A PRELIMINARY STUDY ON ROOT-TO-SHOOT REGENERATION BY ECTOPIC EXPRESSION OF *WUSCHEL* IN *ARABIDOPSIS THALIANA* ROOTS

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NATIONAL UNIVERSITY OF SINGAPORE

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Summary

In plants, new organs and tissues generate from the meristems. The two main meristems located in root apices and shoot apices, namely the shoot apical meristem (SAM) and the root apical meristem (RAM), orchestrate the balance between cell differentiation and cell division with related regulators. For example, the homeodomain protein WUSCHEL (WUS) and its counterpart WOX5 are responsible to maintain the stem cell potency in the SAM and RAM, respectively. *WUS* is first expressed in the 16-cell embryo within the region that will develop into embryonic shoot. Ectopic expression of *WUS* has been shown to induce somatic embryogenesis, indicating that *WUS* can promote the embryonic identity. Intriguingly, when expressed in the root, *WUS* induces shoot stem cell identity and leaf development (without additional cues), floral development (together with LEAFY), or embryogenesis (in response to increased auxin), suggesting that *WUS* establishes stem cells with intrinsic identity.

To elucidate the mechanism underlying stem cell formation and regeneration in plants, we developed a tissue/cell-specific GAL4-GR (GVG)-UAS inducible system to ectopically express WUS in the *Arabidopsis* root. UAS::WUS lines have been generated and crossed with tissue/cell-specific drive lines including pSCR::GVG, pWOX5::GVG, pPIN2::GVG, and pADF5::GVG. Thus, upon DEX application, WUS expression can be ectopically induced in specific root tissues/cells. Our induction experiments showed that, with inducible expression of WUS in pADF5::GVG-UAS::WUS and pADF5::GVG-UAS::WUS-mCherry lines, seedlings induced by DEX for 6 days exhibited a new cluster of stem cells in the root cap region. This formation of the new cluster of stem cells also abolished the root cap cell identity.

Moreover, extended induction of *WUS* expression in the SCR-expressing root endodermis induced leaf formation from the position of lateral roots or at the basal end of lateral roots,

suggesting the involvement of a lateral root development program. In order to test whether ectopic expression of *WUS* in endodermis is sufficient to induce regeneration, we made an artificial J shape of pSCR::GVG-UAS::WUS roots. After 4days of induction with J-shape roots, more leaf primordia formed at the curve of the J shape roots. The lateral root primordia development was also examined with or without induction of *WUS* in endodermis. Our results indicated that with induction of *WUS* in endodermis the lateral root primordia development became different since stage III due to the extra cell divisions in the *WUS*-inducible lines. In addition, the epidermis, cortex, and endodermis specific markers were introduced in the pSCR::GVG-UAS::WUS line. The ectopic expression of *WUS* in endodermis led to extra cell divisions in endodermis, cortex, and epidermis at somewhat extent. Out data also indicated some cells in the cortex lost their identity due to ectopic expression of WUS in endodermis. In the future studies, fluorescence activated cell sorting and microarray assay will be used to unearth the changes in epidermis, cortex, and endodermis cell layers and reveal the molecular framework for leaf regeneration in *Arabidopsis* roots.

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LIST OF ABBREVIATIONS

A	adenosine
AD	activating domain
BD	binding domain
bp	base pair(s)
C-terminal	carboxyl terminal
C	cytidine
CDS	coding sequence
CFP	cyan fluorescent protein
DEX	dexamethasone
DNA	deoxyribonucleic acid
dNTP	deooxyribonucleoside triphosphate
FACS	Fluorescence Activated Cell Sorting
g	grams or gravitational force
G	guanosine
GFP	green fluorescent protein
GR	glucocorticoid receptor
GUS	beta-glucuronidase
h	hour(s)
kb	kilobase(s) or 1000 bp
M	molar
MES	2-[N-morpholino] ethanesulfonic acid
mg	milligram(s)
µ	micro-
µm	micrometer
min	minute(s)
ml	milliliter(s)
mM	millimole
N	any nucleoside
N-terminal	amino terminal
Oligo	oligodeoxyribonucleotide
PPT	phosphinothricin
RFP	red fluorescent protein
RNA	ribonucleic acid
rpm	revolutions per minute
s	second(s)
T	thymidine
UAS	Upstream Activation Sequence
µl	microliter(s)
w/v	weight per volume
wt	wild type
YFP	yellow fluorescent protein

Chapter 1 Introduction

1.1 Overview of regeneration

1.1.1 Regeneration in polyps

In 18th century, it was believed that only plants and several microscopic animals have the ability of regeneration. A scientist named Abraham Trembley discovered, over 260 years ago, that the freshwater polyps could grow into two new polyps if the old polyp was cut into two pieces (Trembley, 1744). This study clearly indicated that the freshwater polyps had the ability in regeneration.

1.1.2 Regeneration in other animal and plant system

The extraordinary discovery demonstrated by Trembley the ability of regeneration in polyps triggered studies in other species by Bonnet (Bonnet, 1779) and Spallanzani (Bonnet, 1779). The ability of regeneration among the metazoans, including earthworms, snails, newts and salamanders, was discovered. Investigations by Sachs (Sachs, 1893) and Goebel (Goebel, 1898) in late 19th century showed that the whole plants could be developed from the cleaved pieces of leaves of pansies and begonias.

1.1.3 Differences and similarities in regeneration between animal and plant systems

These studies suggested that there was a clear difference of regeneration in animal and plant kingdom, where in animals the regeneration process could replace the missed parts

while in plants a complete individual could arise from a piece of plant materials. Although there are differences in regeneration process, the research in plant and animal kingdoms still led to hypothesis that the underlying mechanisms controlling regeneration in plants and animals are probably conserved. The regeneration process must contain two basic processes: (1) acquisition of cellular competence to develop into new organs through cell dedifferentiation or by taking advantage of the previous totipotent cells; and (2) reorganization of the regenerative parts from the severed pieces (Birnbaum and Sanchez Alvarado, 2008). Therefore, it will be interesting to understand the precise steps of regeneration and the factors controlling these steps. Also, understanding the specific stages of the regenerative process both in plants and animals will help to explain the underlying mechanisms of regeneration in both kingdoms.

1.1.4 Difficulties in *in vivo* studies of regeneration

Despite intensive efforts for gaining a detailed knowledge of the principles of regeneration in multicellular organisms, little is known about the precise molecular and cellular basis controlling the regeneration process. This has mainly been due to an inability to carry out *in vivo* studies in the species that have traditionally been used to study regeneration. To overcome this shortcoming, diverse well-established model systems, such as zebrafish, chicks and mice, are currently being used thanks to recent methodological advances, and are beginning to reveal the forces that guide the regeneration and those that prevent it (Davenport, 2005; Sanchez Alvarado and Tsonis, 2006). These vertebrate systems, however, typically only have modest regeneration ability to replace certain missing tissues. In contrast, the ability to regenerate organs or even a whole plant is wide-spread in the plant kingdom,

including *Arabidopsis thaliana*, a small weed that now serves as a model for understanding approximately 250,000 other more complex plants.

1.2 Regeneration in the plant field

1.2.1 Root apical meristem and shoot apical meristem in plants

What will the world be if human did not exist? Certainly, the city would be replaced by plants and animals. Plants would grow in every corner they could. Plants face many kinds of problems: (1) the leaves can be eaten by animals; (2) the branch can break off the tree; (3) the roots may be cut off, etc. These undetermined conditions enforce that the plants must rely on an indeterminate body plan, which is that the number of plant organs is not predetermined, to generate responses to environment conditions (Dinneny and Benfey, 2008). In plants, there are mainly two kinds of meristem which are the proliferative tissues located at the growing apexes. In the shoot, the shoot apical meristem (SAM) is responsible to generate lateral organs such as leaves, flowers and stalk. The root apical meristem (RAM) plays a more specific role, generating differentiated cells which support the growth of the root. Both SAM and RAM are maintained by a specific population of stem cells located in the inner part of the meristematic regions. Stem cell niches constitute the microenvironment that maintains the stem cells by certain developmental signals and stem cell factors (Scheres, 2007). Although there are the structural differences of the shoot apical meristem and root apical meristem, the two share some common characteristics. In the *Arabidopsis* root tips, the stem cells that surround a small group of four organizing cells that rarely undergo cell division, termed the quiescent centre (QC) cells, give rise to distal (columella), lateral (lateral root cap and epidermis) and proximal (cortex, endodermis and stele) cell types. In shoot tips, a zone of three layers of stem cells which consists central zone and peripheral zone is maintained by an

underlying organizing centre (OC) which provides signals and acts as the control of stem cells.

1.2.2 Regeneration of a new root tip

What will happen if the whole root tip is cut off? Using the root-tip regeneration system in *Arabidopsis*, it was shown that respecification of lost cell identities began within hours after excision and that the function of the specialized cells was restored within one day (Sena et al., 2009). The quiescent centre, all surrounding stem cells along with several tiers of daughter cells, and root cap including all the columella and most of the lateral root cap were completely removed by standard excisions at 130 μm from the root tip. The new regenerative organs formed completely after 7 days of excision.

1.2.3 Regeneration of a new quiescent centre in root

Through laser-assisted ablation experiments, Ben Scheres' group has discovered the signaling roles of the quiescent centre in the root stem cell niche. One significant finding is that the existence of quiescent centre can inhibit the cell differentiation of the surrounding cells. Once the quiescent centre was laser ablated, the adjoining stem cells went into differentiation and an auxin maximum recovered and promoted the establishment of a distal organizer (Sabatini et al., 1999; van den Berg et al., 1997) and the surrounding stem cells triggered a local regeneration response which eventually led to the regeneration of a new root tip (Xu et al., 2006). Laser ablation of quiescent centre cells disrupted the flow and distribution of auxin in root tips. The new quiescent centre could be reoccurred in several days after the ablation of the previous quiescent centre. This regeneration study showed

important roles for the auxin-responsive *AP2/EREBP* (APETALA2/ethylene responsive element binding protein) family transcription factors *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) (Aida et al., 2004), and the GRAS family transcription factors *SCARECROW* (*SCR*) (Di Laurenzio et al., 1996) and *SHORTROOT* (*SHR*) (Helariutta et al., 2000) in root stem cell respecification and root regeneration, and provided a regeneration mechanism in which embryonic root stem cell factors respond to and stabilize the distribution of the key phytohormone, auxin, with roles in developmental patterning. Such feedback mechanisms between transcription factors action and auxin distribution may also occur during normal development (Blilou et al., 2005). Thus, like in animals, stem cell respecification and organ regeneration in *Arabidopsis* roots are achieved through the combinational activity of transcription factors. Notably, these transcription factors are members of two plant-specific families, and their activities are intimately linked to local accumulation of the plant hormone auxin, indicating that the exact pathways used to activate regeneration in plants and animals may be specific to each kingdom. Now, the issue arises to what extent regeneration mechanisms have been conserved among different plant organs.

1.2.4 Induced shoot buds from *in vitro* cell culture

The natural ability of roots from many species to form buds that develop into new shoots has been long recognized (Holm, 1925; Raju et al., 1966; Wittrock, 1884). New shoot buds can be induced from roots or root-derived explants (Gordon et al., 2007; Sugimoto et al., 2010; West and Harada, 1993), raising the question of how new organs with different cell lineages and tissue organization are generated within and functionally integrated with existing organs. To address this question, which is also central to regenerative studies in animals, it is important to explore the molecular and cellular mechanisms that underlie the pluripotency of

differentiated or partly differentiated plant cells which enables them to coordinate a new pattern of differentiation.

1.2.5 The structure of shoot apical meristem

The ability of self-renewed shoot meristem is necessary for plants which need repetitive initiation of shoot structures including flowers and leaves during plant development. Previous studies suggest that the shoot meristem is composed of three zones: (1) the central zone located at the shoot apex which contains undifferentiated stem cells that supplement the cells differentiated into primordia initiation cells; (2) a zone underneath (rib meristem) which forms the skeleton of the shoot axis; (3) the peripheral zone (flank meristem) in which leaf and flower primordia initiation occurs with rapidly cell division (Steeves and Sussex, 1989). There are three generative layers in the central zone of the *Arabidopsis* shoot meristem. The rib zone is the organizing centre (OC) of the shoot apical meristem which contains the stem cells that have the full ability to differentiate into the cells of the central zone. The *WUSCHEL* (*WUS*) gene encodes a homeodomain protein which expressed in the organizing centre (OC) (Gordon et al., 2007; Laux et al., 1996; Mayer et al., 1998). The *wus* mutants repetitively initiated defective shoot apical meristems, which led to only a few leaves and discontinued primordia initiation. This mutant eventually showed early terminated flowers. The flowers of this mutant were much fewer compared with wild type and developed into a single central stamen (Laux et al., 1996).

1.2.6 The *WUS/CLV* pathway

Several other mutants have shown the shoot apical meristem structure and function are disrupted. Nearly two decades ago, three genes were discovered with defects in shoot meristem: *CLAVATA1* (*CLV1*), *FASCIATA1* (*FAS1*) and *FASCIATA2* (*FAS2*) (Leyser and

Furner, 1992). The *clv1*, *fas1* and *fas2* mutants showed fasciated flat stems associated with enlarged shoot apical meristems and altered flower development and disrupted phyllotaxy. Flowers of *clv1* mutant not only had increased numbers of organs in all four whorls, but also had some additional whorls not found in wild type plants (Clark et al., 1993). Similar to *clv1* mutants, *clavata3* (*clv3*) mutants also showed enlarged shoot apical meristem even at early mature embryo stage and enlarged floral meristems and more flowers compared with wild type (Clark et al., 1995). The *CLAVATA2* (*CLV2*) gene encodes a receptor-like protein with leucine-rich repeats, and *clv2* mutants showed similar phenotypes with *clv1* mutants, indicating they are in the same pathway in regulating shoot apical meristems and organ development (Jeong et al., 1999; Kayes and Clark, 1998). The enlarged shoot apical meristems and floral meristems were due to mutations in these genes and accumulation of *CLV3*-expressing stem cells. The *CLV3* gene encodes a small peptide which is secreted into the extracellular space. This small peptide acts as a ligand for the *CLV1* receptor-like kinase and transducts the signal of communication between organizing centre and central zone (Clark, 1997; Fletcher et al., 1999; Laufs et al., 1998). The secreted peptide *CLV3* interacts with the *CLV1/CLV2* to maintain the size of the shoot apical meristem. The *CLV* pathway restricts *WUS* expression in the organizing centre to control the size of SAM. In this feedback pathway, WUS acts as a positive signal to maintain the undifferentiated state while the *CLV* acts as the negative signal to regulate the *WUS* expression (Baurle and Laux, 2005; Gross-Hardt and Laux, 2003; Wang and Fiers, 2010; Williams and Fletcher, 2005). Induction of *CLV3* expression rapidly downregulates *WUS* expression, which in turn causes the reduction in the expression of *CLV3* (Muller et al., 2006). Thus, the balance between *WUS* and *CLV3* maintained the size of shoot apical meristem and the balance among the pool of stem cells.

1.2.7 Regeneration caused by ectopic expression of *WUS*

Ectopic induction of *WUS* expression in *Arabidopsis* root tips can induce shoot stem cell identity and leaf development (without additional cues), flower development (together with *LEAFY*, which is a key regulator of flower development (Wagner et al., 2004; Weigel and Nilsson, 1995), or embryogenesis (in response to increased level of auxin) (Gallois et al., 2004). A Cre-*lox*P-based mosaic expression system was used to induce *WUS* expression in *Arabidopsis* root tips in a manner of non-cell-autonomous effects of *WUS* (Gallois et al., 2002; Mayer et al., 1998). The Cre recombinase which is controlled by a heat-shock promoter catalyzed excision of a β-glucuronidase (*GUS*) reporter gene to activate *WUS* expression from the widely expressed 35S promoter. After heat shock, *WUS* expression could be detected in the *Arabidopsis* root tips. The ectopic expression of *WUS* induced *CLV3* expression in the root, but the expression of two genes did not coincide in the same cell, suggesting that the ectopic expression of *WUS* in root tips induced the shoot stem cell identity. The root meristem region was disorganized, indicating the ectopic expression of *WUS* affected other cells. After three or four days post heat-shock induction, *AINTEGUMENTA* (*ANT*, a marker for shoot organ primordia (Elliott et al., 1996) was also detected, suggesting these cells display the shoot stem cell identity (Gallois et al., 2004).

1.2.8 Regeneration caused by ectopic expression of other transcription factors

The *CLASS III HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIP III*) transcription factors acts as master regulators of embryonic apical fate, and ectopic expression of *HD-ZIP III* transcription factors is sufficient to induce a second shoot pole in the root pole region (Smith and Long, 2010). Ectopic expression of a stable version of *REVOLUTA* (*REV*, a *HD-ZIP III* transcription factor (Talbert et al., 1995) under the promoter of *PLETHORA2* (*PLT2*) was able to initiate another shoot pole in the root pole region. Similarly, ectopic expression of

some other *HD-ZIP III* transcription factors, *ICU4* and *PHB*, also induced a second completed shoot pole (Smith and Long, 2010).

1.2.9 Regeneration in Alfalfa

Regeneration is commonly found in several diploid Alfalfa systems: *Medicago littoralis* (Zafar et al., 1995), *M*edicago *lupulina* (Li and Demarly, 1995), *M*edicago *murex* (Iantcheva et al., 1999), *M*edicago *polymorpha* (Iantcheva et al., 1999), *M*edicago *truncatula* (Iantcheva et al., 1999; Nolan et al., 1989; Trieu and Harrisson, 1996). Alfalfa is the most important legume forage crop, cultivated across worldwide (Iantcheva et al., 2001). Regeneration in *Medicago truncatula* can arise from the potential explant tissues in liquid media via direct somatic embryogenesis. A rapid transformation and regeneration method in *Medicago truncatula* was developed and increased the production dramatically. With 12-day cultivation of cotyledonary explants on regeneration medium, shoots began to arise from the cut face of the explants (Iantcheva et al., 2001).

The dramatic regeneration ability in medics raises a question: why do these plants need this regeneration to reproduce the mother plant? One possible explanation is this regeneration method provides protection in the environment at their early development stages and maintains the productivity of the species.

1.2.10 Regeneration in Poplar

The long generation time and seasonal dormancy in Poplar have restricted the demand of biomass production and wood industry. However, the genetic engineering method has offered a short-term breeding program. Through in vitro treatment, micropropagation by proliferation of axillary buds (Peternel et al., 2009; Whitehead and Giles, 1977) or meristem-tip culture (Rutledge and Douglas, 1988) has been reported in several poplar species. *Populus*

balsamifera, commonly known as *P. balsam*, is highly flood-tolerant and is able to form adventitious roots within a few days of a flood. This trait helps it adapt to fire in a forest and it has the ability to generate sprouts from roots, stumps, and buried branches. The dramatic regeneration ability in Populus is quite important for the adaption and reproduction of *Populus*.

Over-expression of a *Populus* Class 1 *KNOX* homeobox gene, *ARBORKNOX1 (ARK1)*, which is orthologous to Arabidopsis *SHOOT MERISTEMLESS (STM)*, or STM in the cell cultures developed well-defined shoots with leaves (Groover et al., 2006). Due to the over-expression of *ARK1* or *STM* which promotes meristematic cell fate and delay terminal differentiation in both the SAM and the cambium, ectopic meristems forming on the adaxial side of leaves, inhibition of leaf development, shortening of internode lengths, and delay of the terminal differentiation of daughter cells derived from the cambium were found in the over-expression *Populus*.

Why does *Populus* need the ability to regenerate in the roots? One obvious advantage is that the generation time shortens due to no seed dormancy. The regeneration breeding method has dramatic agricultural importance. New seedlings arise from a piece of the mother plant, and regenerate into new plants. In agriculture, rapid breeding from pieces of potatoes guarantees the mass production. This rapid breeding method can also help survive from some environmental disaster. Fire or storm might destroy the stem of plants, but the regenerative ability from the roots could facilitate the fast growth of new plants.

1.3 Aims of this study

Though common in nature, the precise underlying mechanisms of regeneration is largely unknown. Through *in vitro* cell culture, new seedlings could arise from the explant tissues in many *Medicago* and *Populus*. With continuing over-expression of *STM* or *ARK1* in *Populus*, new ectopic meristems form in the explants through *in vitro* culture.

Ectopic expression of *WUS* in the *Arabidopsis* root could induce shoot stem cell activity (Gallois et al., 2004). However, it is not clear how *WUS* can overwrite the existing root developmental program even in the presence of master root regulators. Rational manipulation of *WUS* expression (tissue or cell-specific, inducible) in a root context will allow us to dissect at the cellular level how a particular developmental pathway can be initiated within the growing root tips.

To elucidate the mechanism underlying stem cell formation and regeneration in plants, we developed a tissue/cell-specific GAL4-GR (GVG)-UAS inducible system to ectopically express *WUS* in the *Arabidopsis* root. In this study, *WUS* will be cloned and introduced into *Arabidopsis*. The UAS::WUS line will then be crossed with non-inducible and inducible tissue or cell-specific lines. To understand the effect with ectopic expression of *WUS* in different tissues, the induction of *WUS* will be studied.

A combination of newly developed *in vivo* live imaging techniques, fluorescent marker lines, fluorescence activated cell sorting (FACS), microarray expression profiling, regenerative mutant analysis and computational modeling should help us gain a more comprehensive understanding of regeneration mechanisms in plants. In this study, *WUS*-inducible lines will be combined with the epidermis, cortex and endodermis specific markers, respectively.

Based on these studies, we will be able to address: (1) in which types of tissues or cells ectopic expression of *WUS* could induce shoot regeneration; (2) at which time point shoot regeneration could be started; and (3) how long the induction period of WUS would be required to induce shoot regeneration.

These studies will reveal novel aspects of root-to-shoot regeneration and the important roles of key stem cell factors in plant regeneration. In the future, the epigenetic pathways that control the initiation and reprogramming of regeneration process will be elucidated. The ability to manipulate tissue, organ or whole plant regeneration will allow us design novel

propagation techniques and molecular genetic breeding approaches for horticulture and agriculture applications. Moreover, the knowledge and information derived from this study and future studies can be further applied to parallel studies in animals and humans.

Chapter 2 Methods and materials

2.1 Plant growth condition

For all the experiments, *Arabidopsis thaliana* of ecotype Col-0 was used. The temperature for plant growth was 22℃, and the humidity for plant growth was 60%. The photoperiod was 16 hours of light and 8 hours of dark. Seedlings of 5 days-post-germination (dpg) were transferred into pots with soil and grown in the conditions above.

2.2 Seeds sterilization and plating

The seeds were sterilized in microfuge tubes with 10% of bleach for 10 to 15 minutes. During this period, the tubes were shaken three times and centrifuged at 5000 rpm for 30 seconds. After this, the seeds were washed three times by double-distilled water. Then, the seeds were stratified at 4℃ for two days.

After two days at 4℃, the seeds were transferred to Murashige and Skoog medium plate. 2.2 g of Murashige and Skoog medium, 0.5g of 2-(N-morpholino)ethanesulfonic acid and 10 g of sucrose were dissolved into 1 litre of double-distilled water. The pH of the solution was adjusted to 5.8 with 5M KOH. After this, 8 g of plant agar was transferred into the solution and the bottle was autoclaved at 121℃ for 20 minutes. When the medium was cooled to 60℃, it was poured into the growth plates to solidify.

After sowing, the plates were sealed with Parafilm and transferred into the cell culture room at 22℃ with a 16-h-light/8-h-dark photoperiod.

2.3 RNA extraction

Whole seedlings of 4 dpg were collected and 100 mg of plant materials were used. The materials were frozen with liquid nitrogen in the mortar and ground with pestle. The powder was transferred into tubes with 1 ml of TRIZOL reagent and homogenized. Following homogenization, insoluble materials were removed from the homogenate by centrifugation at 12,000 X g for 10 minutes at 2 to 8℃. RNA present in the supernatant was transferred into a new tube. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. The samples were added 0.2 ml of chloroform and shaken vigorously for 15 seconds and incubated at room temperature for 2 to 3 minutes. The samples were then centrifuged at 12,000 X g for 15 minutes at 2 to 8 ℃. RNA present in the upper aqueous phase was transferred into a new fresh tube, added with 0.5 ml isopropanol, incubated at room temperature for 10 minutes and centrifuged at no more than 12,000 X g for 10 minutes at 2 to 8℃. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube. The RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at no more than 7,500 X g for 5 minutes at 2 to 8℃. Finally, the RNA was dissolved in RNase-free water and stored at -80℃.

2.4 cDNA preparation

The RNA samples were reverse-transcribed into cDNA using Bio-Rad iScript^{TM} cDNA Synthesis Kits. The PCR tube was added by following ingredients:

5x reaction mix: 4 μl

RNA sample: 4 μl

iScript transcriptase: 1 µl

Nuclease-free water: 11 µl

The next protocol is:

5 minutes at 25℃,

30 minutes at 42℃,

5 minutes at 85℃,

10 minutes at 16℃.

The cDNA sample was tested by ACTIN (ACT) gene.

2.5 Plasmid construction

Generally, the genes of interest were cloned into pG2NBL-UASpt vector.

For WUSCHEL (WUS), the forward primer is

5'TTAAGCTTATGGAGCCGCCACAGCATCA3' and the reverse primer is

5'GAGGATCCCTAGTTCAGACGTAGCTCAA3'. The restriction enzyme sites are HindIII and BamHI respectively.

For BABYBOOM (BBM), the forward primer is

5'GGTCTAGAATGAACTCGATGAATAACTGG3' and the reverse primer is

5'GTGAGCTCCTAAGTGTCGTTCCAAACTG3'. The restriction enzyme sites are XbaI and SacI respectively.

For FASCIATA (FAS2), the forward primer is

5'GGTCTAGAATGAAGGGAGGTACGATACA3' and the reverse primer is

5'AAGAGCTCTCAGGGGTCAATAGCCATGG3'. The restriction enzyme sites are XbaI and SacI respectively.

For SHOOTMERISTEMLESS (STM), the forward primer is

5'GGTCTAGAATGGAGAGTGGTTCCAACAG3' and the reverse primer is

5'CGGAATTCTCAAAGCATGGTGGAGGAGA3'. The restriction enzyme sites are XbaI and EcoRI respectively.

For IAA30, the forward primer is 5'GATCTAGAATGGGAAGAGGGAGAAGCTC3' and the reverse primer is 5'GCGAATTCTCAGTAGTGATAAGCTCTTG3'. The restriction enzyme sites are XbaI and EcoRI respectively.

For AGAMOUS-LIKE 15 (AGL15), the forward primer is

5'GGTCTAGAATGGGTCGTGGAAAAATCGA3' and the reverse primer is

5'GCGAATTCCTAAACAGAGAACCTTTGTC3'. The restriction enzyme sites are XbaI and EcoRI respectively.

For CLAVATA3/ESR-RELATED 40 (CLE40), the forward primer is

5'GGTCTAGAATGGCGGCGATGAAATACAA3' and the reverse primer is

5'GCGAGCTCCTATGGAGTAAAAGGAATGT3'. The restriction enzyme sites are XbaI and SacI respectively.

In order to amplify the genes of interest, PCR was used. The ingredients for PCR are:

10 x Pfu buffer: 2 µl

dNTP: 0.5 µl

Primer forward: 0.5 µl

Primer reverse: 0.5 µl

cDNA template: 1 µl

Pfu enzyme: 0.5 µl

ddH2O: 15 µl

The protocol for PCR is:

95℃: 3 min

95℃: 45 s

55℃: 45 s

68℃: 2 min

35 cycles for step 2 to 4,

68℃: 10 min

16℃: 5 min

After PCR of the genes of interest, the amplified DNA was purified by QIAGEN QIAquick Gel Extraction Kit.

The purified DNA and pG2NBL-UASpt vector were then digested with double enzymes for 2 hours at 37℃ as follows:

Purified DNA or vector:	15 µl
10x Tango buffer:	3 µl
Enzyme 1:	1 µl
Enzyme2:	1 µl
ddH2O:	10 µl

After digestion for 2 hours, the inserted DNA and vector was purified by QIAGEN QIAquick Gel Extraction Kit respectively.

After purification, the inserted DNA and vector were ligated with Roche T4 DNA ligase for at least 1 hour at room temperature.

After ligation, the ligation product was transformed into 100ul of *E. coli* competent cells. The *E. coli* competent cells were incubated on ice for 10 minutes. The ligation product was added into competent cells and incubated on ice for 30 minutes. The competent cells were then incubated at 42℃ for 90 seconds and immediately added into ice for 2 minutes. After that, 400 ul of liquid Luria-Bertani broth was transferred into the tube. The E. coli was then incubated at 37℃ for 1 hour. The *E. coli* cells were transferred on the LB solid plate with 50 uM kanamycin and incubated for 16 hours at 37℃.

The colonies were confirmed by PCR, and the plasmids were verified by enzyme digestion and subsequently DNA sequencing.

2.6 *Agrobacteria* transformation and plant transformation

The verified plasmids were transformed into GV3101 *Agrobacterium* strains (Koncz et al., 1984; Zhang et al., 2006). The *Agrobacterium* colonies contained the verified plasmid were confirmed again by PCR and transferred into LB liquid with rifampicin, gentamicin and kanamycin. In a 500-ml flask, the *Agrobacterium* strain contained the plasmid were incubated at 28℃ for over 24 hours until the opitical density (OD) value is between 1.5 and 2.0. The *Agrobacterium* cells were collected centrifugation at 4,000 X g for 10 minutes at room temperature, and resuspended in one volume of freshly made 5% (w/v) sucrose solution. The sucrose solution was added with Silwet L-77 to a concentration of 0.02% and gently mixed well. The solution was transferred into a 500-ml beaker.

The wild type Col-0 plants at the stage of flowering were used to perform the plant transformation. The aerial parts of the plants were immersed into the beaker contained the *Agrobacterium* solution for 10 to 30 seconds. The dipped plants were then covered with black bags overnight. The plants were transferred into growth room for seeds. These seeds were the T0 generation of transgenic seeds and needed to be selected.

2.7 Plant selection

The T0 generation of transgenic seeds were sterilized by 10% bleach for 15 minutes and incubated at 4℃ for two days. The seeds were then transferred on the plates with 20 uM phosphinothricin (PPT or BASTA) and grown at 22℃ for 5 or 6 days. The seedlings survived were the transgenic plants of genes of interest. The T0 seedlings survived were transferred into pots.

The T1 seeds were harvested and selected again. T1 seeds were first grown on the plate without PPT for 3 days. After 3days, the T1 seedlings were transferred on the plates contained 20 uM PPT for one week. The seedlings contained the transgene survived, while the seedlings without the transgene dead. The ratio should be 3 to 1, since this indicated the insertion in this line was a single copy. The seedlings survived with a ratio of 3 to 1 were transferred into pots to harvest the T2 seeds.

The T2 seeds were harvested and selected by the same method as T1 seeds. All the seedlings of the homozygous lines survived. The homozygous lines were proliferated and stored.

2.8 Confocal microscopy

The seedlings were grown for several days (detailed in the legend of figures). The roots were stained with propidium iodide (PI) and transferred on the slides covered with a cover slip. The roots were then analysed by confocal microscopy.

2.9 DIC microscopy

The roots were cleared with clearing buffer for 10 minutes. Then, the roots were transferred on a slide and covered with a cover slip.

Chapter 3 Molecular cloning and generation of transgenic plants

3.1 The glucocorticoid inducible GAL4VP16-GR (GVG)/UAS system

It was previously shown that ectopic induction of *WUS* expression in the *Arabidopsis* root induces shoot stem cell identity and leaf development (without additional cues), flower development (together with *LEAFY*), or embryogenesis (in response to increased auxin concentration) (Gallois et al., 2004). In order to be able to track or monitor the initiation and implementation of shoot (leaf and flower) and embryo regeneration processes, a glucocorticoid inducible GAL4VP16-GR (GVG)/UAS system was used to ectopically express WUS.

GAL4/UAS system was first discovered in *Saccharomyces cerevisiae*. GAL4 is a positive regulatory gene in regulation of galactose catabolic enzyme (Klar and Halvorson, 1974). The GAL4/UAS system is a powerful technique which can be used similarly in all organisms. It is first developed by Andrea Brand and Norbert Perrimon in 1993 (Brand and Perrimon, 1993). This system can be used to express UAS-drived genes by the control of promoter of GAL4. This system has two separated components: the *GAL4* gene which encodes yeast transcription activator protein GAL4, and Upstream Activation Sequence (UAS) which functions as a promoter region and can be specifically bound by GAL4 to activate the desired gene expression. This system has the advantage of separation of two lines, by which two different lines can be developed separately and combined together by crosses. GAL4 is a modular protein containing two basic parts: DNA-binding domain (BD) and an activating domain (AD), while UAS is CGG-N_{11}-CCG, where N can be any DNA base (Campbell et al., 2008). Although GAL4 is a protein from yeast, which is not normally present in other organisms, it has been well used as a transcription activator in a variety of

organisms, such as *Drosophila* (Fischer et al., 1988), human cells (Webster et al., 1988), *Arabidopsis* (Aoyama and Chua, 1997), African clawed frog *Xenopus* (Hartley et al., 2002) and *Zebrafish* (Davison et al., 2007), indicating that the mechanisms of gene expression in evolution have been conserved.

In this system, it is often combined with a reporter gene. The UAS is fused with a green fluorescent protein (GFP), a red fluorescent protein (RFP), or beta-glucuronidase (GUS). This helps us monitor the gene expression pattern in the organisms.

In *Arabidopsis*, the GAL4/UAS system was modified as a new inducible system. The DNA-binding domain of the yeast transcription factor GAL4, the transactivating domain of the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor (GR) were constructed together as a chimeric transcription factor (Aoyama and Chua, 1997). In this system, induction of the UAS-drived gene expression needs application of the chemical dexamethasone (DEX), a strong synthetic glucocorticoid. The induction capacity depends on the concentration of DEX, ranging from 0.1μM to 10μM (Aoyama and Chua, 1997). This novel chemical induction system for transcription in plants allowed us to control the ectopic expression of *WUS* spatiotemporally. Different root cell/tissue-specific promoters were combined with the glucocorticoid inducible GAL4-VP16-GR transcription factor. Promoters of SCARECROW (SCR) (in root tips mainly expressed in endodermis and QC) (Di Laurenzio et al., 1996), WUSCHEL-related homeobox 5 (WOX5) (in root tips expressed in QC) (Sarkar et al., 2007), PIN-FORMED 2 (PIN2) (in root tips expressed in epidermis) (Muller et al., 1998) and ACTIN DEPOLYMERIZING FACTOR 5 (in root tips expressed in columella root cap) were fused with GAL4-VP16-GR transcription factor.

3.2 Generation of the UAS-drived constructs

In order to generate the ectopic induction lines, several genes have been cloned into the vector pG2NBL-UASpt. These genes include the shoot stem cell transcription factor WUSCHEL (WUS) (Mayer et al., 1998), Class I knotted-like homeodomain protein SHOOT MERISTEMLESS (STM) (Endrizzi et al., 1996), an AP2-domain containing protein BABY BOOM (BBM) (Galinha et al., 2007), a member of the Aux/IAA family of proteins INDOLE-3-ACETIC ACID INDUCIBLE 30 (IAA30) (Remington et al., 2004), Chromatin Assembly Factor-1 (CAF-1) p60 subunit FASCIATA 2 (FAS2) (Kaya et al., 2001), and a member of the MADS domain family of regulatory factors AGAMOUS-LIKE 15 (AGL15) (Heck et al., 1995).

First, the full length coding sequence (CDS) of WUS is amplified by *Pfu* enzyme from the total cDNA of whole wild-type *Arabidopsis* 4 dpg seedlings. The full length coding sequence of WUS is listed as below.

```
  1 ATGGAGCCGC CACAGCATCA GCATCATCAT CATCAAGCCG ACCAAGAAAG
 51 CGGCAACAAC AACAACAACA AGTCCGGCTC TGGTGGTTAC ACGTGTCGCC
101 AGACCAGCAC GAGGTGGACA CCGACGACGG AGCAAATCAA AATCCTCAAA
151 GAACTTTACT ACAACAATGC AATCCGGTCA CCAACAGCCG ATCAGATCCA
201 GAAGATCACT GCAAGGCTGA GACAGTTCGG AAAGATTGAG GGCAAGAACG
251 TCTTTTACTG GTTCCAGAAC CATAAGGCTC GTGAGCGTCA GAAGAAGAGA
301 TTCAACGGAA CAAACATGAC CACACCATCT TCATCACCCA ACTCGGTTAT
351 GATGGCGGCT AACGATCATT ATCATCCTCT ACTTCACCAT CATCACGGTG
401 TTCCCATGCA GAGACCTGCT AATTCCGTCA ACGTTAAACT TAACCAAGAC
451 CATCATCTCT ATCATCATAA CAAGCCATAT CCCAGCTTCA ATAACGGGAA
501 TTTAAATCAT GCAAGCTCAG GTACTGAATG TGGTGTTGTT AATGCTTCTA
551 ATGGCTACAT GAGTAGCCAT GTCTATGGAT CTATGGAACA AGACTGTTCT
```

601 ATGAATTACA ACAACGTAGG TGGAGGATGG GCAAACATGG ATCATCATTA

651 CTCATCTGCA CCTTACAACT TCTTCGATAG AGCAAAGCCT CTGTTTGGTC

701 TAGAAGGTCA TCAAGAAGAA GAAGAATGTG GTGGCGATGC TTATCTGGAA

751 CATCGACGTA CGCTTCCTCT CTTCCCTATG CACGGTGAAG ATCACATCAA

801 CGGTGGTAGT GGTGCCATCT GGAAGTATGG CCAATCGGAA GTTCGCCCTT

851 GCGCTTCTCT TGAGCTACGT CTGAACTAG

After the amplification of WUS, the PCR products were purified by QIAGEN QIAquick Gel Extraction Kit. The purified products were then confirmed by gel electrophoresis (Fig. 3.1A), showing the fragment length is between 750 bp and 1000 bp, while the actual length of WUS is 879 bp. The confirmed fragments and the pG2NBL-UASpt vector were then digested by two restriction enzymes HindIII and BamHI. After 2 hours incubation at 37℃, the fragments and vector were then purified by QIAGEN QIAquick Gel Extraction Kit. The digested products were then confirmed by gel electrophoresis (Fig. 3.1B) and ligated by Roche T4 DNA ligase. After 1 hour of ligation, the ligation products were then transformed into competent *E. coli* cells. The transformed competent cells were incubated in LB liquid for 1 hour and then transferred on LB agar plate with kanamycin. The plate was incubated at 37℃ for 16 hours. Colonies were inoculated into tubes and incubated on the shaker at 37℃ for another 16 hours. The plasmids of *E. coli* cells in different tubes were extracted and confirmed by digestion with HindIII and BamHI (Fig. 3.1C), showing that the vector with insertion of WUS has two bands by gel electrophoresis. The confirmed plasmid was then sequenced to further confirm that the construct was correct. A single confirmed strain was stored at -80℃. In this way, the vector pG2NBL-UAS::WUS was constructed and confirmed. Using the same methods, pG2NBL-UAS::BBM, pG2NBL-UAS::STM, pG2NBL-UAS::FAS2, pG2NBL-UAS::WUS-mCherry, pG2NBL-UAS::AGL15, pG2NBL-UAS::CLE40, and pG2NBL-UAS::IAA30 were also constructed and confirmed.

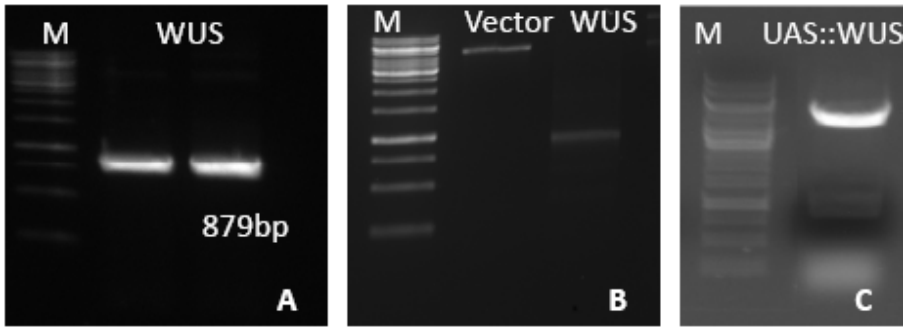

Fig. 3.1. Molecular cloning of pG2NBL-UAS::WUS construct. PCR amplification product of 879 bp of WUS coding sequence was confirmed by gel electrophoresis (A). The digestion products of pG2NBL-UAS vector (middle) and WUS (right) by double enzymes, HindIII and BamHI, were confirmed by gel electrophoresis (B). The digestion of pG2NBL-UAS::WUS by double enzymes, HindIII and BamHI, were confirmed by gel electrophoresis (C).

3.3 Generation of transgenic plants

The pG2NBL-UAS::WUS vector were transferred into GV3101 *Agrobacterium* strains (Koncz et al., 1984; Zhang et al., 2006). The transformation method has been described in previous part. The seeds of the transformed wild type *columbia* plants were harvested and selected by the methods described in previous parts. The T1 seeds had been selected and the results were shown in Table 1-7. According genetics, the single-copy-inserted line should show a 3:1 ratio theoretically. The lines exhibited a 3:1 ratio were then proliferated and selected in the next generation. The homozygous lines were selected and stored for future use.

Talbe 1. Basta selection results of different lines with pG2NBL-UAS::WUS. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::WUS	T1-1	24	20	4	5:1
	T1-2	42	40	2	20:1
	T1-3	35	1	34	1:34
	T1-4	27	27	0	
	T1-5	25	18	7	2.6:1
	T1-6	32	32	0	
	T1-7	29	19	10	1.9:1
	T1-8	29	28	1	28:1
	T1-9	31	26	5	5.2:1
	T1-10	24	0	24	0
	T1-11	25	23	2	11.5:1

Talbe 2. Basta selection results of different lines with pG2NBL-UAS::BBM. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::BBM	T1-1	30	24	6	4:1
	T1-2	32	24	8	3:1
	T1-3	9	8	1	8:1
	T1-4	30	24	6	4:1
	T1-5	26	25	1	25:1
	T1-6	35	30	5	6:1
	T1-7	29	21	8	2.6:1
	T1-8	30	23	7	3.3:1
	T1-9	27	24	3	8:1
	T1-10	30	26	4	6.5:1
	T1-11	31	24	7	3.4:1
	T1-12	27	18	9	2:1
	T1-13	25	21	4	5.3:1

Talbe 3. Basta selection results of different lines with pG2NBL-UAS::FAS2. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::FAS2	T1-1	48	30	18	1.7:1
	T1-2	36	28	8	3.5:1
	T1-3	37	30	7	4.3:1
	T1-4	29	26	3	8.7:1
	T1-5	27	21	6	3.5:1
	T1-6	30	25	5	5:1
	T1-7	34	30	4	7.5:1
	T1-8	31	29	2	14.5:1

Talbe 4. Basta selection results of different lines with pG2NBL-UAS::STM. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::STM	T1-1	31	16	15	1.1:1
	T1-2	23	17	6	2.8:1
	T1-3	27	24	3	8:1
	T1-4	31	28	3	9.3:1
	T1-5	26	0	26	0
	T1-6	27	19	8	2.4:1
	T1-7	18	14	4	3.5:1
	T1-8	29	24	5	4.8:1
	T1-9	31	29	2	14.5:1
	T1-10	27	20	7	2.9:1
	T1-11	24	17	7	2.4:1

Talbe 5. Basta selection results of different lines with pG2NBL-UAS::WUS-mCherry. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::WUS-mCherry	T1-1	45	34	11	3.1:1
	T1-2	30	30	0	
	T1-3	40	25	15	1.7:1
	T1-4	20	18	2	9:1
	T1-5	21	16	5	3.2:1
	T1-6	20	13	7	1.9:1
	T1-7	13	11	2	5.5:1
	T1-8	20	14	6	2.3:1

Talbe 6. Basta selection results of different lines with pG2NBL-UAS::CLE40. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::CLE40	T1-1	31	25	6	4.2:1
	T1-2	28	23	5	4.6:1
	T1-3	32	19	13	1.5:1
	T1-4	37	28	9	3.1:1
	T1-5	25	21	4	5.3:1
	T1-6	31	24	7	3.4:1
	T1-7	32	18	14	1.3:1
	T1-8	31	23	8	2.9:1

Talbe 7. Basta selection results of different lines with pG2NBL-UAS::IAA30. The seeds were germinated on the plates for 4 days and transferred on the 20 μM PPT plates for 7 days. S/D indicates the ratio of survived seedlings to dead seedlings.

	Line	Total No.	No. of Survived seedlings	No. of dead seedlings	Ratio (S/D)
pG2NBL-UAS::IAA30	T1-1	42	37	5	7.4:1
	T1-2	20	16	4	5:1
	T1-3	21	15	6	2.5:1
	T1-4	16	12	3	3:1
	T1-5	24	19	5	3.8:1
	T1-6	27	15	12	1.3:1
	T1-7	33	25	8	3.1:1
	T1-8	31	28	3	8.7:1
	T1-9	27	22	5	4.4:1
	T1-10	24	0	24	
	T1-11	26	19	7	2.7:1
	T1-12	25	0	25	

Chapter 4 Studies of ectopic expression of *WUS* in non-inducible lines

4.1 The GAL4-GFP enhancer trap lines

The *Arabidopsis* root system has exceptionally simple anatomy, and is an excellent system for studying organ formation in plants. In *Arabidopsis* root tips, the stem cells surround a small group of mitotically inactive organizing cells, termed the quiescent centre (QC) cells, and give rise to distal (columella), lateral (lateral root cap and epidermis) and proximal (cortex, endodermis and stele) cell types.

To study the changes of ectopic expression of *WUS* in *Arabidopsis* roots, the non-inducible and dexamethasone (DEX) inducible lines were used.

Dr. Jim Haseloff, MRC Laboratory of Molecular Biology, Cambridge, UK, has invented the GAL4-GFP enhancer trap lines in *Arabidopsis* (Haseloff and Hodge, 2001). In this system, an active CaMV 35S promoter was fused to GAL4 DNA-binding domain which was fused to the plant-suitable transcriptional activation domain from herpes simplex virus (HSV) VP-16. In order to mark the expression region of the GAL4-VP16 and the UAS-drived gene, a UAS-drived modified-GFP sequence was fused to GAL4-VP16 as a reporter marker. To be suitable for translocation into *Arabidopsis* plants, the construct was flanked with the right and left Ti-DNA to enable a random and stable insertion into the genome of a plant cell host. The construct was then transformed into *Arabidopsis* and over 8000 T-DNA transformed *Arabidopsis* plantlets for enhancer-trap driven GAL4 + UAS:mGFP5-ER expression were generated and screened. From which, several hundred of these GAL4-GFP

lines were stable and distinct. In order to name these lines, each line was named with one or two letters and four-digit number described as below. These lines were described as:

Jxxxx = pre-screened for root expression,

Qxxxx = pre-screened for root expression,

KSxxx = pre-screened for shoot and floral expression,

Mxxxx = prescreened for shoot and floral expression.

4.2 Generation and studies of the non-inducible lines

For this project, in order to get insights where the expression of WUS in *Arabidopsis* root specific cell layers or tissues could induce the root-to-shoot transformation, we choose some of the Jxxxx and Qxxxx lines to ectopically express *WUS*. J0951, Q1630, J3411, J0121, and Q2500 were crossed with UAS::WUS.

4.2.1 Studies of ectopic expression of WUS in lateral root cap and epidermis

J0951>>GFP line has tissue-specific GAL4-GFP expression in lateral root cap and epidermis (De Smet et al., 2007). J0951 was crossed with UAS::WUS, and 4-days-postgermination seedlings of F1 generation were used for study. Strong GFP signal could be detected in lateral root cap and weaker in epidermis (Fig. 4.1). The cell organizations in J0951 and J0951>>GFP, UAS::WUS lines had no significant difference. However, the strength of the GFP signals in J0951>>GFP, UAS::WUS line was much weaker than J0951>>GFP line (Fig. 4.1), indicating ectopic expression of *WUS* in this region might change the cell identity to a certain extent. Furthermore, the white arrow (Fig. 4.1, panel B) indicated some cells of epidermis were no longer regular rectangular in outline, indicating

ectopic expression of *WUS* had changed the cell structure and probably also changed the cell identity even at 4 days after germination. In addition, some extra abnormal cell divisions could be detected in the epidermis cells.

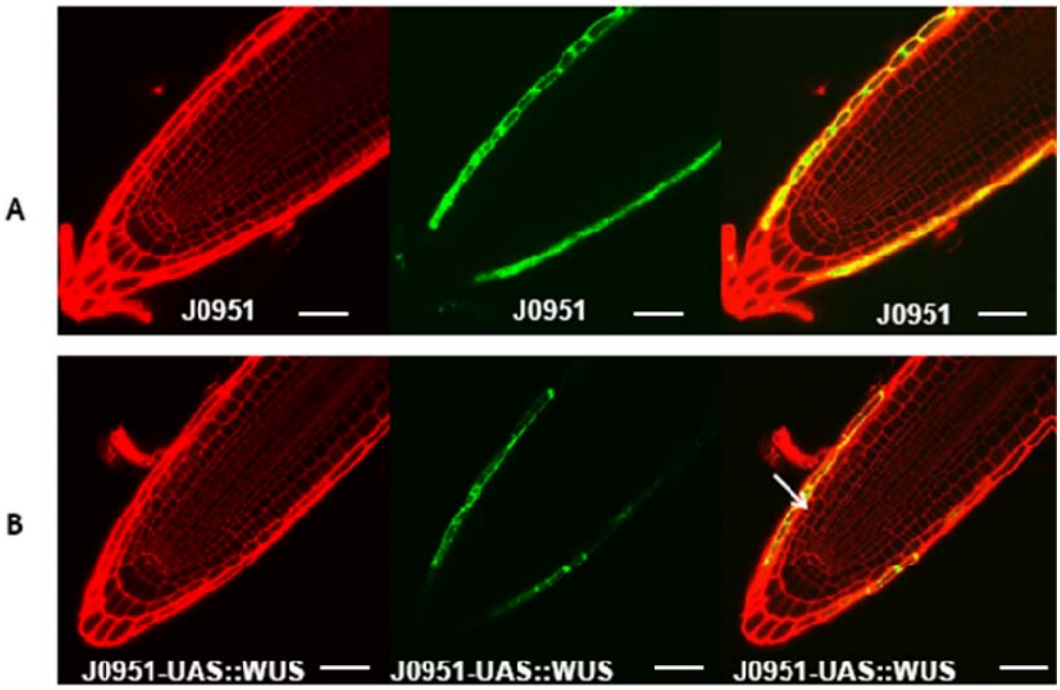

Fig. 4.1. Studies of the changes in root tips with or without expression of WUS in lateral root cap and epidermis. The J0951 (panel A) and J0951-UAS::WUS (panel B) seeds were grown on MS plates for 4 days and undergone the confocal microscopy assay. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. The white arrow indicates the cell shape changed. Bars represent 40μm.

Another similar enhancer trap line, J2301>>GFP, was used to study induction of ectopic shoot tissues in the roots by Gallois, J. L. (Gallois et al., 2004). In this line, the UAS sequence and UAS-drived WUS expression were activated by GAL4-VP16 in the lateral root cap and in the atrichoblasts of the root epidermis. They studied the 4dpg seedlings of J2301>>GFP line and the 7dpg seedlings of J2301>>GFP, UAS::WUS line. The root tips of 7dpg seedlings of J2301>>GFP, UAS::WUS lines showed abnormal cell divisions in both GFP-negative epidermal cells and in the neighboring cells that express GFP.

However, in the 4dpg seedlings of the J0951>>GFP, UAS::WUS line we didn't observe such significant changes in cell divisions (Fig. 4.1). One possible explanation is the abnormal cell divisions need longer time scale of expression of WUS. At 4th day the changes were not obvious, while as longer time window of expression of WUS, in this case, at 7th day, abnormal cell divisions can be observed. Another explanation is the strengths of GAL4-VP16 expression in J0951 and J2301 are different. Compared to their experiments, our results showed at earlier stage (e.g. before 4 dpg) the expression of WUS in lateral root cap and epidermis had very weak changes in the root structure, and at later stage (e.g. after 7 dpg) the seedlings showed abnormal cell divisions and root structure.

Taken together, ectopic expression of *WUS* in lateral root cap and epidermis led to abnormal cell shape and arrangement in these cells and change in cell identity.

4.2.2 Studies of ectopic expression of *WUS* in columella stem cells

Q1630>>GFP line has tissue-specific GAL4-GFP expression in columella stem cells (Fig. 4.2, panel A). Q1630 was crossed with UAS::WUS, and 4 dpg seedlings of F1 generation were used for study. The GFP signal was restricted into the two layers of columella stem cells in 4dpg of Q1630>>GFP lines (Fig. 4.2, panel A). Combined with expression of *WUS* driven by Q1630>>GFP, the GFP signal was much weaker than control (Fig. 4.2). Moreover, the GFP expression region was larger in Q1630>>GFP, UAS::WUS lines than Q1630>>GFP (Fig. 4.2), and the shape of columella stem cells in Q1630>>GFP, UAS::WUS lines was different (Fig. 4.2, panel B). The GFP signal was extended into some epidermal cells in Q1630>>GFP, UAS::WUS lines (Fig. 4.2, panel B). Another change in Q1630>>GFP, UAS::WUS lines is that three layers of columella stem cells could be observed while two layers in control. This indicated that ectopic expression of WUS in columella stem cells led to abnormal cell divisions. Thus, ectopic expression of WUS in columella stem cells might cause the change in cross-talk between columella stem cells and QC, leading to the enlargement of columella stem cells and changes in columella stem cells identity.

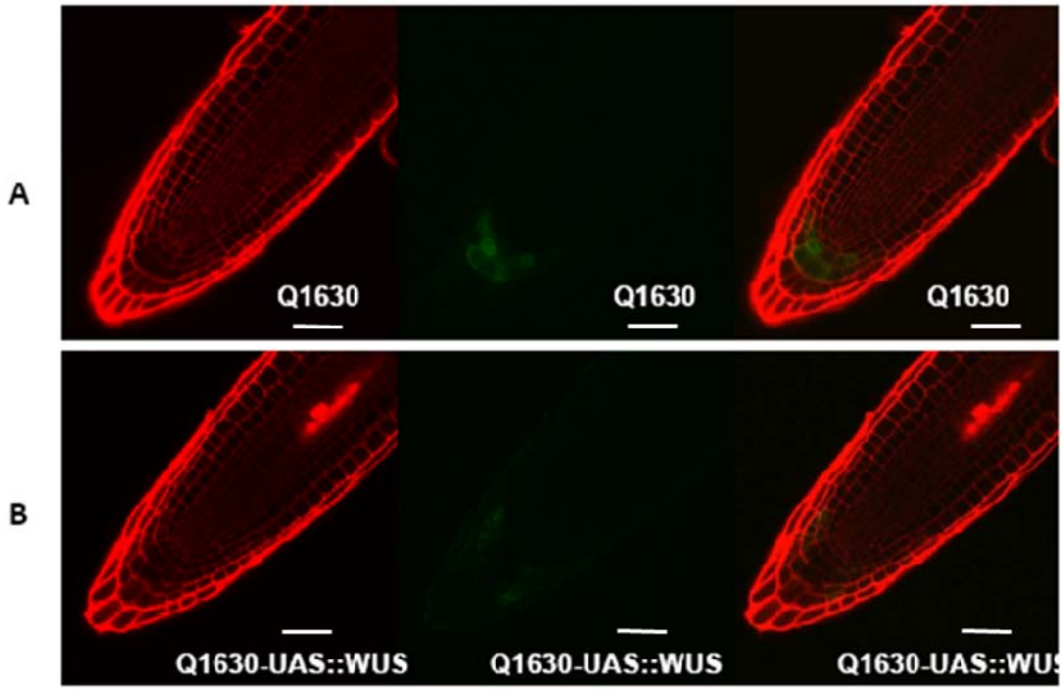

Fig. 4.2. Studies of the changes in root with or without expression of WUS in columella stem cells. The Q1630 (panel A) and Q1630-UAS::WUS (panel B) seeds were grow on MS plates for 4 days and undergone the confocal microscopy assay. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. The insets in A and B are 2X magnifications. Bars represent 40μm.

4.2.3 Studies of ectopic expression of WUS in lateral root cap and columella root cap

J3411>>GFP line has tissue-specific GAL4-GFP expression in columella root cap and lateral root cap (Birnbaum et al., 2003). J3411 was crossed with UAS::WUS, and 4 dpg seedlings of F1 generation were used for study. The GFP signal in J3411>>GFP line was quite strong (Fig. 4.3, panel A). When combined with expression of WUS, the morphological changes in root structure was quite significant at 4 dpg (Fig. 4.3, panel B). Abnormal cell divisions could be observed in the columella root cap and lateral root cap in J3411>>GFP, UAS::WUS lines (Fig. 4.3, panel B). A small bulge could be observed in the side of the columella root cap region in J3411>>GFP, UAS::WUS lines (Fig. 4.3, panel B). The cell size and cell shape in columella root cap were both different in J3411>>GFP, UAS::WUS lines compared with J3411>>GFP lines. More cells with root cap cell identity (shown by GFP signal) and more cells adjacent to the cells which expressed GFP could be observed after ectopic expression of WUS (Fig. 4.3, panel B), indicating ectopic expression of WUS could induce abnormal cell divisions. These abnormal cell divisions might be the establishment of shoot stem cells. Another change was that the roots of J3411>>GFP, UAS::WUS lines showed more cell layers in the root meristem. This might be due to the abnormal cell divisions induced by ectopic expression of WUS. Moreover, the cells in roots of J3411>>GFP, UAS::WUS lines were not well-organized (Fig. 4.3, panel B), that is, more periclinal divisions could be found in the root meristem. Interestingly, the outmost layers of the root with ectopic expression of *WUS* didn't lose its root cap cell identity, while some cell adjacent to the root cap cells showed weaker GFP signal, indicating 4days of expression of WUS in columella root cap cells could be not sufficient to overwrite the cell identity. However, the abnormal cell divisions adjacent to the root cap might indicate that these cells

had already possessed the identity of shoot stem cells. In this case, it might be not enough to form a new shoot organ, however.

With 9 days of ectopic expression of *WUS* in columella root cap, the root tips became callus-like (data not provided). This may indicate that long-term ectopic expression of *WUS* could induce the reprogramming of the cells and overwrite the cell identity. This callus-like cell mass in root tips might need other shoot stem cell factor to induce the root-to-shoot regeneration since in the root tip region some root specific factors might have the inhibition of the function of WUS. Taken together, the ectopic expression of *WUS* in lateral root cap and columella root cap caused abnormal cell divisions and extra cell layers in the meristem.

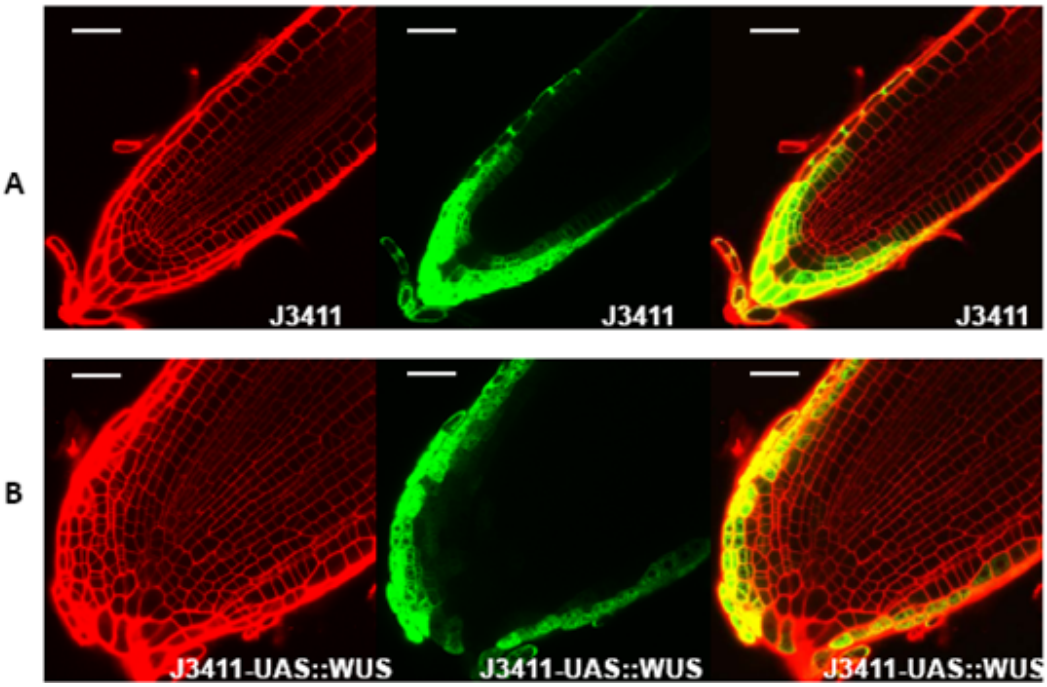

Fig. 4.3. Studies of the changes in root tips with or without expression of WUS in columella root cap. The J3411 (panel A) and J3411-UAS::WUS (panel B) seeds were grown on MS plates for 4 days and undergone the confocal microscopy assay. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. Bars represent 40μm.

4.2.4 Studies of ectopic expression of WUS in pericycle

J0121>>GFP line has tissue-specific GAL4-GFP expression in pericycle cells (Casimiro et al., 2001). J0121 was crossed with UAS::WUS, and 4 dpg seedlings of F1 generation were used for study. In J0121>>GFP lines, GFP signal was detected in pericycle cells of mature zone of root (Fig. 4.4, panel A). Without expression of WUS in pericycle cells, the cell organization in endodermis cells, cortex cells and epidermal cells is well organized (Fig. 4.4, panel A). With ectopic expression of WUS in pericycle cells, the cell shape and organization in outer layers were no longer as wild type (Fig. 4.4, panel B); further confirmed ectopic expression of *WUS* could change the cell identity as well as the cell fate. Taken all together, the cell organization in outer layers changed with ectopic expression of *WUS*.

To test whether ectopic expression of *WUS* in pericycle cells could lead to shoot organs formation (e.g. leaves) in lateral root development, the lateral root development was tracked. Compared with J0121>>GFP lines, the ectopic expression of *WUS* in pericycle cells was not enough to induce shoot organ formation. The J0121>>GFP, UAS::WUS lines seemed normal lateral root development as J0121>>GFP lines (Fig. 4.4, panel C; Fig. 4.5). At early stages, the lateral root development in J0121>>GFP, UAS::WUS lines (Fig. 4.4, panel C) had no obvious differences with J0121>>GFP lines.

With longer time of ectopic expression of *WUS* in pericycle region, the lateral root development still seemed similar with the control lines (data not provided). No shoot-like organs were detected in the lateral root region or root tips. These indicated that ectopic expression of *WUS* in pericycle could not be enough to induce shoot-like organs formation in the root. This may be due to the expression of *WUS* in the late stage of lateral root development. Taken all together, ectopic expression of *WUS* in pericycle was not enough to induce the shoot-like organs formation in the root.

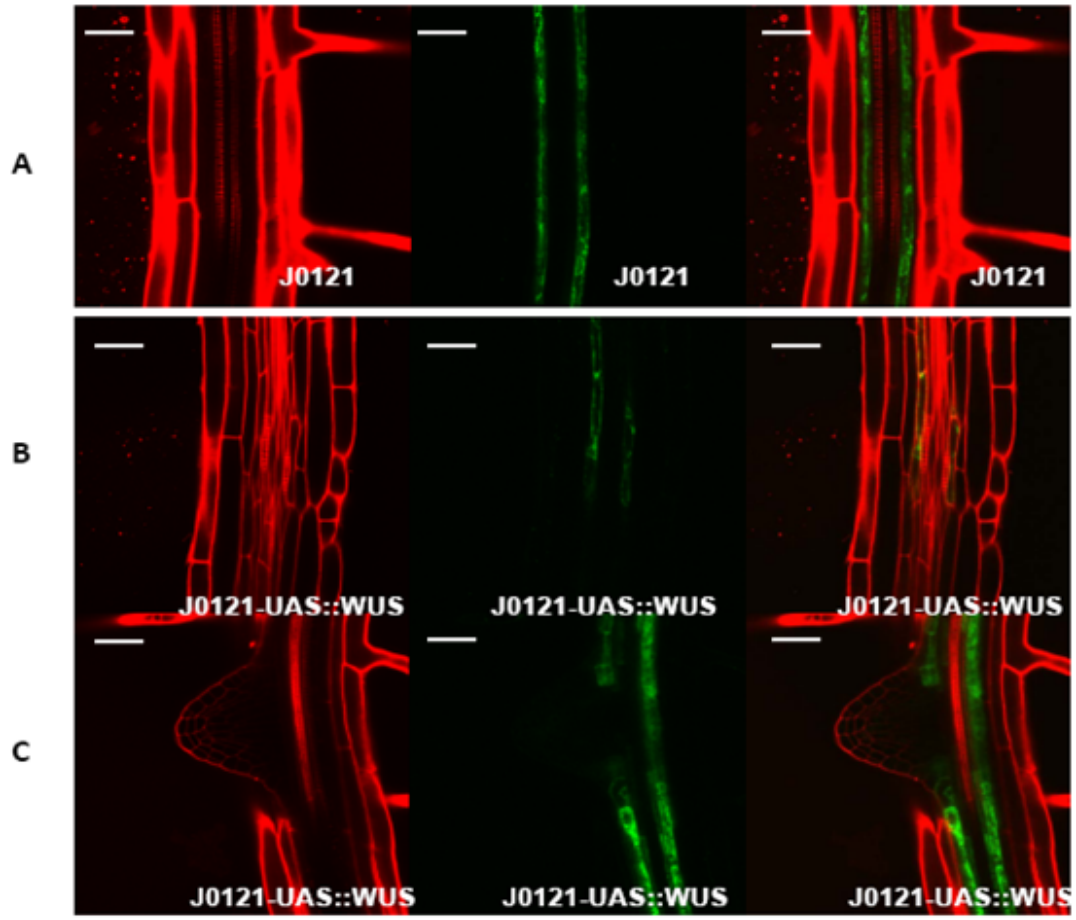

Fig. 4.4. Studies of the changes in root with or without expression of WUS in pericycle. The J0121 (panel A) and J0121-UAS::WUS (panel B) seeds were grow on MS plates for 4 days and undergone the confocal microscopy assay. Panel C was the lateral root development of J0121-UAS::WUS. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. Bars represent 40μm.

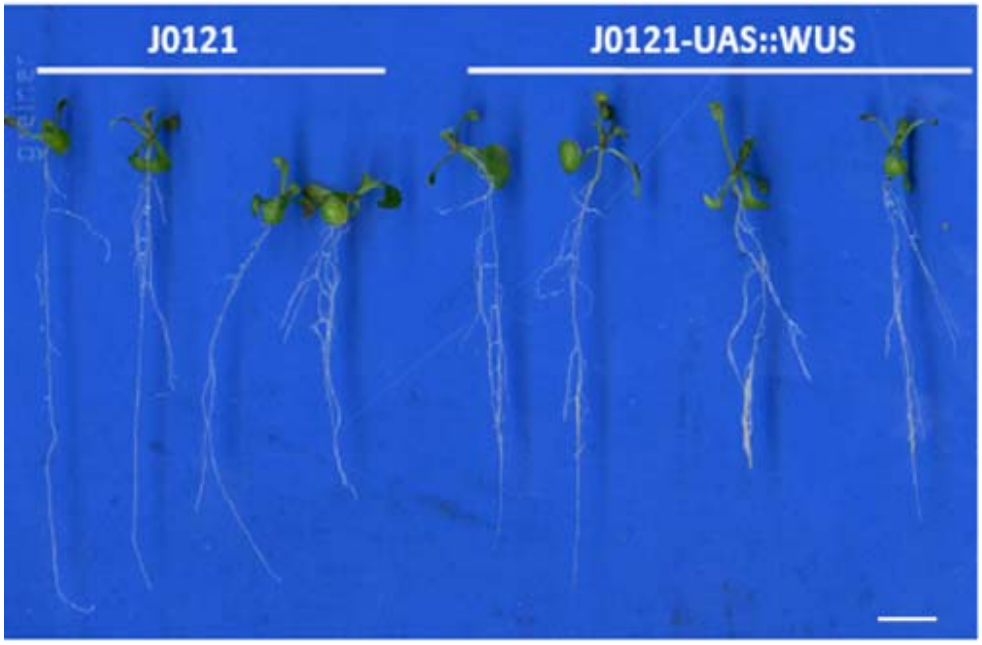

Fig. 4.5. Studies of the changes in lateral root with or without expression of WUS in pericycle. The J0121 (left) and J0121-UAS::WUS (right) seeds were grown on MS plates for 13 days. Bar represents 1cm.

4.2.5 Studies of ectopic expression of WUS in endodermis

Q2500>>GFP line has tissue-specific GAL4-GFP expression in endodermis in root meristem (Fig. 4.6, panel A). Q2500>>GFP was crossed with UAS::WUS, and 4 dpg seedlings of F1 generation were used for study. In Q2500>>GFP lines, GFP signal was detected in endodermis cells and QC cells (Fig. 4.6, panel A). With ectopic expression of WUS driven by Q2500>>GFP lines, the seedlings grown very slow and showed smaller meristem size and shorter root length than Q2500>>GFP lines (Fig. 4.6, panel B). Moreover, the cell in endodermis and cortex had some abnormal periclinal divisions (Fig. 4.6, panel B). In addition, the cell organization and cell shape had some changes (Fig. 4.6, panel B), which was consistent with the results in chapter 3.

The lateral root development was also studied. Without expression of *WUS* in endodermis, Q2500>>GFP lines showed normal lateral root development and at 13days after germination, the lateral roots were bushy and plentiful (Fig. 4.7, panel A). While with ectopic expression of *WUS* in endodermis driven by Q2500>>GFP lines, the lateral root development was quite slow (Fig. 4.7, panel B). Even at 13 days after germination, the number of lateral roots was quite smaller (Fig. 4.7, panel B). Although some seedlings did have several lateral roots, most of the seedlings had no lateral root formation, indicating the expression of *WUS* in endodermis driven by Q2500>>GFP lines could inhibit lateral root development at somewhat extent. Interestingly, the lines with ectopic expression of *WUS* in endodermis driven by Q2500>>GFP also showed longer hypocotyls, curled leaves and less leaves than control (Fig. 4.7, panel B). Although the changes in primary root and lateral root development were obvious, no shoot organs formed at two weeks after germination (Fig. 4.7, panel B). The Q2500>>GFP, UAS::WUS lines could not flower and have no seeds due to the strong phenotype induced by expression of *WUS*.

Taken all together, ectopic expression of *WUS* in endodermis leads to no shoot-like organs formation in the root.

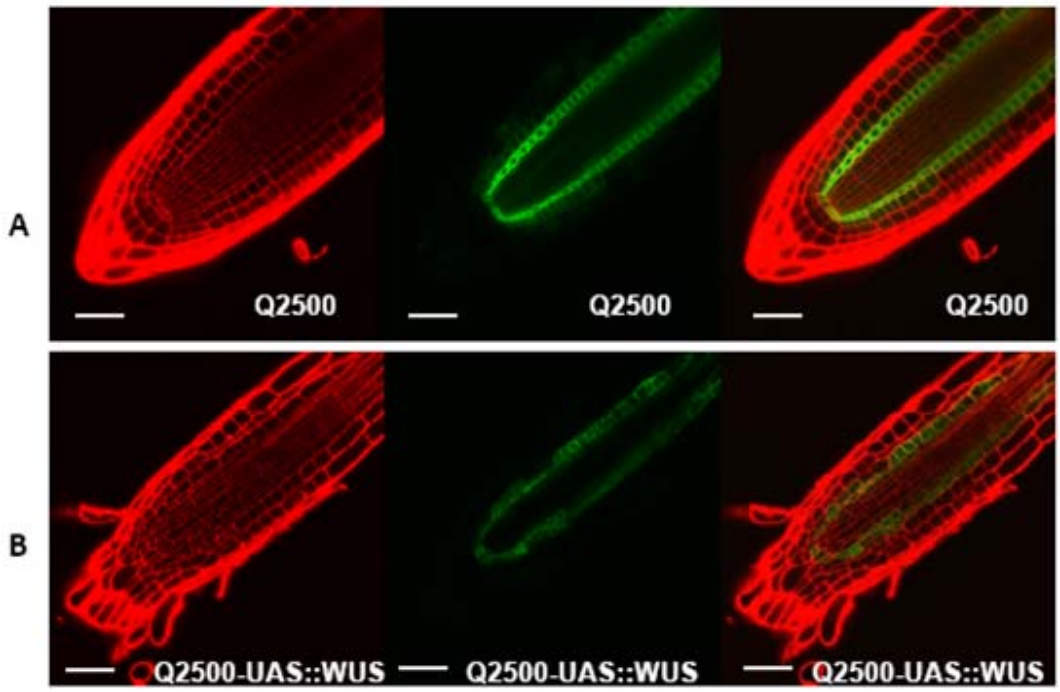

Fig. 4.6. Studies of the changes in root with or without expression of WUS in endodermis. Q2500 (panel A) and Q2500-UAS::WUS (panel B) seeds were grown on MS plates for 4 days and subjected to the confocal microscopy assay. Panel C was the lateral root development of J0121-UAS::WUS. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. Bars represent 40μm.

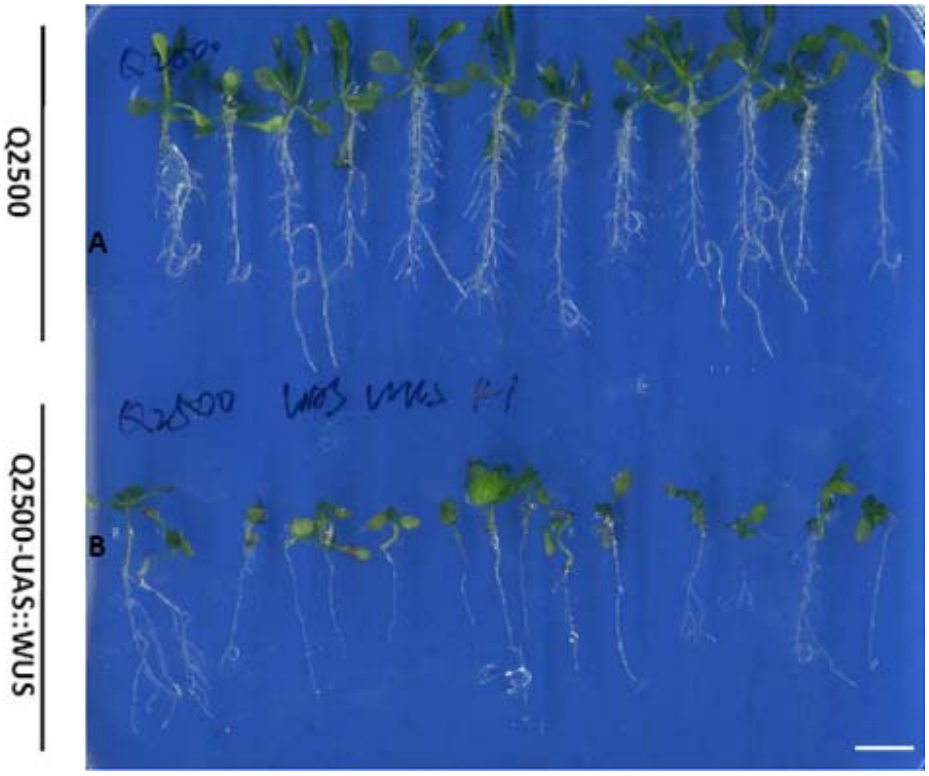

Fig. 4.7. Studies of the changes in lateral root with or without expression of *WUS* in endodermis. The Q2500 (panel A) and Q2500-UAS::WUS (panel B) seeds were grown on MS plates for 13 days. Bar represents 2cm.

4.3 Discussion

WUS, a transcriptional factor with homeodomain, is mainly expressed in the organizing centre of shoot apex. Ectopic induction of *WUS* expression in *Arabidopsis* root tips can induce shoot stem cell identity and leaf development (without additional cues), flower development (together with LEAFY, which is a key regulator of flower development) (Wagner et al., 2004; Weigel and Nilsson, 1995), or embryogenesis (in response to increased level of auxin) (Gallois et al., 2004).

For all of the non-inducible lines with ectopic expression of *WUS*, some changes in cell shape, cell organization and cell divisions could be observed in both the cells with expression of *WUS* and the cells adjacent to the cells where *WUS* was expressed.

WUS is a shoot stem cell transcription factor, which helps maintain the shoot meristem. The expression of WUS is located in the organizing centre. The ectopic expression of *WUS* in other cells replaced the previous cell identity and established the new identity with shoot meristem.

Our data indicated ectopic expression of *WUS* in epidermis, cortex, quiescent centre, columella stem cells, columella root cap, lateral root cap, and pericycle did lead to aberrant cell arrangement in the some meristem cells, abnormal cell shape and extra cell divisions in both the cells with expression of *WUS* and the adjoining cells. However, ectopic expression of *WUS* in these cells was not sufficient to induce the shoot-like organs formation in the root although some cells with shoot stem cell identity were induced in the root. The ectopic expression of *WUS* in columella root cap and lateral root cap induced much more extra cell divisions and some cells with shoot stem cell identity. These indicated that, during

regeneration process, the expression of *WUS* reprogrammed the cell identity and promoted the establishment of shoot meristem.

However, with 9 days of ectopic expression of *WUS* in the columella root cap and lateral root cap led to a callus-like cell masses (data not provided). Previously, it was shown that the expression of *WUS* in the organizing centre induced the expression of *CLV*, an inhibitor of *WUS* (Gallois et al., 2004). The *WUS*/*CLV* negative feedback loop controls the size of shoot apical meristem. In the future, several specific markers showing the identity of shoot stem cells are necessary to combined with the ectopic expression of *WUS* lines. The new induced cells might already have the cell identity of shoot stem cells due to the continuous expression of *WUS* in the root.

There are two possible explanations for these non-inducible lines with ectopic expression of *WUS* which failed forming shoot-like organs: (1) some other transcriptional factors expressed in the root can inhibit the reprogramming of *WUS* in some extent; (2) the root-to-shoot regeneration need the support of the neighboring cells. In the future, the microarray analysis will be performed to unearth some genes that may respond to the ectopic expression of *WUS*.

Chapter 5 Studies of ectopic expression of WUS in inducible lines

5.1 The GVG/UAS system and advantages of GVG/UAS system

To be able to control the induction of *WUS* in time and space scales, the GVG/UAS system was used. In this system, induction of the UAS-drived gene expression needs to apply with DEX, a strong synthetic glucocorticoid. This system has the following advantages: (1) the GVG lines and the UAS lines can be generated separately, so this system can generate many combinations; (2) this system allows the in vivo studies of genes of interest, so this is more realistic in nature; (3) this system allows the artificial control of expression of genes of interest in time and space scales, so this is better for the developmental studies of *Arabidopsis*; (4) this system allows to study the gene expression pattern of some specific tissues or cells by using the Fluorescence Activated Cell Sorting (FACS) approach and the combination with other tissue/cell specific markers, so this system facilitates the dissection of molecular events underlying the regeneration process.

5.2 Generation and studies of WUS-inducible lines

5.2.1 Generation of GVG lines and WUS-inducible lines

Promoters of SCARECROW (SCR) (in root tips mainly expressed in endodermis and QC) (Di Laurenzio et al., 1996), WUSCHEL-related homeobox 5 (WOX5) (in root tips expressed in QC) (Sarkar et al., 2007), PIN-FORMED 2 (PIN2) (in root tips expressed in epidermis) (Muller et al., 1998) and ACTIN DEPOLYMERIZING FACTOR 5 (in root tips expressed in Columella root cap) were fused with GAL4-VP16-GR transcription factor by Dr. Xu Jian. The lines of pSCR::GVG, pADF5::GVG, pWOX5::GVG, and pCO2::GVG have been selected and crossed with the UAS::WUS line. We have generated and studead the following lines: pSCR::GVG-UAS::WUS, pADF5::GVG-UAS::WUS, pADF5::GVG-UAS::WUS-mCherry, pWOX5::GVG-UAS::WUS, and pCO2::GVG-UAS::WUS.

5.2.2 Studies of ectopic expression of WUS in columella root cap

To study the induction of *WUS* in the expression region of ADF5, the WT col seeds and pADF5::GVG-UAS::WUS seeds were sterilized, incubated at 4℃ for 2days, grown on Murashige and Skoog medium for 3 days, transferred on MS medium with 5 uM DEX for 6days. As in Fig. 5.1., under the QC, there were two layers of columella stem cells and four layers of columella root cap. The cells of columella root cap contain granules of starch, so these cells can be stained by lugol solution. Without induction of WUS in columella root cap, these cells maintained their own cell identity and the starch granules accumulated in these cells. However, with induction of *WUS* in columella root cap for 6 days, these cells in columella root cap were no longer stained, indicating the cell identity was clearly changed. Moreover, a cluster of cells accumulated at the region where the columella root cap should form (Fig. 5.1), suggested that more cell divisions occurred in this region. These abnormal cell divisions were induced by the ectopic expression of *WUS*, which might indicate the roles of *WUS* in promoting the cell divisions and maintaining cell competence of meristem both in the WUS-expressing cells and their neighbouring cells.

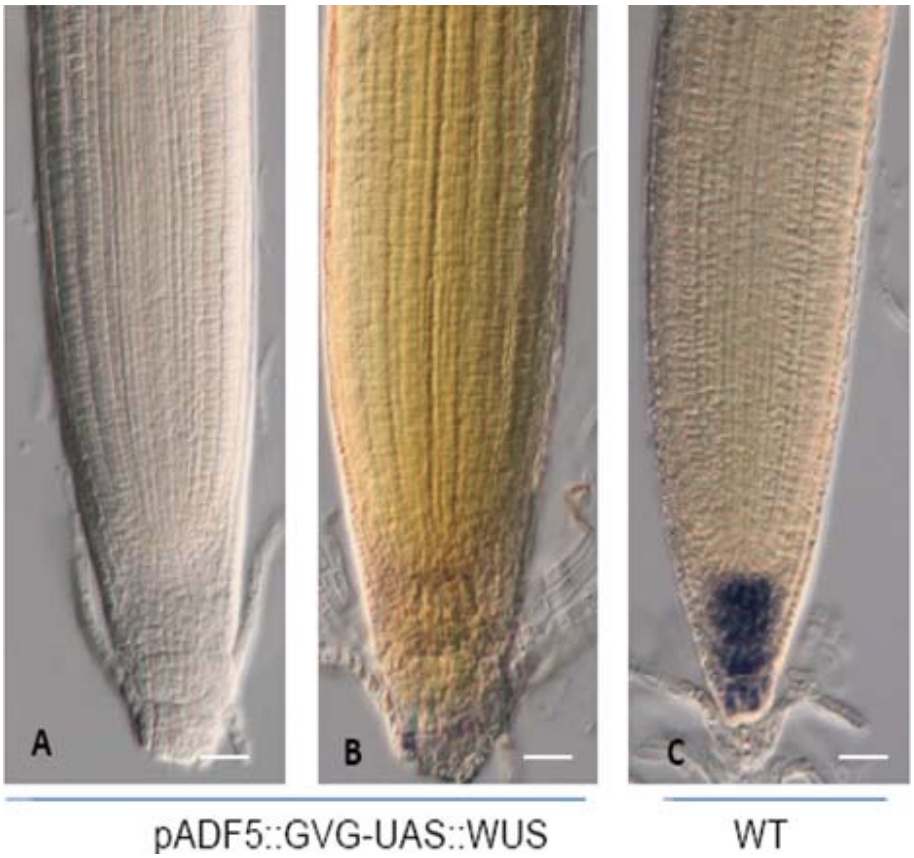

Fig. 5.1. Studies of ectopic expression of *WUS* in columella root cap. The WT and pADF5::GVG-UAS::WUS seeds were grown on MS plate for 3 days and transferred on the 5μM DEX plates for another 6 days. The pADF5::GVG-UAS::WUS (A & B) and WT (C) seedlings were tested by Differential Interference Contrast microscopy (DIC). The WT (C) and pADF5::GVG-UAS::WUS (B) seedlings were stained with lugol staining buffer. Bars represent 40μm.

To further confirm the induction of extra cell divisions in columella root cap, the pADF5::GVG-UAS::WUS-mCherry lines, in which the coding sequence of mCherry was fused with the C-terminal of *WUS*, were generated. With 3 days induction of WUS-mCherry in columella root cap by 5uM DEX, a cluster of cells could be observed in the region where the columella root cap should form (Fig. 5.2), indicating that the protein fusion of *WUS* with mCherry at C terminal still maintained at least the roles of WUS in promoting cell divisions.

The pADF5::GVG and pADF5::GVG-UAS::WUS-mCherry lines were also analyzed by confocal microscopy. With 3 days induction of WUS-mCherry in columella root cap by 5uM DEX, a cluster of cells could be observed in columella root cap. The outermost cells and several cells just below the columella stem cells still maintained their own cell identity (indicated by the GFP signal), however, in the centre of the cluster, the GFP signal could not be detected (Fig. 5.3), indicating the cell identity of these cells had been changed although the true identity of these cells was unclear.

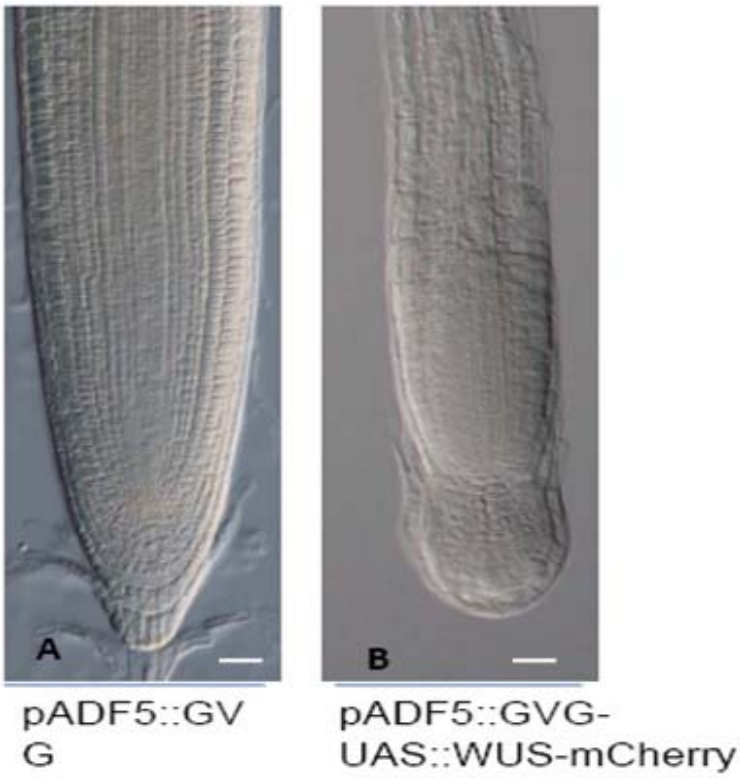

Fig. 5.2. Studies of ectopic expression of WUS-mCherry in columella root cap. The pADF5::GVG and pADF5::GVG-UAS::WUS-mCherry seedlings were grown on MS plates for 3 days and transferred on 5μM DEX plates for another 3 days. The pADF5::GVG (A) and pADF5::GVG-UAS::WUS-mCherry (B) seedlings were examined by Differential Interference Contrast microscopy (DIC). Bars represent 40μm.

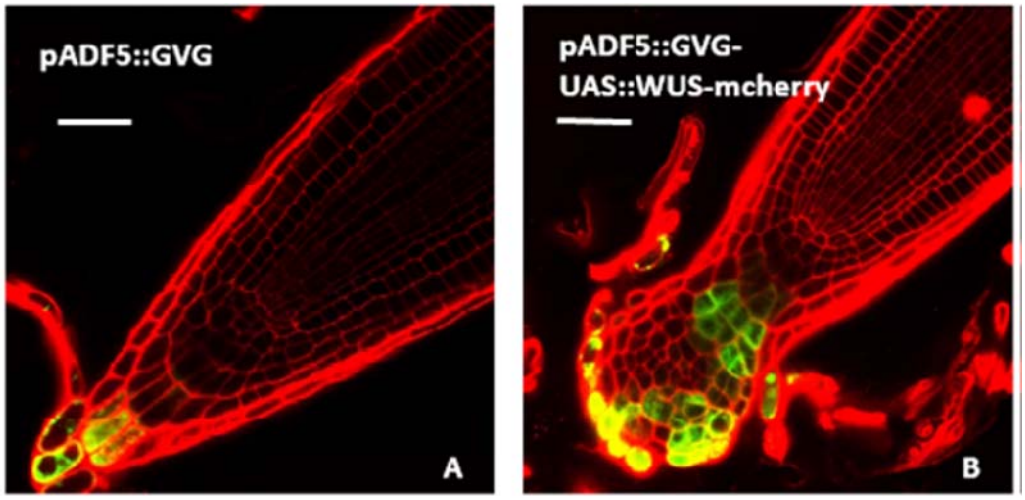

Fig. 5.3. Studies of confocal microscopy images of extopic expression of WUS-mCherry in columella root cap. The pADF5::GVG and pADF5::GVG-UAS::WUS-mCherry seedlings were grown on MS plates for 3 days and transferred on 5μM DEX plates for another 3 days. The pADF5::GVG (A) and pADF5::GVG-UAS::WUS-mCherry (B) seedlings were examined by confocal microscopy. Bars represent 40μm.

WUS activates *CLV3* through a negative feedback loop in the shoot apex, which controls the size of stem cell pool (Brand et al., 2000; Clark et al., 1995; Fletcher et al., 1999; Schoof et al., 2000). Thus, *CLV3* is used as a specific marker of shoot stem cell identity (Laux, 2003). With ectopic expression of *WUS* in *Arabidopsis* roots, *CLV3* expression could be detected after 36 hours of *WUS* expression and another marker of shoot organ primordia, *AINTEGUMENTA* (*ANT*) (Elliott et al., 1996), could be detected after 3 or 4 days of *WUS* expression and the root tips contained green tissues after 6 days of *WUS* expression (Gallois et al., 2004). Thus, 3 days of *WUS* expression in columella root cap might be enough to change the cell identities and induce a new shoot organ (in this case, a cluster of cells). However, to further confirm the identity of these cells in the cluster, some specific markers (e.g. CLV3) should be tested.

5.2.3 Studies of ectopic expression of WUS in endodermis

To study the induction of *WUS* in the endodermis, the expression of *WUS* was introduced into the expression region of *SCARECROW* (*SCR*) (Di Laurenzio et al., 1996). In this case, the pSCR::GVG-UAS::WUS line was generated. With induction of WUS since seed germination, the pSCR::GVG-UAS::WUS line showed curled leaves, longer hypocotyls and shorter roots than the pSCR::GVG line (Fig. 5.4). The root length of each line after 6 days of germination on the 5uM DEX plates was quantified and it showed that for the pSCR::GVG line, the root length was 1.92±0.26cm (n=40), and that for the pSCR::GVG-UAS::WUS line, the root length was 0.86±0.12cm (n=47) (Fig. 5.4). This data showed that ectopic expression of *WUS* in endodermis delayed the root growth. At 6 days after germination on DEX plate, no green tissue was observed. This indicated that during early development of primary root some specific transcription factors might inhibit the function of WUS.

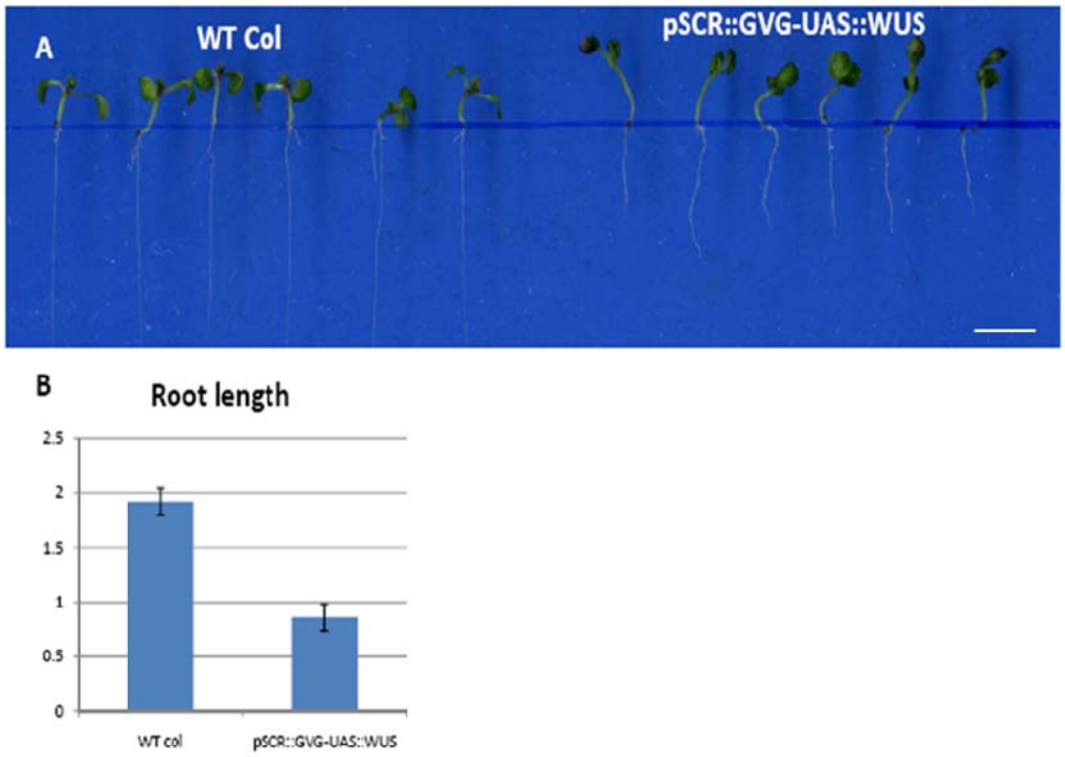

Fig. 5.4. Phenotype studies of ectopic expression of WUS in endodermis. (A) The WT (left) and pSCR::GVG-UAS::WUS (right) seeds were grown on 5μM DEX plates for 6 days. (B) The root length of WT col and pSCR::GVG-UAS::WUS seedlings of 6 dpg on DEX plates. Error bars indicate the standard divisions. Bar represents 5mm.

From 9 days of induction of *WUS* in endodermis, the leaf formation could be detected (Fig. 5.5) in the lateral root region. With about two weeks of induction of *WUS*, leaves were induced at the lateral root region and the lines with induction of *WUS* in endodermis showed almost no lateral root development (Fig. 5.5). Previously it was reported that between 2 weeks and 3 weeks after expression of WUS in root, leaf-like organs were developed in the primary root with an about 50% frequency, or a green callus-like tissue formed in the primary root (Gallois et al., 2004). Our data indicated with 4 to 6 days of ectopic expression of WUS in endodermis, green stuffs (leaf primordia) could be detected in the lateral root region; with 9 days of ectopic expression of *WUS* in endodermis, leaves can be detected in the lateral root region.

With induction of *WUS* in endodermis, three types of induced tissues were observed (Fig. 5.6). The first type was a lateral root with a new shoot-like organ (Fig. 5.6A), which may be due to the induction of *WUS* after the lateral root had been determined. The second type was several shoot-like primordia with green stuffs (Fig. 5.6B), which may be due to the induction of *WUS* before the lateral root had been determined. The third type was a basal part of lateral root with two or three protrusions which could be developed into new leaves (Fig. 5.6C). These data may indicate that the induction of shoot organs could be formed at the lateral root formation region.

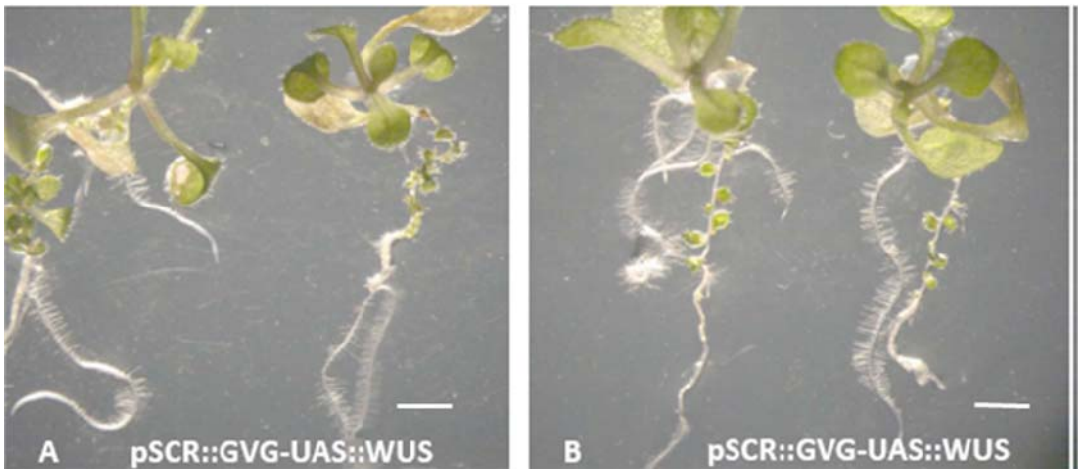

Fig. 5.5. Long-term effects of induction of *WUS* in endodermis. The pSCR::GVG-UAS::WUS seeds were grown on MS plates for 3 days and transferred on 5 μM DEX plates for two weeks. Bars represent 20mm.

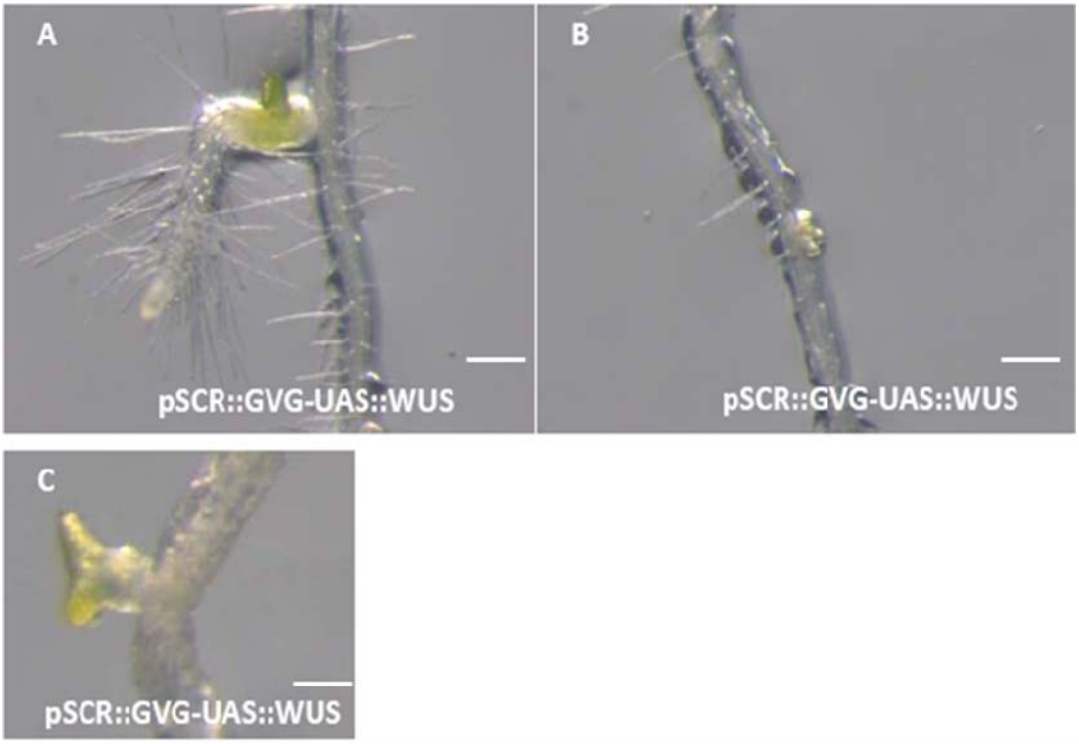

Fig. 5.6. Three main types of primordia in lateral root region by induction of *WUS* in endodermis: (A) a lateral root with a shoot-like organ, (B) several primordia in the lateral root region, and (C) two green primordia with a basal part. The pSCR::GVG-UAS::WUS seeds were grown on MS plates for 3days and transferred on 5μM DEX plates for another 4 days. Bars: A-B, 15mm; C, 10mm.

To test whether the induced leaf-like organs were formed at the lateral root region, a J-hook method was used (Laskowski et al., 2008). In this method, the 6 dpg seedlings were transferred on the 5uM DEX plates with a J-shape at the root tip region. After 4 days of treatment with DEX, the induction of *WUS* in endodermis caused the leaf-like organs in the curved root (Fig. 5.7, panel A). Without induction of *WUS*, the root showed normal lateral root (Fig. 5.7, panel B). With induction of *WUS*, no lateral root formed, suggesting that the lateral root primordium had been transformed into leaf-like organs.

Compared with previous data, the leaf-like organs formation in the root by ectopic expression of *WUS* in endodermis occurred in the lateral root region. Thus, it is necessary to dissect that at which stage of the lateral root primordium development changes occurred and lead to the root-to-shoot transformation.

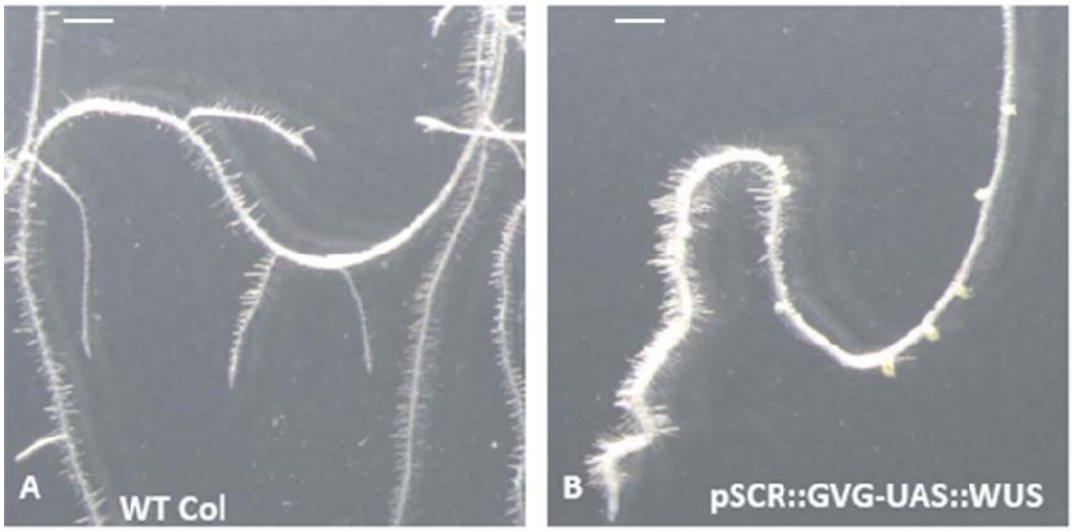

Fig. 5.7. Phenotypes in lateral root region by induction of WUS in endodermis with J-hook in root tips. The WT (A) and pSCR::GVG-UAS::WUS (B) seeds were grown on MS plates for 6days and transferred on 5μM DEX plates for another 4 days with a J-hook in the root tip region. Bars represent 5mm.

To study the changes after induction of *WUS* in endodermis, confocal microscopy was used to follow the changes of induction of *WUS*. Normally, the seedlings were grown on Murashige and Skoog medium for 3 days. After this, the seedlings were treated with 5uM DEX for one day or two days. For the pSCR::GVG lines, the meristem of primary root was well-organized (Fig. 5. 8A). The primary roots were composed of epidermis, cortex, endodermis, and stele from outside to inside. The cell organization was orderly and the cell shape was rectangular without expression of *WUS* (Fig. 5.8A). With one day induction of *WUS* in endodermis, the cells of endodermis layer divided periclinally. These periclinally divided cells still hold the same cell identity with endodermis cells. For the cortex layer, periclinal divisions also occurred, and the cell shape and arrangement changed (Fig. 5.8B). With two days induction of *WUS* in endodermis, more severe changes were observed: the increased frequencies of periclinal cell divisions in endodermis and cortex. The daughter cells of some cells in endodermis layer hold the cell identity of endodermis. Outside of the endodermis layer, there were more cell layers, in which the cortex cells could not be determined (Fig. 5.8C). The arrangement of the cells in square frame (Fig. 5.8C) was clearly complicated and non-orderly. Due to the abnormal cell divisions and the extra cell division induced by the ectopic expression of *WUS*, the root had more cell layers and showed wider root width compared to control (Fig. 5.8C).

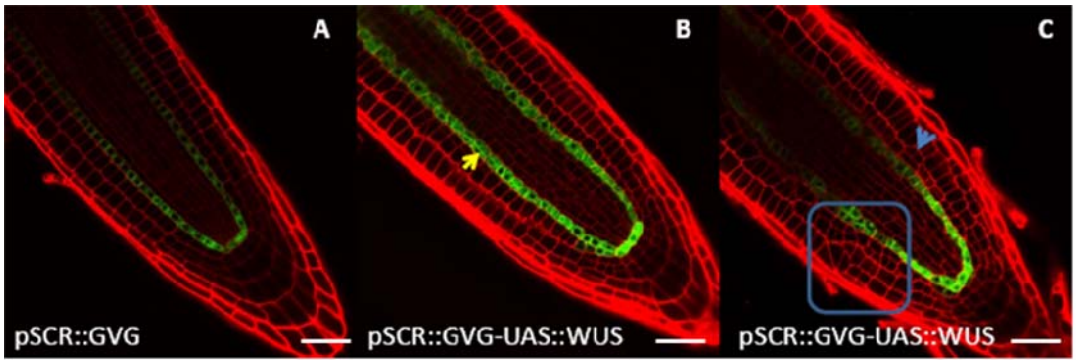

Fig. 5.8. Confocal images of primary roots with induction of *WUS* expression in endodermis. The pSCR::GVG and pSCR::GVG-UAS::WUS seeds were grow on MS plates for 3 days. Then, the pSCR::GVG (A) and pSCR::GVG-UAS::WUS (B &C) were transferred on 5μM DEX plates for 1 day (A &B) or 2 days (C). Yellow arrow indicates periclinal cell divisions in endodermis. Blue arrow indicates periclinal divisions in cortex. Blue square frame indicates abnormal cell divisions in epidermis, cortex and endodermis. Bars represent 40μm.

The *Arabidopsis* lateral root results from the pericycle founder cells (Dolan et al., 1993). The lateral root development can be divided into 8 different stages (Peret et al., 2009). Lateral root are initiated when the pericycle founder cells undergo a few anticlinal divisions to form a single layer of ten small cells with the equal length which is the lateral root primordia (termed Stage I; Fig. 5.9a, b, c, and d) (Casimiro et al., 2001; Dubrovsky et al., 2001; Malamy and Benfey, 1997). In the next step, a periclinal division occurs in the lateral root primordia and creates two layers: an inner layer (IL) and an outer layer (OL) (termed Stage II; Fig. 5.9b, c, and d). A further periclinal division in outer layer creates a three layer primordia comprising of OL1, OL2, and IL (termed Stage III; Fig. 5.9b, c, and d). Next, a further periclinal division in inner layer generates a total of four cell layers (OL1, OL2, IL1, IL2) (Stage IV; Fig. 5.9b, c, and d). In next step, a central cell in both OL1 and OL2 undergoes an anticlinal division to create a four-cell cuboidal shape (Stage V; Fig. 5.9b, c, and d). The cells in OL1, OL2, and IL2 also divide. Now, the lateral root development reaches to the midway. In next stage, cells of OL2 undergo a periclinal division generating a new layer and the four central cells in OL1 also undergo a periclinal division (Stage VI; Fig. 5.9b, c, and d). In the next step, the lateral root primordia are similar with the mature root tip containing 3 layers that are epidermis, cortex and endodermis (Stage VII; Fig. 5.9b, c, and d). In the final step, the lateral root primordia eventually emerges from the primary root (Stage VIII; Fig. 5.9b, c, and d) (Casimiro et al., 2001; Dubrovsky et al., 2001; Malamy and Benfey, 1997).

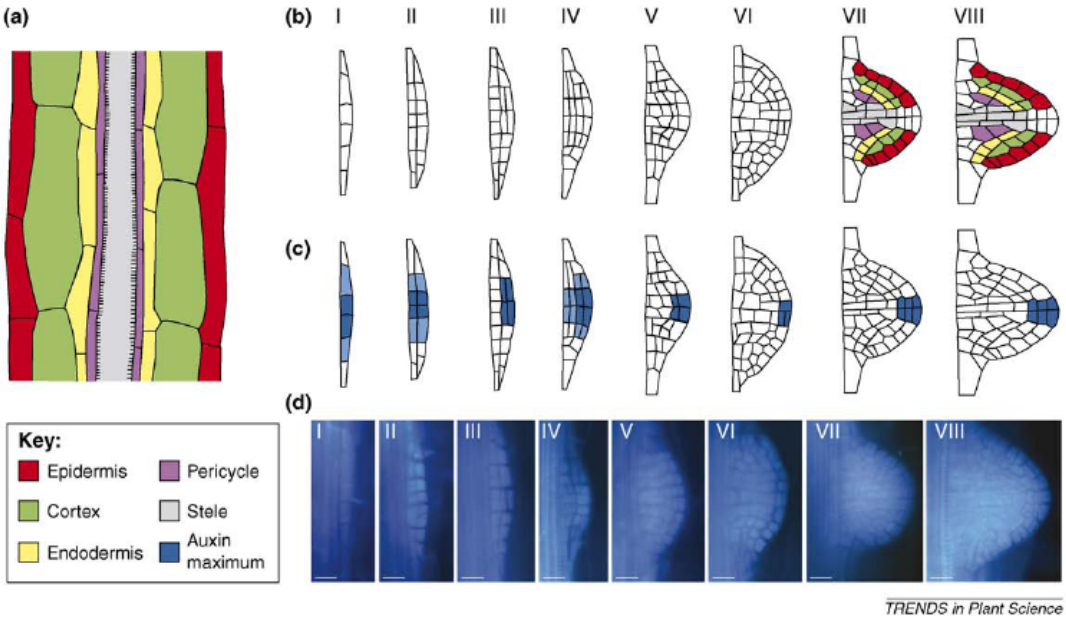

Fig. 5. 9. Morphological changes during lateral root development. Lateral roots originate deep within the primary root from the pericycle cells (a). The eight stages of primordium development (roman numbers) (Malamy and Benfey, 1997)are shown (b) as well as establishment of the auxin signaling maximum as demonstrated with the DR5:GUS reporter (blue gradient (c)) (Benkova et al., 2003). The cartoons were drawn from aniline-blue-stained roots for each stage of lateral root development (d). The scale bars represent 20 mm (Peret et al., 2009).

To track the changes with induction of WUS, the different stages of the lateral root development were studied. As shown in Fig. 5.10., with induction of *WUS*, no difference could be detected before Stage III of lateral root primordia development. However, from stage III onwards, differences could be observed in the line with induction of *WUS* (Fig. 5.10). Induction of *WUS* caused extra cell divisions in lateral root primordia. This could be clearly observed in stage VIII, that is, after lateral root had emerged from the primary root. In the basal part of the lateral root primordia, extra cell divisions occurred more frequently, thus this may indicate the early establishment of the leaf-like organs.

After the 'lateral root' emerged out from the primary root, there were two or three small tips in the lateral root region. The yellow arrows (Fig. 5.11) indicated the extra cell divisions occurred in the basal part of the tips. We also detected some red dots in the extra protrusions in the lateral root region (white arrows in Fig. 5.11). These red dots indicated the chloroplast in the leaf-like primordia. The small tips then developed into leaves primordia and leaves in the root. These data indicated continuous ectopic expression of *WUS* in the lateral root primordia overwritten the cell identity, established the root-to-shoot transformation, and led to leaf development in the lateral root region.

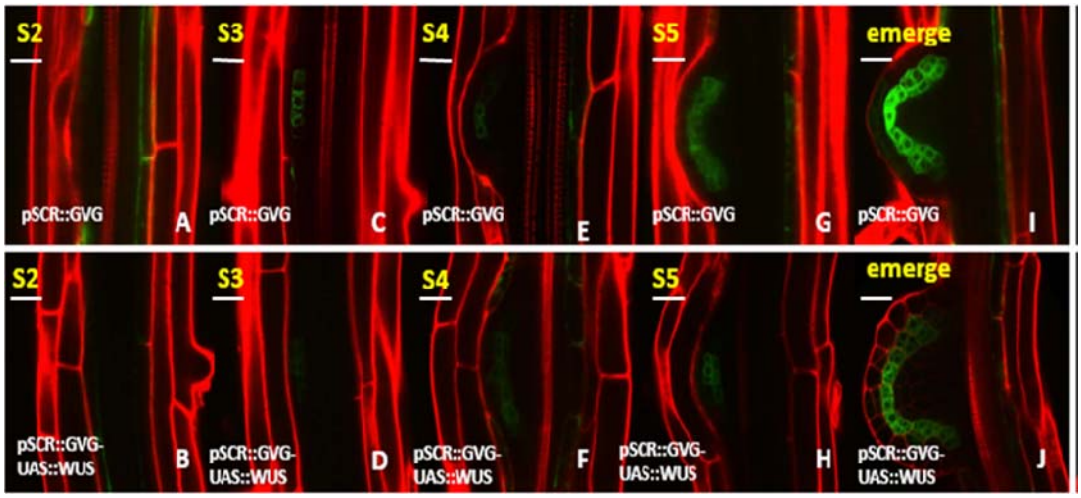

Fig. 5.10. Confocal images of different stages of lateral root primordia development: stage 2 (A&B), stage 3 (C&D), stage 4 (E&F), stage 5 (G&H), emerge (I&J). The pSCR::GVG and pSCR::GVG-UAS::WUS seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 4 days. Bars represent 20μm.

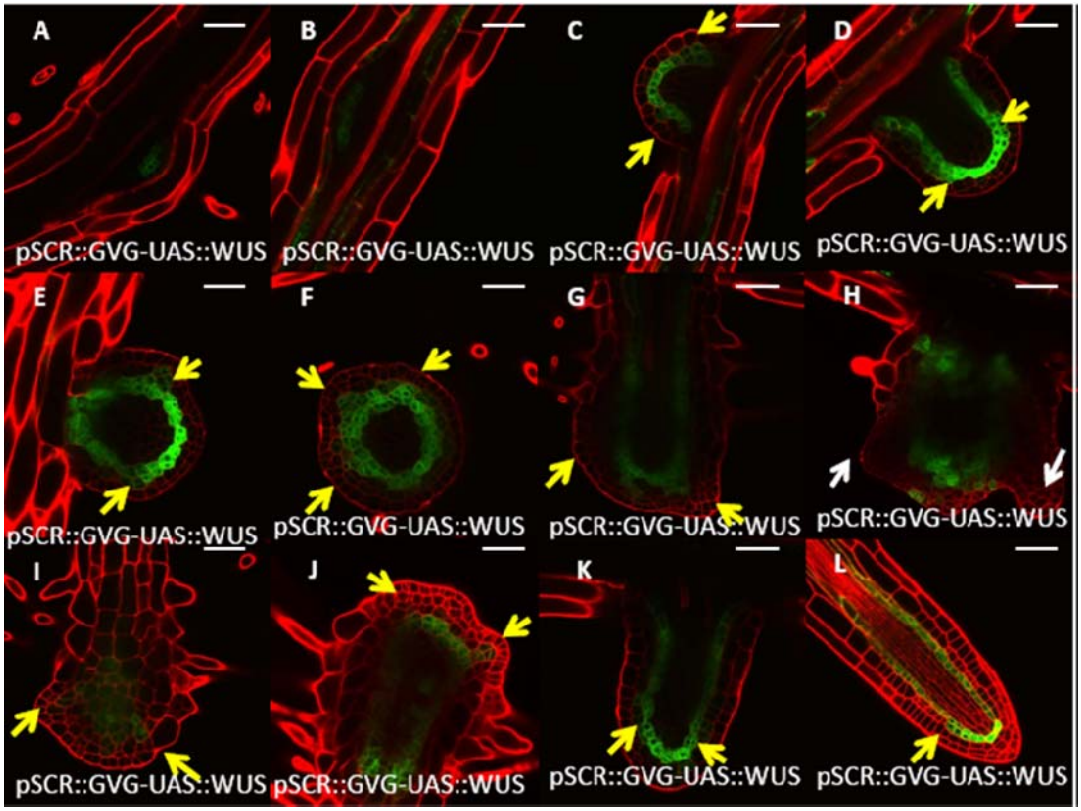

Fig. 5.11. Confocal images of different stages of lateral root development with induction of *WUS* in endodermis. (A) and (B) indicate lateral root primordia development. (C) indicates the emerging of lateral root. (D-L) indicate the lateral root development after emerging out from primary root. Yellow arrows indicate the extra cell divisions occurred in the lateral root. White arrows indicate red dots occurred in the cells. The pSCR::GVG and pSCR::GVG-UAS::WUS seeds were grow on MS plates for 3 days and transferred on 5µM DEX plates for 4 days. Bars represent 40µm.

Since the confocal images of lateral root primordia were difficult to reveal the inner cell arrangement in lateral root primordia, differential interference contrast microscopy (DIC) was used to track the lateral root development in both pSCR::GVG and pSCR::GVG-UAS::WUS lines. This experiment also confirmed that from stage III onwards extra cell divisions occurred in the basal part of the LRP (Fig. 5.12). From stage III to stage VIII, due to the extra cell divisions in basal part, the shape of lateral root primordia was flatter in the line with induction of *WUS* than in the line without induction of WUS. With longer time of induction, the 'lateral root' contained three or more tips (Fig. 5.13) and the cells in this region might acquire the identity of shoot apex cells since at later stage chloroplasts could be observed in the new formed tips. These tips were the leaf primordia (the yellow arrows in Fig. 5.13) and would develop into leaves in the root. In the middle part, there was the establishment of the shoot apical meristem (the red arrows in Fig. 5.13). As shown in Fig. 5.11., the red dots inside the cells were actually the chloroplast and the new formed tips in lateral root region became green. Thus, the induction of WUS induced the leaf-like organ formation and this establishment of shoot organs destroyed the lateral root development. Our data indicated that successful root-to-shoot transformation had occurred in the lateral root region and required continuous induction of *WUS* expression in endodermis following the establishment of lateral root primordia.

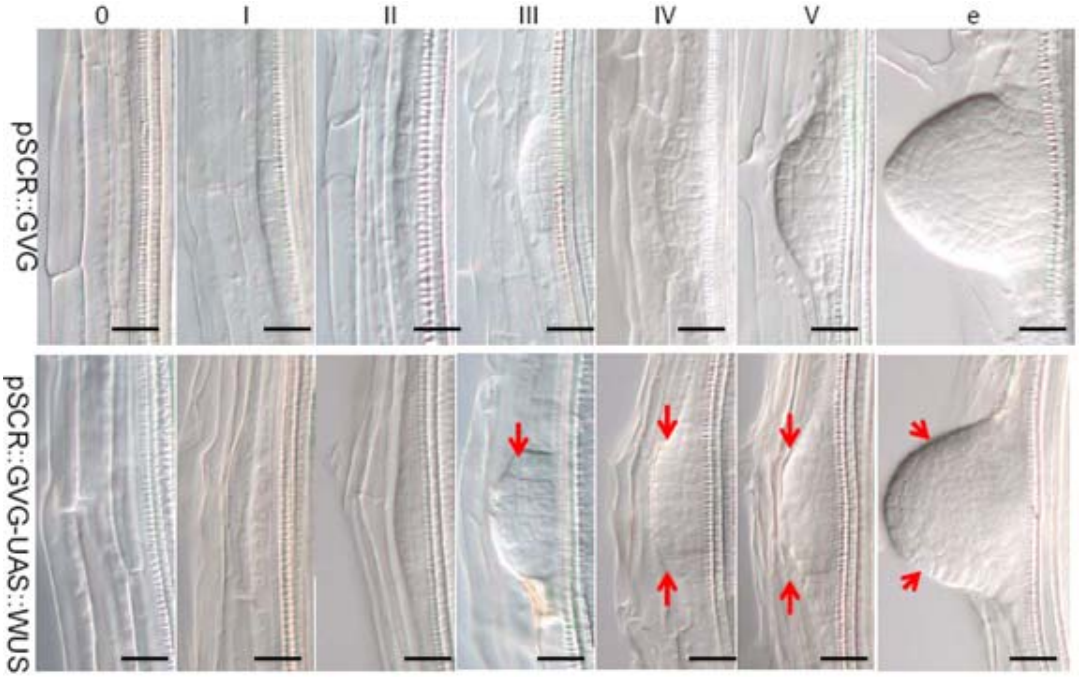

Fig. 5.12. DIC images of different stages of lateral root development. Red arrows indicate the extra cell divisions occurred in the lateral root primordia. The pSCR::GVG and pSCR::GVG-UAS::WUS seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 2 days. Bars represent 20μm.

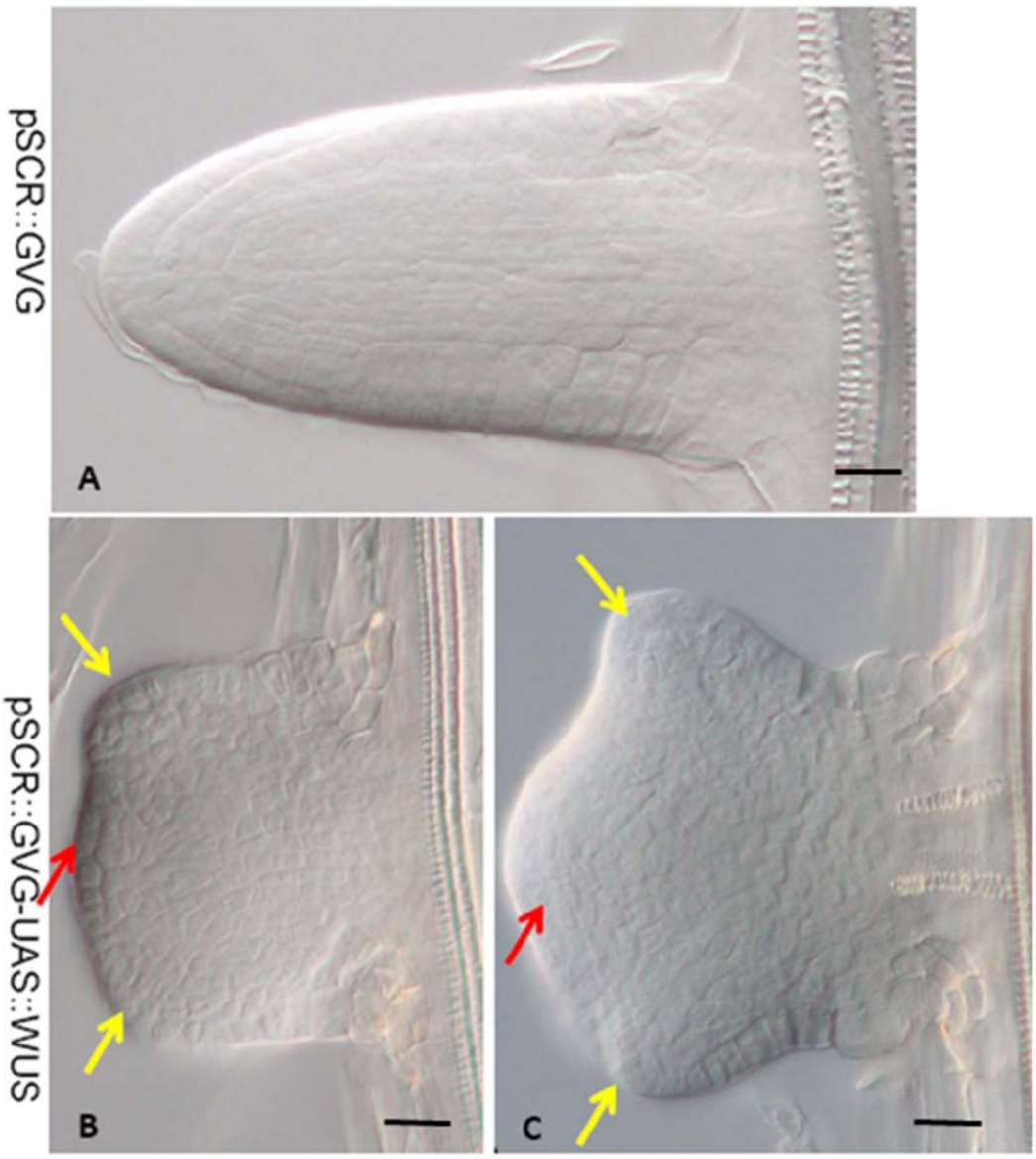

Fig. 5.13. Phenotype studies of long-term treatment with DEX. The pSCR::GVG (A) and pSCR::GVG-UAS::WUS (B&C) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 4 days. Yellow arrows indicate the leaf primordia formation in the lateral root region. Red arrows indicate the shoot apical meristems in the shoot-like organs. Bars represent 20μm.

5.2.4 Studies of ectopic expression of WUS in cortex

Although the induction of *WUS* in endodermis region by promoter of SCR could induce significant changes in primary root even after one day of induction, the changes induced by the induction by promoter of CO2 was much weaker after two days. This may be due to the weaker induction ability by promoter of CO2 indicated by the weaker GFP signal in the cortex layer (Fig. 5.14). The cell arrangement and cell shape in the cortex layer and apical region of epidermis were slightly different from the control. In the apical region of root meristem, the cells with expression of *WUS* were slightly larger than the control, and there were several cells which had the irregular cell shape and extra cell divisions. The induction with longer time was not performed; however, the changes in cell divisions and cell arrangement could be still predicted.

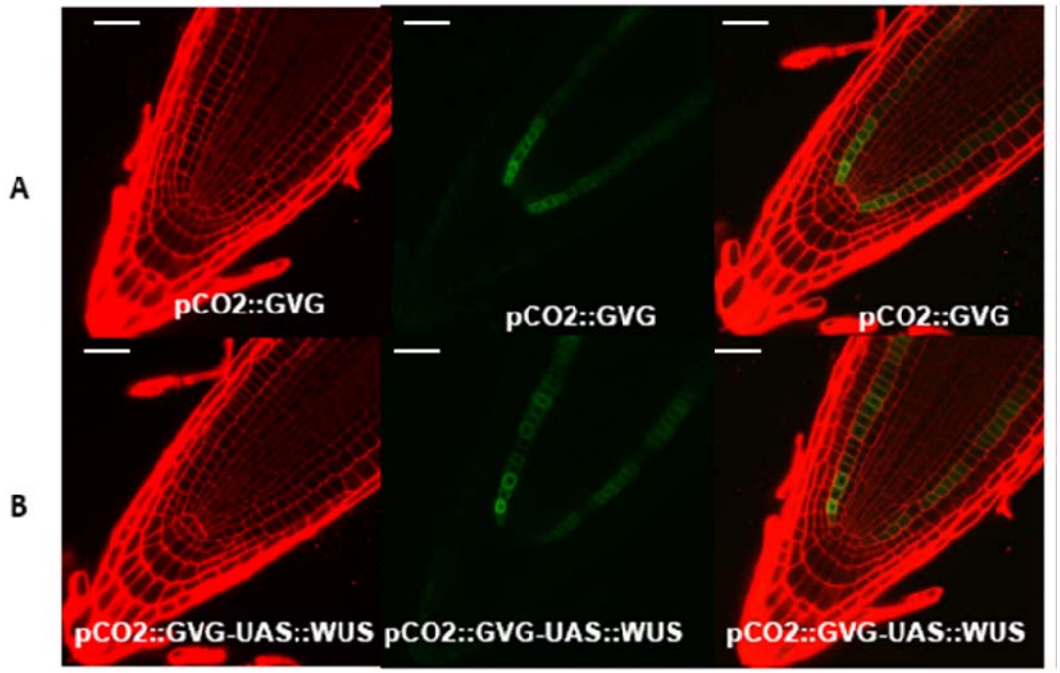

Fig. 5.14. Studies of the cortex cells with or without expression of WUS in cortex by DEX. The pCO2::GVG (panel A) and pCO2::GVG-UAS::WUS (panel B) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 2 days. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. Bars represent 40μm.

5.2.5 Studies of ectopic expression of *WUS* in quiescent centre

To study whether expression of *WUS* in QC could induce the leaf formation in the root, the pWOX5::GVG lines were used to ectopically express *WUS* in the QC. The expression of *WUS* in QC had weak effects: the strength of the expression was weaker in the *WUS* induction line; the cell shape and arrangement in columella stem cells, columella root cap, cortex/endodermis initials and some epidermal cells had changed (Fig. 5.15). The weak effects caused by *WUS* may be due to the weak expression of *WUS* and short time range of induction by DEX.

However, long term induction of *WUS* still was not sufficient to induce leaf formation in the lateral root region, although the *WUS* expression line did have less lateral roots (Fig. 5.16). This may be due to the weak expression pattern of *WUS*, and maybe some other root specific factors (e.g. CLE40) could inhibit the function of *WUS*.

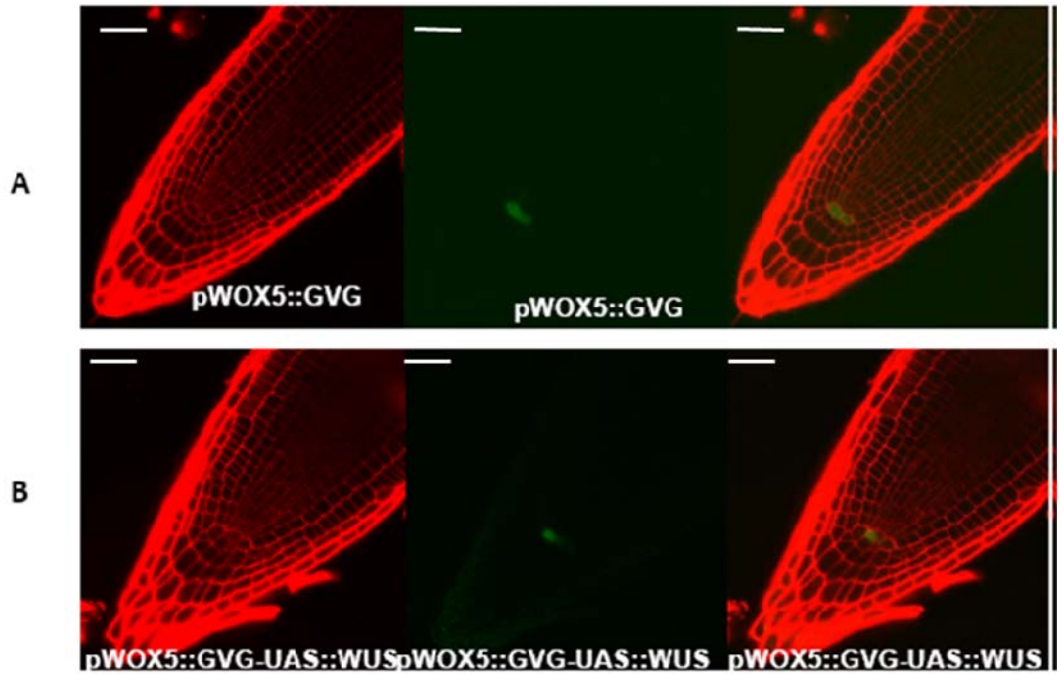

Fig. 5.15. Studies of the QC cells with or without expression of WUS in QC by DEX. The pWOX5::GVG (panel A) and pWOX5::GVG-UAS::WUS (panel B) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 1 day. The red signal indicates the cell membrane. The green signal indicates the GAL4-GFP expression. Bars represent 40μm.

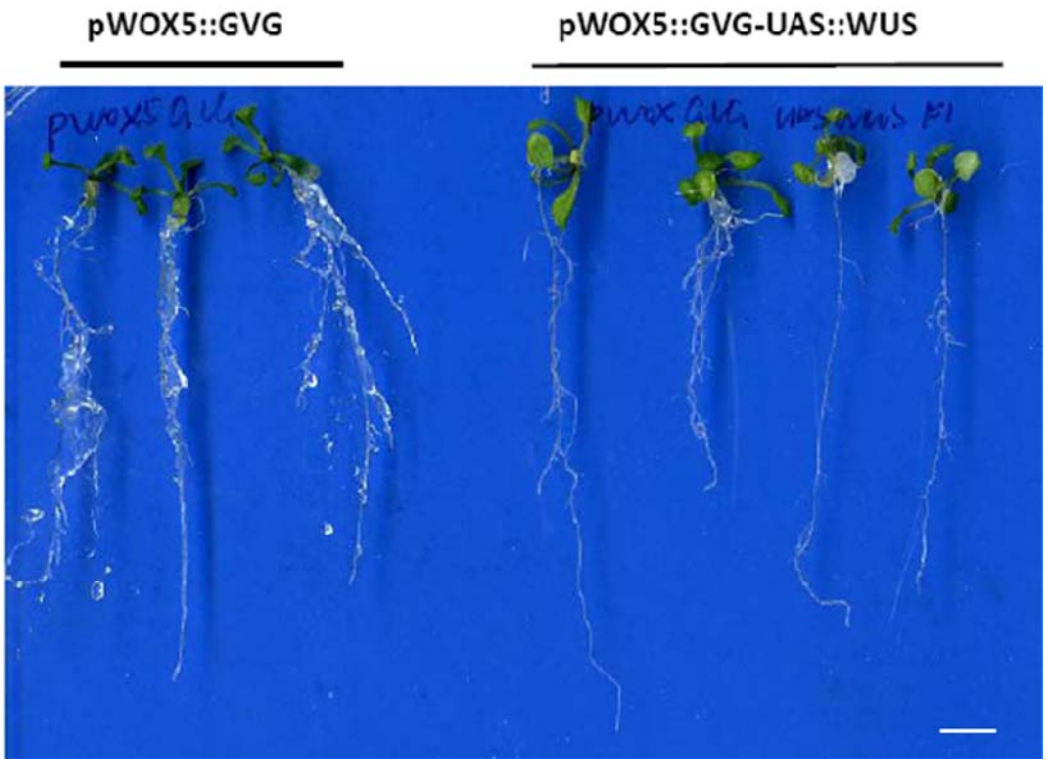

Fig. 5.16. Long-term studies of the QC cells with or without expression of WUS in QC by DEX. The pWOX5::GVG (left) and pWOX5::GVG-UAS::WUS (right) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 9 days. Bar represents 10mm.

5.3 Studies of changes in endodermis, cortex, epidermis when ectopic expression of WUS in endodermis by specific markers

To track the changes in the different cell layers (epidermis, cortex, endodermis and pericycle), some specific markers were combined with the *WUS* inducible lines. Combining fluorescence activated cell sorting methodologies (FACS) with genomic approaches, analysis of gene expression pattern in the three layers will shed light on how induction of *WUS* in endodermis affect the cells with expression of *WUS* and the adjacent cells, what cluster of genes have been upregulated or downregulated by ectopic expression of WUS in endodermis, and what genes have been the key regulators in the regeneration process. The pWER::CFP (expressed in epidermis), pCO2::H2BYFP (expressed in cortex) and pSCR::H2BYFP (expressed in endodermis) lines were crossed with pSCR::GVG-UAS::WUS, respectively.

5.3.1 Studies of changes in endodermis when ectopic expression of WUS in endodermis by pSCR::H2BYFP

When pSCR::GVG-UAS::WUS was combined with the endodermis specific marker pSCR::H2BYFP, the cells of YFP expression was consistent with the cells of GFP expression, indicating that these cells with YFP and GFP signals maintain the identity of endodermis. Consistent with the results of pSCR::GVG-UAS::WUS lines, the periclinal division occurred in both endodermis and cortex (Fig. 5.17, panel B). The periclinal divisions which occurred in endodermis generated daughter cells with cell identities of endodermis, while the periclinal divisions which occurred in cortex generated daughter cells without identities of endodermis (Fig. 5.17, panel B). This showed the ectopic expression of *WUS* in endodermis affected some changes in gene expression pattern in cortex cells. To study these gene expression pattern changes in the endodermis, the FACS (sorting the cells with YFP expression in the nucleus) and microarray assay will be performed.

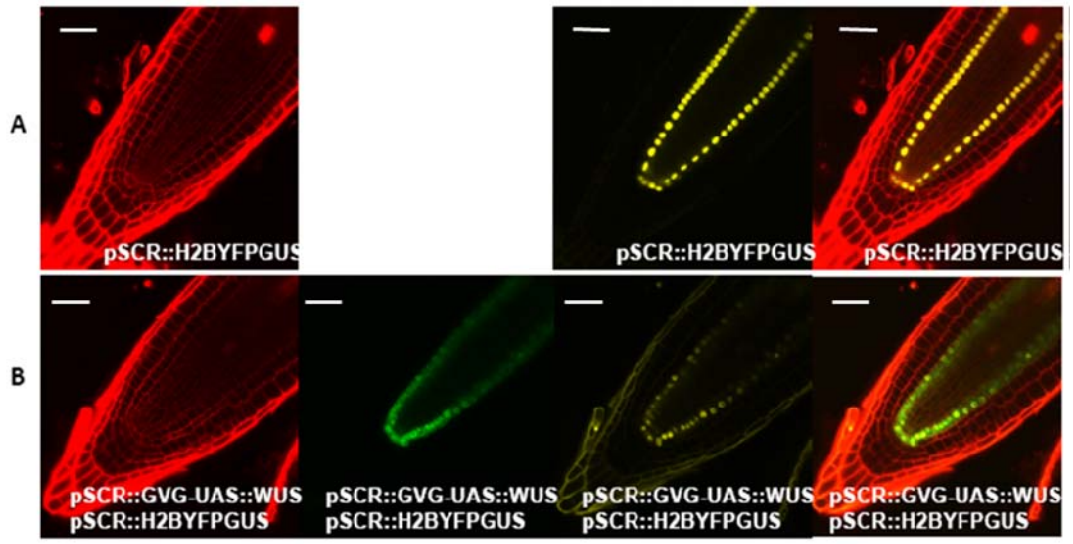

Fig. 5.17. Assay of endodermis layer by an endodermis specific marker (pSCR::H2BYFP) with DEX treatment. The pSCR::H2BYFP (panel A) and pSCR::GVG-UAS::WUS pSCR::H2BYFP (panel B) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 1 day. The red, green and yellow signals indicate the cell membrane, GAL4-GFP expression and the nucleus respectively. Bars represent 40μm.

5.3.2 Studies of changes in cortex when ectopic expression of WUS in endodermis by pCO2::H2BYFP

When combined with the cortex specific marker, pCO2::H2BYFP, the cell identity of cortex cells was affected by one day induction of *WUS* in endodermis (Fig. 5.18). Due to the extra cell divisions in the outer region of endodermis, these cells had lost the identity of cortex cells in the lower part of the meristem (Fig. 5.18B&C). However, some upper cells in the cortex region did maintain the cell identity of cortex cells (Fig. 5.18B&C). The periclinal divisions in cortex or endodermis created some cells with neither identity of cortex and endodermis (the white arrow in Fig. 5.18B). This may indicate that the periclinal divisions generated more cell layers and some cells which might maintain the cell identity of shoot stem cells.

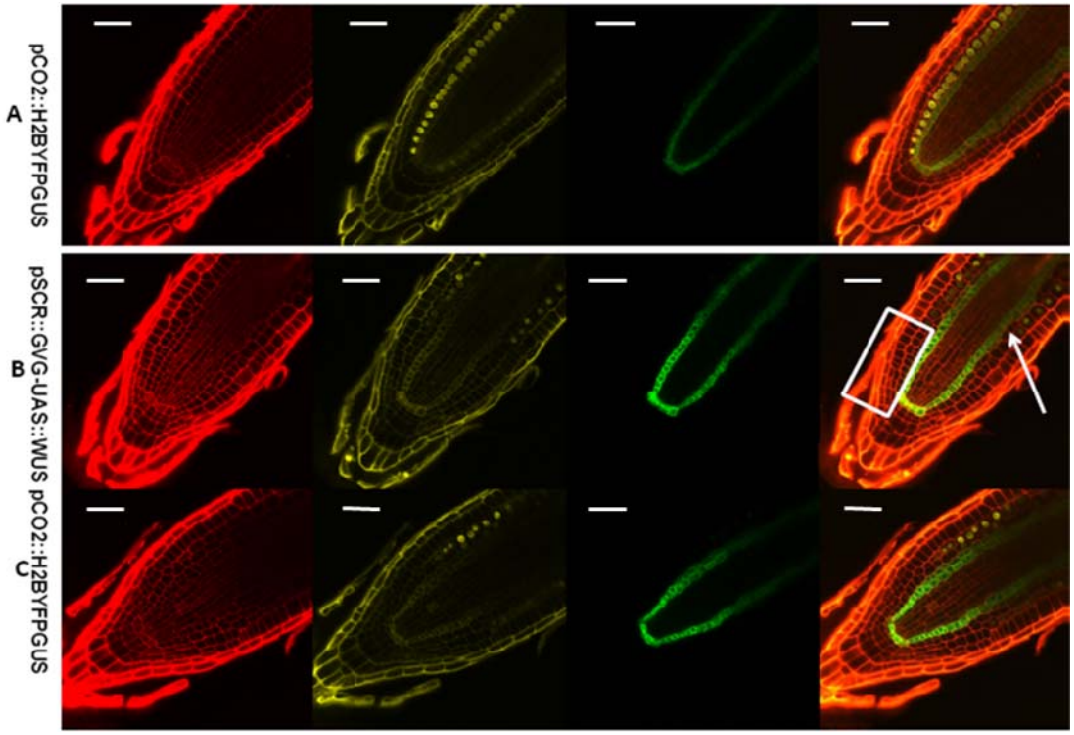

Fig. 5.18. Assay of cortex layer by a cortex specific marker (pCO2::H2BYFP) with DEX treatment. The pCO2::H2BYFP (panel A) and pSCR::GVG-UAS::WUS pCO2::H2BYFP (panel B&C) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 1 day. The red, green and yellow signals indicate the cell membrane, GAL4-GFP expression and the nucleus respectively. The white arrow indicates cells with no GFP and YFP occurred between endodermis and cortex. The white square frame indicates cells lost the identity of cortex. Bars represent 40μum.

5.3.3 Studies of changes in epidermis when ectopic expression of *WUS* in endodermis by pWER::H2BYFP

When combined with the epidermis marker, pWER::CFP, with one day induction of WUS in endodermis the pSCR::GVG-UAS::WUS lines showed much weaker changes in the epidermis. The strength of CFP signal in the WUS-inducible line was much weaker than the strength of CFP signal in pSCR::GVG line (Fig. 5.19). One possible explanation is that one day of the ectopic expression of *WUS* in endodermis do affect the cell identity in cortex layer, however, the induction of *WUS* is not enough to change the cell identity in epidermis layer. The changes in epidermis layer may need longer time (e.g. 2 days) of induction to affect the cells in epidermis.

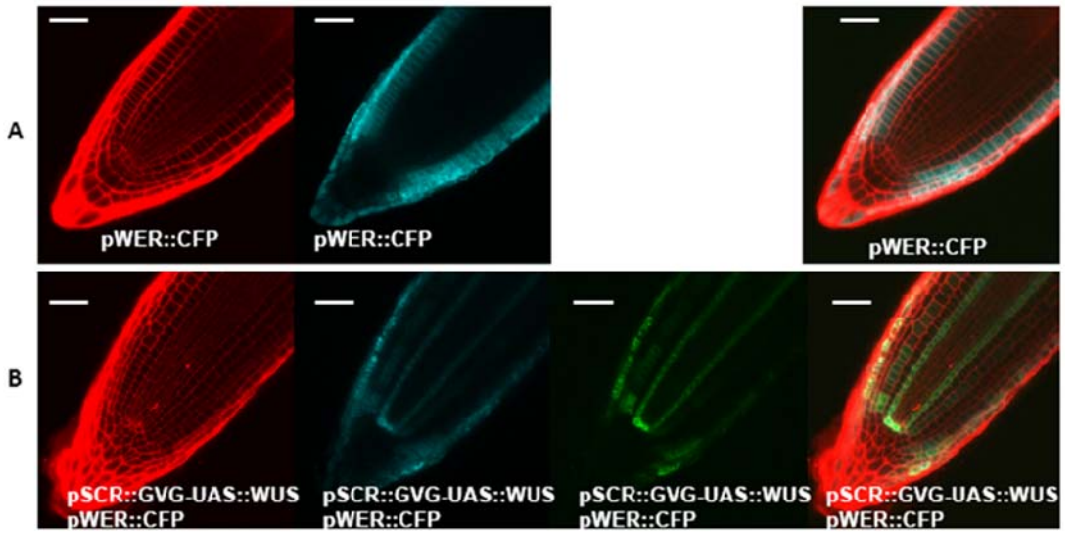

Fig. 5.19. Assay of epidermis layer by an epidermis specific marker (pWER::CFP) with DEX treatment. The pWER::CFP (panel A) and pSCR::GVG-UAS::WUS pWER::CFP (panel B) seeds were grow on MS plates for 3 days and transferred on 5μM DEX plates for 1 day. The red, green and cyan signals indicate the cell membrane, GAL4-GFP expression and epidermis respectively. Bars represent 40μm.

5.4 Discussion

5.4.1 Ectopic expression of *WUS* in root caused meristem cell fate and shoot regeneration

In this study, the glucocorticoid inducible GAL4VP16-GR/UAS system was used to ectopically express *WUS* in some specific tissues and cells in *Arabidopsis* root.

Ectopic expression of *WUS* in columella root cap led to strong phenotypes in both non-inducible and inducible lines. In non-inducible lines, ectopic expression of *WUS* in columella root cap led to callus-like root tip, while in inducible line it led to a cluster of cells in the root cap. These induced cells lost the cell identity of their mother cells.

Ectopic expression of *WUS* in endodermis also led to strong phenotypes in both lines: shorter root length, longer hypocotyls and curled leaves. For the non-inducible lines, ectopic expression of *WUS* in endodermis led to delayed development and no flower development. For inducible lines, ectopic expression of *WUS* in endodermis led to no lateral root development but induced leaf formation in the lateral root region. Our results indicated the ectopic expression of *WUS* in early stage of lateral root primordia development could lead to the root-to-shoot transformation.

Ectopic expression of *WUS* in endodermis induced extra cell divisions in endodermis, cortex, and epidermis. The induced cell divisions led to new cell identity.

The root-to-shoot regeneration in lateral root primordia was different from control since the stage 3 onwards. From the emerging of ‘lateral root’, extra cell divisions could be detected in the sides which may be the leaf primordia. After the emerging of ‘later root tip’, the leaf primordia and shoot apical meristem could be found in this tip.

Our data indicate the expression of *WUS* in endodermis since lateral root primordia formation could induce the root-to-shoot regeneration. Continuous induction of *WUS* in the

lateral primordia led to overwrite the cell identity of lateral root and establish the shoot meristem formation and the leaf primordia.

The overwriting of cell fate is under the epigenetic regulation. The evolutionarily conserved SWI/SNF ATPase complexes control the developmental gene expression (Kwon et al., 2005). It was reported SNF2 ATPases played important roles in cell division, differentiation and embryo patterning in *Drosophila* and *Mice* (Bultman et al., 2000; Kennison and Tamkun, 1988; Reyes et al., 1998). Regulation of many transcription factors is dependent on SNF2-containing chromatin-remodeling complexes (Martens and Winston, 2003; Peterson and Workman, 2000). The complexes, which control the access of trans-acting transcriptional regulators or of components of the general transcriptional machinery to the condensed eukaryotic genome (Emerson, 2002; Narlikar et al., 2002), are recruited to *cis*-regulatory DNA regions by sequence-specific transcriptional activators/repressors or by specific histone modification code at these sites(Martens and Winston, 2003; Peterson and Workman, 2000). *SPLAYED* (*SYD*) is a member of the *SNF2* ATPases subfamily of transcriptional coregulators (Verbsky and Richards, 2001). The molecular and genetic studies of *SYD* revealed that it played a role in regulation of the stem cell pool in the SAM primarily via direct transcriptional control of the master SAM regulator *WUS* (Kwon et al., 2005). In addition, loss of function FAS1 or FAS2, which are implicated in chromatin assembly (Kaya et al., 2001), led to ectopic expression of *WUS*. These together indicated the regulation of *WUS* was through the epigenetic regulation. Skipping the epigenetic regulation of *WUS* by ectopic induction of *WUS* can lead to the organ regeneration.

5.4.2 Can WUS-related homeobox genes lead to organ regeneration?

WUS itself is expressed in the organizing centre cells and maintains the stem cell pool. In *Arabidopsis thaliana*, *WUS* has 14 othologs, namely *WOX1-14* (van der Graaff et al., 2009). For instance, *WOX5* is expressed in the RAM and maintains the root meristem. The

functions of *WOX* family genes are stem cell maintenance, lateral organ formation, embryo patterning, or regulation in cell proliferation. Our studies showed that ectopic expression of *WUS* in root induced the shoot organ regeneration. This raises one question: whether ectopic expression of *WUS*-related homeobox genes could induce the organ regeneration? Over-expression of *WUS* in the QC showed no significant induction of cell division and no organ regeneration. One possible explanation is some specific inhibitor could restrict both *WOX5* and *WUS* in some specific region. The fact that Over-expression of *WOX1* by gain-of-function mutation in *WOX1* showed down-regulation in *CLV3* and smaller organ size and cell size in leaves than wild type (Zhang et al., 2011), suggested cell expansion and division is possibly affected in order to have partially retarded the organ development. However, what extent and how the *WOX* family genes could induce organ regeneration still remained to be explored. In order to address this question, some future studies using similar approaches to ectopic expression of *WOX* family genes in the specific tissue or cells will shed light on the underlying mechanisms of organ regeneration.

5.4.3 How do auxin and cytokinin cross-talk in the regeneration process?

Plant hormones played important roles in controlling organ growth and differentiation. Two most common plant hormones are auxin and cytokinin. It was reported that a high auxin-to-cytokinin concentration ratio could promote the root formation (including lateral root development), while a low auxin-to-cytokinin concentration ratio could promote shoot development (Muraro et al., 2011). Auxin was accumulated in the lateral root founder cells (Benkova et al., 2003) and promoted differentiation of the lateral root primordia. Thus, during the root-to-shoot regeneration by ectopic expression of *WUS* in endodermis, when and how the auxin and cytokinin cross-talk and the concentration ratio swithes remains to be investigated.

5.4.4 Regeneration in animals

Compared with plants and some animals, i.e. fishes and amphibians, humans have little capacity to regenerate lost appendages. Despite extensive studies in plants, we still have no idea why mammals lost the regeneration ability. The cells located within the inner cell mass (ICM) of the developing blastocyst, can be explanted and embryonic stem (ES) cells lines established from them that can be cultured in vitro, essentially indefinitely (Chambers and Tomlinson, 2009). Some transcriptional factors are essential to maintain the pluripotency of ES cells: Oct4, Sox2, and Nanog (Chambers et al., 2003; Chambers et al., 2007; Masui et al., 2007; Niwa et al., 2000). The homeodomain protein Nanog is reported to play roles in mediating acquisition of both embryonic and induced pluripotency (Silva et al., 2009). Compared with plant homeodomain protein WUS, Nanog might function as a similar pathway in maintenance of the stem cells.

Our studies in regeneration in plants might shed light on the underlying mechanisms of regeneration and also apply to parallel studies in animals and humans.

Chapter 6 General conclusions and future work

6.1. General conclusions

Recent studies on regeneration in *Arabidopsis* have revealed many new findings, such as: (1) ectopic expression of the *Arabidopsis* class-1 *KNOX* gene, *KNAT2*, could restore carpel development to *stm* mutants (Scofield, 2008); (2) ectopic expression of *STM* and *WUS* activated a subset of meristem functions, including cell division, *CLAVATA1* expression and organogenesis (Gallois et al., 2002); (3) ectopic induction of *WUS* expression in *Arabidopsis* root tips could induce shoot stem cell identity and leaf development (without additional cues), flower development (together with LEAFY, which is a key regulator of flower development) (Wagner et al., 2004; Weigel and Nilsson, 1995), or embryogenesis (in response to increased level of auxin) (Gallois et al., 2004); (4) ectopic expression of a stable version of *REVOLUTA* (*REV*, a *HD-ZIP III* transcription factor) (Talbert et al., 1995)under the promoter of *PLETHORA2* (*PLT2*) was able to initiate another shoot pole in the root pole region (Smith and Long, 2010); (5) once the quiescent centre is laser ablated, the adjoining stem cells goes into differentiation and an auxin maximum recovers and promotes the establishment of a distal organizer (Sabatini et al., 1999; van den Berg et al., 1997) and the surrounding stem cells triggers a local regeneration response which eventually leads to the regeneration of a new root tip (Xu et al., 2006); (6) new shoot buds can be induced from roots or root-derived explants (Gordon et al., 2007; Sugimoto et al., 2010; West and Harada, 1993) and the regeneration from callus from multiple tissues occurs via a root development pathway (Sugimoto et al., 2010).

Our data indicated ectopic expression of *WUS* in epidermis, cortex, quiescent centre, columella stem cells, columella root cap, lateral root cap, pericycle did lead to aberrant cell arrangement in the some meristem cells, abnormal cell shape and extra cell divisions in both the cells with expression of *WUS* and the adjoining cells. However, ectopic expression of *WUS* in these cells was not sufficient to induce the shoot-like organs formation in the root although some cells with shoot stem cell identity were induced in the root.

In this study, the glucocorticoid inducible GAL4VP16-GR/UAS system was used to ectopically express *WUS* in some specific tissues and cells in *Arabidopsis* root.

Ectopic expression of *WUS* in columella root cap led to strong phenotypes in both non-inducible and inducible lines. In non-inducible lines, ectopic expression of WUS in columella root cap led to callus-like root tip, while in inducible line it led to a cluster of cells in the root cap. These induced cells lost the cell identity of their mother cells.

Ectopic expression of *WUS* in endodermis also led to strong phenotypes in both lines: shorter root length, longer hypocotyls and curled leaves. For the non-inducible lines, ectopic expression of *WUS* in endodermis led to delayed root development and no flower development. For inducible lines, ectopic expression of WUS in endodermis led to no lateral root development but leaf formation in the lateral root region.

Ectopic expression of *WUS* in endodermis induced extra cell divisions in endodermis, cortex, and epidermis. The induced cell divisions led to new cell identity.

The root to shoot regeneration in lateral root primordia was different from control since the stage 3 onwards. From the emerging of ‘lateral root’, extra cell divisions could be detected in the sides which may be the leaf primordia. After the emerging of ‘later root tip’, the leaf primordia and shoot apical meristem could be found in this tip.

Our data indicate the expression of *WUS* in endodermis after the primary root development but before lateral root primordia formation could induce the root to shoot

regeneration. During the regeneration process, the *WUS*-induced cell divisions and shoot stem cell identity promote the establishment of shoot apical meristem and leaf primordia.

6.2. Future work

Ectopic expression of *WUS* in *SCR*-expressing cells was demonstrated to induce the root to shoot regeneration at the position where lateral root forms, but the precise roles of WUS in the root to shoot regeneration remain to be elucidated and the regulation of downstream genes through *WUS* should be studied further. The existing microarray data have used the whole plants or parts of the plants (root, apex, leaf, or flower) in WT plants and 35S::WUS lines (Busch et al., 2010). A better way would be analysis of gene profiling in some specific tissues or cells. Recent technological advances combining FACS of cell/tissue-specific fluorescent marker lines and genomic approaches have led to a comprehensive understanding of cell/tissue-specific gene expression patterns in the *Arabidopsis* root, which is on the developmental time scale with unprecedented resolution (Birnbaum et al., 2003; Brady et al., 2007; Dinneny and Benfey, 2008; Dinneny et al., 2008; Sena et al., 2009). Thus, a combinational of new developed *in vivo* live imaging techniques, fluorescent marker lines, FACS, microarray expression profiling, regenerative mutant analysis and computational modeling should help us to gain a more complete understanding of regeneration mechanisms in plants. A time scale of induction of *WUS* (3 hours, 6 hours, 12 hours, and 24 hours) in endodermis with combining FACS and microarray expression profiling will be necessary to identify the gene regulation in endodermis, cortex and epidermis.

It will be necessary to induce the expression of *WUS* in pericycle in an-inducible manner since the pericycle founder cells develop into the lateral root. To further study the ability of root to shoot regeneration, the plant phytohormones might also play some roles during this process and it is necessary to study their roles in the regeneration process. Thus, combining with some auxin markers will be necessary.

As the root to shoot regeneration is related to lateral root development, combining with the lateral root deficient mutant will help us understand the regeneration process. Thus, to further study how the ectopic expression of WUS affects lateral root development, the slr-1d (no lateral root development) mutant will be crossed with pSCR::GVG-UAS::WUS lines. Moreover, since long-term induction of WUS in endodermis could induce leaf formation in the lateral root region, combining with the leaf formation deficient mutant will help us understand the regeneration process. Thus, to further study how the ectopic expression of WUS affects leaf formation in the root, some mutants which cannot form leaves will be crossed with pSCR::GVG-UAS::WUS lines.

References

Aida, M., Beis, D., Heidstra, R., Willemsen, V., Blilou, I., Galinha, C., Nussaume, L., Noh, Y.S., Amasino, R., and Scheres, B. (2004). The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. Cell *119*, 109-120.

Aoyama, T., and Chua, N.H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J *11*, 605-612.

Baurle, I., and Laux, T. (2005). Regulation of WUSCHEL transcription in the stem cell niche of the Arabidopsis shoot meristem. Plant Cell *17*, 2271-2280.

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591-602.

Birnbaum, K., Shasha, D.E., Wang, J.Y., Jung, J.W., Lambert, G.M., Galbraith, D.W., and Benfey, P.N. (2003). A gene expression map of the Arabidopsis root. Science *302*, 1956-1960.

Birnbaum, K.D., and Sanchez Alvarado, A. (2008). Slicing across kingdoms: regeneration in plants and animals. Cell *132*, 697-710.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature *433*, 39-44.

Bonnet, C. (1779). Oeuvres d'Histoire Naturelle et de Philosophie de Charles Bonnet (Neuchatel, Switzerland: Samuel Fauche).

Brady, S.M., Orlando, D.A., Lee, J.Y., Wang, J.Y., Koch, J., Dinneny, J.R., Mace, D., Ohler, U., and Benfey, P.N. (2007). A high-resolution root spatiotemporal map reveals dominant expression patterns. Science *318*, 801-806.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M., and Simon, R. (2000). Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science *289*, 617-619.

Bultman, S., Gebuhr, T., Yee, D., La Mantia, C., Nicholson, J., Gilliam, A., Randazzo, F., Metzger, D., Chambon, P., Crabtree, G., *et al.* (2000). A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes. Mol Cell *6*, 1287-1295.

Busch, W., Miotk, A., Ariel, F.D., Zhao, Z., Forner, J., Daum, G., Suzaki, T., Schuster, C., Schultheiss, S.J., Leibfried, A., *et al.* (2010). Transcriptional control of a plant stem cell niche. Dev Cell *18*, 849-861.

Campbell, R.N., Leverentz, M.K., Ryan, L.A., and Reece, R.J. (2008). Metabolic control of transcription: paradigms and lessons from Saccharomyces cerevisiae. Biochem J *414*, 177-187.

Casimiro, I., Marchant, A., Bhalerao, R.P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P.J., *et al.* (2001). Auxin transport promotes Arabidopsis lateral root initiation. Plant Cell *13*, 843-852.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L., and Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. Nature *450*, 1230-1234.

Chambers, I., and Tomlinson, S.R. (2009). The transcriptional foundation of pluripotency. Development *136*, 2311-2322.

Clark, S.E. (1997). Organ formation at the vegetative shoot meristem. Plant Cell *9*, 1067-1076.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1993). CLAVATA1, a regulator of meristem and flower development in Arabidopsis. Development *119*, 397-418.

Clark, S.E., Running, M.P., and Meyerowitz, E.M. (1995). CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as CLAVATA1 Development *121*, 2057-2067.

Davenport, R.J. (2005). What controls organ regeneration? Science *309*, 84.

Davison, J.M., Akitake, C.M., Goll, M.G., Rhee, J.M., Gosse, N., Baier, H., Halpern, M.E., Leach, S.D., and Parsons, M.J. (2007). Transactivation from Gal4-VP16 transgenic insertions for tissue-specific cell labeling and ablation in zebrafish. Dev Biol *304*, 811-824.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., *et al.* (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. Development *134*, 681-690.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M.G., Feldmann, K.A., and Benfey, P.N. (1996). The SCARECROW gene regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell *86*, 423-433.

Dinneny, J.R., and Benfey, P.N. (2008). Plant stem cell niches: standing the test of time. Cell *132*, 553-557.

Dinneny, J.R., Long, T.A., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J., and Benfey, P.N. (2008). Cell identity mediates the response of Arabidopsis roots to abiotic stress. Science *320*, 942-945.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organisation of the Arabidopsis thaliana root. Development *119*, 71-84.

Dubrovsky, J.G., Rost, T.L., Colon-Carmona, A., and Doerner, P. (2001). Early primordium morphogenesis during lateral root initiation in Arabidopsis thaliana. Planta *214*, 30-36.

Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q., Gerentes, D., Perez, P., and Smyth, D.R. (1996). AINTEGUMENTA, an APETALA2-like gene of Arabidopsis with pleiotropic roles in ovule development and floral organ growth. Plant Cell *8*, 155-168.

Emerson, B.M. (2002). Specificity of gene regulation. Cell *109*, 267-270.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J.Z., and Laux, T. (1996). The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. Plant J *10*, 967-979.

Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M. (1988). GAL4 activates transcription in Drosophila. Nature *332*, 853-856.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R., and Meyerowitz, E.M. (1999). Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science *283*, 1911-1914.

Galinha, C., Hofhuis, H., Luijten, M., Willemsen, V., Blilou, I., Heidstra, R., and Scheres, B. (2007). PLETHORA proteins as dose-dependent master regulators of Arabidopsis root development. Nature *449*, 1053-1057.

Gallois, J.L., Nora, F.R., Mizukami, Y., and Sablowski, R. (2004). WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. Genes Dev *18*, 375-380.

Gallois, J.L., Woodward, C., Reddy, G.V., and Sablowski, R. (2002). Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in Arabidopsis. Development *129*, 3207-3217.

Goebel, K. (1898). Organographie der Pflanzen (Jena: I. Allgemeine Organographie).

Gordon, S.P., Heisler, M.G., Reddy, G.V., Ohno, C., Das, P., and Meyerowitz, E.M. (2007). Pattern formation during de novo assembly of the Arabidopsis shoot meristem. Development *134*, 3539-3548.

Groover, A.T., Mansfield, S.D., DiFazio, S.P., Dupper, G., Fontana, J.R., Millar, R., and Wang, Y. (2006). The Populus homeobox gene ARBORKNOX1 reveals overlapping mechanisms regulating the shoot apical meristem and the vascular cambium. Plant Mol Biol *61*, 917-932.

Gross-Hardt, R., and Laux, T. (2003). Stem cell regulation in the shoot meristem. J Cell Sci *116*, 1659-1666.

Hartley, K.O., Nutt, S.L., and Amaya, E. (2002). Targeted gene expression in transgenic Xenopus using the binary Gal4-UAS system. Proc Natl Acad Sci U S A *99*, 1377-1382.

Haseloff, J., and Hodge, S. (2001). Targeted gene expression in plants using GAL4 US Patent *6*, 558.

Heck, G.R., Perry, S.E., Nichols, K.W., and Fernandez, D.E. (1995). AGL15, a MADS domain protein expressed in developing embryos. Plant Cell *7*, 1271-1282.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.T., and Benfey, P.N. (2000). The SHORT-ROOT gene controls radial patterning of the Arabidopsis root through radial signaling. Cell *101*, 555-567.

Holm, T. (1925). On the development of buds upon roots and leaves. Ann Bot *39*.

Iantcheva, A., Vlahova, M., Bakalova, E., Kondorosi, E., Elliott, M.C., and Atanassov, A. (1999). Regeneration of diploid annual medics via direct somatic embryogenesis promoted by thidiazuron and benzylaminopurine. Plant Cell Reprod 904-910.

Iantcheva, A., Vlahova, M., Trinh, T.H., Brown, S.C., Slater, A., Elliott, M.C., and Atanassov, A. (2001). Assessment of polysomaty, embryo formation and regeneration in liquid media for various species of diploid annual Medicago. Plant Science, 621-627.

Jeong, S., Trotochaud, A.E., and Clark, S.E. (1999). The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell *11*, 1925-1934.

Kaya, H., Shibahara, K.I., Taoka, K.I., Iwabuchi, M., Stillman, B., and Araki, T. (2001). FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. Cell *104*, 131-142.

Kayes, J.M., and Clark, S.E. (1998). CLAVATA2, a regulator of meristem and organ development in Arabidopsis. Development *125*, 3843-3851.

Kennison, J.A., and Tamkun, J.W. (1988). Dosage-dependent modifiers of polycomb and antennapedia mutations in Drosophila. Proc Natl Acad Sci U S A *85*, 8136-8140.

Klar, A.J., and Halvorson, H.O. (1974). Studies on the positive regulatory gene, GAL4, in regulation of galactose catabolic enzymes in Saccharomyces cerevisiae. Mol Gen Genet *135*, 203-212.

Koncz, C., Kreuzaler, F., Kalman, Z., and Schell, J. (1984). A simple method to transfer, integrate and study expression of foreign genes, such as chicken ovalbumin and alpha-actin in plant tumors. EMBO J *3*, 1029-1037.

Kwon, C.S., Chen, C., and Wagner, D. (2005). WUSCHEL is a primary target for transcriptional regulation by SPLAYED in dynamic control of stem cell fate in Arabidopsis. Genes Dev *19*, 992-1003.

Laskowski, M., Grieneisen, V.A., Hofhuis, H., Hove, C.A., Hogeweg, P., Maree, A.F., and Scheres, B. (2008). Root system architecture from coupling cell shape to auxin transport. PLoS Biol *6*, e307.

Laufs, P., Grandjean, O., Jonak, C., Kieu, K., and Traas, J. (1998). Cellular parameters of the shoot apical meristem in Arabidopsis. Plant Cell *10*, 1375-1390.

Laux, T. (2003). The stem cell concept in plants: a matter of debate. Cell *113*, 281-283.

Laux, T., Mayer, K.F., Berger, J., and Jurgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development *122*, 87-96.

Li, X.Q., and Demarly, Y. (1995). Characterisation of factors affecting plant regeneration frequency of Medicago lupulina L. Euphytica, 143-148.

Malamy, J.E., and Benfey, P.N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development *124*, 33-44.

Martens, J.A., and Winston, F. (2003). Recent advances in understanding chromatin remodeling by Swi/Snf complexes. Curr Opin Genet Dev *13*, 136-142.

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., *et al.* (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol *9*, 625-635.

Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., and Laux, T. (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell *95*, 805-815.

Muller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998). AtPIN2 defines a locus of Arabidopsis for root gravitropism control. EMBO J *17*, 6903-6911.

Muller, R., Borghi, L., Kwiatkowska, D., Laufs, P., and Simon, R. (2006). Dynamic and compensatory responses of Arabidopsis shoot and floral meristems to CLV3 signaling. Plant Cell *18*, 1188-1198.

Muraro, D., Byrne, H., King, J., Voss, U., Kieber, J., and Bennett, M. (2011). The influence of cytokinin-auxin cross-regulation on cell-fate determination in Arabidopsis thaliana root development. J Theor Biol *283*, 152-167.

Narlikar, G.J., Fan, H.Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475-487.

Niwa, H., Miyazaki, J., and Smith, A.G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet *24*, 372-376.

Nolan, K.E., Rose, R.J., and Gorst, J.R. (1989). Regeneration of Medicago truncatula from tissue culture: increased somatic embryogenesis using explants from regenerated plants. Plant Cell Rep, 278-281.

Peret, B., De Rybel, B., Casimiro, I., Benkova, E., Swarup, R., Laplaze, L., Beeckman, T., and Bennett, M.J. (2009). Arabidopsis lateral root development: an emerging story. Trends Plant Sci *14*, 399-408.

Peternel, S., Gabrovšek, K., Gogala, N., and Regvar, M. (2009). In vitro propagation of European aspen (Populus tremula L.) from axillary buds via organogenesis. Sci Hortic, 109-112.

Peterson, C.L., and Workman, J.L. (2000). Promoter targeting and chromatin remodeling by the SWI/SNF complex. Curr Opin Genet Dev *10*, 187-192.

Raju, M.V.S., Steeves, T.A., and Coupland, R.T. (1966). On the occurrence of root buds on perennial plants in Saskatchewan. Can J Bot *44*, 33-37.

Remington, D.L., Vision, T.J., Guilfoyle, T.J., and Reed, J.W. (2004). Contrasting modes of diversification in the Aux/IAA and ARF gene families. Plant Physiol *135*, 1738-1752.

Reyes, J.C., Barra, J., Muchardt, C., Camus, A., Babinet, C., and Yaniv, M. (1998). Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha). EMBO J *17*, 6979-6991.

Rutledge, C.B., and Douglas, G.C. (1988). Culture of meristem tips and micropropagation of 12 commercial clones of Poplar in vitro. Physiol Plant, 367-373.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, N., Weisbeek, P.*, et al.* (1999). An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell *99*, 463-472.

Sachs, J. (1893). Physiologische Notizen, I. Ueber einige Beziehungen derspecifischen Grosse der Pflanzen zu ihrer Organisation. Flora, 49-81.

Sanchez Alvarado, A., and Tsonis, P.A. (2006). Bridging the regeneration gap: genetic insights from diverse animal models. Nat Rev Genet *7*, 873-884.

Sarkar, A.K., Luijten, M., Miyashima, S., Lenhard, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R., and Laux, T. (2007). Conserved factors regulate signalling in Arabidopsis thaliana shoot and root stem cell organizers. Nature *446*, 811-814.

Scheres, B. (2007). Stem-cell niches: nursery rhymes across kingdoms. Nat Rev Mol Cell Biol *8*, 345-354.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F., Jurgens, G., and Laux, T. (2000). The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell *100*, 635-644.

Scofield, S., Dewitte, W., Murray, J.A.H. (2008). A model for Arabidopsis class-1 KNOX gene function. Plant Signaling & Behavior *3*, 257-259.

Sena, G., Wang, X., Liu, H.Y., Hofhuis, H., and Birnbaum, K.D. (2009). Organ regeneration does not require a functional stem cell niche in plants. Nature *457*, 1150-1153.

Silva, J., Nichols, J., Theunissen, T.W., Guo, G., van Oosten, A.L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., and Smith, A. (2009). Nanog is the gateway to the pluripotent ground state. Cell *138*, 722-737.

Smith, Z.R., and Long, J.A. (2010). Control of Arabidopsis apical-basal embryo polarity by antagonistic transcription factors. Nature *464*, 423-426.

Steeves, T.A., and Sussex, I.M. (1989). Patterns in Plant Development. Cambridge: Cambridge University Press.

Sugimoto, K., Jiao, Y., and Meyerowitz, E.M. (2010). Arabidopsis regeneration from multiple tissues occurs via a root development pathway. Dev Cell *18*, 463-471.

Talbert, P.B., Adler, H.T., Parks, D.W., and Comai, L. (1995). The REVOLUTA gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of Arabidopsis thaliana. Development *121*, 2723-2735.

Trembley, A. (1744). Me´ moires pour Servir a L'histoire d'un Genre de Polypesd'eau Douce, a Bras en Forme de Cornes (Leiden: Jean and Herman Verbeek).

Trieu, A.T., and Harrisson, M.J. (1996). Rapid transformation of Medicago truncatula via shoot organogenesis. Plant Cell Rep, 6-11.

van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P., and Scheres, B. (1997). Short-range control of cell differentiation in the Arabidopsis root meristem. Nature *390*, 287-289.

van der Graaff, E., Laux, T., and Rensing, S.A. (2009). The WUS homeobox-containing (WOX) protein family. Genome Biol *10*, 248.

Verbsky, M.L., and Richards, E.J. (2001). Chromatin remodeling in plants. Curr Opin Plant Biol *4*, 494-500.

Wagner, D., Wellmer, F., Dilks, K., William, D., Smith, M.R., Kumar, P.P., Riechmann, J.L., Greenland, A.J., and Meyerowitz, E.M. (2004). Floral induction in tissue culture: a system for the analysis of LEAFY-dependent gene regulation. Plant J *39*, 273-282.

Wang, G., and Fiers, M. (2010). CLE peptide signaling during plant development. Protoplasma *240*, 33-43.

Webster, N., Jin, J.R., Green, S., Hollis, M., and Chambon, P. (1988). The yeast UASG is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator. Cell *52*, 169-178.

Weigel, D., and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature *377*, 495-500.

West, M., and Harada, J.J. (1993). Embryogenesis in Higher Plants: An Overview. Plant Cell *5*, 1361-1369.

Whitehead, H.C.M., and Giles, K.L. (1977). Rapid propagation of poplars by tissue culture methods. N Z J For Sci, 40-43.

Williams, L., and Fletcher, J.C. (2005). Stem cell regulation in the Arabidopsis shoot apical meristem. Curr Opin Plant Biol *8*, 582-586.

Wittrock, V.B. (1884). Om Rootskott hos Ortartade Vaxter, med Sarskild Hansyn till deras olika Biologiska Betydelse. Botaniska Notiser *34*, 21-37.

Xu, J., Hofhuis, H., Heidstra, R., Sauer, M., Friml, J., and Scheres, B. (2006). A molecular framework for plant regeneration. Science *311*, 385-388.

Zafar, Y., Nenz, E., Damiani, F., Pupili, F., and Arcioni, S. (1995). Plant regeneration from explant and protoplast derived calluses of Medicago littoralis. Plant Cell Tissue Organ Culture, 41-48.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., and Chua, N.H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc *1*, 641-646.

Zhang, Y., Wu, R., Qin, G., Chen, Z., Gu, H., and Qu, L.J. (2011). Over-expression of WOX1 leads to defects in meristem development and polyamine homeostasis in Arabidopsis. J Integr Plant Biol *53*, 493-506.