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Project Final report

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Summary

In plants the shoot apical meristem (SAM), located at the tip of the stem, is the source of all above ground post embryonic organs. The SAM arises during embryogenesis and operates throughout the life of the plant to maintain a self-renewed population of undifferentiated stem cells, and to generate different types of organs. Lateral organs, such as leaves and flowers, follow a regular pattern, or phyllotaxis, that can be described mathematically and defines part of the plant architecture.

One of the most abundant lateral organ pattern in nature is the spiral phyllotaxis, found in the plant model *Arabidopsis thaliana*. This pattern is described by successive organs along the stem being separated by the so called "golden angle" 137.5°. Recently we reported that in *Arabidopsis*, the triple loss-of-function mutant *plethora3, plethora5, plethora7* (*plt3plt5plt7*) is defective in establishing spiral phyllotaxis and preferentially adopts a distichous pattern of lateral organ initiation where organs form sequentially separated by 180° [1]. *PLT3*, *PLT5* and *PLT7* genes encode for AP2 domain transcription factors. Phyllotaxis is known to be dependent on the phytohormone auxin polarised transport, however what parameters control precise switch in phyllotaxis are unknown.

Using a domain specific complementation approach, I demonstrated that PLT's function in the centre of the meristem is required and sufficient for organ patterning. During this project I showed that auxin biosynthesis is reduced in the apex of *plt3plt5plt7*, and mutants with reduced auxin biosynthesis leads to a similar phenotype as *plt3plt5plt7*. Moreover expression of the auxin biosynthetic gene *YUCCA4* in the centre of *plt3plt5plt7* meristem fully complements the triple mutant phyllotactic phenotype. Therefore PLT transcription factors regulate auxin biosynthesis throughout the meristem, controlling the quantity of auxin available for organ initiation and patterning. The observation of central zone markers showed that the stem cell niche size is reduced in *plt3plt5plt7* mutant background. Increasing the size of the stem cell niche by increasing the phytohormone cytokinin biosynthesis in *plt3plt5plt7* background is also sufficient to rescue the organ patterning defect of the mutant. Expression analysis of marker genes showed that the rescue of *plt3plt5plt7* observed with cytokinin biosynthesis is not correlated to a larger stem cell niche, but to a higher auxin production. Together these data show that PLT-mediated control of auxin biosynthesis in the SAM regulates phyllotaxis. Furthermore this work emphasizes the tight cross regulation between cytokinin and auxin biosynthesis to couple stem cell fate to organogenesis and phyllotaxis.

Project context and objectives

Scientific background

In higher plants the shoot apical meristem is required to maintain a population of pluripotent stem cells that will provide daughter cells to be incorporated into lateral organ primordia. A balance between the number of stem cells and the number of daughter cells departing to lateral organs is necessary to preserve the integrity of the meristem and the proper development of lateral organs such as leaves and flowers.

The stem cells, located at the apex of the meristem, in the central zone (CZ), are slow dividing cells. The daughter cells are misplaced, after division, at the periphery of the meristem, or peripheral zone (PZ), where they divide more rapidly and can be incorporated into organ primordia after exposure to a developmental signal, the phytohormone auxin [2-4]. The cells of the meristem are maintained undifferentiated by the expression of the class I *Knotted*-like homeobox (KNOX) gene *SHOOTMERISTEMLESS* (*STM*) [5, 6]. Stem cell identity and number is regulated by a two-component mechanism constituted of the homeodomain protein WUSCHEL (WUS) and the secreted glycopeptide CLAVATA3 (CLV3) [7, 8]. *WUS* is expressed in the organizing centre, below the stem cell niche [4, 9]. The WUS protein diffuses to the overlying stem cells to activate the *CLV3* expression [10]. In turn the CLV pathway restricts *WUS* expression to maintain constant the size of the stem cell niche [11]. This feedback regulatory loop allows a tight control of the number of stem cells in the CZ of the meristem (Figure 1). The loss-of-function mutants *stm* and *wus* cannot maintain a shoot apical meristem [5, 6, 12], whereas *clv3* mutants have an enlarged meristem.

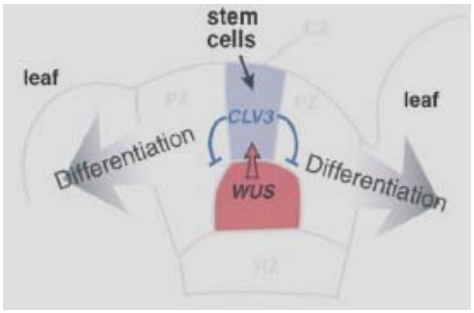


Figure 1: Representation of the feedback regulatory loop between *CLV3* and *WUS* for stem cell maintenance at the shoot apical meristem. The organizing centre (red) specifies stem cell identity in the stem cell niche (blue). Stem cell identity requires *WUS* expression. Stem cells express *CLV3* which in turn repress *WUS* expression in the organizing centre. (Figure from Schoof et al., 2000)

The lateral organs, leaves and flowers, are initiated following a regular pattern, or phyllotaxis. The plant model *Arabidopsis*, as many other species, has organs organised as a spiral along the stem which are separated by about 137.5°. Another pattern frequently found in nature is the decussate pattern, when two organs are initiated simultaneously as an opposite pair, shifted 90° with respect to

the previous pair. A distichous phyllotaxis, as observed in maize, defines organs initiating sequentially and separated by 180°.

At the shoot apical meristem organogenesis is triggered by the accumulation of the phytohormone auxin (Reinhardt et al., 2000). Based on the expression patterns of auxin biosynthesis genes such as *YUCCA1* (*YUC1*), *YUCCA2* (*YUC2*), *YUCCA4* (*YUC4*), *YUCCA6* (*YUC6*), and *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 TAA1*, auxin is thought to be produced throughout the meristem dome [13-15] and redistributed in incipient primordia mainly via the active efflux transporter PIN-FORMED1 (PIN1) [3, 16-18]. In the absence of a functional PIN1 protein, no floral organs are formed due to a failure to accumulate auxin at the PZ of the shoot meristem, giving rise to plants with pin-like phenotype. Influx and efflux auxin transporters are also required for organ positioning [1, 18, 19]. Early experiments demonstrated that the position of a new primordium depends on the position of the older ones [20]. The current models for phyllotaxis are relying on a lateral inhibition process where each organ primordium acts as an auxin sink and creates an inhibitory field in its vicinity where no other organ can initiate, as auxin will not be able to accumulate [18]. In computational models auxin accumulation or flux feeds back on *PIN1* expression and / or polarity to amplify its accumulation in incipient organs [21-23].

Organ patterning defects are also highly correlated to changes in meristem size or / and organization [24, 25]. The size of the meristem is positively influenced by the phytohormone cytokinin [26]. In rice, loss-of-function of *LONELY GUY* (*LOG*), a cytokinin biosynthetic gene, displays a *wus*-like phenotype. In maize the loss-of-function of *ABERRANT PHYLLOTAXY1* (*ABPHYL1*), a type A response regulator (A-ARR) of the cytokinin signalling pathway, displays switches from distichous phyllotaxis to decussate in 20% of the mutant population [25, 27, 28]. Environment is also playing a role in plant organ initiation and patterning, as it was shown that light can activate the cytokinin signalling pathway to supply a source of stem cells required for organogenesis [15].

Recently, in the laboratory of Pr. Scheres, we reported that the *plt3plt5plt7* triple mutant is defective in establishing spiral phyllotaxis and preferentially adopts a distichous pattern of lateral organ initiation [1] (Figure 2). Contrary to *plt3plt5plt7* mutant, many mutants with phyllotaxis defects display random patterns of organ formation instead of specific switches from one regular pattern to another. Moreover defects in phyllotaxis are usually part of a pleiotropic phenotype, complicating the distinction between direct and indirect effects. Therefore the aim of the project is to understand the molecular mechanisms controlling directly phyllotaxis in *Arabidopsis* using *plt3plt5plt7* mutant as a new and unique experimental tool.

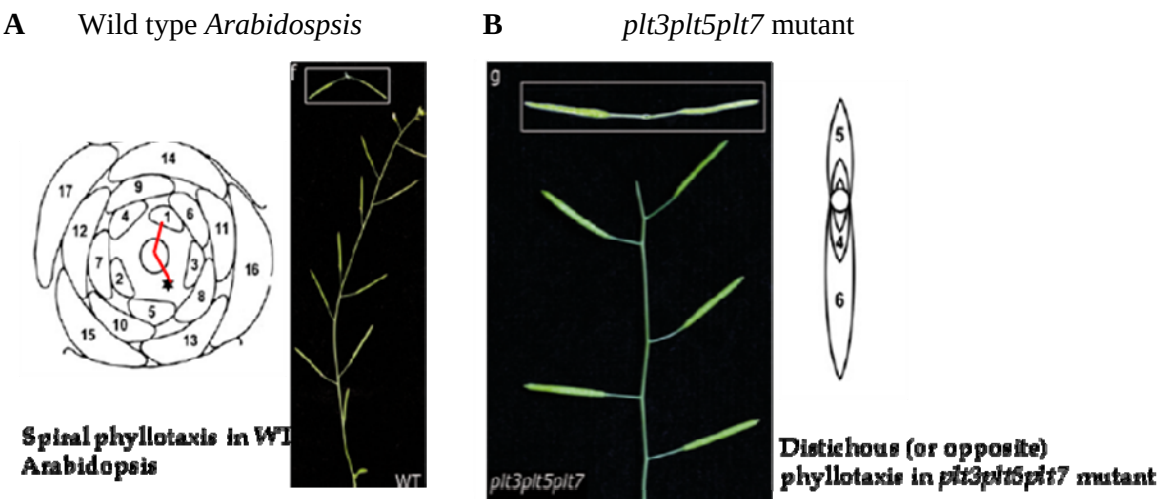


Figure 2: Phyllotaxis phenotype in *plt3plt5plt7* mutant. (**A**) On the left: schematic top view of a wild type shoot apex with the CZ in the centre, surrounded by lateral organ primordia. The angle separating two consecutive primordia is about 137.5°. On the right: wild-type inflorescence with later organs arranged in a spiral. In the close up two consecutive organs are separated by the golden angle 137.5°. (**B**) On the left: *plt3plt5plt7* inflorescence with lateral organs arranged in an alternate, or distichous pattern. The close up shows consecutive organs separated by 180°.

The PLETHORA 3, 5, and 7 AP2 domain transcription factors have overlapping expression domains in the centre of the shoot meristem. They are also expressed at different stages of organ development. We demonstrated earlier that PLT3, PLT5 and PLT7 regulate phyllotaxis partly by modulating *PIN1* expression at the periphery of the meristem [1]. However the overlapping domain of expression between the three *PLT* genes is the centre of the meristem, suggesting that *PLTs* could regulate meristem architecture, and by then phyllotaxis.

The objectives of this research project were:

-To determine the domain of *PLT* expression, at the shoot apical meristem, necessary and sufficient to reestablish a spiral phyllotaxis. This information, together with data available on transcriptomic response after short induction of PLT transcription factors, could direct us to a specific function of *PLT* genes in the regulation of phyllotaxis.

-To analyse meristem architecture in *plt3plt5plt7* triple mutant background by using fluorescent markers combined with the technique of live imaging at the shoot apical meristem by confocal microscopy.

In *Arabidopsis*, *PLT* genes are the only regulators that were shown to specifically modulate phyllotactic patterns. Therefore understanding how PLTs control phyllotaxis is essential and could provide, for the first time, a regulatory mechanism explaining changes of phyllotaxis observed in nature, and might then designate an evolutionary, regulatory module for plant architecture. This research can also provide a link between phyllotaxis / organogenesis and meristem architecture, and

shed light on how the balance between the number of stem cells and the number of cells departing into organ primordia is controlled.

Results and discussion

1/ PLETHORA activity in the centre of the meristem is required for phyllotaxis.

PLETHORA activity is required for the efflux auxin transporter *PIN1* upregulation at sites of incipient primordia, suggesting that PLT's control of phyllotaxis might be exerted in the primordia [1]. However the greatest overlap of *PLT* expression domains lies at the centre of the meristem. In order to understand where PLT's function is predominantly required for spiral phyllotaxis, I conducted a domain specific complementation of *plt3plt5plt7* triple mutant using shoot specific promoters: *STM* promoter is expressed both in the PZ and the CZ but excluded from organ primordia; in the CZ, *WUS* promoter is active in the organizing centre (OC), below the stem cell niche, and *CLV3* promoter is expressed in the stem cell niche; *ANTEIGUMENTA* (*ANT*) promoter is expressed specifically in organ primordia.

The correct expression of each construct was verified (Figure 3) and accumulation of recombinant proteins was never observed outside their expected domains.

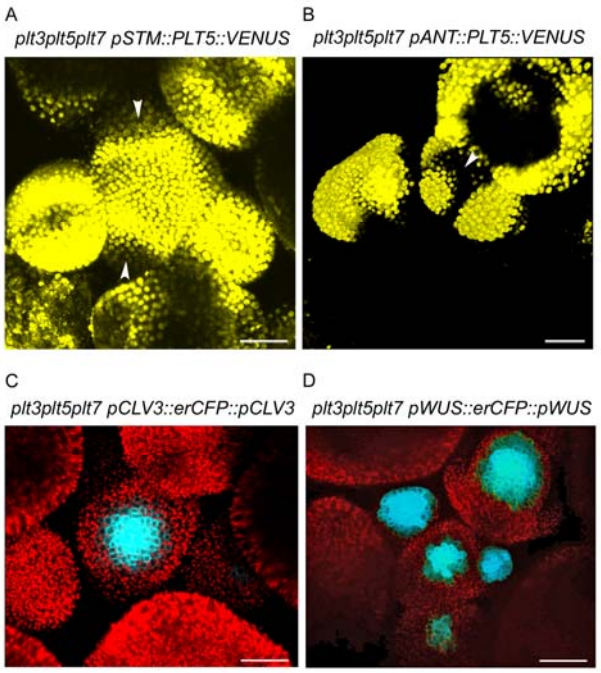


Figure 3: Expression patterns of the complementation constructs, or of the promoters utilized for the complementation experiment. (**A-D**) Confocal 3D projections of inflorescence meristems expressing pSTM::PLT5::VENUS (**A**); pANT::PLT5::VENUS (**B**); pCLV3::erCFP::pCLV3 (**C**) and pWUS::erCFP::pWUS (**D**) in *plt3,5,7* mutant background. The white arrow heads in (A) point to flower primordia where PLT5 is depleted, as expected by using *STM* promoter. In (B) the arrow head points to the centre of the inflorescence meristem where PLT5 protein is absent, as expected by using *ANT* organ specific promoter. The red channel in (D) and (E) visualizes autofluorescence. Scale bars represent 20 µm.

The phyllotaxis phenotype was analysed by measuring the angle separating the organs along the stem of the main inflorescence (Figure 4). The results show that spiral phyllotaxis was completely restored when *PLT5* was expressed throughout the meristem except in organ primordia, under *STM* promoter

(Figure 3A; Figures 4A, B and C). Expression of *PLT5* in the stem cells of the CZ using *CLV3* promoter also complements *plt3plt5plt7* phyllotaxis defect (Figure 3C; Figure 4D). Partial complementation was observed using *WUS* promoter which is active in the cells of the OC (Figure 3D; Figure 4E). Surprisingly driving expression of *PLT5* from the organ specific *ANT* promoter failed to complement *plt3plt5plt7* phenotype (Figure 3B; Figure 4F). The experiment was also conducted expressing *PLT3* and *PLT7* genes under *STM*, *CLV3*, *WUS* and *ANT* promoters in *plt3plt5plt7* (data not shown). The three *PLT* genes could rescue the phyllotaxis phenotype of *plt3plt5plt7* triple mutant with similar strength for similar domains, suggesting redundant function to control phyllotaxis. To conclude PLT transcription factors act in the centre of the meristem to control organ patterning, rather than in primordia.

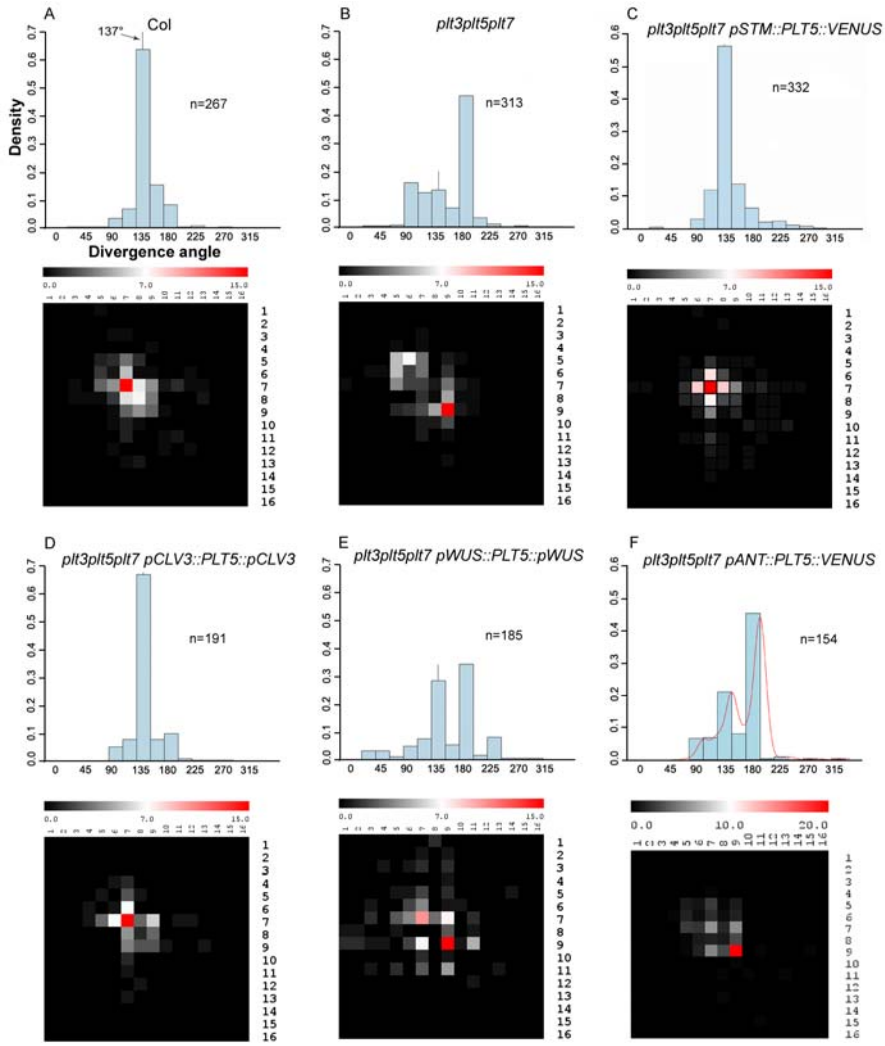


Figure 4. Expression of *PLT5* in the centre of the meristem is sufficient to complement *plt3,5,7* phyllotaxis defect. (**A**) Silique divergence angle distribution in Col inflorescences, angle classes are defined by their midpoint (upper panel). The most frequent divergence angle between two organs falls in the category comprising the angle 137.5º. The lower panel shows, in a 2D heat map, the distribution of patterns in successive silique divergence angles in Col. For each angle class on the x axis, the occurrence of each class in

the following internode, plotted on the y axis, is represented by color intensity. The angles have been divided into 16 classes separated by 22.5º (axis x and y) . The angle class 7 comprises the golden angle 137.5º, the class 9 includes the angle 180º. In Col, the preferred angle between succesive organs is 137.5º. (**B**) Silique divergence angle distribution in *plt3plt5plt7* mutant (upper panel). In *plt3plt5plt7* the most frequent divergent angle between two organs falls in the category comprising the angle 180°. The corresponding lower panel display the distribution of patterns in consecutive silique divergence angles. In the triple mutant the spiral pattern is not stable whereas the distichous pattern is. (**C**-**F**), Silique divergence angle distribution in *plt3plt5plt7 pSTM::PLT5::VENUS* (**C**) ; *plt3plt5plt7 pCLV3 ::PLT5 ::pCLV3* (**D**); *plt3plt5plt7 pWUS::PLT5::pWUS* (**E**); *plt3plt5plt7 pANT::PLT5::VENUS* (**F**) upper panels ; corresponding lower panels display the distribution of patterns in successive silique divergence angles. n: number of silique divergence angles.

2/ Increasing auxin in the centre of the meristem can restore a spiral phyllotaxis in the mutant *plt3plt5plt7*.

Transcript profiling analyses after PLT induction have indicated that auxin biosynthesis genes are potential targets of the PLT transcription factors (personal communication from Renze Heisdra). *YUCCA1* (*YUC1*) and *YUC4*, which encode flavin-containing monooxygenases involved in a rate limiting step of auxin biosynthesis [29, 30], are expressed both in the centre of the meristem and in organ primordia, overlapping with *PLT* expression domains [13]. The expression levels of *YUC1* and *YUC4* were both reduced in *plt3plt5plt7* mutant shoot apices (Figures 5A and B), suggesting that PLTs control *YUC* expression. We also observed an upregulation of *YUC4* transcript level after four hours of PLT5 induction, indicating that auxin biosynthesis can be rapidly activated by PLT activity (Figure 5C).

If decreased auxin production at the shoot apex was responsible for the phyllotaxis defect observed in *plt3plt5plt7*, then we might expect low auxin mutants to display similar phenotypes. We thus assessed the phyllotactic pattern among the progeny of *yuc1/+ yuc4* individual, as *yuc1yuc4* double mutant fail to generate lateral organs [13]. Similarly to *plt3plt5plt7*, we observed a shift from spiral phyllotaxis to a stable distichous pattern in this population (Figures 5F, G and H). This result suggests that PLTs control phyllotaxis via YUC-mediated control of auxin biosynthesis in the SAM. To confirm this hypothesis we restored *YUC4* expression in *plt3plt5plt7* using *STM* promoter, and the organ specific *FILAMENTOUS FLOWER* (*FIL*) promoters (Figures 5D, E). When *YUC4* was expressed under the *STM* promoter, the phyllotaxis of the mutant was fully rescued to a spiral pattern (Figures 5F, G and I). Similarly to the complementation using *PLT* genes, driving *YUC4* into organ primordia by *FIL* promoter did not complement the organ patterning defect of *plt3plt5plt7* (Figures 5F, G and J), suggesting that auxin synthesized at the SAM is of greater importance in determining phyllotaxis than that synthesized in lateral organs.

Taken together these results demonstrate that PLTs positively regulate auxin biosynthesis at the SAM, and further suggest that PLTs control phyllotaxis predominantly through controlling the amount of auxin available at the SAM.

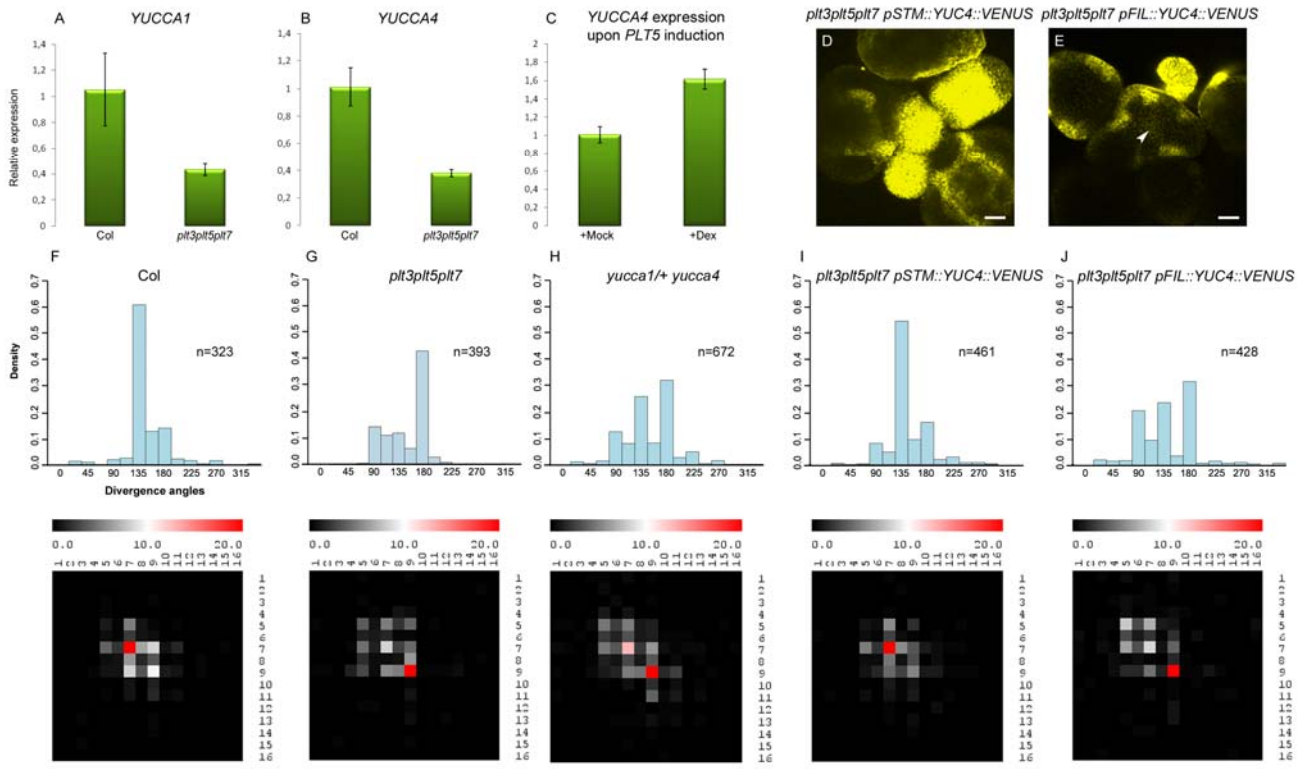


Figure 5. PLTs regulate phyllotaxis through the transcription of the auxin biosynthetic genes *YUC1* and *YUC4*. (A-B) *YUC1* (A) and *YUC4* (B) expression levels measured by quantitative RT-PCR in Col and *plt3plt5plt7* dissected 10 do vegetative shoot apices. (C) *YUC4* expression level after 4h PLT5 induction. Error bars represent the standard error from three independent experiments. (D-E) Expression pattern of pSTM::YUC4::VENUS (D) and pFIL::YUC4::VENUS (E) in *plt3plt5plt7* inflorescence meristems by confocal laser scanning microscopy. In (E) the arrowhead marks the meristem centre where pFIL::YUC4::VENUS expression is absent. (F-J) Distribution of silique divergence angles (upper panels), and corresponding silique divergence angle pattern distribution (lower panels) in Col (D); *plt3plt5plt7* (E), *yuc1/+ yuc4* (F); *plt3plt5plt7 pSTM::YUC4::VENUS* (G); *plt3plt5plt7 pFIL::YUC4::VENUS* (H). n: number of divergence angles. Scale bars represent 20 µm.

I have demonstrated here that increasing auxin production in the centre of the shoot meristem causes the distichous phyllotaxis pattern of *plt3plt5plt7* mutants to switch to a stable wild type spiral pattern. Low auxin *yuc* mutants display similar phyllotaxis defects as the triple *plt3plt5plt7* mutant. The expression levels of *YUC1* and *YUC4* at the shoot apical meristem are reduced in the mutant compared to wild type, and after four hours of PLT5 induction *YUC4* expression was increased. These results indicate that in *plt3plt5plt7* mutants auxin has become a limiting factor for organ patterning. One possibility is that a primordium, acting as an auxin sink in a low auxin context, might

deplete auxin from a larger area in its surrounding, and then increases the inhibitory field where no auxin can accumulate. Therefore it is possible that in *plt3plt5plt7* auxin accumulates further away from the previous primordium compared to wild-type, i.e oppositely. When *PLTs* or *YUC4* genes are expressed throughout the SAM with *STM* promoter in *plt3plt5plt7*, they might increase auxin levels in the SAM and reduce the size of the inhibitory field back to wild type. In contrast, *ANT* and *FIL* promoters, which are specifically active in organ primordia, might only allow the auxin produced to be sequestered in the developing organ, where it would remain unavailable to the meristem, and not contributing to organ patterning. Unlike *STM* and *CLV3* promoters, the *WUS* promoter driving *PLT3*, *PLT5* or *PLT7* can only partially rescue *plt3plt5plt7* phyllotaxis, despite its activity in the centre of the meristem. Analysis of the promoter activities showed that expression levels of pWUS::erCFP::pWUS are much lower compared to pCLV3::erCFP::pCLV3 in the SAM (data not shown). Moreover the auxin is not produced within the L1 where the auxin transporters are present, and therefore its redistribution throughout the meristem might be less efficient.

Computational models of spiral phyllotaxis implementing variation on the threshold for organ initiation, or on the range of inhibition, yield to an increased frequency of distichous angles, reminiscent of *plt3plt5plt7* mutants [31]. One possibility to introduce variation in the model leading to distichous patterns is to modify auxin production / degradation, supporting our hypothesis that local auxin biosynthesis in the centre of the meristem is an essential parameter for phyllotaxis, modulation of which can lead to switches from spiral to distichous patterns.

3/ PLETHORAs' role in phyllotaxis in the centre of the meristem does not involve positive regulation of *PIN1* expression.

In the laboratory of Pr. Scheres we have previously shown that increasing the amount of PIN1 efflux carrier in organ primordia can decrease the frequency of opposite angles in *plt3plt5plt7* triple mutants. *PIN1* is expressed in the outermost layer of cells (L1) in the meristem, both in organ primordia and in the centre of the meristem. Therefore PLT activity in the centre of the meristem might also be involved in the regulation of *PIN1* expression. To investigate if PLT function in the SAM is carried out in part via PIN1, I expressed *PIN1GFP* under *CLV3* and *STM* promoters in *plt3plt5plt7* mutants (Figures 6E and F). Neither construct was sufficient to restore spiral phyllotaxis in *plt3plt5plt7* (Figures 6A to D). I conclude that PLTs do not promote spiral phyllotaxis by regulating positively *PIN1* expression at the SAM.

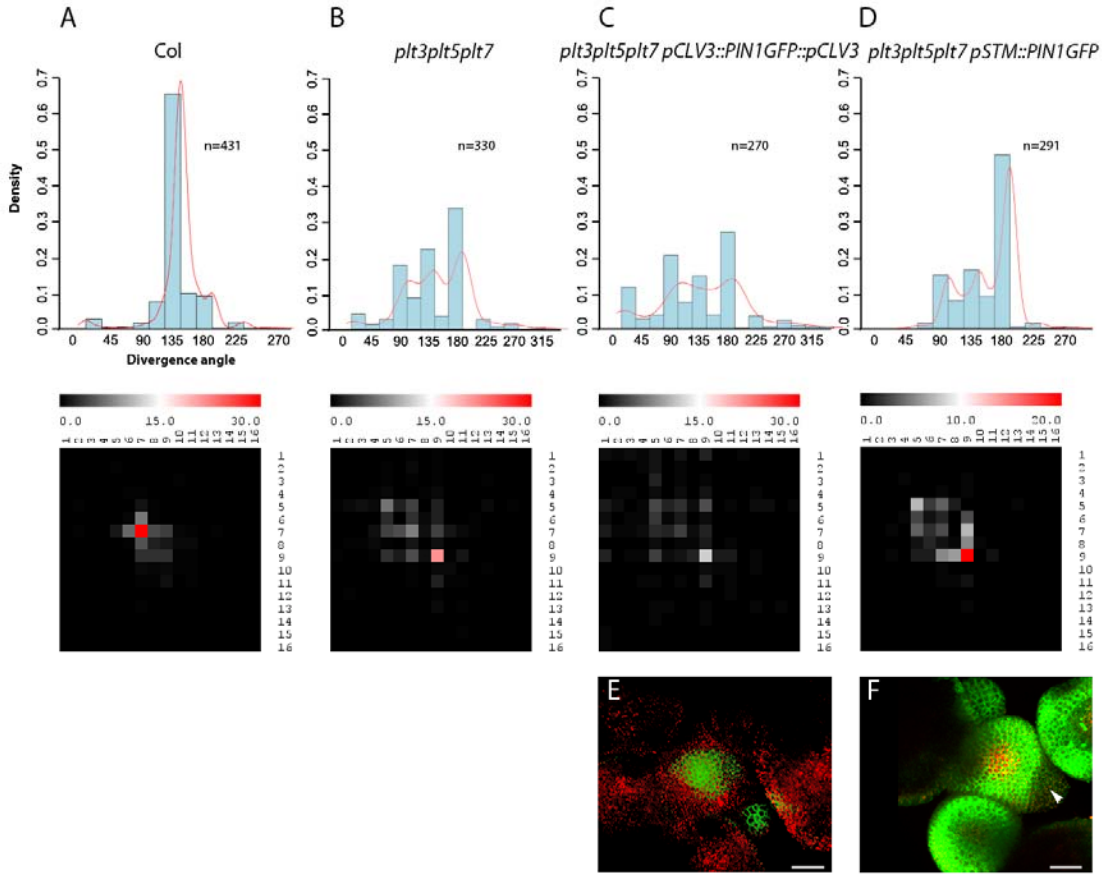


Figure 6. Expression of PIN1::GFP in the centre of the meristem does not complement *plt3plt5plt7* phyllotaxis defect. (A-D) Silique divergence angle distribution (upper panels), and corresponding silique divergence angle pattern distribution (lower panels) in Col (A); *plt3plt5plt7* (B); *plt3plt5plt7 pCLV3::PIN1GFP::pCLV3* (C); *plt3plt5plt7 pSTM::PIN1GFP* (D). (E-F) Expression pattern of pCLV3::PIN1GFP::pCLV3 (E) and pSTM::PIN1GFP (F) constructs generated in *plt3plt5plt7* background using confocal laser scanning microscopy. The arrow head on the confocal surface projection of *plt3plt5plt7 pSTM::PIN1GFP* inflorescence meristem points to an organ primordium where PIN1 is downregulated, as expected using the *STM* promoter. n : number of divergence angles. Scale bars represent 20 µm.

In Prasad et al. (2011), we previously postulated that *PLT* genes control phyllotaxis partially through the regulation of *PIN1* expression at the incipient primordia. Nevertheless our domain specific complementation assays clearly point to a function for PLTs in the centre of the meristem. The auxin efflux carrier PIN1 is also expressed in the meristem centre where it might play an important role in auxin redistribution through the apex. However using *CLV3* and *STM* promoters to express *PIN1* in the SAM did not complement the *plt3plt5plt7* phyllotaxis phenotype. Therefore PLT's control of phyllotaxis does not involve positive regulation of PIN1 expression in the centre of the meristem. As postulated above the auxin pool might be low in *plt3plt5plt7* SAMs, resulting in reduced auxin accumulation at incipient primordia that is nevertheless sufficient to drive organ inception. This reduction would impact PIN1 expression within a primordium. In this way the decreased expression of *PIN1* in *plt3plt5plt7* primordia might be an indirect consequence of low auxin production at the

apex. In agreement with this idea, *plt3plt5plt7* mutants interact synergistically with *pin1*/+ [1], and similarly the *pin1* phenotype is enhanced by *yuc1yuc4* mutations [32].

4/ PLETHORAs are involved in the maintenance of the stem cell niche.

As PLT activity is required in the centre of the meristem to control phyllotaxis, I investigated the meristem structure of *plt3plt5plt7* by analysing the cell-type specific fluorescent markers *WUS*, *CLV3*, *STM* and *ANT*. The CZ size of the meristem is reduced in the triple mutant *plt3plt5plt7* (Figures 7A and B). At vegetative stage the size of pWUS::GUS::pWUS expression domain was decreased (Figure 7A). At reproductive stage *WUS* expression domain was reduced by 20% through longitudinal confocal sections in *plt3plt5plt7* inflorescences (Figure 7A lower panels; Figure 8A). Also the number of cells marked with pWUS::erCFP::pWUS in the median transverse confocal section were reduced by 35% (Figure 8B). The pCLV3::erCFP::pCLV3 longitudinal domain was reduced by about 35% in *plt3plt5plt7* triple mutant (Figure 7B; Figure 8C). Furthermore expression levels of *WUS* and *CLV3* were reduced in vegetative *plt3plt5plt7* apices (Figures 7C and D), however no obvious changes in the expression level or domain of an *STM* reporter gene were observed (Figure 7C; Figure 8E). Using an inducible *PLT5* expression system [1], I observed an increased of *WUS* expression after four hours of *PLT5* induction, however no change in *STM* expression was seen after four and eight hours of *PLT5* induction (Figures 8F and G). Those data suggest that PLT5 can act rapidly and specifically on *WUS* expression level, and then on the size of the CZ. Although *PIN1* expression is reduced at sites of incipient primordia in *plt3plt5plt7* [1], I did not observe any defect in the accumulation of pANT::H2B::VENUS marker in lateral organs (Figure 7D).

These results show that PLT activity is required to maintain the size of *CLV3* and *WUS* domains, and overall meristem and primordium organization are not severely affected in *plt3plt5plt7* triple mutant.

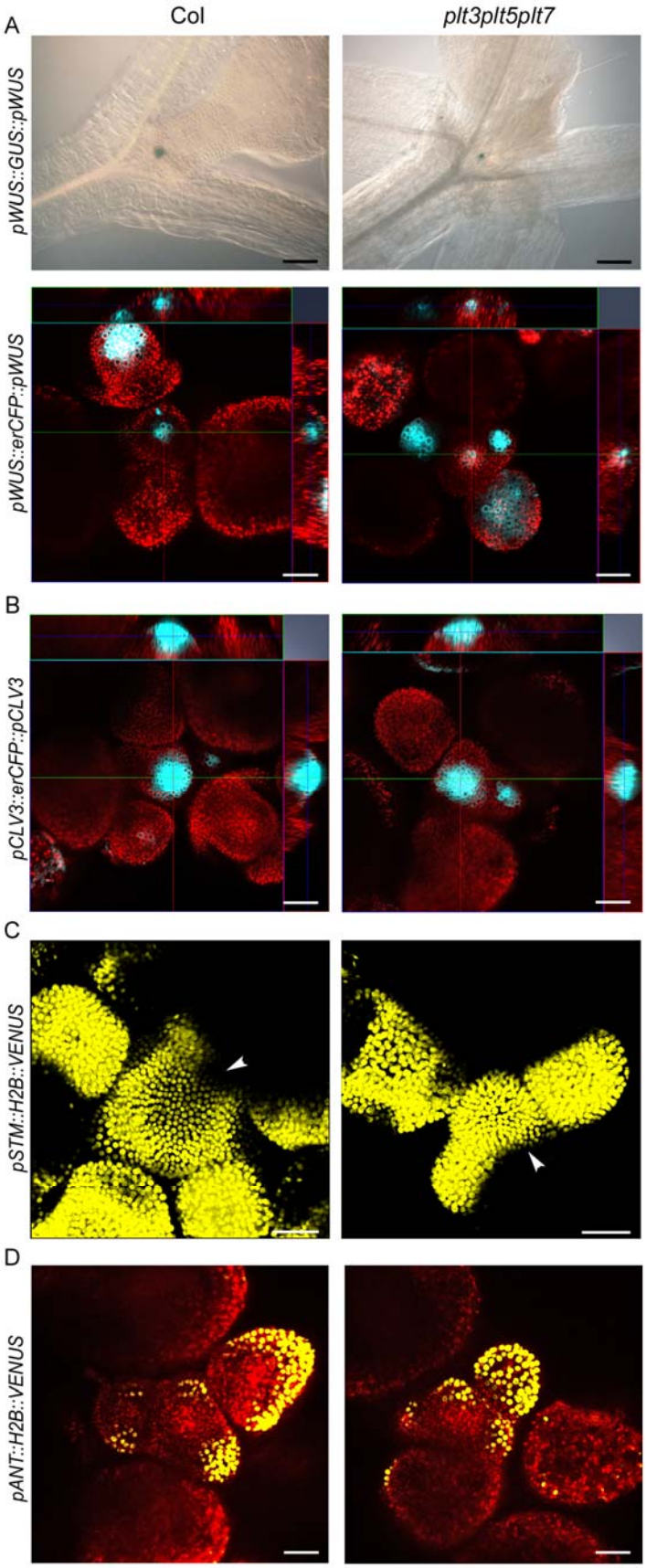


Figure 7. *PLT3*, *PLT5* and *PLT7* genes are required to maintain the size of the stem cell niche. (A) The upper panels display 10 days old (do) seedlings expressing pWUS::GUS::pWUS in Col and *plt3plt5plt7* backgrounds. The lower panels show confocal surface projections of Col and *plt3plt5plt7* inflorescence meristems expressing pWUS::erCFP::pWUS. (B) Confocal surface projections of inflorescence meristems expressing pCLV3::erCFP::pCLV3 in Col and *plt3plt5plt7* mutant. (C) Confocal 3D projections of Col *pSTM::H2B::VENUS* and *plt3plt5plt7 pSTM::H2B::VENUS* inflorescence meristems. (D) Confocal surface projections of meristem expressing pANT::H2B::VENUS in Col and *plt3plt5plt7* mutant. The vertical red lines in (A-B) confocal scanning micrographs mark the orientation of the longitudinal optical sections presented in the above attached panels. The horizontal green lines show the orientation of the longitudinal optical sections attached on the right panels. The arrow heads in (C) display early flower primordia where pSTM::H2B::VENUS protein fusion is downregulated. The red channel in (A), (B) and (D) visualizes autofluorescence. Scale bars represent 100 µm in (A) upper panels, 25 µm in (A) lower panels and in (B-D).

A

A: number of transverse sections with CFP signal in the inflorescence meristem
B: number of cells with CFP signal in the median transverse section of the inflorescence meristem

	A	B
Line1	12	13
Line1	15	6
Line1	9	18
Line2	13	15
Line2	11	18
Line3	13	14
Line4	17	11
Line4	13	17
Line4	13	13
Average	12,89	13,89

Col *pWUS::erCFP::pWUS*
p-value for A:: 0,005
p-value for B: 0,01

	A	B
Line1	9	14
Line1	9	10
Line1	9	15
Line2	10	4
Line3	10	7
Line3	11	13
Line3	13	7
Line3	8	6
Line4	12	7
Line4	10	6
Average	10,1	8,9

plt3plt5plt7 pWUS::erCFP::pWUS

B

	A
Line1	17
Line1	20
Line1	16
Line1	13
Line2	16
Line2	17
Line3	15
Line3	13
Line3	11
Av.	15,33

Col *pCLV3::erCFP::pCLV3*
p-value for A: 0,0001

	A
Line1	9
Line1	9
Line1	9
Line1	10
Line2	10
Line2	11
Line3	13
Line3	8
Line3	12
Av.	10,11

plt3plt5plt7 pCLV3::erCFP::pCLV3

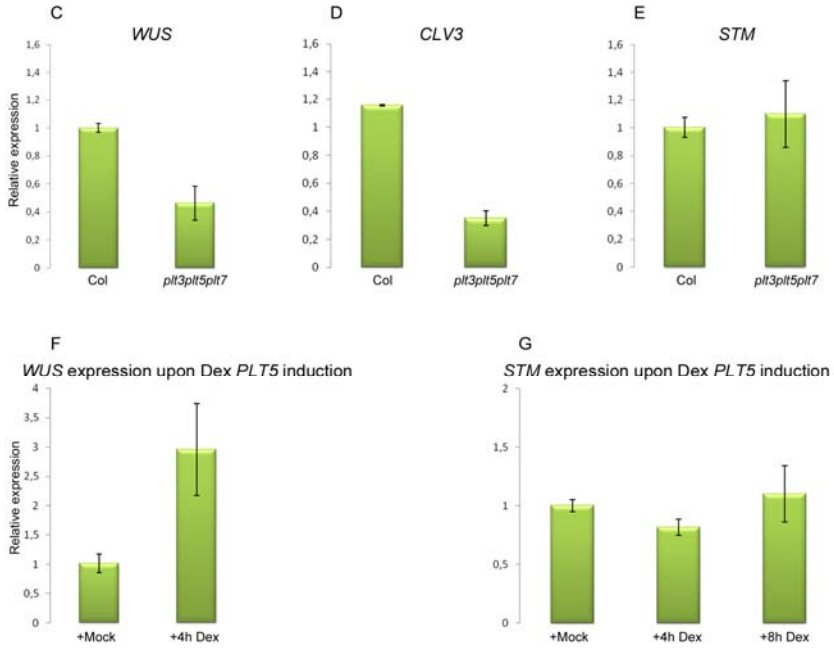


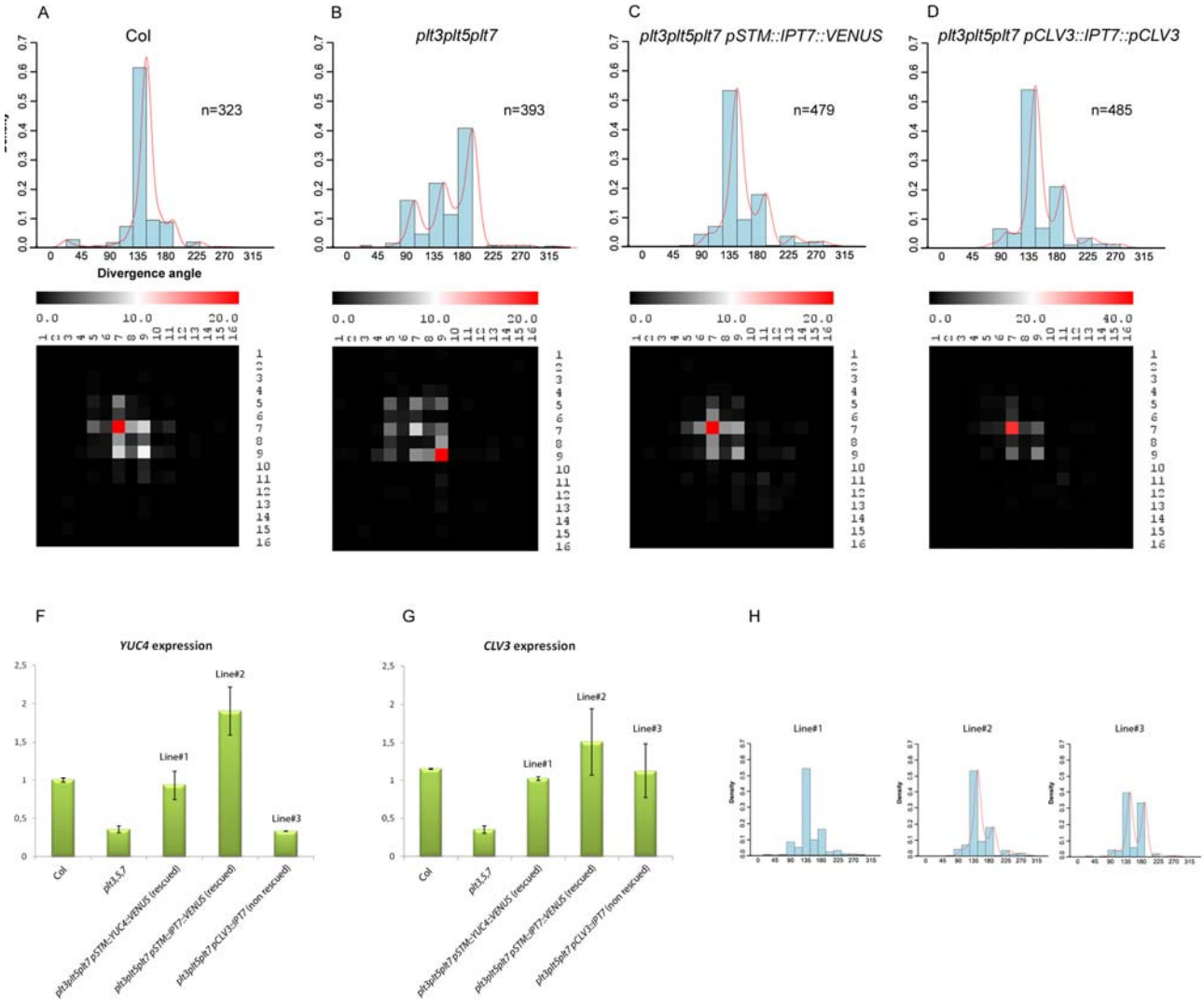
Figure 8. *PLT* genes are required to maintain the size of the stem cell niche. (A) Analysis of confocal scanning micrographs through the z axis of inflorescence meristems in individuals of 4 independent lines of Col *pWUS::erCFP::pWUS* and *plt3plt5plt7 pWUS::erCFP::pWUS*. For each line was counted the number of transverse scanning micrographs with CFP signal in the inflorescence meristem (column A) and the number of cells with CFP signal in the median transverse section of the inflorescence meristem (column B). (B) Based on the confocal scanning projections through the inflorescence meristems of Col *pCLV3::erCFP::pCLV3* and *plt3plt5plt7 pCLV3::erCFP::pCLV3* independent lines, the number of transverse sections with CFP signal was deducted (column A). ANOVA statistical analyses were performed, and p-values < 0.05 were considered to be

significant. (C-E) Expression levels of *WUS*, *CLV3* and *STM* in Col and *plt3plt5plt7* dissected 10 do vegetative shoot apices, measured by quantitative RT-PCR. Error bars represent the standard error from 3 independent experiments.

5/ Cytokinin biosynthesis in the centre of the meristem can also rescue *plt3plt5plt7* phyllotaxis defect.

As *plt3plt5plt7* mutants display a smaller CZ than wild type plants (Figures 7 and 8), and that changes in meristem size are often correlated with phyllotaxis defects [24, 25], I investigated if *plt3plt5plt7* phyllotaxis phenotype could be restored to wild type by increasing the size of the CZ. Signalling through the hormone cytokinin controls the size of the CZ [33]. Increasing specifically cytokinin production, and by then CZ size, can be achieved by expressing the cytokinin biosynthetic gene *ISOPENTENYLTRANSFERASE 7* (*IPT7*) [34, 35]. I used this strategy by expressing *IPT7* under *STM* and *CLV3* promoters in *plt3plt5plt7* and found that both constructs can restore a stable spiral pattern in *plt3plt5plt7* (Figures 9A to D; Figure 9I). I therefore conclude that increasing specifically auxin or cytokinin production in the meristem centre, can trigger a switch back from the distichous pattern, highly stable in *plt3plt5plt7*, into the spiral pattern prevalent in wild type. This result raises the following question: are the rescues of *plt3plt5plt7* phenotype by auxin and cytokinin production due to higher auxin levels, to an increase in meristem size, or both?

To answer this question I conducted an expression analysis of *YUC4* to estimate rate of auxin production, and *CLV3* to estimate the size of the stem cell pool in *plt3plt5plt7* plants showing varying degrees of complementation from the *pSTM::YUC4::VENUS*, *pSTM::IPT7::VENUS* and *pCLV3::IPT7::pCLV3* constructs. Dissected shoot apices from 10 days old seedlings from the fully rescued lines *plt3plt5plt7 pSTM::YUC4::VENUS* and *plt3plt5plt7 pSTM::IPT7::VENUS* showed wild type or higher expression levels of *YUC4* (Figures 9F to H Line#1 and Line#2). However the line *plt3plt5plt7 pCLV3::IPT7::pCLV3*, which does not show a full complementation (Figure 9H Line#3), displays reduced *YUC4* transcript levels, similar to *plt3plt5plt7* mutant (Figure 9F Line#3). Therefore *YUC4* expression level correlates with the degree of rescue of the selected lines.



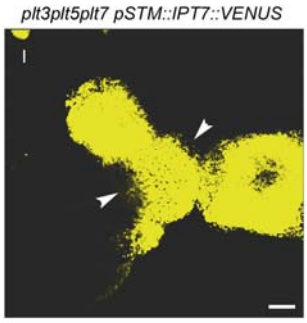


Figure 9. Increasing cytokinin biosynthesis in the centre of the meristem is sufficient to rescue *plt3plt5plt7* phyllotaxis defect. (A-E) Distribution of silique divergence angles (upper panels), and corresponding silique divergence angle pattern distribution (lower panels) in Col (A); *plt3plt5plt7* (B); *plt3plt5plt7 pSTM::IPT7::VENUS* (C); *plt3plt5plt7 pCLV3::IPT7::pCLV3* (D); *plt3plt5plt7 pFIL::IPT7::VENUS* (E). (F-H) Expression levels of *YUC4* (F) and *CLV3* (G) measured by quantitative RT-PCR in Col; *plt3plt5plt7* ; *plt3plt5plt7 pSTM::YUC4::VENUS* line with a distribution of silique divergence angles similar to wild type (H, Line#1); *plt3plt5plt7 pSTM::IPT7::VENUS* line with a distribution of silique divergence angles similar to wild type (H, Line#2); and *plt3plt5plt7 pCLV3::IPT7::pCLV3* line with a distribution of silique divergence angles closer to *plt3plt5plt7* (H, Line#3) using 10 do dissected vegetative shoot apices. (I) Expression of pSTM::IPT7::VENUS in *plt3plt5plt7* meristem inflorescence. The arrow heads point to incipient flower primordia marked by a depletion of pSTM::IPT7::VENUS. Error bars show the standard error from 3 independent experiments. n: number of silique divergence angles. Scale bar represents 20 µm.

The phyllotaxis phenotype of *plt3plt5plt7* is fully complemented by the expression of *IPT7*, which can increase cytokinin biosynthesis, under shoot meristem specific promoters. Cytokinin production is known to positively regulate the size of the stem cell niche: therefore *plt3plt5plt7* complementation might be due to an increase of the meristem size. However the above results indicate that the degree of *plt3plt5plt7* complementation does not correlate with increased central zone size (according to *CLV3* expression as a stem cell marker), but with the level of *YUC4* expression, thus with auxin production. It has been shown that overexpression of *IPT* genes leads to increased auxin biosynthesis [36]. Here I also showed that expression of *IPT7* in the SAM induces an increase of *YUC4* expression level, independently of PLTs (Figure 9F). Moreover *CLV3* expression level in the rescued line *plt3plt5plt7 pSTM::YUC4::VENUS* is similar to wild type, suggesting that auxin, in parallel to its role in organ formation and patterning, is involved into a feedback loop with cytokinin activity, in the meristem centre, to couple lateral organogenesis with stem cell activity. Auxin has been implicated in promoting WUS expression through CK signalling [29]. Furthermore Yoshida et al. (2011) demonstrated that light activates meristem activity by inducing cytokinin signalling, which correlates with increased auxin biosynthesis in the central zone of the meristem. Together those data suggest that auxin concentration is sensed in the SAM to control stem cell niche size. *PLT* genes, via their regulation of auxin biosynthesis, might indirectly affect cytokinin activity or synthesis in the centre of the meristem, and therefore the size of the stem cell niche. Both events might contribute to the establishment of phyllotaxis. However, PLT-mediated control of auxin biosynthesis in the meristem centre is a critical process for phyllotaxis, as lines with rescued level of *CLV3* expression, but with low *YUC4* RNA levels, do not complement the phyllotaxis defect. This work, for the first time, assigns a role for local auxin production in controlling phyllotaxis and in coordinating the balance between stem cell niche and organogenesis.

Local auxin biosynthesis controls many developmental processes as demonstrated by the defects observed in *yuc* mutants in floral organs, vascular tissue development, embryogenesis, leaf formation, and gynoecium patterning [29]. The molecular mechanisms regulating patterns of auxin biosynthesis are hitherto not well understood. This study now implicates the PLT transcription factors in the control of auxin biosynthesis at the shoot apex and reveals its importance in the regulation of phyllotaxis. Until now mathematical models of auxin-dependent processes such as phyllotaxis mainly focus on polar auxin transport. My work shows that the quantity and the localization of auxin production can orchestrate organ distribution at the shoot apex, and it will be interesting to implement local auxin biosynthesis into phyllotaxis models.

Project Impact

The aim of the European Research Area is to invest in innovation and knowledge for a competitive knowledge-based EU economy, to increase mobility for career development and to encourage young scientists to take up positions within Europe.

The "PLT" project certainly contributes to European excellence and competitiveness as understanding phyllotaxis in the genetic plant model *Arabidopsis* means understanding evolution and establishment of plant morphogenesis. Although research on apical meristem function is fundamental and falls under the scope of systemic biology, it certainly has agricultural relevance. For instance the fine tuning of the mechanisms controlling plant architecture can be utilized to optimize plant morphology for agricultural purposes. Manipulating auxin biosynthesis specifically at the shoot apex of agricultural species can potentially modify organ patterning, as in *Arabidopsis*. Such changes might be relevant to optimize fruit harvesting for example. Indeed if fruits or vegetables are arising opposite to each other, they will form ordered rows, allowing efficient harvesting. This knowledge can also contribute to the elaboration of sophisticated virtual plant models which may be useful in obtaining production forecasts (biomass, fruit, seed) for different conditions. Therefore the results of this project will be of a general interest not only in the European community but internationally, contributing to an increase in the science European leadership.

During the project I acquired new expertise in analyzing live and *in planta* gene expression, utilizing a cutting-edge technique where the principal expertise is in the US (Prof. Meyerowitz's laboratory), but which is starting to be developed in Europe, notably in a group in France (Prof. Jan Traas) and in Germany (Dr Marcus Heisler). This new technique is essential to understand the dynamics of non-linear gene networks, which is not possible by using classical genetic approaches. Therefore it is in the interest of the European Research Area to promote the implementation of such a technique in order to maintain its competitiveness.

I have been working in three different European countries during my different trainings in research, to sharpen my mobility and adaptability capacities. Thanks to this project, funded through the 7th framework program, I could share experiences and establish connections with a large number of international students and post doctoral fellows in the host group. The project, as proposed two years ago, sheds light on the understanding of plant architecture and stem cell maintenance by using cutting edge techniques, all contributing to a complete training toward an independent career and to fulfill the objectives of Intra European Fellowship.

During the time of the fellowship I also had the opportunity to communicate the results of the project in national and international conferences during oral presentations. This experience was essential to

create connections with other groups sharing similar research interest, and to disseminate the outcome of this European funded project.

Oral presentations that I have been given during the fellowship (dissemination activities):

-ALW Meeting Experimental Plant Sciences 2012. Lunteren, The Netherlands.

Authors: **V. Pinon**, K. Prasad, and B. Scheres. Title: "PLETHORA transcription factors control auxin biosynthesis in the centre of the shoot meristem to regulate phyllotaxis in *Arabidopsis*."

Type of audience: Plant Biology community of the Netherlands with European keynote speakers.

Size of audience: about 300.

-Plant Growth Biology and Modeling 2011. Elche, Spain.

Authors: V. Pinon, K. Prasad, S.P. Grigg, and B. Scheres. Title: "Unravelling PLETHORA transcription factors function in the control of phyllotaxis in *Arabidopsis*."

Type of audience: International Plant Biologists involved in Systems Biology projects.

Size of audience: about 200.

-ALW Meeting Experimental Plant Sciences 2012. Lunteren, The Netherlands.

Authors: **V. Pinon**, K. Prasad, S.P. Grigg, and B. Scheres. Title: "Control of phyllotaxis by PLETHORAs: PIN1 auxin transport or / and shoot meristem regulation?."

Type of audience: Plant Biology community of the Netherlands with European keynote speakers.

Size of audience: about 300.

Scientific (peer reviewed) publications realized during the project:

-V. Pinon, K. Prasad, G.F. Sanchez-Perez, S.P. Grigg, R. Heidstra and B. Scheres. **2012**. Local auxin biosynthesis regulation by PLETHORA transcription factors controls phyllotaxis in Arabidopsis. Preparation for submission in Genes & Development.

- K. Prasad**,** S. P. Grigg**,** M. Barkoulas**,** R. K. Yadav**,** G. F. Sanchez-Perez**, V. Pinon,** I. Blilou**,** H. Hofhuis**,** P. Dhonukshe**,** C. Galinha**,** A. P. Mähönen**,** W. Muller**,** S. Raman**,** A. J. Verkleij**,** B. Snel**,** G. V. Reddy**,** M. Tsiantis and B. Scheres. **2011**. *Arabidopsis* PLETHORA transcription factors control phyllotaxis. **Current Biology**, 21, 13:1123-1128.

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