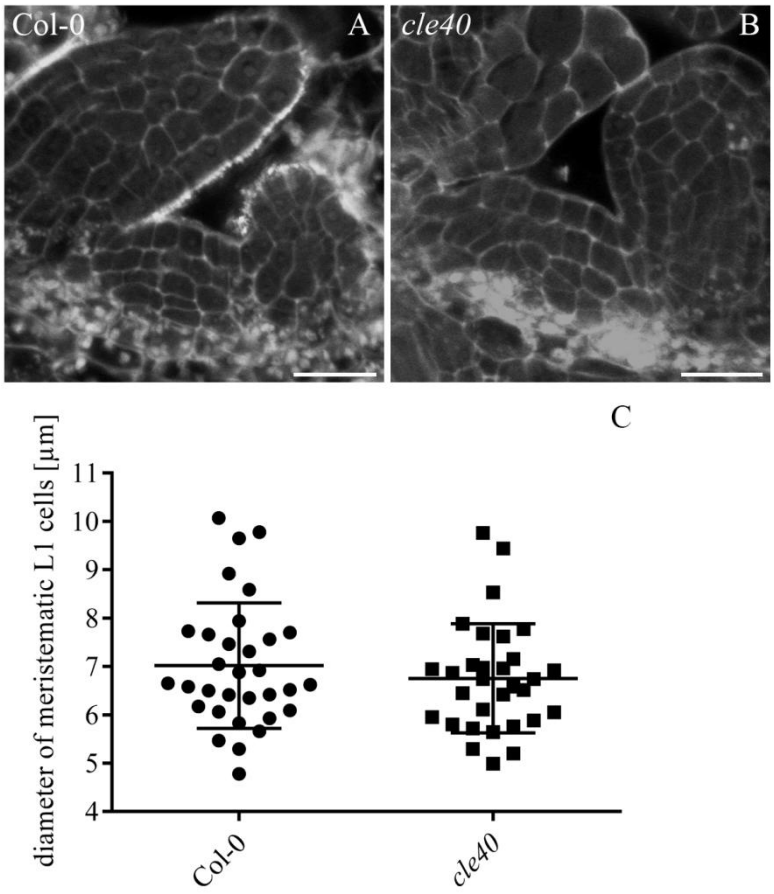



**Figure 12: Col-0 and *cle40* SAMs differ in their cell number, not in their cell size.**

**A, B: Longitudinal optical sections of SAMs of Col-0 (A) and *cle40* (B) seedlings 4dag stained with propidium iodide. Scale bars represent 20 µm. C: Size of Col-0 and *cle40* meristematic L1 cells. Dots and squares show size of individual cells. Horizontal bars represent mean values (long bars) and standard deviations (short bars). p = 0.39.**

## 9.9 CLE40 is a positive regulator of floral meristem size, in the absence of CLV1

To investigate if CLE40 also plays a role after floral transition, *cle40* mutants were analysed regarding carpel number per flower as a readout for floral meristem size, a method that was

previously established (Ni and Clark, 2006). Wild type flowers consist of two carpels. An increase in floral meristem size, like in *clv* mutants, leads to the formation of additional carpels (Koornneef *et al.*, 1983; Clark *et al.*, 1993; Kayes and Clark, 1998; Clark *et al.*, 1995). If CLE40 is a positive regulator of floral meristem size, floral meristems should be decreased in size in *cle40* mutants, resulting in a reduction of carpel number. But in comparison to Col-0, carpel number was not altered in *cle40* mutants (Figure 13 A). Loss of CLE40 might result in only a minor reduction of floral meristem size. The remaining meristem might still be sufficiently sized to produce two carpels per flower. However, the decrease caused by loss of CLE40 might become visible in a mutant background where meristem size is increased, like in *clv* mutants (Clark *et al.*, 1993; Kayes and Clark, 1998; Clark *et al.*, 1995).

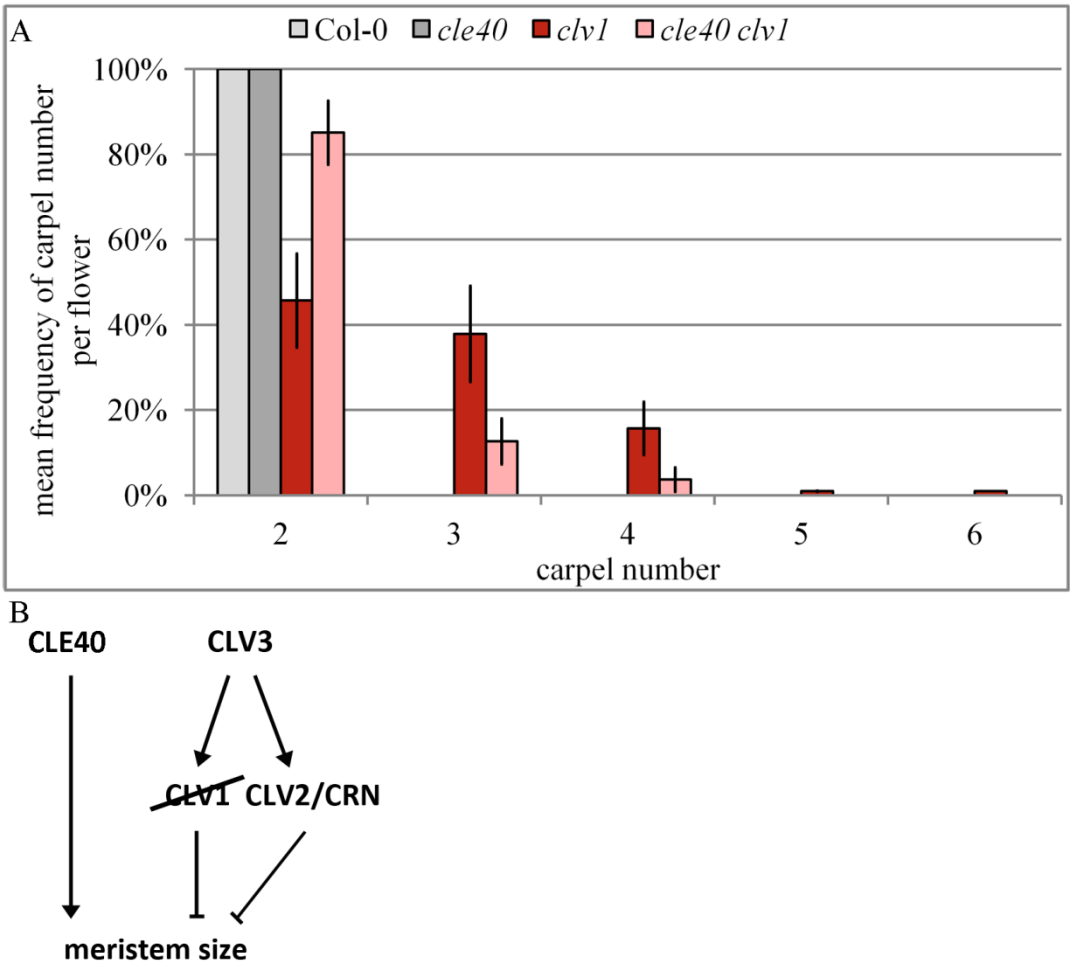



**Figure 13: CLE40 is a positive regulator of floral meristem size in the absence of CLV1.**

**A: Mean frequency distribution of carpel number per flower of different genotypes. Bars show mean values of five independent measurements. Error bars show standard deviation. B: Model of a signalling pathway showing positive function of CLE40 on meristem size in the absence of CLV1. The number of all samples used for genetic analyses can be found in Supplemental Table 1.**

Thus, *cle40 clv1* double mutants were analysed, expecting that if CLE40 is a positive regulator of floral meristem size, loss of CLE40 in the *clv1* background would suppress the *clv1* single mutant phenotype.

As expected, *clv1* single mutants showed an increase in carpel number, due to partial loss of CLV3 signal transmission (Müller *et al.*, 2008; Clark *et al.*, 1995; Ogawa *et al.*, 2008) (Figure 13A, Supplemental Table 1A). About 45 % of the *clv1* mutant flowers exhibited the wild type phenotype, while 38 % and 16 % had three and four carpels respectively. Loss of CLE40 in the *clv1* background suppressed this phenotype. 85 % of the double mutant flowers had two carpels and 13 % had three carpels (Figure 13 A).

Thus, CLE40 is also a positive regulator of floral meristem size. Furthermore, we conclude that the CLE40 signal can be transmitted independently of CLV1, because the positive effect of CLE40 on meristem size was found in the *clv1* mutant background (Figure 13 B).

## 9.10 CLE40 can be transmitted independently of CLV1 and CLV2

Finding that the CLE40 signal can be transmitted independently of CLV1 in floral meristems (Figure 13) and the SAM (Figure 11) and also independently of CLV2 in the SAM, raised the question, if in the absence of CLV1, CLE40 acts via CLV2 and vice versa, meaning that the two receptors would function in parallel in the CLE40 pathway, as they do in the CLV pathway (Müller *et al.*, 2008) (Figure 14 B, C). To answer this question, carpel number per flower was counted for *clv2*, *clv1 clv2*, and for the respective combinations with *cle40.*

If CLE40 is transmitted independently of CLV2, the double mutant *clv2 cle40* should show a suppression of the *clv2* single mutant phenotype. If CLE40 is transmitted independently of both receptors, CLV1 and CLV2, a suppression of the *clv1 clv2* double mutant phenotype should be detectable in *clv1 clv2 cle40* triple mutants.

Analyses of *clv2* and *cle40 clv2* revealed a suppression effect in the double mutant (Figure 14 A, Supplemental Table 1 B), similar to that seen in *clv1 cle40* mutants (Figure 13 A, Supplemental Table 1 A). The mean number of flowers with two carpels was increased in the double mutant to about 80 % instead of about 60 % in the *clv2* single mutant. However, individual experiments showed a rather high variability, *e.g.* in two experiments, the frequency of double mutant flowers with two carpels ranged from 58 % to over 90 % of the respective single mutant flowers with two carpels (Supplemental Table 1 B).

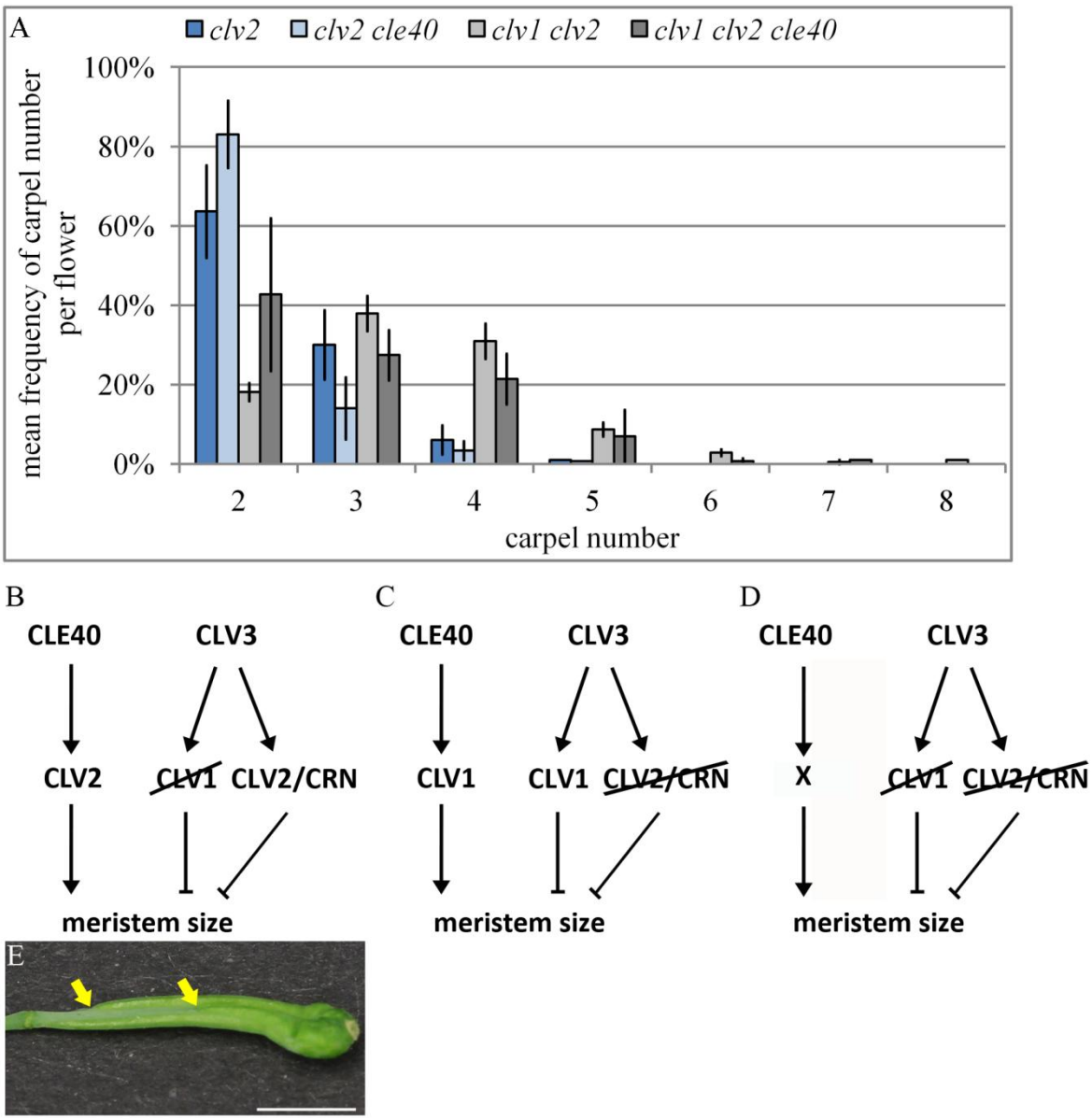



**Figure 14: CLE40 acts independently of CLV1 and CLV2.**

**A: Mean frequency distribution of carpel number per flower of different mutants. Bars show mean values of five independent measurements. Error bars show standard deviation. B-D: Models of signalling pathways showing three possible scenarios of positive function of CLE40 on meristem size through CLV2 (B), CLV1 (C), and an unknown receptor X (D). E: Silique of a *clv2* mutant with partial valves. Yellow arrows point at the end of two partial valves. Scale bar is 0.2 cm.**

In *clv2* single mutants, flowers with four or five carpels were observed, but were not found or less frequent in the double mutant (Figure 14 A, Supplemental Table 1 B).

Three of five measurements resulted in p-values for *clv2 cle40* and *clv2* that indicated a clearly significant difference (Supplemental Table 1 B). *clv2* mutants are known to exhibit the valveless phenotype, the strength of which varies depending on the observed *clv2* allele. This

phenotype is characterized by the presence of gynoecia that partially or even totally lack valves (Kayes and Clark, 1998) (Figure 14 E). It is possible that this phenotype obscures the actual carpel number in *clv2* mutant combinations, leading to ambiguous results.

In *cle40 clv1 clv2* triple mutants there was a significant suppression of the *clv1 clv2* phenotype detectable in two of three measurements (Supplemental Table 1 C). While on an average 18 % of the *clv1 clv2* double mutant flowers had two carpels, flowers with two carpels could be detected with a mean frequency of 43 % in the triple mutants (Figure 14 A). Although in one of three measurements, the differences seen between the double and the triple mutant were not significant, we observed the same tendency of suppression (Supplemental Table 1 C).

However, because of the clear suppression of the *clv1 clv2* phenotype by loss of CLE40 in two independent measurements, we assume that in the floral meristem, the function of CLE40 is similar to its function in the SAM, where CLE40 acts independently of CLV1 and CLV2.

We conclude that, in the floral meristem, the CLE40 signal can be transmitted at least in part independently of both CLV1 and CLV2 via one or more unknown receptors (Figure 14 D).

## 9.11 CLE40 promotes meristem size antagonistically to the CLV pathway

Because loss of CLE40 suppresses the *clv1 clv2* double mutant phenotype, we suggested that CLE40 acts antagonistically to the CLV pathway in the floral meristem. To test our hypothesis, *clv3 cle40* double mutants were analysed regarding carpel number per flower.

If CLE40 and CLV3 act antagonistically in the floral meristem, the double mutant would be expected to show a suppression of the *clv3* single mutant phenotype.

In fact, comparison between *clv3* and *clv3 cle40* carpel number gave ambiguous results (Figure 15 A, Supplemental Table 1 D).

One measurement indicated that the frequency of flowers with lower carpel numbers was decreased and that of flowers with higher carpel numbers was increased in the double mutant (d in Figure 15 A), allowing us to conclude that the double mutant exhibits a shift of carpel number towards higher numbers compared to the single mutant. Two further measurements showed differences between the two mutants that did not follow any clear pattern (a and c in Figure 15 A). In one case the frequency of flowers with two and six carpels was strongly increased while that of flowers with three to five carpels was slightly increased. In the other

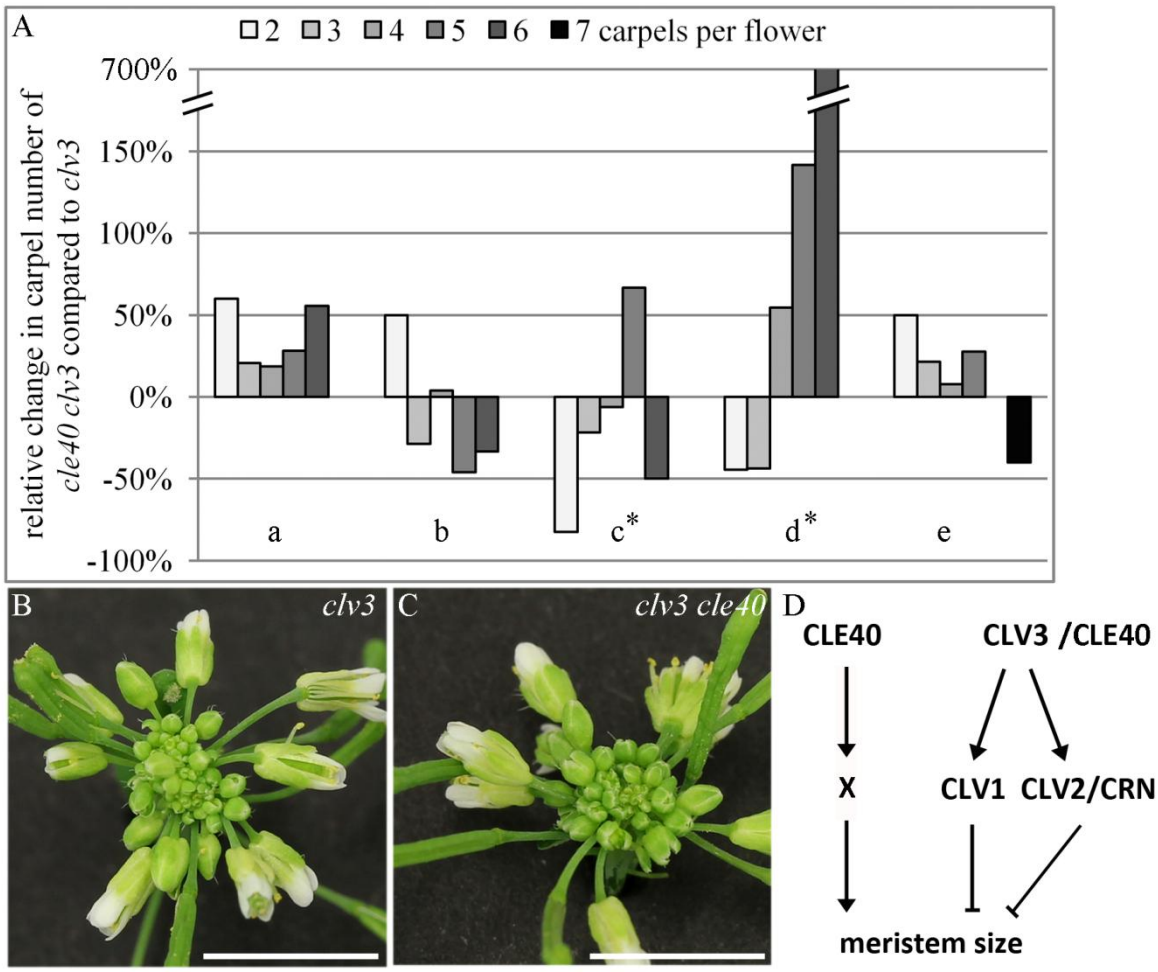



**Figure 15: Genetic studies do not reveal a clear function of CLE40 in the CLV pathway.**

**A: Relative change in carpel number per flower of *clv3 cle40* compared to *clv3*. a-e are five independent measurements. Asterisks represent statistically significant differences between *clv3 cle40* and *clv3* within one measurement. p-value (a) = 0.9, p-value (b) = 0.3, p-value (c) = 0.02, p-value (d) = $0.4 \times 10^{-4}$, p-value (e) = 0.5; B and C: Shoot apices of *clv3* and *clv3 cle40* respectively showing no detectable difference in inflorescence apex phenotype. Scale bars represent 0.5 cm. D: Model of a signalling pathway showing possible function of CLE40 through the CLV pathway and an antagonistic pathway, in which X represent the unknown receptor that transmits the CLE40 signal.**

case, flowers with two or six carpels were decreased while the others were increased or only very slightly decreased. In two further measurements, we found that in the double mutant, there was rather a shift towards lower carpel numbers, but the distribution of carpel numbers differed between these measurements (b and d in Figure 15 A).

In two of these five measurements, we observed significant differences. However, the outcomes of these two measurements differed from each other (c and d in Figure 15 A).

Examination of inflorescence apex phenotype also could not reveal any difference between *clv3* and *clv3 cle40*, whereas both mutants differed strongly from the wild type and the aphenotypic inflorescence apex of the *cle40* single mutant (Figure 15 B, C, Figure 18 A, B).

Although the suppression of the *clv1 clv2* phenotype by loss of CLE40 points towards an antagonistic function of CLE40 and CLV3, there was no suppression of the *clv3* mutant phenotype detectable in *clv3 cle40*. Instead, one measurement showed a shift of carpel number frequency towards higher carpel numbers in the double mutant. This could be interpreted as a consequence of a parallel function of CLE40 and CLV3, but this result was not reproducible (Figure 15 A, Supplemental Table 1 D). Possibly, CLE40 functions through the CLV pathway and also through the antagonistic CLE40 pathway (Figure 15 D), but these genetic analyses cannot fully answer this question.

However, independent of a possible function of CLE40 in the CLV pathway, CLE40 positively regulates floral meristem size via an antagonistic pathway, because loss of CLE40 function suppresses the *clv1 clv2* double mutant phenotype (paragraph 9.10).

## 9.12 Under heat stress conditions, CLE40 contributes significantly to the CLV pathway

Because comparison of *cle40 clv3* with *clv3* did not lead to any reproducible results, we thought that maybe there are slight variations within the growth conditions that affect the function of the peptides, causing ambiguous results. We hypothesized that such slight variations, e.g. of the temperature, into one or the other direction, could potentially cause a shift in the balance of the CLV pathway and the antagonistic CLE40 pathway to one or the other direction. Thus, fluctuations in growth conditions could result in ambiguous expression of the phenotype, depending on the severity and direction of a potential fluctuating factor that the plants were exposed to.

To test our hypothesis, *clv3* and *clv3 cle40* mutants were compared concerning carpel number per flower, after having been exposed to heat stress. Plants were grown at a temperature of 30 °C for ten days. If under these heat stress conditions, CLE40 is acting in the CLV pathway, we would expect the double mutants to show an enhanced carpel number phenotype compared to *clv3* single mutants. If on the other hand, CLE40 is acting only in the antagonistic pathway under these conditions, we would expect a suppression of the *clv3* single mutant phenotype in the *clv3 cle40* double mutant.

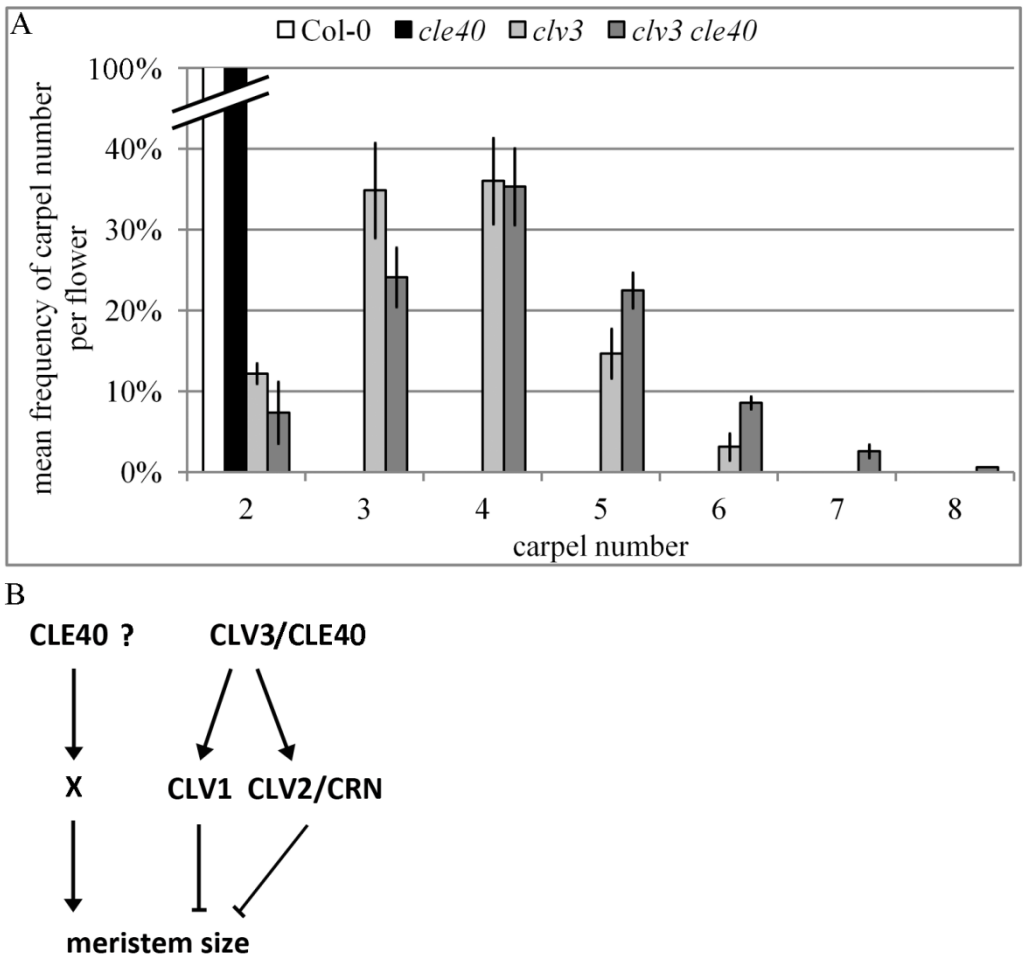



**Figure 16: Under heat stress conditions, CLE40 acts in parallel to CLV3 as a negative regulator of meristem size.**

**A: Mean frequency distribution of carpel number per flower of plants grown at 30 °C. Bars show mean values of three independent measurements. Error bars show standard deviation. B: Model of a signalling pathway showing function of CLE40 through the CLV pathway under heat stress conditions and a possible parallel function in the antagonistic pathway including an unknown receptor X. Question mark indicates that under heat stress, it is not known if the meristem size-promoting CLE40 pathway is active at all.**

*cle40 clv3* mutants that were grown at 30 °C showed an enhancement of the carpel number phenotype that was observed in *clv3* single mutants. The double mutants showed a decrease in the frequency of flowers with two, three, and four carpels and at the same time, the frequency of flowers with five and six carpels was increased. Occasionally, seven or eight carpels per flower were observed in the double mutant, but not in the single mutant, indicating that under heat stress conditions, CLE40 acts in parallel to CLV3 to restrict floral meristem size (Figure 16 A, B, Supplemental Table 1 E).

*clv3* single mutants and *cle40 clv3* double mutants, which were grown at the same time, but were not exposed to heat stress, did not exhibit this differences in carpel numbers (e in Figure 15 A). Neither did Col-0 wild type plants and *cle40* single mutants that were exposed to heat treatment show any altered phenotype (Figure 16 A).

We concluded from these findings that under heat stress conditions, the function of CLE40 is shifted, away from the meristem size-promoting pathway towards the meristem-size restrictive CLV pathway (Figure 16 B). These finding indicates that under heat stress conditions, a reorganization of the meristem controlling pathways may occur, which ensures robustness and plasticity of meristem growth control.

## 9.13 CLE40 positively regulates floral meristem size by suppression of the negative regulator BAM1

Although CLE40 also functions as a repressor of floral meristem size under heat stress, under standard growth conditions its main function in the floral meristem seems to be the promotion of meristem size. This function was found in the *clv1* and *clv2* mutant background. Interestingly, CLV1 was described to repress the transcription of its close homologue *BAM1* in the rib meristem (Nimchuk *et al.*, 2015). While in the wild type, *BAM1* expression is restricted to the L1, in the *clv1* mutant background, *BAM1* is up-regulated in the rib meristem and BAM1 functions as a substitute for CLV1 in the CLV pathway (Nimchuk *et al.*, 2015).

On the basis of these findings, we asked if the floral meristem size-promoting function of CLE40 might be BAM1 dependent. The relation between *BAM1*, *CLE40*, and *CLV1* was examined by genetic analyses to test this hypothesis (Figure 17, Supplemental Table 1 F). *clv1 bam1* double mutants were described before to exhibit an enhanced carpel number phenotype compared to *clv1* single mutants, due to the function of BAM1 in the CLV pathway in the absence of CLV1 (DeYoung and Clark, 2008; Durbak and Tax, 2011; Nimchuk *et al.*, 2015).

If the meristem size-promoting effect of CLE40 is BAM1 dependent, loss of BAM1 should abolish the suppression of the *clv1* mutant phenotype in *clv1 cle40.* Consequently, *clv1 bam1 cle40* triple mutants should not show any suppression of the *clv1 bam1* double mutant phenotype.

Neither the null mutant *bam1-3* (DeYoung *et al.*, 2006), nor *bam1 cle40* double mutants exhibited a carpel phenotype that differed from wild type or *cle40* single mutants (Figure

17 A, Supplemental Table 1 F, I). Consistent with published data, *clv1 bam1* double mutants showed an enhancement of the carpel number phenotype observed for *clv1* single mutants. While on an average, 99 % of the *clv1* mutant flowers had two, three or four carpels, the majority of *clv1 bam1* flowers had four to seven carpels, which were observed with a mean frequency of about 20 % each (Figure 17 A, Supplemental Table 1 F).

The triple mutant *clv1 bam1 cle40* did not show any suppression of the *clv1 bam1* double mutant phenotype, but rather a slight enhancement. The average frequency of flowers with two to six carpels was reduced in the triple mutant and the frequency of flowers with higher

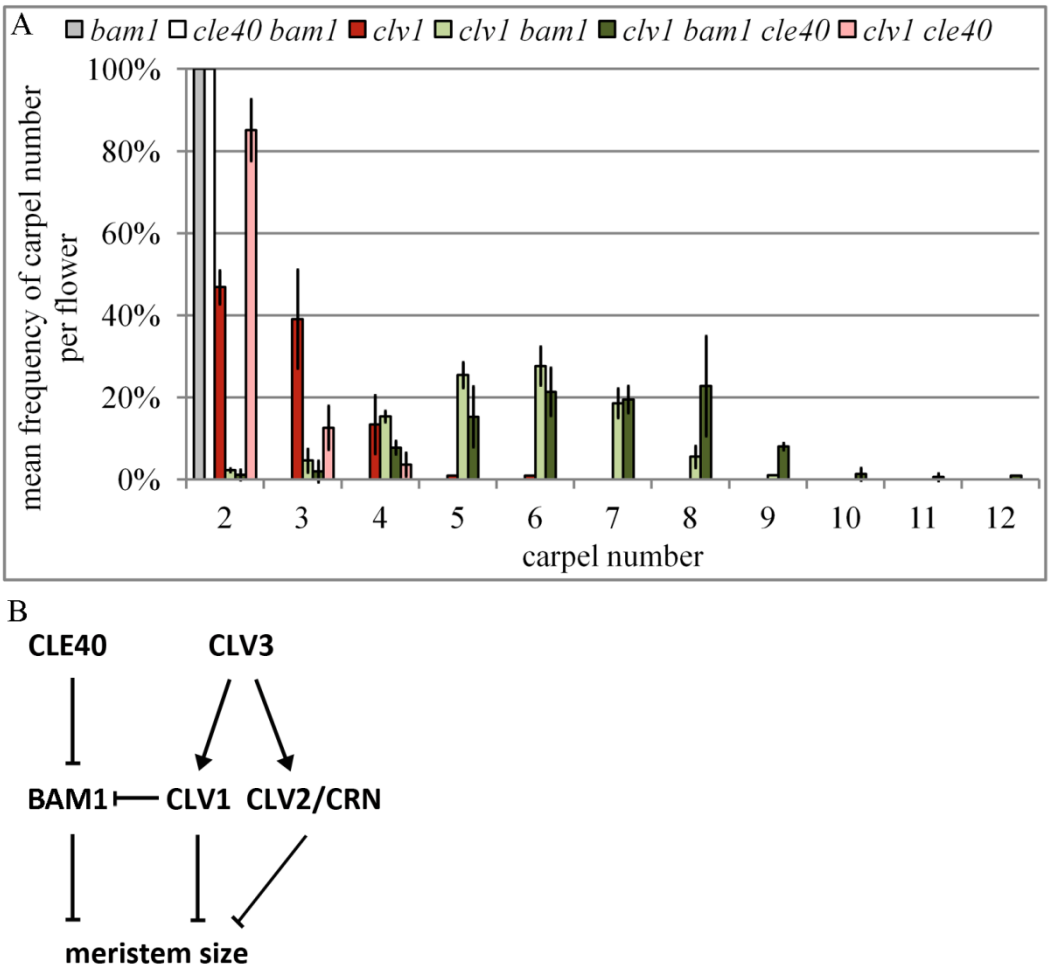



**Figure 17: BAM1 is epistatic to CLE40 in the meristem size-promoting pathway.**

**A: Mean frequency distribution of carpel number per flower of different mutant combinations. Bars show mean values of three independent measurements. Error bars show standard deviation. B: Model of a signalling pathway showing repression of BAM1 by CLE40 that explains the positive function of CLE40 on meristem size upon loss of CLV1 function. Bar pointing from CLV1 to BAM1 indicates the repression of CLV1 expression in the rib meristem via BAM1.**

numbers of carpels was increased (Figure 17 A, Supplemental Table 1 F). Consequently, BAM1 is epistatic to CLE40 concerning the floral meristem size-promoting effect of CLE40 (Figure 17 B).

The enhancement of the *clv1 bam1* carpel number phenotype observed in *clv1 bam1 cle40* might be due to the loss or shift of a feedback regulation.

Taken together, the meristem size-promoting effect of CLE40 was abolished upon loss of BAM1, and this led to the conclusion that CLE40 positively regulates floral meristem size by suppressing the function of the negative floral meristem regulator BAM1 (Figure 17 B).

## 9.14 Does CLE40 promote the size of the inflorescence meristem?

The so-far described functions of CLE40 refer to the SAM and the floral meristem. To find out if CLE40 also plays a role in the regulation of inflorescence meristem size, replicates of inflorescence apices were produced and analysed using a scanning electron microscope (SEM).

If CLE40 positively regulates inflorescence meristem size, *cle40* mutants should exhibit smaller meristems than wild type plants. However, comparison of *cle40* and Col-0 revealed no difference in inflorescence meristem size or architecture (Figure 18 A, A', B, and B'). We concluded that if there is a function of CLE40 in the regulation of inflorescence meristem size at all, loss of this function does not cause a drastic phenotypic change in the *cle40* single mutant.

Because the floral meristem size-promoting effect of CLE40 was detected in the absence of CLV1 and CLV2, where meristems are enlarged, we hypothesized that in the inflorescence meristem, a phenotypic change caused by loss of CLE40 might also be detectable in the corresponding mutant backgrounds.

To test this hypothesis, *clv1* single mutants and *clv1 cle40* double mutants were used for further analyses. Because *clv1* mutants compared to Col-0 are increased in both, height and width of the meristem (Figure 18 C, C'), the volume of the meristem would have to be measured to allow detection of slight differences between two genotypes. To avoid this, mutants were compared by counting the number of siliques formed over a certain length of the inflorescence stem, assuming that a difference in inflorescence meristem size would cause differences in organ number. This method also has the advantage that differences that occur

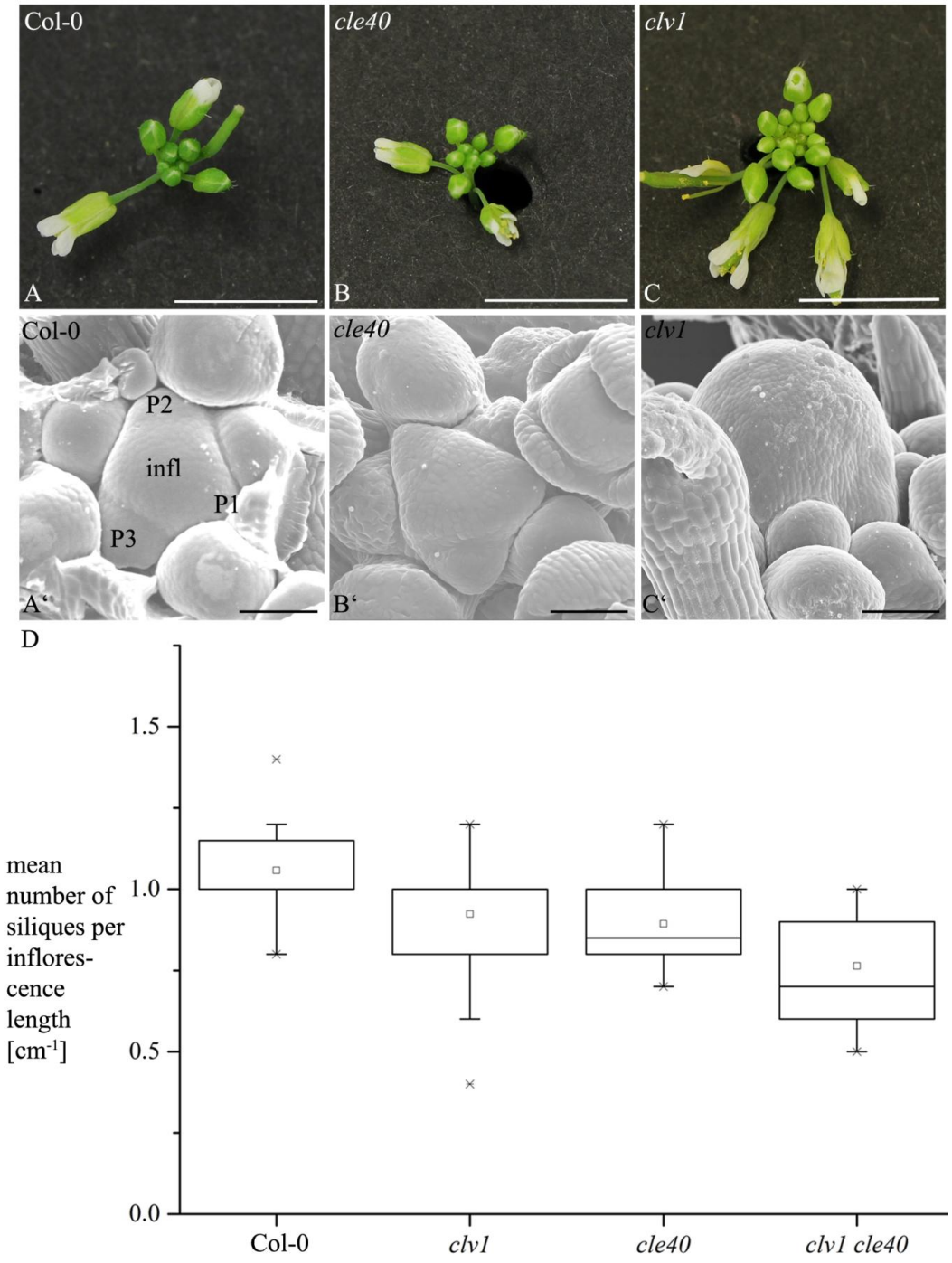



**Figure 18: *cle40* inflorescence meristem phenotypes**

**A and B: There is no detectable difference between inflorescence apex architecture of Col-0 (A) and *cle40* (B). C: inflorescence apex of *clv1*; A'-C': SEM pictures of Col-0 (A'), *cle40* (B'), and *clv1* (C'), showing no difference in inflorescence meristem size or architecture between Col-0 and *cle40* (A' and B'). Side view of a typical *clv1* inflorescence meristem (C') showing difference in height and width compared to Col-0. infl = inflorescence meristem; P1-P3 = the three youngest primordia; D: number of siliques per shoot length [$cm^{-1}$]; Comparison of the genotypes revealed a significant difference between all mutants analysed. p-values are: Col-0/*clv1* = 0.035, Col-0/*cle40* = 0.005, *clv1*/*clv1 cle40* = 0.012, and *cle40*/*clv1 cle40* = 0.038. Error bars in D show standard deviation. Scale bars represent 0.5 cm in A-C and 50 µm in A'-C'.**

over time would be detected, while the SEM pictures can only show a snapshot of the meristem.

If CLE40 is a positive regulator of inflorescence meristem size, the average number of siliques formed on a certain length of inflorescence should be decreased upon loss of CLE40. Analyses of *clv1* and *clv1 cle40* silique number revealed a significant difference between the two mutants (Figure 18 D). For *clv1* and *clv1 cle40*, the mean number of siliques per 1 cm shoot length was 0.92 and 0.76, respectively, thus *cle40 clv1* double mutants formed on an average 83 % of the number of siliques that *clv1* single mutants formed on the same length of inflorescence (p-value 0.012).

However, for *clv1* single mutants there was also a significant reduction in silique number detectable compared to Col-0. While wild type plants generated on an average 1.06 siliques per 1 cm shoot length, *clv1* single mutants generated 0.92 siliques (p-value 0.035). The loss of CLV1 function was expected to cause an increase in silique number compared to the wild type due to the partial loss of CLV3 signal transduction. Interestingly, *cle40* single mutants formed fewer siliques than Col-0. While the wild type generated on an average 1.06 siliques per 1 cm shoot length, *cle40* mutant generated on an average 0.89 siliques (p-value 0.005), consistent with the hypothesis, that CLE40 promotes meristem size.

Maybe the reduction in silique number that was observed comparing Col-0 and *clv1* is due to an increase in total shoot length of *clv1* compared to Col-0. If this holds true, *clv1* mutants could generate in total more siliques than Col-0, but could still have less siliques per cm shoot length. Thus, further analyses will be necessary to answer the question, if CLE40 also promotes the size of the inflorescence meristem.

## 9.15 ACR4 is a positive regulator of floral meristem size, which acts independently of CLV1 and CLV2

It was previously described that the receptor kinase ACR4 is involved in CLE40 signal transduction in the root and that expression of *ACR4* can be found in the L1 throughout development, partially overlapping with the expression domain of *CLE40* (Stahl *et al.*, 2009; Gifford *et al.*, 2003) (Figure 6). Thus, we asked if CLE40 might be transmitted via ACR4 in the shoot.

To investigate this hypothesis, *acr4-2* knockout mutants (Gifford *et al.*, 2003) were used for genetic analyses of floral meristems (Figure 19). If CLE40 and ACR4 act in the same pathway, *acr4* and *cle40* should exhibit the same or similar mutant phenotypes.

Like *cle40* single mutants, *acr4* single mutants did not show any mutant carpel number phenotype (Figure 19 A, Supplemental Table 1 G). We hypothesized that mutant phenotypes caused by loss of ACR4 function might become visible in a *clv* mutant background, like those of *cle40* mutants. Thus, further analyses concentrated on combinations of *acr4* with *clv* mutants.

In the *clv1* mutant background, loss of ACR4 led to a suppression of the *clv1* carpel number phenotype. On an average, over 80 % of the *clv1 acr4* double mutant flowers had two carpels per flower while about half of the *clv1* single mutant flowers formed two carpels. 1 % of the double mutant flowers had four carpels, while 15 % of the single mutant flowers had four carpels and even five or six carpels per flower could be detected in the single mutant (Figure 19 A, Supplemental Table 1 A). Consequently ACR4, like CLE40, is a positive regulator of floral meristem size in the absence of CLV1.

Comparison of *acr4 clv2* double mutants with *clv2* single mutants revealed a suppression of the *clv2* single mutant phenotype in the double mutant, but one of four measurements did not reveal a significant difference, similar to the results obtained with *clv2* and *clv2 cle40*, suggesting that the valveless phenotype obscures the results (Figure 19 A, Supplemental Table 1 B, Figure 14 A, E). However, comparison of *clv1 clv2* with *clv1 clv2 acr4* revealed a suppression of the *clv1 clv2* carpel number phenotype. On an average, about 20 % of the double mutant flowers formed two carpels, while in the triple mutant about 70 % of the flowers had two carpels. The frequency of flowers with three to five carpels was reduced in the triple mutant and more than five carpels were never observed. In contrast to this, we found up to eight carpels per flower in the double mutant (Figure 19 A, Supplemental Table 1 C). The suppression of the *clv1 clv2* double mutant phenotype by loss of ACR4 suggests that the positive effect of ACR4 on floral meristem size is independent of CLV1 and CLV2, comparable to the effect of CLE40 on floral meristem size (Figure 19 B, Figure 14 A).

Interestingly, *acr4 clv3* double mutants showed a suppression of the *clv3* single mutant carpel number phenotype, which was not observed in the *cle40 clv3* double mutant (Figure 15, Figure 19 A, Supplemental Table 1 D), suggesting that ACR4 acts antagonistically to CLV3.

Taken together, ACR4 is a positive regulator of floral meristem size that acts independently of the CLV pathway and its mutant combinations with *clv1* and *clv2* exhibit very similar

phenotypes like *cle40* combinations with *clv1* and *clv2*, supporting the hypothesis that CLE40 and ACR4 could act in the same pathway to positively regulate floral meristem size.

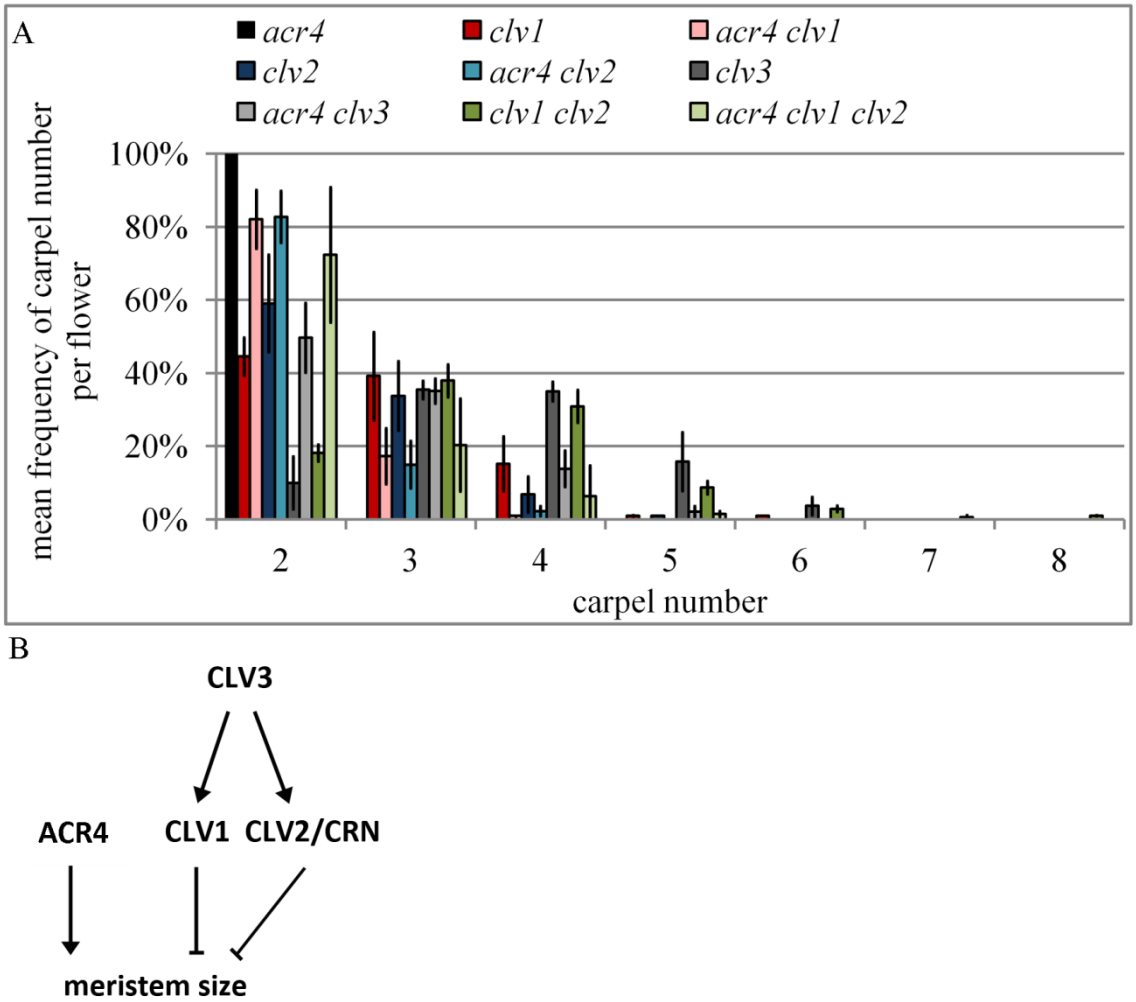



**Figure 19: ACR4 has a positive effect on floral meristem size that is independent of CLV1 and CLV2.**

**A: Frequency distribution of carpel number per flower of different mutant combinations. Bars show mean values of three independent measurements. Error bars show standard deviation. B: Model of a signalling pathway showing positive function of ACR in a pathway that acts antagonistically to the CLV pathway in the floral meristem.**

## 9.16 *ACR4* is expressed in the L1 of the inflorescence meristem

Our results show that CLE40 and ACR4 could act in the same pathway to promote floral meristem size. Thus, we analysed the expression of *ACR4* and compared it to that of *CLE40*, with the aim to identify the exact overlap in expression and find out, from which layer the meristem size-promoting pathway functions.

*ACR4* was previously described to be expressed in the L1 throughout development (Gifford *et al.*, 2003), but *CLE40* expression is much broader than the described expression of *ACR4* (Figure 6). With an *ACR4::H2B-TDTOMATO* line, we found expression of *ACR4* exclusively in the L1, consistent with previous descriptions. We detected *ACR* expression in the inflorescence meristem including developing primordia (Figure 20 A'-C'), as well as in floral meristems and floral organ anlagen (Figure 20 D). Thus, *CLE40* expression overlaps with that of *ACR4* exclusively in the L1.

We concluded from our findings that if CLE40 and ACR4 are part of the same pathway, this pathway would be acting from the L1 to control meristem size.

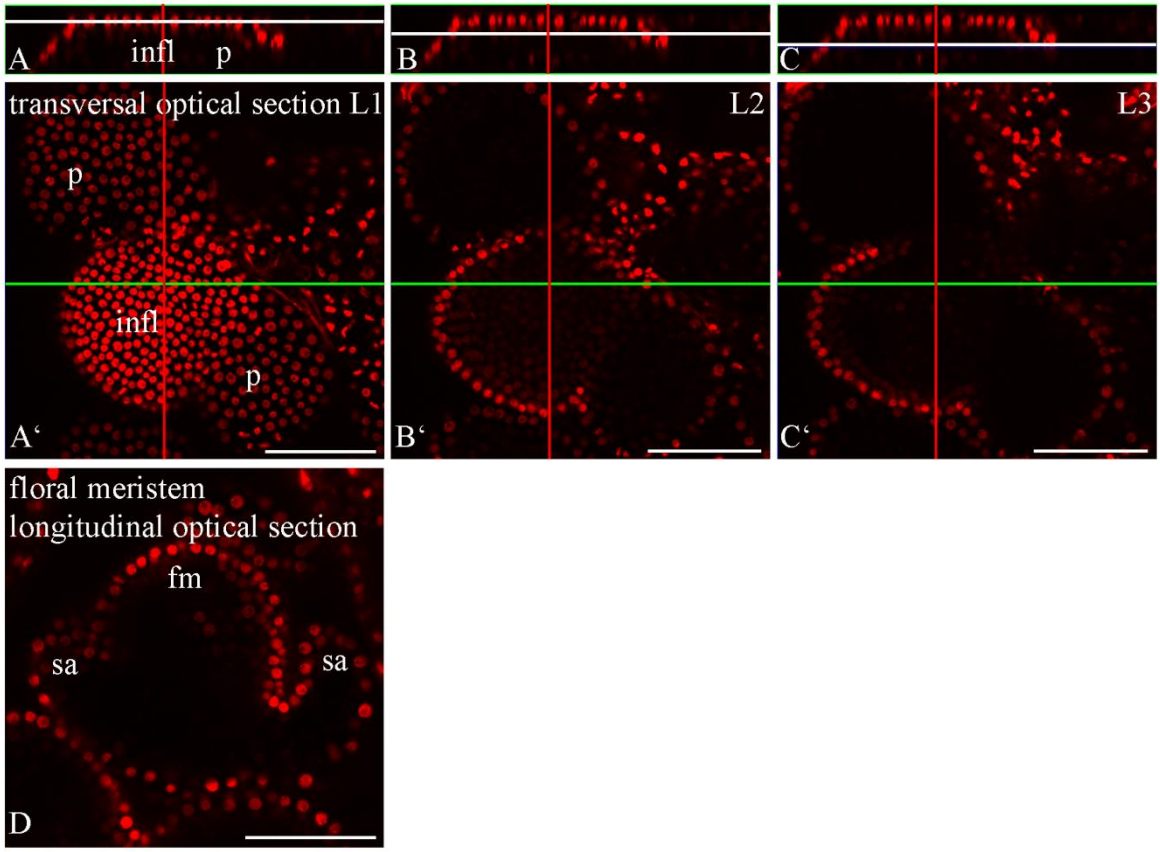



**Figure 20: *ACR4::H2B-TDTOMATO* expression in the L1 of an inflorescence and a floral meristem**

**Transversal optical sections of the L1 (A'), L2 (B'), and L3 (C') of an inflorescence meristem (infl) with developing primordia (p) and longitudinal optical section of a floral meristem (fm) (D) with sepal anlagen (sa) show expression of *ACR4::H2B-TDTOMATO* in the L1. Pictures in A-C show reconstructed longitudinal optical sections, each containing a white line that marks the plane of the respective transversal optical section. Note that the meristem is flattened due to the pressure of the cover slide, causing the expression to appear broader than it is, e.g. what looks like weak L2 expression in C' actually originates from the L1 (compare C' to C). Red and green lines in A'-C' show planes that were used to generate longitudinal optical sections, those generated in the "green plane" are shown in A-C. Scale bars represent 50 µm.**

## 9.17 ACR4 acts downstream of CLE40 to promote floral meristem size

Similar mutant shoot phenotypes caused by loss of either CLE40 or ACR4, as well as an overlap in expression, support the hypothesis that CLE40 and ACR4 could act in the same pathway to promote floral meristem size. To further investigate this hypothesis, *cle40 acr4* double mutants were analysed for carpel number phenotypes.

If CLE40 and ACR4 act in the same pathway, their double mutants would be expected to not exhibit an enhancement of the single mutant phenotypes.

*cle40 acr4* double mutants did not show any mutant carpel number phenotype, like the single mutants (Figure 21 A, Supplemental Table 1 G, H). However, because the *cle40* and the *acr4* mutant phenotype became visible in the *clv* mutant background, *cle40 acr4* double mutants were analysed in the *clv1 clv2* mutant background, too.

We found that *cle40 clv1 clv2* and *acr4 clv1 clv2* mutants exhibit similar carpel number phenotypes, but we noticed that the effects on carpel number caused by loss of ACR4 were stronger than those caused by loss of CLE40 in the *clv1 clv2* background (Figure 21 A, Supplemental Table 1 C).

Analyses of *acr4 cle40 clv1 clv2* quadruple mutants and the triple mutants *acr4 clv1 clv2* and *cle40 clv1 clv2* revealed, that the quadruple mutant resembles the *acr4 clv1 clv2* double mutant. Comparison of *clv1 clv2 cle40* with *clv1 clv2 cle40 acr4* revealed a significant difference in two of three measurements, but comparison of *clv1 clv2 acr4* with *clv1 clv2 cle40 acr4* never showed any significant difference, indicating that ACR4 acts downstream of CLE40 (Figure 21, Supplemental Table 1 C).

Taken together, loss of either CLE40 or ACR4 in the *clv1 clv2* background suppresses the double mutant phenotype, but the effects caused by loss of ACR4 are stronger. If ACR4 function is lost in the *clv1 clv2* mutant background, loss of CLE40 does not have any consequences.

From these findings we concluded, that CLE40 and ACR4 have the potential to act in the same pathway. In this pathway, ACR4 would act downstream of CLE40 and have the potential to function as the CLE40 receptor (Figure 21 B). We postulate, that in this pathway, there are further factors besides CLE40 that promote floral meristem size directly or indirectly through ACR4, because loss of ACR4 alone or ACR4 and CLE40 have stronger effects than loss of CLE40 alone.

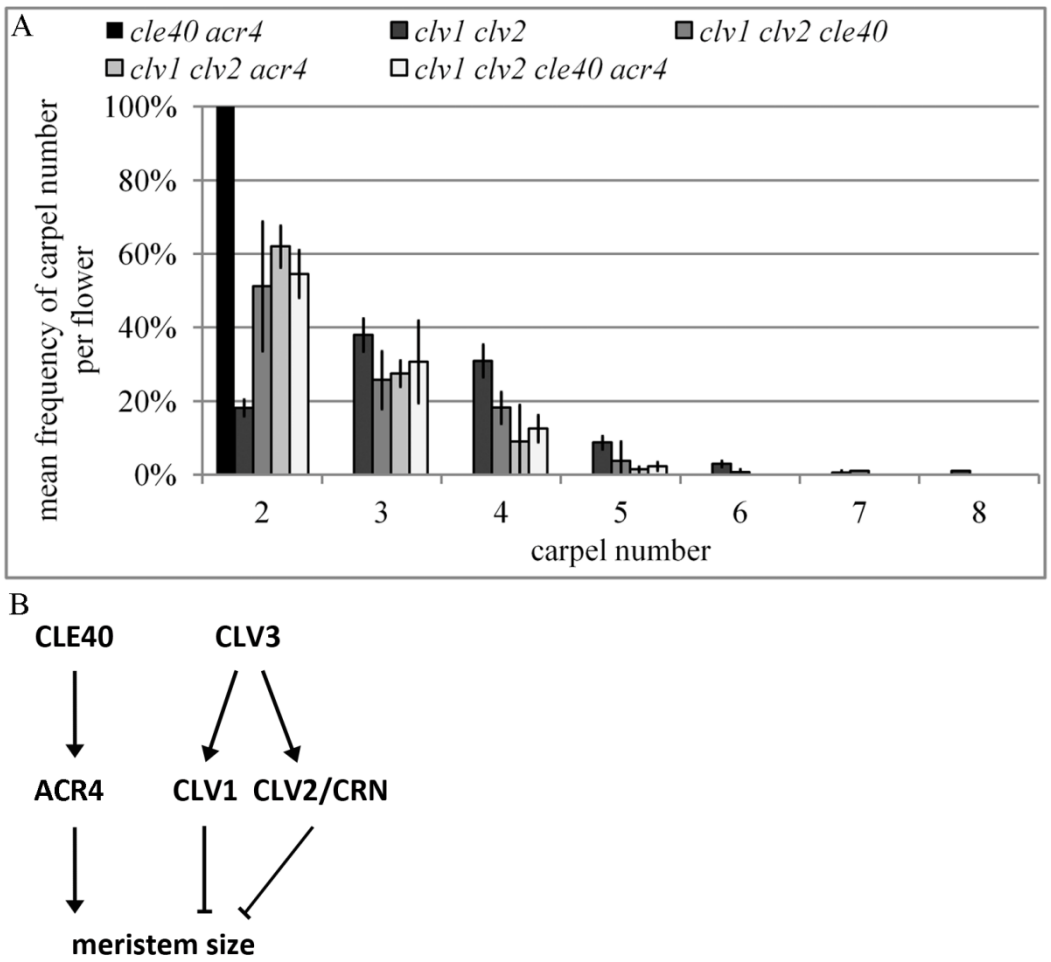



**Figure 21: ACR4 acts downstream of CLE40 to promote floral meristem size.**

**A: Mean frequency distribution of carpel number per flower. Bars show mean values of three (*cle40 acr4* and *clv1 clv2*) and two (*clv1 clv2 cle40*, *clv1 clv2 acr4*, and *clv1 clv2 cle40 acr4*) independent measurements. Error bars show standard deviation. B: Model of a signalling pathway depicting possible function of CLE40 and ACR4 in the meristem size promoting pathway, which acts antagonistically to the CLV pathway.**

# 10 Discussion

## 10.1 *CLE40* differs from *CLV3* in expression strength, pattern, and regulation

This work reveals the function of CLE40 as a positive regulator of meristem size during different stages of shoot development of *Arabidopsis thaliana*, acting antagonistically to its close homologue CLV3 by controlling the number of meristematic cells (Hobe *et al.*, 2003).
Comparison of *CLE40* and *CLV3* expression in the shoot revealed, that *CLE40* has a broad expression domain, but *CLV3* is restricted to stem cells, consistent with previous publications, which in addition described the expression of *CLE40* as weaker than that of *CLV3* (Hobe *et al.*, 2003; Sharma *et al.*, 2003; Fletcher *et al.*, 1999). *CLE40* was detected in seedlings 4 dag in the L1 of leaves and in the vasculature of the hypocotyl. Due to its broad expression in leaves, it was not possible to distinguish the SAM from the surrounding tissue, because in this developmental stage the SAM is overlain by developing leaves (paragraph 9.1). In the reproductive stage, *CLE40* expression was detected in the inflorescence and the floral meristem. Interestingly in inflorescence meristems, *CLE40* expression in the L1 was found to be down-regulated upon organ formation and was restored in later stages of organ development, a pattern that is reminiscent of an inverse auxin-signalling pattern (Vernoux *et al.*, 2011). In the L3, such a pattern was not observed for *CLE40* (paragraph 9.2). Neither was a pattern like this described or observed for the expression of *CLV3* (Fletcher *et al.*, 1999).
The L1 specific *CLE40* expression pattern indicates that in the shoot, *CLE40* expression might be down-regulated by auxin in the L1, but not in the L3. Strikingly in the root, the opposite effect was described. *CLE40* expression is up-regulated in the proximal and the distal root meristem upon auxin treatment (Wink, 2013). Different phytohormones were previously described to exert contrary functions in the shoot and in the root. While for example exogenous auxin treatment causes cell expansion in almost all shoot tissues, it leads to inhibition of cell elongation in roots (Perrot-Rechenmann, 2010). Thus, it would not be surprising if in the shoot, in contrast to the root, *CLE40* expression was negatively regulated by auxin.
Further studies on this subject will be necessary to eventually answer this question. If in the shoot, *CLE40* is down-regulated by high concentrations of auxin, plants carrying a mutant allele of the auxin transporter gene *PIN-FORMED 1* (*PIN1*) would be expected to show an

equal distribution of *CLE40* expression within the L1, due to loss of polar auxin transport within the shoot apex towards organ primordia (Okada *et al.*, 1991; Gälweiler *et al.*, 1998; Wisniewska *et al.*, 2006; Petrásek *et al.*, 2006). Exogenous application of auxin to the apex of such a plant should be followed by a local decrease in *CLE40* expression. Beyond this, a reporter line expressing a *CLE40* reporter construct together with an auxin signalling marker could show, if the decrease in *CLE40* expression is preceded by an increase of auxin, and if local *CLE40*-minima overlap exactly with local auxin-maxima.

It is known that for proper function of auxin signalling, the formation of local auxin-maxima and -minima is crucial. In the shoot, inhibition of polar auxin transport, like in *pin1* transporter mutants, results in the formation of 'naked' inflorescences that lack the capacity to initiate flowers (Okada *et al.*, 1991; Bennett *et al.*, 1995; Gälweiler *et al.*, 1998; Wisniewska *et al.*, 2006; Petrásek *et al.*, 2006). In the root, inhibition of polar auxin transport causes a shift of the auxin maximum that results in a disrupted distal root pattern (Sabatini *et al.*, 1999).

Interestingly, CLE peptides are known to function in a dosage dependent manner. *cle40* mutants are known to exhibit shorter roots, but exogenous application of CLE40 peptide results in even shorter roots instead of restoring the wild type phenotype (Pallakies and Simon, 2014). This result indicates that an accurate control of CLE peptide dosage and distribution might be necessary to ensure proper function of CLE peptide signalling; and this control might be achieved by the regulation of *CLE40* by auxin. Consequently, addition of exogenous CLE40 peptide to *cle40* mutants would not be sufficient to rescue the mutant phenotype due to the lack of proper regulation of CLE40 concentration and distribution within the tissue.

Besides the differences between *CLE40* and *CLV3* in the L1 specific pattern, analyses of transcriptional interdependence of *CLE40* and *CLV3* revealed that *CLV3* expression is not regulated by CLE40 signalling, but *CLE40* expression might be regulated by CLV3 signalling.

In *cle40* mutants, the *CLV3* expression domain does not differ from the wild type *CLV3* expression domain (paragraph 9.6). In contrast, the expression of *CLE40* might be regulated by CLV3, because *CLE40* expression was detected more often in the L2 of *clv3* mutants than in the L2 of wild type plants (paragraph 9.5). It is possible, that this up-regulation of *CLE40* expression in the L2 is due to a general upregulation of *CLE40* expression in *clv3* mutants and that it correlates with the postulated substitution of CLV3 function by CLE40. It is known from previous studies that CLE40 can actually substitute for CLV3 function if expressed under the control of the *CLV3* promoter (Hobe *et al.*, 2003). However, the broader expression

domain of *CLE40* as compared to *CLV3*, as well as the differences reported in expression strength and the potential differences in regulation of *CLE40* and *CLV3* indicate that despite the high similarity in their sequences, both peptides might fulfil different functions in the shoot and CLE40 might have further functions besides buffering of the CLV3 level.

## 10.2 *CLE40* expression overlaps with expression of different receptor candidates

CLV3 represses the positive stem cell regulator *WUS* by acting via two parallel pathways, one involving homomeric complexes of the receptor-like kinase CLV1 and another one, including heteromeric complexes of the receptor-like protein CLV2 and the kinase CRN (Brand, 2000; Schoof *et al.*, 2000; Mayer *et al.*, 1998; Bleckmann *et al.*, 2010; Somssich *et al.*, 2015; Müller *et al.*, 2008). Potentially, CLV1-CLV2-CRN multimeric complexes also transmit the CLV3 signal, but they might also be inactive and formed upon excess of CLV3 (Somssich *et al.*, 2015). Besides this, the receptor-like kinase RPK2 and the CLV1 homologues BAM1, BAM2, and BAM3 are also involved in CLV3 signal transduction in the shoot (Kinoshita *et al.*, 2010; DeYoung *et al.*, 2006; DeYoung and Clark, 2008; Durbak and Tax, 2011).

In the root, CLE40 was described to negatively regulate the WUS homologue WOX5, which promotes columella stem cell fate, in a pathway that also includes the receptor-like kinase ACR4 and CLV1 (Haecker *et al.*, 2004; Stahl *et al.*, 2009; Tanaka *et al.*, 2002; Stahl *et al.*, 2013).

In the vasculature, proliferation of stem cells in the procambium is promoted by a signalling module composed of the CLE peptide TRACHERY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), the CLV1 related LRR receptor kinase TDIF RECEPTOR/ PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) and the WUS homologue WOX4 (Cock and McCormick, 2001; Ito *et al.*, 2006; Hirakawa *et al.*, 2008; Hirakawa *et al.*, 2010; Fisher and Turner, 2007; Haecker *et al.*, 2004).

Thus in different plant meristems, signalling modules composed of CLE peptides, receptor-like kinases, and WUS clade members of the WOX family that function as targets are involved in the control of meristem regulation (Aichinger *et al.*, 2012; van der Graaff *et al.*, 2009).

This fact and the high similarity of CLE40 and CLV3 gave rise to the hypothesis that in the shoot, CLE40 might be transmitted via the receptors of the CLV pathway. This hypothesis is

supported by a previous publication, in which binding of both CLV3 and CLE40 to CLV1, CLV2, and BAM1 was shown *in vivo* (Guo *et al.*, 2010). Besides this, CLE40 might also act via ACR4, which is not a known receptor of the CLV pathway in the shoot, but the potential CLE40 receptor in the root. Shoot specific expression of *ACR4* was previously described to be found in the L1 (Gifford *et al.*, 2003).

To further elucidate these hypotheses, CLV1, CLV2, CRN, and ACR4 were analysed concerning their shoot specific expression to find out to what extent their expression overlaps with that of *CLE40*.

Consistent with previous descriptions, *ACR4* expression was detected in the L1 of the inflorescence and the floral meristem, thus partially overlapping with *CLE40* expression (Gifford *et al.*, 2003) (paragraph 9.2 and 9.16). The expression domains of *CLV1*, *CLV2*, and *CRN* largely overlapped with that of *CLE40*, with the exception of *CRN* that was present in the L2, where *CLE40* was found only in the minority of the analysed wild type plants (paragraph 9.2, 9.3, and 9.4). Thus, all analysed receptors have the potential to be involved in the transmission of the CLE40 signal in the shoot. Interestingly for *CLV1* and *CLV2*, the same L1 specific pattern was observed like for *CLE40* expression, indicating that *CLV1* and *CLV2* might be subjects of the same control mechanism that regulates *CLE40* expression, or the similarities observed in their expression pattern might also be due to a mutual regulation among them. However, the CLV2 pattern was not visible in early stages of primordium initiation in contrast to that of CLV1. For *CRN* and *ACR4*, this pattern was not detected. But, the presence of H2B in those reporters might obscure the exact expression pattern, because the high stability of H2B might prevent fluorophores from degradation. Previous studies on *ACR4* expression and protein localization described an equal distribution within the L1, with the exception of polar localization of ACR4 protein towards the basal membrane of the L1. A spatial difference in *ACR4* expression within the L1 similar to that observed for *CLE40, CLV1*, and *CLV2* was not described. However, those *ACR4* expression studies were either also done with a H2B-fluorophore-fusion or, if done with an ACR4-GFP protein fusion, they did not describe any expression of the protein fusion in inflorescence meristems with emerging flower primordia, structures in which the L1 specific pattern of *CLE40*, *CLV1* and *CLV2* expression was found (Gifford *et al.*, 2003). To study the change over time in expression strength of ligands and receptors in the L1 at high resolution, it will be necessary to analyse reporter lines, which lack the H2B or contain a destruction box (Glotzer *et al.*, 1991).

Interestingly, previous studies on *CLV1* expression did either not reveal any expression in the L1, or the L1 expression was found only in the minority of the analysed plants at a low level and no spatial differences in *CLV1* expression within the L1 were mentioned (Clark *et al.*, 1997; Nimchuk *et al.*, 2015). However, the reporter analysis, in which *CLV1* was occasionally detected in the L1 at a low level, was carried out with a confocal microscope that is not as sensitive as the LSM780, which was used for the experiments performed for this dissertation (Nimchuk *et al.*, 2015). This example shows the importance of high sensitive confocal microscopy to unravel exact spatial and temporal distributions of different factors involved in plant development at high resolution as a basis to study their function in detail.

## 10.3 The CLE40 pathway positively regulates shoot meristem size

To study the function of CLE40 in the shoot, genetic analyses of CLE40 and its potential receptors were performed. Mis-expression analyses could not be performed on *CLE40*, because of the dosage dependent functions of CLE peptides, which obscure the results of such studies.

Genetic analyses revealed that CLE40 is a positive regulator of meristem size in the vegetative SAM (paragraph 9.7) and the floral meristem (paragraph 9.9), and that CLE40 acts antagonistically to the CLV pathway by positively regulating the number of meristematic cells (paragraph 9.8, 9.11). The meristem size promoting function of CLE40 could be detected in the absence of CLV1 and CLV2; consequently CLE40 can function at least in part independently of CLV1 and CLV2 to promote meristem size (Figure 22) (paragraph 9.10 and 9.11). However, it is possible that in wild type plants, CLE40 also functions via CLV1 and/or CLV2 and that their function is substituted by other receptors in their absence. In the same manner, the function of CLV1 is substituted by the function of its homologues BAM1 and BAM3 in the absence of CLV1 while in the presence of CLV1, BAM1 and BAM3 do not contribute to the CLV pathway (DeYoung and Clark, 2008; Durbak and Tax, 2011; Nimchuk *et al.*, 2015). In wild type plants, *BAM1* and *BAM3* are solely expressed in the L1 of the meristem and in the developing vascular strands below emerging primordia respectively, but in *clv* mutants, their expression is up-regulated in the rib meristem, facilitating the substitution of CLV1 function (Nimchuk *et al.*, 2015).

Consequently in wild type plants, CLE40 might partially act via the same receptors that perceive CLV3 to fulfil an antagonistic function.

Binding of two peptides with antagonistic functions to the same receptor was recently described in the context of stomata development. EPIDERMAL PATTERNING FACTOR 2 (EPF2) and Stomagen/EPFL9, two structurally related cysteine-rich peptides, compete for binding the LRR receptor kinase ERECTA (ER) (Hara *et al.*, 2009; Hunt and Gray, 2009; Ohki *et al.*, 2011; Lee *et al.*, 2015; Torii *et al.*, 1996). However, while binding of EPF2 to ER activates a MAPK signalling cascade, which eventually leads to the inhibition of stomata initiation, binding of Stomagen to ER prevents the signal transduction, thus positively affecting stomata development (Lee *et al.*, 2012; Lee *et al.*, 2015; Shpak *et al.*, 2005; Wang *et al.*, 2007; Hara *et al.*, 2009; Hunt and Gray, 2009; Hunt *et al.*, 2010; Sugano *et al.*, 2010). The authors conclude that this competitive binding is a molecular mechanism that optimizes stomatal patterning and that such a concept of fine-tuning signal transduction by related endogenous peptides that assume opposing functions may extend to other peptide families (Lee *et al.*, 2015).

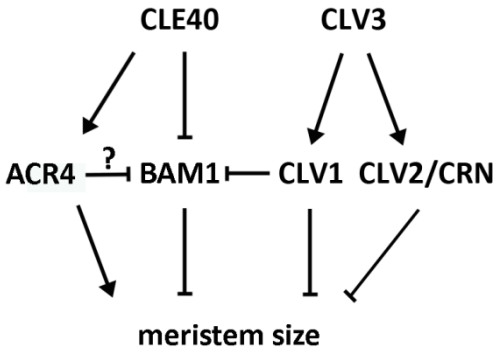



**Figure 22: The CLE40 pathway functions antagonistically to the CLV pathway via ACR4 and BAM1.**

## 10.4 The CLE40 pathway involves ACR4 and BAM1

Among the receptors that were analysed in this study, ACR4 and BAM1 were found to be involved in the CLE40 pathway. Genetic studies revealed that like CLE40, ACR4 has a meristem size promoting function and acts downstream of CLE40 (paragraph 9.15 and 9.17). Like CLE40, ACR4 can function at least partially independent of CLV1 and CLV2, because the meristem size promoting function of ACR4 is detectable in the absence of CLV1 and CLV2. Thus, in the shoot as well as in the root, CLE40 and ACR4 might be a ligand receptor pair (Figure 22), though the evidence for direct binding of CLE40 to ACR4 is missing. Consequently, ACR4 might also act downstream of another unknown CLE40 receptor in the CLE40 pathway or function as a co-receptor for a CLE40 receptor.

It is known that receptor complex formation is a common feature of plant signalling pathways controlling different processes (Tax and Kemmerling, 2012).

The perception of brassinosteroids, a group of steroidal plant hormones, by their receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) activates complex formation of BRI1 with BRI1 ASSOCIATED RECEPTOR KINASE1 (BAK1) (Clouse *et al.*, 1996; Li and Chory, 1997; Wang *et al.*, 2001; Kinoshita *et al.*, 2005; Li *et al.*, 2002). Complex formation of BRI1 and BAK1 is necessary for the regulation of brassinosteroid target genes (Nam and Li, 2002; Wang *et al.*, 2005).

Pathogen defence involves complex formation of FLAGELLIN-SENSITIVE 2 (FLS2) with BAK1, which is triggered by binding of the FLS2 ligand flagellin (Gómez-Gómez and Boller, 2000; Heese *et al.*, 2007; Chinchilla *et al.*, 2006; Chinchilla *et al.*, 2007; Somssich *et al.*, 2015).

In the *Arabidopsis* root, control of distal meristem fate includes ACR4 and CLV1, which form different complexes depending on their localization. While both form hetero-dimers at plasma membranes rather than at plasmodesmata, ACR4 homomeric complexes are formed at plasmodesmata in addition to heteromeric complexes with CLV1 (Stahl *et al.*, 2013). The exact function of the different complexes is not completely understood, but it was hypothesized that the complexes at plasmodesmata control movement of molecules influencing cell-to-cell communication in the distal root meristem (Stahl and Simon, 2013).

These examples show that receptor complexes are composed of different receptors, some of which are directly involved in signal transduction without binding the ligand of the co-receptor.

Thus in the shoot, ACR4 could be directly involved in the transmission of the CLE40 signal, even if it was not the primary receptor of CLE40. This hypothesis is supported by a previous publication, which reported that the kinase activity of ACR4 is not required for ACR4 function, because loss of kinase function in an ACR4 transgenic construct did not disable this construct to restore the wild type seed and ovule phenotype in *acr4* mutants. The authors concluded, that ACR4 forms heteromers with kinase-active partners during signalling (Gifford *et al.*, 2005).

Besides ACR4, BAM1 was also found to be involved in the CLE40 pathway. Genetic analyses showed that the meristem size promoting function of CLE40 is depending on the presence of BAM1, because the suppression of the *clv1* phenotype upon loss of CLE40 was abolished if *BAM1* was mutated (paragraph 9.13). Because BAM1 and CLE40 have antagonistic effects in the *clv1* mutant background and *BAM1* was shown to be epistatic to

*CLE40,* CLE40 was placed upstream of BAM1 as a repressor of BAM1 function (Figure 22). Consequently, CLE40 has a positive effect on meristem size by repressing the function of the negative meristem regulator BAM1. It is not clear how this suppression of BAM1 function by CLE40 is achieved.

Because binding of CLE40 to BAM1 was previously shown, it is possible that CLE40 inhibits BAM1 by competing with another BAM1 ligand, like CLV3 that would cause meristem size restriction upon binding to BAM1 (Guo *et al.*, 2010; DeYoung and Clark, 2008).

Considering the aforementioned receptor complex formations, some of which are depending on ligand-receptor-binding, it also seems possible that binding of CLE40 to BAM1 could either cause complex formation of BAM1 that would result in an inactivation of BAM1, or it could prevent complex formation of BAM1 that would result in activation of BAM1 downstream signalling. However, if the effect of CLE40 on BAM1 is direct or if CLE40 affects BAM1 function indirectly, e.g. by a subset of downstream signalling events that might involve ACR4, remains to be elucidated.

## 10.5 Does the CLE40 pathway function in a specific layer?

It is not yet known, what the potential connection between ACR4 and BAM1 in the CLE40 pathway could look like. One possibility is that CLE40 acts via ACR4 to repress BAM1 function (Figure 22). *ACR4* expression and *CLE40* expression were shown to overlap in the L1 (Gifford *et al.*, 2003) (paragraph 9.2 and 9.16). Interestingly, BAM1 is present in the L1 in wild type plants. Thus in the L1, CLE40 could function as a short range signal that acts via ACR4 on BAM1 to promote meristem size. In the past, several publications have addressed the necessity of a signal coming from the L1 to maintain the shoot apical meristem (Reinhardt *et al.*, 2003; Kessler *et al.*, 2006; Chickarmane *et al.*, 2012). The CLE40 signal might fulfil this function, because it positively regulates meristem size and its potential receptor ACR4 is located in the L1.

However, a repressive function of BAM1 on meristem size from the L1 was to date not described. In contrast, previous studies reported, that BAM1 functions as a negative regulator of meristem size from the rib meristem, where it is ectopically expressed in *clv* mutants (Nimchuk *et al.*, 2015). Strikingly, *cle40* and *acr4* mutant phenotypes are only visible in *clv* mutant backgrounds, correlating with the presence of ectopic BAM1 in the rib meristem (paragraph 9.9 and 9.15).

Originally, *cle40* mutants were crossed into *clv* mutants to find out if subtle phenotypes caused by loss of CLE40 would become visible in plants with enlarged meristems. However, the correlation of ectopic *BAM1* expression in the rib meristem with *cle40* and *acr4* mutant phenotypes raises the question, if CLE40 functions together with ACR4 on BAM1 solely in the rib meristem, but not necessarily on BAM1 in the L1. To answer this question, it will be necessary to analyze *cle40* and *acr4* mutants in other mutant backgrounds, where the meristem is enlarged, but *BAM1* expression is not changed in comparison to wild type plants. Recently, *phb phv cna* triple mutants, which exhibit an increase in meristem size, were described to not show any ectopic *BAM1* expression in the shoot (Nimchuk *et al.*, 2015). The three homeodomain transcription factors PHABULOSA, PHAVOLUTA, and CORONA were described to act additively to the CLV pathway (Green *et al.*, 2005; Williams *et al.*, 2005).

It would be interesting to see, if in these mutant backgrounds, the sole increase of meristem size is sufficient to visualize *cle40* and *acr4* mutant phenotypes. If loss of *CLE40* function in the *phb phv cna* triple mutant causes a suppression of the *phb phv cna* triple mutant phenotype, CLE40 function would not depend on BAM1 presence in the rib meristem, but it might depend on BAM1 presence in the L1. If in the same background, the suppression effect caused by loss of CLE40 would be abolished upon knockout of *BAM1*, which in the triple mutant *phb phv cna* would be expected to be present only in the L1, this would mean that in the L1 BAM1 also functions downstream of CLE40 as a negative regulator of meristem size (Figure 23 B).

However, if the *cle40* mutant phenotype exclusively occurred in mutant backgrounds that correlate with ectopic *BAM1* expression in the rib meristem, the negative function of BAM1 on meristem size in the L3 would be necessary for CLE40 function. If this holds true, CLE40 could function either from the L1 via ACR4 to activate downstream signalling that eventually would repress BAM1 function in the rib meristem (Figure 23 C pathway 1, 2), or it could function via two separate pathways, one in which CLE40 would function via ACR4 to promote meristem size from the L1 (Figure 23 C pathway 1), and another one including BAM1 in the L3. The latter could be addressed by CLE40 from the L1 (Figure 23 C pathway 3a) and the L3 (Figure 23 C pathway 3b), because CLE40 is a diffusible peptide, but it would not necessarily have to include ACR4 (Figure 22, Figure 23 C) (Sharma *et al.*, 2003).

However, the differences found in the *CLE40* expression pattern between the L1 and the L3, which point to different forms of regulation of *CLE40* expression in these layers, and the fact that the potential CLE40 receptor ACR4 is solely found in the L1, imply that CLE40 might have diverse functions in the different layers. Thus the meristem size promoting function of

CLE40 might originate from the L1, where its potential receptor ACR4 is located, to eventually address BAM1 in the L1 or in the rib meristem, while CLE40 in the L3 might function in other processes.

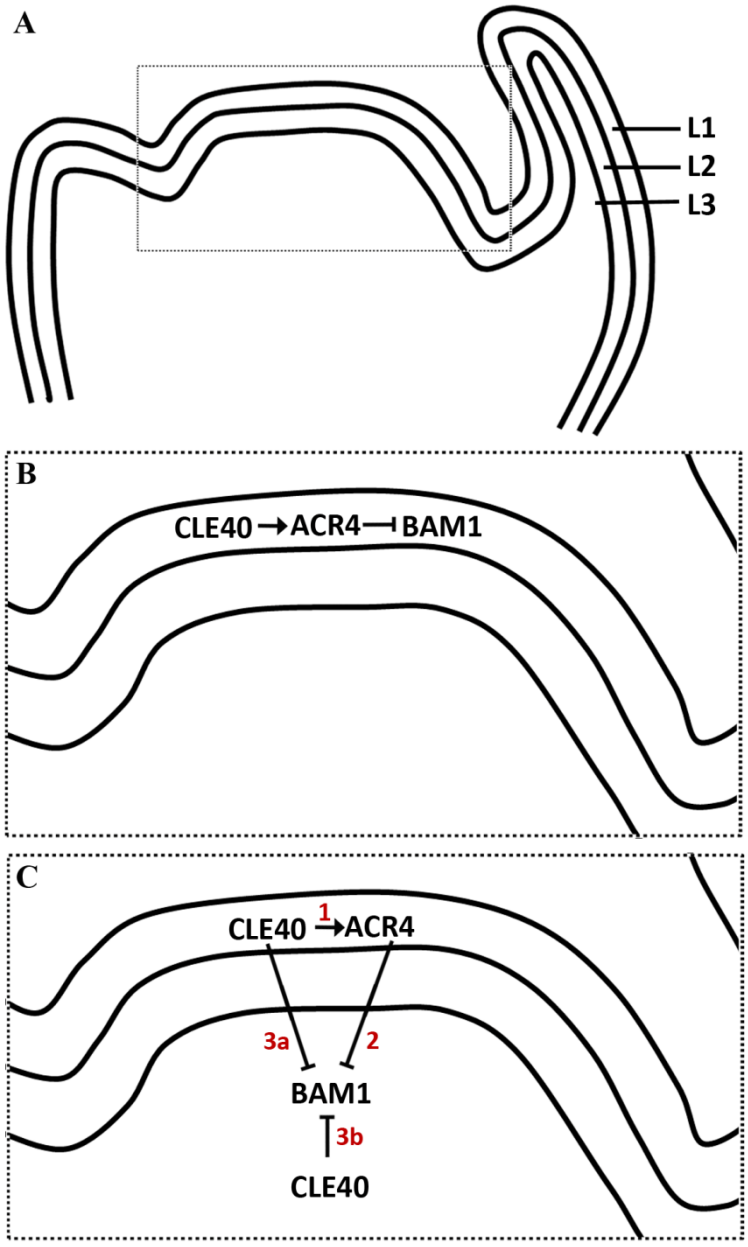



**Figure 23: Localization of the CLE40 pathway**

**A: Overview of a shoot meristem with the three layers L1, L2, and L3; dotted box shows region that is highlighted in B and C; B: In the L1, CLE40 could function via ACR4 to repress BAM1 function, thus promote meristem size. C: CLE40 signalling via ACR4 in the L1 could activate downstream signalling in the L1 that would eventually promote meristem size (1); CLE40 signalling via ACR4 in the L1 could activate downstream signalling in other layers that would eventually cause meristem size increase due to repression of BAM1 function in the L3 (1, 2); CLE40 could function either from the L1 (3a) or from the L3 (3b) to repress BAM1 function in the L3 without signalling via ACR4.**

## 10.6 CLE40 functions in the CLV pathway under heat stress conditions

Assuming that CLE40 signals from the L1 to promote meristem size, despite the possibility that targets like BAM1 could be addressed in the L3, raises the question if CLE40 also has a function in the L3, where *CLE40* expression was detected too.

One possible function of CLE40 in the L3 could be the participation in the CLV pathway, together with CLV3. Previous publications reported that *CLE40*, if expressed under the control of the *CLV3* promoter, has the potential to restore the wild type phenotype in a *clv3* mutant background (Hobe *et al.*, 2003).

However under standard growth conditions, genetic studies could not reveal any function of CLE40 in the CLV pathway. Comparison of *cle40* single mutants with *cle40 clv3* double mutants could not reveal any reproducible suppression or enhancement of the *clv3* single mutant phenotype upon loss of CLE40 function. But under heat stress conditions, CLE40 signalling significantly contributes to the CLV pathway. Compared to *clv3* single mutants, *cle40 clv3* double mutants exhibited an enhancement of the floral meristem phenotype (paragraph 9.12).

Consequently, CLE40 has the potential to function in antagonistic pathways, depending on environmental influences; and the meristem size-promoting function of CLE40 might actually originate from the L1, while in the L3, the function of CLE40 might be buffering of the CLV pathway against environmental influences like heat stress (Figure 24). The need for a buffering mechanism was previously postulated. It is known that variations in CLV3 expression over a range of ten-fold can be tolerated in meristem homeostasis (Müller *et al.*, 2006). Other authors have speculated that this effect can be explained by the turnover of plasma membrane localized CLV1 upon binding of CLV3 or by the formation of CLV3 induced inactive multimeric CLV1-CLV2-CRN complexes (Nimchuk *et al.*, 2011; Somssich *et al.*, 2015). However, this would only explain how a meristem can deal with an excess of CLV3. Assuming that CLV3 level would drop due to environmental influences, substitution of CLV3 by CLE40 would provide a mechanism to quickly compensate such fluctuations without the need to increase the number of *CLV3* expressing stem cells. If the expression of *CLV3* depends on any environmental influence, like temperature, remains to be investigated.

Taken together, under standard growth conditions, no function of CLE40 in the CLV pathway could be revealed by genetic studies, but under heat stress conditions, CLE40 contributes to the CLV pathway.

However, some of the results obtained by genetic analyses that were performed under standard growth conditions seem to contradict these findings. If under standard growth

conditions, CLE40 functions solely from the L1, where its potential receptor ACR4 is expressed, to promote meristem size, *acr4 clv3* double mutants would be expected to resemble *cle40 clv3* double mutants under standard growth condition. However, while loss of ACR4 suppresses the *clv3* mutant phenotype, no reproducible effect could be detected in *cle40 clv3* double mutants compared to *clv3* single mutants (paragraph 9.11 and 9.15). Consequently, it is possible that under standard growth conditions, CLE40 has a function in the L3, which is antagonistic to the potential L1 function, and which upon loss of CLE40 in *clv3* mutants is masked by the simultaneous loss of both functions. If this holds true, the effects seen under heat stress conditions would be caused by a shift of the balance of the two CLE40 functions. Under heat stress conditions, the restrictive function of CLE40 on meristem size in the CLV pathway would be more dominant than the meristem size-promoting function of CLE40 (Figure 24).

For detailed analyses of the location of CLE40 pathways, it will be necessary to perform expression analyses under different environmental influences, like heat stress. Possibly, a function of *CLE40* in the L3 under heat stress conditions correlates with an up-regulation of *CLE40* expression in this region or with a down-regulation of CLV3 level.

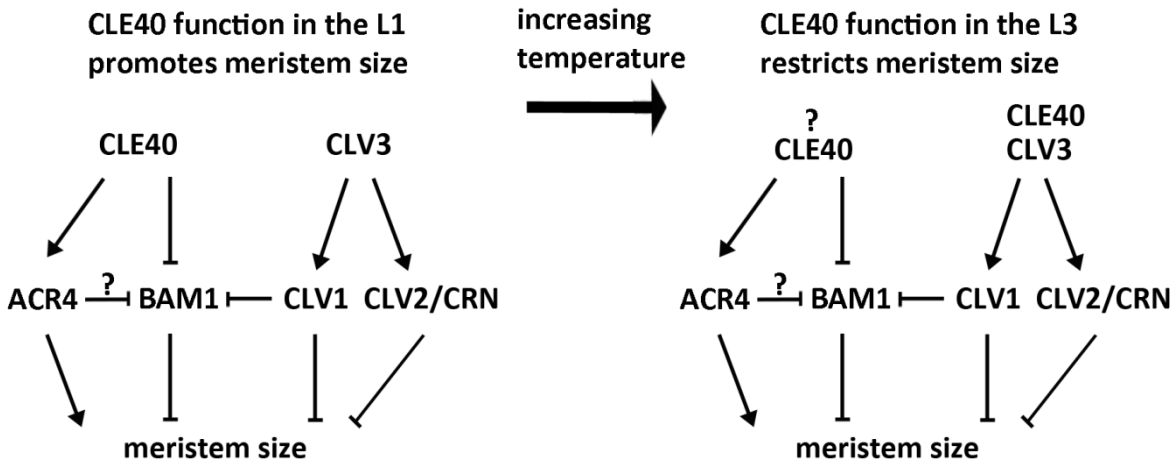



**Figure 24: With increasing temperature CLE40 function shifts towards restriction of meristem size.**

**Question mark above CLE40 indicated that it is not clear if under heat stress conditions, the meristem size promoting pathway is active at all.**

## 10.7 Does CLE40 function via ACR4 in the epidermis?

Besides the expression of *CLE40* in the L1 and L3 of different shoot meristems, *CLE40* expression was also detected in the L1 of leaves and in the hypocotyl of seedlings 4 dag, as well as in developing sepal anlagen (paragraph 9.1). Because the potential CLE40 receptor ACR4 is expressed in the L1 throughout development, starting in early embryonic stages, it is possible that CLE40 and ACR4 generally function together in the epidermis (Gifford *et al.*, 2003).

Interestingly for ACR4, a function in the epidermis in regulation of cellular organization was previously described for sepal margins and ovule integuments, both of which are derived from L1 tissue (Gifford *et al.*, 2003; Jenik and Irish, 2000).

Sepal margins of *acr4* mutants exhibit a disorganized cellular structure and in some regions the cuticle is missing. Integuments were described to appear disorganized and show growth retardations. In case fertilization is possible, defects remain in developing seeds. Defects in the organization of the developing embryo were not described, but in those seeds that exhibited severe defects in integument organization, development of embryo and endosperm were described to be delayed (Gifford *et al.*, 2003).

The described *acr4* mutant phenotypes are rather subtle, thus at first glance, *acr4* mutant shoots appear aphenotypic. The sepal margin phenotype was only detectable by SEM analyses. Unfertilized ovules and aborted seeds, which resulted from cellular disorganization of ovule integuments, ranged between 40-85 %. Thus, a quite high variability in the number of viable seeds is produced by *acr4* mutants, and differences in seed numbers could easily be traced back to slight differences in growth conditions or even be overlooked.

Consequently, it is possible that these phenotypes also appear in *cle40* mutants without having been noticed yet. Besides this, it is also possible that the phenotypes observed in *acr4* mutants occur in a weaker form in *cle40* mutants, because the signalling pathway in which ACR4 acts in the L1 does not necessarily have to include only one peptide that uses ACR4 as receptor. Several peptides could be involved in the regulation of cellular organization via ACR4 in the L1; hence knockout of one potential ligand would not necessarily cause the same phenotype that is caused by loss of its receptor. Further studies on *cle40* mutant phenotypes will be necessary to find out, if CLE40 participates in the regulation of L1 specific cellular organization that also involves ACR4.

## 10.8 Targets of CLE40

### 10.8.1 *WUS* as potential target of CLE40

CLE40 was shown to be a key regulator of meristem size that acts during different stages of shoot development and has the potential to be involved in further processes like the regulation of cellular organization within the L1, due to its broad expression pattern.

The L1 specific expression pattern of *CLE40* indicates that it might be target of regulation by the phytohormone auxin (paragraph 9.2). However, known factors acting downstream of CLE40 are restricted to the receptor-like kinases ACR4 and BAM1, but further target genes located downstream of the CLE40 pathway remain unknown.

As described above, regulation of different plant meristems involves signalling modules that contain WUS clade members of the WOX family as targets (Aichinger *et al.*, 2012) (chapter 10.2). Thus, it is plausible that the CLE40 pathway that promotes meristem size in different shoot meristems could also target members of the WUS clade.

*WUS* itself is expressed in the organizing centre of the SAM, the inflorescence meristem, and the floral meristem, from where it is secreted to stem cells located in overlying layers to promote meristem fate and to restrict its own level by activation of the *WUS* repressor CLV3 (Mayer *et al.*, 1998; Yadav *et al.*, 2011; Daum *et al.*, 2014).

Thus, its expression overlaps with that of *CLE40*, consequently CLE40 might positively act on *WUS* to promote meristem size. This hypothesis is supported by the finding that CLE40 acts upstream of BAM1, a negative regulator of meristem size that contributes to the CLV pathway upon loss of CLV1 (DeYoung and Clark, 2008; Durbak and Tax, 2011; Nimchuk *et al.*, 2015). Consequently, CLE40 could function positively on meristem size by repression of the negative meristem-size regulator BAM1 that in turn would repress *WUS*, thus CLE40 would eventually have an indirect positive function of *WUS*.

The negative regulation of *WUS* in the CLV pathway results in an expansion of the *WUS* mRNA domain if a component of the CLV pathway is lost, or in loss of *WUS* transcripts upon over-expression of CLV3 (Brand, 2000; Schoof *et al.*, 2000).

Whether loss of BAM1 in the *clv1* mutant background, where BAM1 functions in the CLV pathway, leads to a further expansion of the *WUS* expression domain compared to *clv1* single mutants remains to be investigated.

If CLE40 promotes meristem size by positively regulating *WUS*, over-expression of *CLE40* should lead to an expansion of the *WUS* domain, like that observed in *clv* mutants, regardless

of whether it acts via BAM1 to promote meristem size or if it acts via a pathway excluding BAM1 (Figure 23). However, it is known that over-expression of *CLE40* under the control of the *35S* promoter leads to effects comparable to that caused by over-expression of *CLV3*. Probably, due to the very high similarity of CLE40 and CLV3, CLE40 interferes with the CLV pathway in case its expression increases strongly (Hobe *et al.*, 2003).

Thus, to answer the question if CLE40 positively regulates *WUS* to promote meristem size, it will be necessary to analyze inducible *CLE40* over-expression lines at different time points after induction of *CLE40* expression. Shortly after transgene induction, the expression level of *CLE40* would be expected to be lower than at later time points of induction. Consequently, if CLE40 positively acts on *WUS*, the *WUS* domain would be expected to first be expanded shortly after transgene induction and then to vanish due to increasing levels of CLE40 that mimic effects caused by increasing levels of CLV3.

Besides this, expression analyses of *WUS* reporter constructs in *cle40* mutant backgrounds would reveal, if the level of *WUS* transcripts is decreased upon loss of CLE40 function. However, in *cle40* single mutants, mutant phenotypes were only observed in the vegetative SAM, which is difficult to access by confocal microscopy. In the inflorescence and the floral meristem, both of which are rather easy to access, phenotypic effects caused by loss of CLE40 function became visible in *clv* mutants only (paragraph 9.7 and 9.9). Because it is not yet known, if this is due to the sole increase in meristem size in these mutant backgrounds, or if CLE40 function depends on the loss of CLV function and on the ectopic expression of *BAM1* that correlates with loss of CLV1 function, it would be necessary to perform the *WUS* reporter analyses in the *clv1* mutant background.

### 10.8.2 Phytohormone signalling pathways as potential targets of CLE40

Besides the possibility that CLE40 addresses *WUS* as a target, it is also possible that CLE40 functions on different signalling pathways that are controlled by phytohormones, which previously were shown to be potential targets of CLE40 in the root (Pallakies and Simon, 2014). The authors found that in the *cle40-2* transcriptome, several components of auxin signalling are mis-regulated. However, they state that no specific direction of regulation of auxin targets could be assigned, because they were adversatively affected upon loss of CLE40 function. Besides the mis-regulated auxin target genes, the authors also found that genes inhibiting cytokinin signalling were down-regulated, while genes promoting cytokinin signalling were up-regulated in roots of *cle40* mutants.

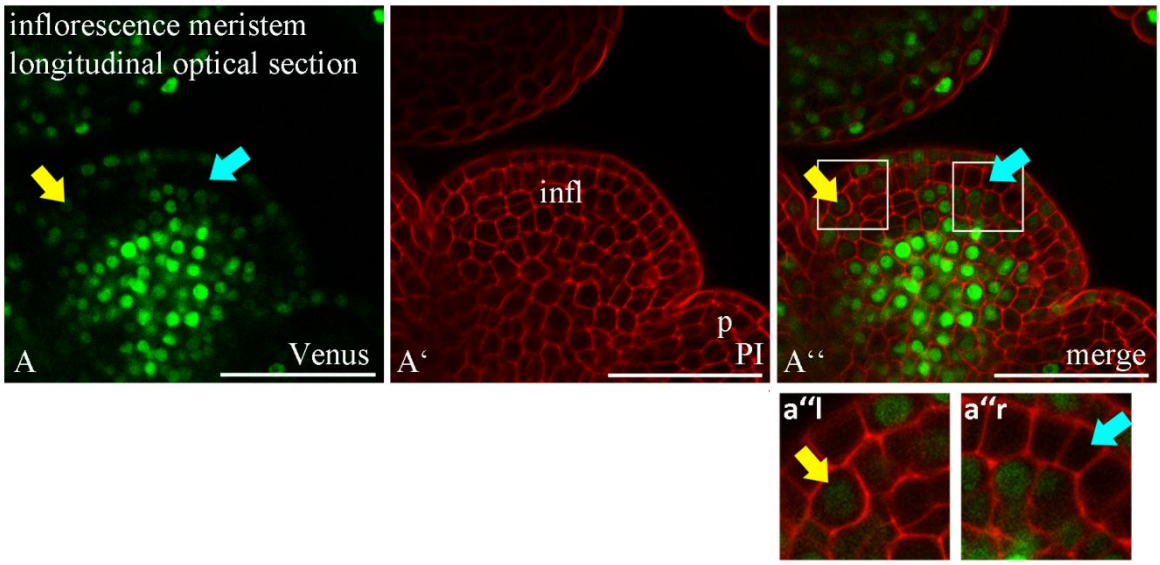
In the shoot, cytokinin was described to positively affect SAM growth and cell division, comparable to the function of CLE40 (Higuchi *et al.*, 2004; Nishimura *et al.*, 2004). Thus in the shoot, CLE40 might have a positive effect on cytokinin signalling. Interestingly, the cytokinin biosynthesis enzyme gene *LONELY GUY 4* (*LOG4*) is expressed in the L1 of the shoot meristem, like *CLE40*; and apically derived cytokinin, together with CLV signalling was shown to control the position of the *WUS* domain within the stem cell niche (Kuroha *et al.*, 2009; Chickarmane *et al.*, 2012). This gives rise to the hypothesis, that CLE40 might positively affect cytokinin signalling by controlling components involved in cytokinin biosynthesis to control the position of the *WUS* domain, thus positively affecting meristem size. Further studies will be necessary to reveal, if CLE40 is involved in the regulation of cytokinin biosynthesis in the shoot and if it is involved in the regulation of other phytohormone signalling pathways.

## 10.9 Conclusion

Taken together, this work provides evidence that CLE40 is a positive regulator of meristem size acting during different stages of shoot development. It is part of a complex network of antagonistic pathways that function together to ensure robustness and plasticity of meristem maintenance. CLE40 itself was shown to have antagonistic functions, the balance of which depends on environmental influences like temperature. This control of the balance of promotive and restrictive signalling upon different environmental influences provides a level of regulation of meristem robustness and plasticity, which is necessary to ensure plant growth and development in response to changing environmental influences.

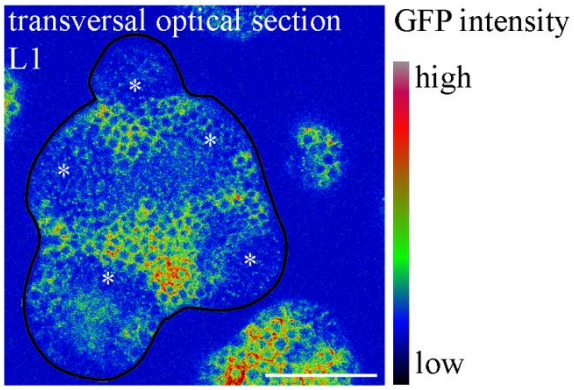
# 11 Supplemental Material

## 11.1 Supplemental Figures



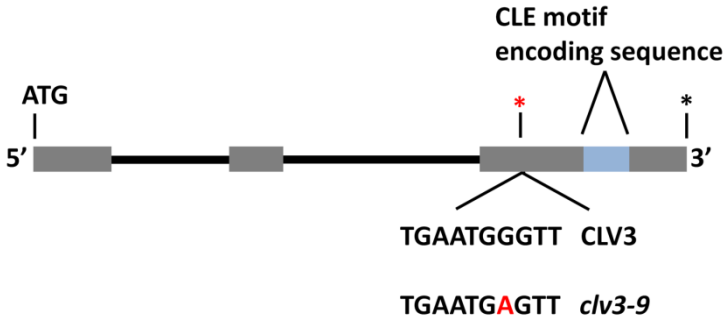

**Supplemental Figure 1: *CLE40::Venus-H2B* is occasionally found in the L2.**

**A-A'': Longitudinal optical sections of an inflorescence meristem (infl) with emerging primordia (p) showing expression of *CLE40::Venus-H2B* in Col-0. A: Venus-H2B; A': PI; A'': merged pictures; a''l and a''r are magnifications of the regions that are highlighted by the left (l) and right (r) box in A'' respectively. Yellow arrows point at L2 cells that express *CLE40::Venus-H2B*. Blue arrows point at L2 cells without any detectable expression. Scale bars represent 50 µm.**




**Supplemental Figure 2: Intensity transformation image of *CLV1::CLV1-2xGFP* shows that GFP is lowest in developing primordia.**

**Transversal optical section of the L1 of the inflorescence meristem that is shown in Figure 7A'; Colours ranging from blue to red show relative intensity of GFP ranging from low to high respectively. Asterisks mark the position of primordia, which correlate with low GFP intensity. Black line surrounds the outer limits of the meristem and the youngest primordia.**




**Supplemental Figure 3: Gene structure of *CLV3* and *clv3-9***

**G->A transition at position 412 downstream of ATG (position 1) results in a stop codon upstream of the region encoding the conserved CLE motif in *clv3-9*. Grey boxes: exons, black lines: introns, blue box: CLE motif encoding sequence, black asterisk: stop codon in the *CLV3* wild type allele, red asterisk: stop codon in the *clv3-9* allele.**

## 11.2 Supplemental Tables

A

| cn | *clv1* | | | | | *cle40 clv1* | | | | | *acr4 clv1* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.33 | 0.62 | 0.41 | 0.50 | 0.42 | 0.88 | 0.87 | 0.91 | 0.89 | 0.72 | 0.73 | 0.75 | 0.85 | 0.73 | 0.88 |
| 3 | 0.46 | 0.26 | 0.40 | 0.27 | 0.51 | 0.09 | 0.13 | 0.09 | 0.11 | 0.22 | 0.26 | 0.21 | 0.14 | 0.26 | 0.12 |
| 4 | 0.21 | 0.12 | 0.18 | 0.21 | 0.07 | 0.04 | | 0.01 | | 0.06 | 0.01 | 0.04 | 0.01 | 0.01 | |
| 5 | | | 0.01 | 0.01 | | | | | | | | | | | |
| 6 | | | | 0.01 | | | | | | | | | | | |
| n | 100 | 151 | 363 | 105 | 73 | 105 | 121 | 254 | 63 | 93 | 100 | 188 | 443 | 100 | 99 |
| p | | | | | | <0.001 | <0.001 | <0.001 | <0.001 | 0.002 | <0.001 | 0.001 | <0.001 | <0.001 | <0.001 |

B

| cn | *clv2* | | | | | *cle40 clv2* | | | | | *acr4 clv2* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.67 | 0.74 | 0.49 | 0.74 | 0.54 | 0.72 | 0.89 | 0.84 | 0.77 | 0.93 | 0.88 | 0.80 | 0.77 | 0.91 |
| 3 | 0.28 | 0.21 | 0.40 | 0.23 | 0.39 | 0.21 | 0.07 | 0.13 | 0.23 | 0.06 | 0.08 | 0.16 | 0.21 | 0.08 |
| 4 | 0.06 | 0.05 | 0.12 | 0.02 | 0.07 | 0.07 | 0.03 | 0.03 | | 0.01 | 0.04 | 0.04 | 0.02 | 0.01 |
| 5 | | | | 0.01 | | | 0.01 | | | | 0.01 | | | |
| n | 108 | 175 | 306 | 100 | 90 | 80 | 127 | 315 | 70 | 84 | 179 | 379 | 106 | 86 |
| p | | | | | | 0.603 | 0.019 | <0.001 | 0.368 | <0.001 | 0.017 | <0.001 | 0.449 | <0.001 |

C

| cn | *clv1 clv2* | | | *cle40 clv1 clv2* | | | *acr4 clv1 clv2* | | | *cle40 acr4 clv1 clv2* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.19 | 0.16 | 0.20 | 0.64 | 0.39 | 0.26 | 0.93 | 0.58 | 0.66 | 0.59 | 0.50 |
| 3 | 0.34 | 0.38 | 0.42 | 0.20 | 0.31 | 0.31 | 0.06 | 0.25 | 0.30 | 0.23 | 0.39 |
| 4 | 0.35 | 0.32 | 0.26 | 0.15 | 0.21 | 0.28 | 0.01 | 0.16 | 0.02 | 0.15 | 0.10 |
| 5 | 0.09 | 0.11 | 0.07 | 0.00 | 0.08 | 0.13 | | 0.01 | 0.02 | 0.03 | 0.01 |
| 6 | 0.04 | 0.03 | 0.02 | 0.00 | 0.01 | 0.01 | | | | | |
| 7 | 0.01 | 0.00 | 0.01 | 0.01 | | 0.01 | | | | | |
| 8 | | 0.01 | 0.01 | | | | | | | | |
| n | 140 | 95 | 99 | 99 | 80 | 97 | 116 | 100 | 50 | 66 | 70 |
| p | | | | <0.001 | 0.002 | 0.963 | <0.001 | <0.001 | <0.001 | 0.014 | <0.001 |
| p | | | | | | | | | | 0.87 | 0.077 |

D

| cn | *clv3* | | | | | *cle40 clv3* | | | | | *acr4 clv3* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.08 | 0.03 | 0.17 | 0.10 | 0.02 | 0.10 | 0.05 | 0.04 | 0.05 | 0.03 | 0.61 | 0.45 | 0.44 |
| 3 | 0.33 | 0.33 | 0.37 | 0.37 | 0.16 | 0.31 | 0.29 | 0.36 | 0.16 | 0.18 | 0.31 | 0.38 | 0.36 |
| 4 | 0.37 | 0.34 | 0.33 | 0.38 | 0.45 | 0.34 | 0.44 | 0.39 | 0.46 | 0.44 | 0.08 | 0.16 | 0.17 |
| 5 | 0.18 | 0.25 | 0.09 | 0.14 | 0.21 | 0.19 | 0.17 | 0.19 | 0.26 | 0.24 | | 0.01 | 0.03 |
| 6 | 0.04 | 0.06 | 0.04 | 0.01 | 0.09 | 0.04 | 0.05 | 0.03 | 0.06 | 0.08 | | | |
| 7 | | | | | 0.06 | 0.01 | | | | 0.03 | | | |
| | a | b | c | d | e | a | b | c | d | e | | | |
| n | 315 | 150 | 100 | 87 | 86 | 249 | 120 | 80 | 110 | 96 | 147 | 98 | 94 |
| p | | | | | | 0.988 | 0.335 | 0.018 | <0.001 | 0.534 | <0.001 | <0.001 | <0.001 |

E

| cn | *clv3* | | | *cle40 clv3* | | |
|---|---|---|---|---|---|---|
| 2 | 0.13 | 0.13 | 0.11 | 0.04 | 0.11 | 0.07 |
| 3 | 0.42 | 0.32 | 0.31 | 0.24 | 0.21 | 0.28 |
| 4 | 0.33 | 0.33 | 0.42 | 0.40 | 0.31 | 0.35 |
| 5 | 0.12 | 0.18 | 0.14 | 0.24 | 0.24 | 0.20 |
| 6 | | 0.04 | 0.02 | 0.08 | 0.09 | 0.08 |
| 7 | | | | | 0.03 | 0.02 |
| 8 | | | | | 0.01 | |
| n | 245 | 326 | 102 | 241 | 159 | 100 |
| p | | | | <0.001 | <0.001 | 0.016 |

F

| cn | *clv1* | | | *clv1 bam1* | | | *clv1 bam1 cle40* | | |
|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.48 | 0.50 | 0.42 | 0.02 | 0.02 | 0.03 | 0.02 | 0.01 | 0.00 |
| 3 | 0.40 | 0.27 | 0.51 | 0.06 | 0.06 | 0.01 | 0.00 | 0.01 | 0.05 |
| 4 | 0.12 | 0.21 | 0.07 | 0.16 | 0.16 | 0.14 | 0.09 | 0.09 | 0.06 |
| 5 | | 0.01 | | 0.28 | 0.26 | 0.22 | 0.07 | 0.17 | 0.22 |
| 6 | | 0.01 | | 0.28 | 0.23 | 0.32 | 0.15 | 0.25 | 0.25 |
| 7 | | | | 0.15 | 0.22 | 0.19 | 0.22 | 0.21 | 0.16 |
| 8 | | | | 0.03 | 0.05 | 0.08 | 0.37 | 0.18 | 0.14 |
| 9 | | | | 0.01 | | | 0.07 | 0.08 | 0.09 |
| 10 | | | | | | | 0.00 | 0.01 | 0.03 |
| 11 | | | | | | | 0.01 | | 0.00 |
| 12 | | | | | | | | | 0.01 |
| n | 105 | 105 | 73 | 95 | 97 | 72 | 82 | 101 | 101 |
| p | | | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | 0.006 |

G

| cn | *cle40* | | | | | | *acr4* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| n | 98 | 89 | 101 | 100 | 100 | 77 | 100 | 76 | 100 | 100 | 100 | 99 |

H

| cn | *cle40 acr4* | | | | | Col-0 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| n | 99 | 100 | 66 | 71 | 100 | 100 | 95 | 88 | 100 | 88 | 70 |

I

| cn | *bam1* | | | *cle40 bam1* | | |
|---|---|---|---|---|---|---|
| 2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| n | 100 | 93 | 97 | 100 | 100 | 72 |

**Supplemental Table 1: Summary of all frequencies of carpel number per flower used in this study**

***e.g.* for *clv1* in A: Five columns represent five independent measurements. Same colours represent values from same measurements and different colours represent independent measurements. cn = carpel number, n = number of samples, p = p-value; A-D, F-I: Values obtained for plants that grew at 21 °C, E: values for plants that grew at 30 °C, p-values of double mutants refer to the respective single mutant, which is shown in the same table. p-values of triple mutants refer to the respective double mutant, which is shown in the same table. Upper and lower p-values of the quadruple mutant *acr4 cle40 clv1 clv2* refer to *cle40 clv1 clv2* and *acr4 clv1 clv2* respectively.**

# 12 Materials and Methods

## 12.1 Plant material

*acr4-2, bam1-3*, *cle40-2*, *clv1-20*, and *clv2-101* were described before (Gifford *et al.*, 2003; DeYoung *et al.*, 2006; Stahl *et al.*, 2009; Durbak and Tax, 2011; Kinoshita *et al.*, 2010). *bam1-3 clv1-20* double mutants were kindly provided by the lab of Dr. Frans Tax. *clv3-9* mutants were generated in 2003 by the lab of Rüdiger Simon (personal communication Rüdiger Simon). *clv3-9* is in the Col-*er* background and to exclude effects on meristem size caused by the mutant *erecta* allele (Mandel *et al.*, 2014), *clv3-9* was crossed with Col-0 to generate *clv3-9 ER* (+/+). All genetic analyses described in this work were performed with the *clv3-9 ER* (+/+) allele. The *CLE40::Venus-H2B* line, which consists of a 2291bp long *CLE40* promoter that drives expression of *Venus-H2B*, was generated and provided by René H. Wink (Wink, 2013). The double marker line *CRN::mCherry-H2B CLV2::Venus-H2B* was generated and provided by Andrea Bleckmann. *CRN::mCherry-H2B* was described before as well as the vectors pAB160 and pAB146, which were used to generate *CLV2::Venus-H2B* (Bleckmann, 2010). The double marker line was created by crossing of plants that expressed *CRN::mCherry-H2B* and *CLV2::Venus-H2B* respectively. The *CLV1::CLV1-2xGFP clv1-11* line was kindly provided by the lab of Dr. Zack Nimchuk and was described before (Nimchuk *et al.*, 2011). *CLV3::H2B-YFP* was generated in the lab of Rüdiger Simon based on the vector pUB14 (Brand *et al.*, 2002).

## 12.2 Working with *Arabidopsis thaliana*

### 12.2.1 Plant growth conditions

All plants were grown under continuous light and 21 °C. For growth on soil, seeds were sown on soil and kept at 4 °C in the dark before they were shifted to continuous light to induce simultaneous germination. For heat stress experiments, plants were grown under standard conditions until they started bolting. Primary inflorescences were cut before plants were shifted to 30 °C for formation of secondary inflorescences that were used to count carpels per flower. For vegetative meristem analyses, seeds were sterilized for two hours with chlorine gas and incubated for two days in 0.5 % (w/v) agarose at 4 °C in the dark. For germination,

seeds were placed on plates containing 0.5 x Murashige and Skoog (MS) medium with Gamborgs no. 5 vitamins, 0.5 g/l MES (2-(N-morpholino) ethanesulfonic acid), 1 % sucrose, and 1.2 % plant agar. The pH of the medium was set to 5.6 - 5.8 with KOH. Seeds were checked for germination each morning and evening after plating, and only those that had germinated within the first two days after plating were used to measure the meristem size 4 dag.

### 12.2.2 Seed sterilization

Seeds were sterilized in case they were placed on plates with growth medium for germination. For sterilization, seeds were placed in open tubes and exposed to chlorine gas for two hours. Chlorine gas was produced by mixing 100 ml of sodium hypochlorite with 3 ml of 37 % (v/v) HCl.

### 12.2.3 Generation of multiple mutants

To generate multiple mutant combinations, standard crossing techniques were used. The F2 generation was genotyped to screen for homozygous multiple mutant combinations. For primers used for genotyping see Table 1.

### 12.2.4 Generation of transgenic plants

#### 12.2.4.1 *ACR4::H2B-TDTOMATO*

To generate plants expressing *ACR4::H2B-TDTOMATO,* the TOPO® cloning system and the gateway® cloning system were used. The sequence of *TDTOMATO* was amplified via PCR using a Clontech vector as template. The sequence was ligated into a pMDC99 based gateway destination vector, 3' of the attR2 site, via restriction sites. The new vector was named pMASNF2.

The sequence of *H2B* was amplified via PCR using the vector pAB146 (Bleckmann, 2010) as template. The *H2B* sequence was ligated into the gateway destination vector pMASNF2, 5' of the *TDTOMATO* sequence, via restriction sites. The new vector was named pAH21.

1925 bp upstream of the *ACR4* start codon were amplified via PCR and cloned into pENTR/D-TOPO. pAH21 and the pENTR/D-TOPO vector that contained the *ACR4* promoter were used to generate the expression vector encoding *ACR4::H2B-TDTOMATO* in an LR reaction. This vector was transformed into *E.coli* and positive transformants were selected by PCR. After verification of its sequence, *Agrobacterium tumefaciens GV3101* was transformed with the vector and transgenic plants were created by floral dip (Clough and Bent, 1998).

### 12.2.5 Generation of transgenic mutants

To generate *CLE40::Venus-H2B clv3-9* and *CLV3::H2B-YFP cle40-2*, homozygous mutants were crossed with transgenic plants, which were each tested for expression in the shoot prior to crossing. F2 plants were grown on plates with germination medium, which was supplemented with the respective antibiotic, until resistant transgenic plants could be distinguished from sensitive plants. Transgenic F2 plants were transferred to soil and screened for homozygous mutants. Homozygous mutant F3 plants were grown on plates with antibiotics to screen for lines that segregated in a 3:1 ratio. From those lines, resistant plants were transferred to soil and used for microscopic analyses.

### 12.2.6 Genetic analyses

For analyses of carpel numbers, the oldest 10 - 15 siliques per plant were used. Each carpel was counted as one, independent of its size. Diagrams show mean values of carpel number frequency per flower from several independent measurements. For each measurement, the number of samples is mentioned in Supplemental Table 1.

For analyses of silique number per inflorescence stem length, the number of siliques on main and side shoots was counted over a distance of 8 - 10 cm starting with the oldest silique at position 0 cm.

### 12.2.7 Measurement of cell size in the SAM

To determine cell size, seedlings were stained with PI 4 dag and the cell diameter in the radial axis was measured for up to three adjacent L1 cells per meristem. Confocal analyses were done with a Zeiss LSM510 laser scanning microscope. Instead of counting all cells of the

meristem, only those that were clearly located in the same plane were counted and their size was measured.

## 12.3 Working with DNA

### 12.3.1 Isolation of genomic DNA

Isolation of genomic DNA from leaves was performed according to a previously published protocol (Dellaporta *et al.*, 1983).

### 12.3.2 Amplification of DNA via PCR

#### 12.3.2.1 Standard PCR protocol used for genotyping

Standard PCRs were performed according to the following protocol. For amplification of *CLE40* prior to restriction hydrolysis, 40 cycles were run to increase the amount of PCR product.

| | | |
|---|---|---|
| 94°C | 3min | |
| 94 °C | 30 sec | 35 x |
| primer specific annealing temperature | 30 sec | |
| 72 °C | 1 min/ 1000 bp | |
| 72 °C | 5 min | |
| 12 °C | ∞ | |

#### 12.3.2.2 Primer sequences

For genotyping, primers listed in Table 1 were used. For genotyping of T-DNA insertion lines, two PCRs per allele were performed.

| allele | forward primer 5'-3' | reverse primer 5'-3' | T-DNA primer 5'-3' |
|---|---|---|---|
| *acr4-2* | TTGTGAACTTCGTGT GACTCG | GTGAGAACTCCGCAA GTGAAG | TAGCATCTGAATTTC ATAACCAATCTCGAT ACA (Sail LB) |
| *cle40-2* | GGAGAAACACAAGA TACGAAAGCCATG | ATTGTGATTTGATAC CAACTTAAAA | - |
| *clv1-20* | TTTGAATAGTGTGTG ACCAAATTTGA | TCCAATGGTAATTCA CCGGTG | TGGTTCACGTAGTGG GCCATCG (SalkLba1) |
| *clv2-101* | CACCATGATAAAGAT TGCAGAT | TGAGCAAAAGATACC TAACA | ATATTGACCATCATA CTCATTGC (gabi left border) |
| *clv3-9* | ATGGATTCGAAGAGT TTTCTGCTAC | TCAAGGGAGCTGAA AGTTGTTTCT | - |
| *bam1-3* | CTAACGACTCTCCGG GAGCT | TAAGGACCACAGAG ATCAGGATTAC | TGGTTCACGTAGTGG GCCATCG (SalkLba1) |
| *ER* | AAGAAGTCATTCAAA GATGTGA | AGAAATTTCAGGTTT GGAATCTGT | - |
| *er-105* | AAGAAGTCATTCAAA GATGTGA | AGCTGACTATACCCG ATACTGA | - |

**Table 1: List of primers used for genotyping**

**For genotyping of *clv3-9*, PCR products were sequenced with the primers used for amplification.**

### 12.3.3 Restriction hydrolysis of PCR products

To distinguish between *CLE40* and *cle40-2*, PCR products were hydrolysed with FD *Ase*I (Thermo Fisher Scientific) according to the protocol for digestion of PCR products that was provided by the supplier.

### 12.3.4 Gel electrophoresis

For visualization of PCR products, either 0.7 % (v/w) or 2 % (v/w) agarose gels were used, depending on the size of the expected product. Ethidium bromide was added to a final concentration of 100 µg/l.

## 12.4 Histological methods

### 12.4.1 Preparation of seedlings for bright field microscopy of SAMs

Seedlings were prepared by removal of chlorophyll through incubation with ascending concentrations of ethanol and subsequent incubation with histoclear. To avoid disruption of plant tissue, seedlings were first incubated in 30 % (v/v) ethanol for 15 min before incubation was continued in 50 % (v/v) EtOH, 75 % (v/v) EtOH, and 85 % (v/v) EtOH for one hour each. An overnight incubation in 85 % (v/v) EtOH was followed by incubation in a mixture of EtOH and histoclear (1:1). Seedlings were stored in histoclear at 4 °C and incubated in immersion oil over night prior to microscopy.

### 12.4.2 mPS-PI staining

PI staining of seedlings was performed like described before (Truernit *et al.*, 2008).

## 12.5 Microscopy

### 12.5.1 Bright field microscopy

For bright field microscopy, a Zeiss Axioscope II microscope was used together with the software AxioVision.

### 12.5.2 Confocal microscopy and sample preparation

Confocal analyses of shoot apices were carried out with a Zeiss LSM780 laser scanning microscope. Shoot apices were cut with a razor blade and placed on double-side adhesive tape on a microscopy slide. For imaging without counter staining, apices were covered with 0.1 % (v/v) Tween20 to avoid inclusion of air bubbles. Then, water was added before apices were covered with a cover slide. For counter staining of cell walls, water was replaced by propidium iodide (final concentration 2.5 mM). Image analyses were carried out with the software Zen.

### 12.5.3 Scanning electron microscopy and preparation of samples

For preparation of SEM prints, older flowers were removed from shoot apices with forceps until the inflorescence meristem was visible as a dark green spot under the binocular microscope. Younger flowers were not removed but pushed to the side to avoid excessive leakage of wound fluid. The uppermost 1 cm of the inflorescence apex was removed and pushed upside down into a two-component vinyl polysiloxane impression material (Express™ 2Ultra Light Body Quick, 3M ESPE) that would polymerize shortly after having been mixed and form a negative print of the inflorescence apex. To produce a replicate of the inflorescence apex, the negative print was filled with epoxy and air inclusions were removed by careful application of pressure with forceps. After an incubation of 24 hours, shoot replicates could be used for SEM, which was done with the help of a SEM Leo 1430 VP.

## 12.6 Statistics

For statistical analyses, an unpaired two-sample student's t-test was used. A p-value of $< 0.05$ was interpreted as indicator for significant differences.

## 12.7 Alignment of CLE40 and CLV3 CLE motif

The alignment was performed with Vector NTI using the 13 amino acid CLE motif of CLV3 that was previously described (Ohyama *et al.*, 2009).

# 13 Appendix

## 13.1 Author Contributions

Julia B. Schmid and Rüdiger Simon designed the experiments. Julia B. Schmid carried them out and wrote the manuscript. Marc Somssich generated the vector pMASNF2. Adrian Hülsewede cloned the vector encoding *ACR4::H2B-TDTOMATO* and generated transgenic lines with this vector.

## 13.2 Nomenclature

Names of proteins are written in capital letters (e.g. CLE40), names of genes are written in capital italic letters (*CLE40*). Mutants are written with small italic letters (*cle40)*.
Names of reporter construct are written e.g. *CLE40::H2B-Venus*, with a hyphen indicating protein fusion of H2B and Venus, and two colons indicating expression of the protein fusion under the control of the *CLE40* promoter.

# 14 Summary

In this thesis, the function of the small secreted peptide CLE40 in the shoot of *Arabidopsis thaliana* is characterized.

*CLE40* is expressed in the L1 and the L3 of the inflorescence meristem and the floral meristem. In the L1 of the inflorescence meristem, its expression strength changes during organ development. At positions of organ initiation, *CLE40* expression is absent or less strong than before organ initiation, but increases in later stages of flower development. The underlying mechanism driving these changes is not known, but this pattern resembles an inverse auxin signalling pattern, thus *CLE40* might be a target of auxin signalling.

The expression domain of *CLE40* largely overlaps with those of the CLV pathway receptors *CLV1*, *CLV2*, and *CRN*, and it partially overlaps with that of *ACR4* in the L1 of the shoot.

*CLV1* and *CLV2* expression decreases at positions of organ initiation, similar to the changes in *CLE40* expression, indicating that *CLE40*, *CLV1*, and *CLV2* might be subjects of the same control mechanism or the similarities observed in their expression pattern might be due to a mutual regulation among them. Finding that the CLV receptors have much broader expression patterns than the target *WUS*, leads to the conclusion that they might be involved in more signalling processes in the shoot than only the CLV pathway.

However, CLE40 function in the shoot is not depending on CLV1, neither does it depend on CLV2. CLE40 is a positive regulator of meristem size in the vegetative SAM and in the floral meristem, controlling the number of meristematic cells. CLE40 promotes floral meristem size by suppressing the function of the negative meristem regulator BAM1. Besides this, it functions upstream of ACR4 that also regulates floral meristem size independently of CLV1 and CLV2. It is possible that CLE40 functions via ACR4 on BAM1, but it could also function via two separate pathways including one or the other RLK. It is not clear, if the meristem size promoting function of CLE40 is restricted to the L1 or the L3, or if it originates in both layers.

Under heat stress, the promotive function of CLE40 shifts towards a restrictive function, probably via the CLV pathway. These two opposing functions of CLE40 might originate in different layers. The location of the putative CLE40 receptor ACR4 in the L1 and the expression of the CLV target *WUS* in the L3 point towards localization of the promotive pathway in the L1 and the restrictive pathway in the L3. The possibility to shift the balance of these two pathways towards each other represents a level of regulation of meristem maintenance and organogenesis that enables a plant to quickly respond to changing environmental influences.

# 15 Zusammenfassung

Diese Arbeit befasst sich mit der Funktion des Signalpeptides CLE40 im Spross von *Arabidopsis thaliana.*

*CLE40* ist in der L1 und der L3 des Infloreszenz- und Blütenmeristems exprimiert. In der L1 des Infloreszenzmeristems variiert die Expressionsstärke während der Organogenese. In Primordien ist sie relativ gering verglichen mit der Expressionsstärke im Meristem, steigt aber in späteren Stadien der Blütenentwicklung. Der zugrundeliegende Kontrollmechanismus dieser Variabilität ist unbekannt, aber die Ähnlichkeit dieses Musters zum inversen Auxinmuster deutet auf eine Kontrolle von *CLE40* durch Auxin hin.

Die Expressionsdomäne von *CLE40* überlappt größtenteils mit denen von *CLV1*, *CLV2* und *CRN*, den Rezeptoren des CLV Signalweges. Teilweise überlappt sie mit der Expressionsdomäne von *ACR4* in der L1 des Sprosses.

*CLV1* und *CLV2* zeigen das gleiche L1 spezifische Expressionsmuster wie *CLE40*, was auf eine mögliche Regulation durch denselben Kontrollmechanismus oder eine gegenseitige Regulation hindeuten könnte. Das im Vergleich zu *WUS* relativ breite Expressionsmuster der Rezeptoren des CLV-Signalweges lässt vermuten, dass diese im Spross an weiteren Signalprozessen neben dem CLV-Signalweg beteiligt sein könnten.

Allerdings ist die Funktion von CLE40 im Spross unabhängig von CLV1 und CLV2. CLE40 fördert Meristemgröße im SAM und im Blütenmeristem, durch die Kontrolle der Zellzahl. Es fördert die Größe des Blütenmeristems durch die Suppression der negativen Wirkung von BAM1. CLE40 ist in seiner Funktion der Funktion von ACR4 vorgeschaltet, das ebenfalls die Größe des Blütenmeristems unabhängig von CLV1 und CLV2 fördert. Möglicherweise hemmt CLE40 über ACR4 die Funktion von BAM1. Es könnte aber auch über zwei unabhängige Signalwege über ACR4 oder BAM1 wirken. Unklar ist ob die positive Wirkung von CLE40 aus der L1, der L3 oder aus beiden Lagen stammt. Unter Hitzestress wird die positive Wirkung von CLE40 auf das Meristem zugunsten einer negativen Wirkung, wahrscheinlich über die Rezeptoren des CLV Signalweges, verschoben.

Diese antagonistischen Funktionen von CLE40 könnten in verschiedenen Lagen ihren Ursprung finden. Die Lokalisation des putativen CLE40 Rezeptors ACR4 in der L1 und die Expression des CLV Zielgenes *WUS* in der L3 deuten darauf hin, dass der positiv wirkende Signalweg in der L1 und der negativ wirkende Signalweg in der L3 lokalisiert sein könnten. Die Möglichkeit die Ausprägung beider Signalwege zugunsten des einen oder anderen zu verschieben weist eine Ebene der Regulation des Gleichgewichts von Meristemerhalt und

Organogenese auf, die es Pflanzen ermöglicht, schnell auf sich verändernde Umweltbedingungen zu reagieren.

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