The Association of Homeotic Gene Expression with Stem Cell Formation and Morphogenesis in Cultured *Medicago truncatula*

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STATEMENT OF ORIGINALITY

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

ABSTRACT

Somatic embryogenesis (SE) can be induced *in vitro* in *Medicago truncatula* 2HA by auxin and cytokinin but not in wild type Jemalong. Wild-type Jemalong will only form callus in the presence of auxin and cytokinin and both Jemalong and 2HA will form roots in response to auxin alone. The F2 analysis of 2HA X Jemalong crosses suggest that a single gene may open the way to SE but additional genes are required to maximise the process. Auxin and cytokinin are required for 2-3 weeks for SE and for *de novo* root formation auxin is essential for about one week. Abscisic acid (ABA) and ethylene, both stress related hormones, enhance SE induction but inhibit callus and *de novo* root formation.

The *WUSCHEL* (*WUS*) gene was investigated in *M. truncatula* (*Mt*) and identified by the similarity with *Arabidopsis WUS* in amino acid sequence, phylogeny, promoter element patterns, and expression patterns *in planta*. *MtWUS* is induced by cytokinin after 24-48 h in embryogenic cultures and maximum expression occurs after 1 week which coincides with totipotent stem cell induction. *MtWUS* expression, as illustrated by promoter-GUS studies, subsequently localises to the embryo and corresponds to the onset of *MtCLV3* expression. RNAi studies show that *MtWUS* expression is essential for callus and somatic embryo production. There is evidence based on the presence of *MtWUS* promoter binding sites that *MtWUS* is required for the induction of *MtSERF1* which appears to have a key role in the signalling required for SE induced in 2HA.

MtWOX5, as for *MtWUS*, was identified by similarity to *Arabidopsis WOX5* based on amino acid sequence, phylogeny, promoter element patterns, and expression patterns *in planta*. *MtWOX5* expresses in the auxin induced root primordium and root meristem and appears to be involved in pluripotent stem cell induction. GA suppresses the *MtWOX5* expression in the root apex and suppresses the root primordium induction, consistent with the importance of *MtWOX5* in *in vitro* root formation.

The evidence is discussed that the homeotic genes *MtWUS* and *MtWOX5* are “hijacked” for stem cell induction which is key to somatic embryo and *de novo* root induction. In relation to SE, a key role for *WUS* in the signalling involved in induction is discussed and a model developed.

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2HA	*Medicago truncatula* cv. Jemalong 2HA
ABA	Abscisic Acid
ABAR	ABSCISIC ACID RECEPTOR
ABI	ABA INSENSITIVE
ABF	Auxin Binding Factor
ACC	1-aminocyclopropane-1-carboxylic acid
AGL15	AGAMOUS-LIKE 15
AHK	*Arabidopsis* Histidine Kinase
AHP	*Arabidopsis* Histidine-Phosphotransfer
APRT	Adenine phosphoribosyltransferase
ARF	Auxin Response Factor
ARR	*Arabidopsis* response regulator
AVG	Aminoethoxyvinylglycine
BA	Benzyladenine
BBM	BABY BOOM
CHASE	Cyclases/histidine-kinases-associated sensory extracellular
CKX	Cytokinin oxidase/dehydrogenase
CLV	CLAVATA
cZ	*cis*-zeatin
DELLA	DELLA-domain, named after the first five amino acids
DMAPP	Dimethylallyl diphosphate
DPI	Diphenyleneiodonium sulfate
dZ	Dihydrozeatin
FUS3	FUSCA3
GA	Gibberellin
GA_3	Gibberellic Acid 3
GA2ox	GA 2β-hydroxylase
GA3ox	GA 3β-hydoxylase
GA20ox	GA 20-oxidase
GPA1	G protein *Arabidopsis* α subunit
HMBDP	Hydroxymethylbutenyl diphosphate
IAA	Indole-3-acetic acid
iP	*N*6-(Δ2-isopentenyl)-adenine
IPA	Indole-3-pyruvic acid
IPT	Adenosine phosphate-isopentenyltransferase (ISOPENTYL TRANSFERASE)
IPOx	Indole-3-acetaldoxime
JA	Jasmonic acid

ABBREVIATIONS

LEC	LEAFY COTYLEDON
LOG	LONELY GUY
LRR	Leucine Rich Repeat
MADS	Domain found in MCM1, AGAMOUS, DEFICIENS and SRF
MemT	*meta*-methoxytopolin
MeoT	*ortho*-methoxytopolin
MEP	Methylerythritol phosphate
mT	*meta*-topolin
MtSK1	*Medicago truncatula* serine/threonine kinase 1
NAA	α- Napthalene Acetic Acid
OC	Organising Centre
oT	*ortho*-topolin
PEM	Proembryogenic masses
PIN	PIN-FORMED
PKL	PICKLE
QC	Quiescent Centre
QHB	QUIESCENT-CENTER-SPECIFIC HOMEOBOX
RAM	Root Apical Meristem
RDP	Riboside 5'-diphosphate
RMP	Riboside 5'-monophosphate
ROS	Reactive oxygen species
RTP	Riboside 5'-triphosphate
SA	Salicylic acid
SAM	Shoot Apical Meristem
SCF	Complex named for its components; SKP1, cullin and F-Box
SCR	SCARECROW
SE	Somatic embryogenesis
SEs	Somatic embryos
SERK	SOMATIC EMBRYOGENESIS RECEPTOR KINASE
SERF	SOMATIC EMBRYO RELATED FACTOR
SPY	SPINDLY
STM	SHOOTMERISTEMLESS
TF	Transcription factor
TIR1	Transport Inhibitor Response 1
Trp	Tryptophan
tZ	trans-zeatin
WOX	WUSCHEL-related homeobox
WUS	WUSCHEL

CHAPTER 1

Introduction

1.1 General Introduction

Somatic embryogenesis (SE) is the process whereby non-zygotic cells form embryos and the embryos pass through characteristic embryological stages ultimately producing a new plant. Somatic embryos (SEs) can be induced *in vitro* from somatic cells by the utilization of hormone signals and a suitable genotype.

SE occurs naturally in some plant species such as *Kalanchoë* (Garcês et al., 2007). A type of SE known as apomixis also occurs *in vivo*. In apomixis, embryos develop in the ovule without fertilisation, producing seed with the same genotype as the parent.

Somatic embryogenesis is a useful model for investigating embryo development because of the similarity of the developmental processes between somatic and zygotic embryogenesis (Zimmerman, 1993; Dodeman et al., 1997). Embryos induced *in vitro* from somatic cells allow the supply of substances exogenously and the embryos can be accessed more easily (Fehér et al., 2003).

In modern plant science research, genetic transformation is a powerful tool for functional genomics. Besides shoot regeneration, somatic embryo formation is also an important method for regenerating plants *in vitro*. SE has been studied since the work of Steward et al. (1958) and Reinert (1958), and provides a pathway for *Agrobacterium*-mediated transformation in plants.

Legumes are not only important food crops but are also important as a natural source of nitrogen because of their symbiotic association with the rhizobia bacteria in their nodules. Because of the ability for nitrogen fixation, small genome size, self-fertility, and ability to be transformed, the legume species *Medicago truncatula* has become an international model legume species for genetic and molecular studies and its whole genome is being sequenced (Cook, 1999; Young and Shoemaker, 2006).

Most species of legumes, including *M. truncatula*, have difficulty in regenerating *in vitro*. SEs can be induced in *M. truncatula* cv Jemalong (Jemalong) in culture, but the number is very low. Therefore, our group selected a genetic line which could produce SEs in high numbers (Nolan et al., 1989). *M. truncatula* cv Jemalong 2HA (2HA)

obtained via a cycle of tissue culture and subsequent selection through seed for four generations is a highly embryogenic mutant. 2HA produces 500 times more SEs than its wild type progenitor Jemalong when cultured, and it is nearly isogenic with respect to Jemalong (Rose et al., 1999). Little information is available about the 2HA mutation, but a mutant that can increase the induction of SEs 500 times and regenerate normal plants is of value in understanding SE mechanisms.

1.2 Zygotic Embryogenesis

In Arabidopsis, the morphology of zygotic embryogenesis has been investigated in most detail (Berleth et al., 2002). The embryo development from a fertilized egg cell to a full embryo can be separated into discrete stages: zygote stage, globular stage, heart stage, torpedo stage and bent cotyledon stage.

After fertilisation, the zygote elongates and divides asymmetrically producing an apical daughter cell and a basal daughter cell (Fig. 1.1). After several longitudinal and transverse divisions the apical daughter cell forms the major part of the globular embryo with the apical embryo domain, which will differentiate to form the shoot apical meristem and the cotyledons, and the central embryo domain which will form the hypocotyl. The basal daughter cell also divides to form the hypophysis, which will form the quiescent centre (QC) of the root apical meristem, the root cap, and the suspensor which connects the embryo with the maternal tissue. Suspensor cells can be seen at each developmental stage. The structure of shoot apical meristem and root apical meristem can be identified at the heart stage. The vascular differentiation in the cotyledon and hypocotyl can be first seen at the torpedo stage, and becomes clearer in the bent cotyledon stage (Tykarska, 1976, 1979; Laux et al., 2004).

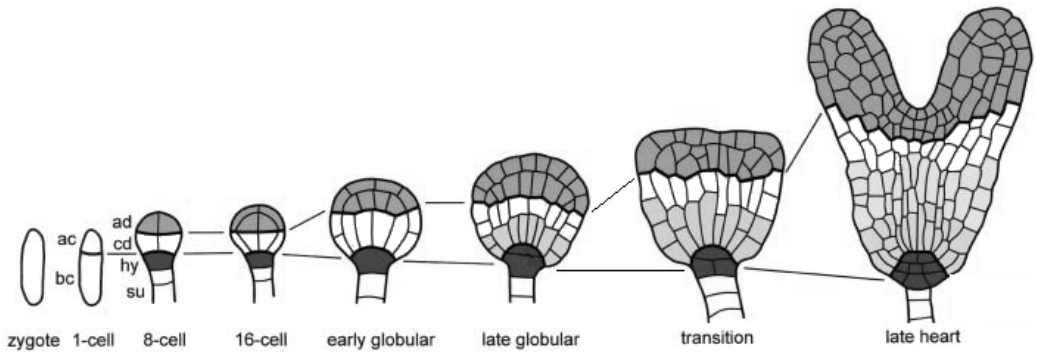


Figure 1.1 The *Arabidopsis* embryo development process. Cells with different colours indicate the different domains of the embryo: Gray (ad), apical embryo domain; white (cd), central embryo domain; dark gray (hy), hypophysis. Ac, apical daughter cell; bc, basal daughter cell; su, suspensor. The diagram is modified from Laux et al., 2004.

Some genes expressed in the zygote can serve as markers. Both *WUSCHEL-related homeobox 2* (*WOX2*) and *WOX8* are expressed in the zygote. After the first division, *WOX2* localizes to the apical daughter cell and its descendants until the 16-cell stage;

and *WOX8* localizes to the basal daughter cell and its descendants including the hypophysis and suspensor until the early globular stage (Haecker et al., 2004).

In the globular and heart stage, some key genes signify important developmental events in Arabidopsis. *WUSCHEL* (*WUS*) expresses in the organizing centre (OC) under the central zone of the shoot apical meristem and starting from the 16-cell stage embryo (Fig. 1.2) indicates the formation of the shoot apical meristem primordium. *WUS* is required for maintaining stem cells in an undifferentiated state (Mayer et al., 1998). The *CLAVATA 3* (*CLV3*) gene expresses in the central zone of the shoot apex above the organizing centre where the stem cells are located (Fig. 1.2). The *CLV3* gene is required to regulate the *WUS* expression. The *CLV3* expression starts after *WUS* expression, at the late heart stage, indicating that the shoot apical meristem has formed completely (Laux et al., 2004). The *SHOOTMERISTEMLESS* (*STM*) gene is another marker indicating that the shoot apical meristem structure has been completed. It starts to express in the epidermis of the central zone of the shoot apical meristem at the late heart stage, outside of the *CLV3* expression region. *STM* expresses in cells which act as a transition zone (Fig. 1.2) between the stem cells and differentiating cells, and restricts the organ initiation until enough cells from stem cells are produced to facilitate subsequent organ formation (Lenhard et al., 2002).

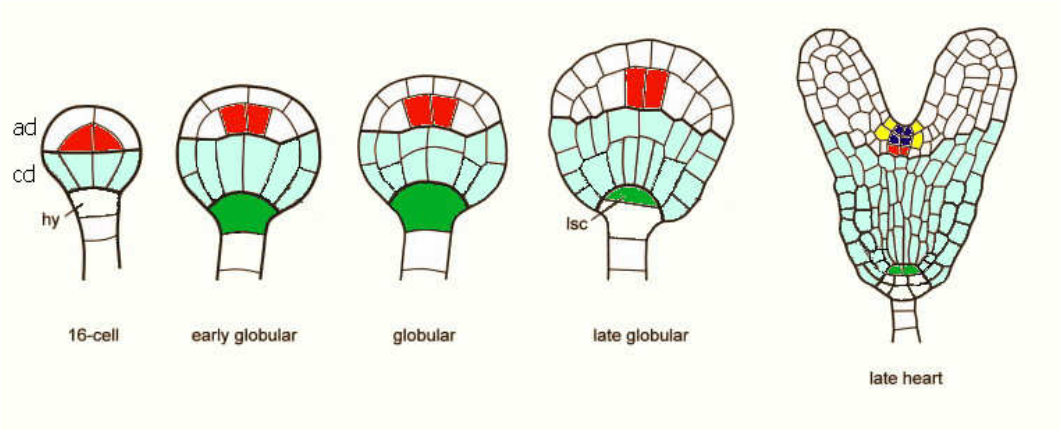


Figure 1.2 Expression pattern of *WUS*, *WOX5*, *CLV3* and *STM* genes in the different stages of embryogenesis in *Arabidopsis*. *WUS* expression is in red and starts at the 16-cell stage. *WOX5* expression is in green and starts at the early globular stage. *CLV3* expression is in blue, and *STM* expression is in yellow, and both of them start at the late heart stage. ad, apical embryo domain; cd (light blue), central embryo domain; hy, hypophysis; lsc, lens-shaped cell. The diagram is modified from Laux et al., 2004.

The root apical meristem QC maintains the undifferentiated state of the surrounding stem cells (Sabatini et al., 2003). In zygotic embryos, the QC is derived from the hypophysis cell at the globular stage (Mansfield et al., 1991) and high auxin is required in the hypophyseal region for QC initiation (Friml, 2003). *WOX5* expression (Fig. 1.2) starts in the hypophyseal region and locates in the QC (Haecker et al., 2004). *WOX5* is associated with auxin accumulation (Gonzali et al., 2005) and is required to maintain the stem cell population (Sarkar et al., 2007). *SCARECROW* (*SCR*) plays a role in specifying the QC identity and also can be used as a marker to indicate QC formation in embryogenesis (Laux et al., 2004; Tucker and Laux, 2007).

After the bent cotyledon stage, the embryo is mature and the dormancy processes start. However, in some crops, a prematurely sprouting embryo may sometimes occur when there is germination on the plant without dormancy. Abscisic acid (ABA) is involved in the induction and regulation of the dormancy and inhibits precocious germination and improves the quality of the seed (Kermode, 2005).

1.3 Somatic Embryogenesis

Somatic embryogenesis as previously indicated is a form of asexual reproduction from somatic or gametic cells (Zimmerman, 1993). It occurs naturally *in vivo* in some species like *Kalanchoë* (Garcês et al, 2007) and Bryophyllum (Yarbrough, 1932), but not in most other species. To obtain SEs *in vitro*, and regenerate plants, many strategies have been used. Hormones, stress, nutrition, explant type, special genotypes, and environmental factors such as light and temperature are usually investigated in different species to find the best somatic embryo induction and regeneration rate. Different species usually have some special mix of factors and some special procedures may be required, but usually auxin, stress, special genotypes and basic nutritional support are the basic requirements for SE (Merkle et al., 1995). The processes of SE may be divided into three stages: induction, development, and germination. The induction stage involves the processes which initiate the embryogenic program, the development stage involves the embryo development to the cotyledon stage, and the germination stage occurs after the cotyledon stage when small plantlets are formed (Merkle et al., 1995). Even with very specific strategies, the micro-environments regulating *in vitro* development are still not as well defined as *in vivo* for zygotic embryogenesis.

Molecular biology opens up new approaches for somatic embryogenesis investigation. One approach is in overexpressing embryo-related genes like the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK*) gene, or genes like *WUS* and *LEAFY COTYLEDON* (*LEC*) to help understand the pathway of somatic embryo induction (Hecht et al., 2001; Zuo et al., 2002; Harada, 2001). Another approach is to use hormone specific or functionally specific genes or markers, like *PIN-FORMED1* (*PIN1*) and *M. truncatula SERINE/THREONINE KINASE 1* (*MtSK1*) genes, to analyse the regulation of hormones and stress in SE (Bassuner et al., 2007; Nolan et al., 2006). These approaches are discussed in reviews by Rose and Nolan (2006a) and by Namasivayam, 2007.

1.3.1 Induction

The induction process is required for the somatic cells to obtain embryogenic competence for somatic embryogenesis (Dodeman et al., 1997). Once the somatic cells

acquire embryogenic competence they proliferate to form proembryogenic masses (PEM) which will give rise to SEs in the explant tissue (Jiménez, 2001a). The PEM may be thought of as a group of totipotent stem cells. Explants from tissue such as immature zygotic embryos or somatic embryo tissue where stem cell exist, SEs can be produced without a callus stage. Otherwise, the induction process may be associated with several divisions and there is a callus phase (Williams and Maheswaran, 1986). More detailed consideration of factors involved in somatic embryo induction, such as hormones and stress, will be presented in Section 4.

1.3.2 Development

In suspension cultures, somatic embryos show similar stages to zygotic embryogenesis with globular, heart, torpedo, cotyledon and plantlet stages. Suspensor-like cells can also be found, but only in some reports (Yeung, 1995).

Before the globular stage, there are several differences in morphology between zygotic and somatic embryos, especially in cell division patterns and suspensor formation (Yeung, 1995). These differences might be dependent on the microenvironment in which embryo development occurs. The zygote is attached to a suspensor, and receives nutrients via these cells. The suspensor is not usually normal in SE and in the early development the embryo in tissue explants is surrounded by cells of the callus and the relationship to surrounding cells is more complicated. After the globular or early heart stage, SEs usually grow on the callus surface and maintain a loose attachment with callus cells, and the microenvironment is more similar to zygotic embryogenesis (Yeung, 1995; Bassuner et al., 2007). Therefore, the morphology between the two types of embryogenesis is more similar after the heart stage.

Somatic embryogenesis shows similar morphological changes to zygotic embryogenesis during the globular to cotyledon stage (Yeung, 1995), and it is believed they have similar gene regulation patterns (Zimmerman, 1993). Therefore, in molecular genetics, SE can also provide understanding of zygotic embryogenesis such as in the case of *SERK* (Schmidt et al., 1997) and *Mt SOMATIC EMBRYO RELATED FACTOR 1* (*MtSERF1*) (Mantiri et al., 2008a). Similarly, the knowledge from zygotic embryogenesis from genetic models like *Arabidopsis* is frequently used to investigate

SE (Fehér et al., 2003).

Root apical meristem formation in somatic embryos is not so easily identified. In zygotic embryos, the root meristem structure can be seen at the heart stage. Root meristem-like structures can frequently be clearly recognised in the fully developed somatic embryo. For the earlier embryo-like structures which have greater attachment to the callus, root apical meristem structures are forming around the base of the structure even after detachment from the callus (Bassuner et al., 2007). Therefore the marker genes like *WOX5* and *SCR* which indicated root apical meristem formation in zygotic embryogenesis may show different expression patterns in SE.

Exogenous hormones are usually decreased in concentration or not supplied in the medium during somatic embryo development. High concentrations of auxin inhibit embryo development and may cause secondary embryos to form, but low concentrations of auxin may be necessary in some species (Ammirato, 1983). Endogenous cytokinin levels decrease in the developing zygotic embryo, therefore it is not surprising that exogenous cytokinin in SE causes abnormal development such as multiple shoots in some cases (Merkle et al., 1995).

1.3.3 Germination

The major events in germination processes are shoot and/or root development from the embryo (Merkle et al., 1995). After the cotyledon stage, zygotic embryos will go into dormancy and form the seed, but SEs usually germinate to form plantlets without undergoing dormancy. However, in many systems, the success rate of germination in SE is quite low and may be caused by embryos not being fully mature. This may be caused by (1) lack of hormone support by the somatic embryo itself and where exogenous cytokinin can help (Ammirato, 1983), or by (2) premature germination. In zygotic embryogenesis, ABA can induce dormancy, inhibit germination, and improve the quality of the embryo in the germination stage (Kermode, 2005). Therefore, exogenous ABA is also used in SE to improve the quality of the embryo for many species (Ammirato, 1983). Some SEs may go into dormancy and gibberellic acid (GA_3) which is frequently used in the medium to help somatic embryo germination may help to break the somatic embryo dormancy (Ammirato, 1983; Gaj, 2004).

1.4 Regulation of the Induction of Somatic Embryogenesis

In this section, major factors influencing the induction of embryogenic cells are considered in some detail. These factors are: hormones, stress, and some developmental genes.

Genotype and explants type can influence the capability of somatic embryo induction. Immature embryos are regularly used for somatic embryo induction in many species such as wheat, soybean and *Arabidopsis* (Merkle et al., 1995). For some strongly embryogenic genome types, like *Medicago sativa* Regen S and *M. truncatula* 2HA and R108, expanded leaves or seedling roots can also be used for somatic embryo induction (Rose, 2004).

1.4.1 Hormones

The major hormones which can influence the induction process are auxin, cytokinin and GA. GA has been implicated as a suppressor of embryogenesis (Thakare et al., 2008). In this section I have included background on hormone metabolism, not often considered in relation to SE.

- **Auxin**

- **Auxin regulation of plant function**

The plant hormone auxin was discovered in the 1930s (see Callis, 2005) and has been extensively researched. Auxin is involved in many plant growth and development processes, including cell growth and cell division (Callis, 2005), vascular tissue differentiation (Mattsson et al., 2003), lateral root formation (Wilmoth et al., 2005), control of apical dominance (Booker et al., 2003), the phototropic response (Friml et al., 2002b) as well as embryogenesis. The biology of auxin is very complex, even after several decades of research it is still not fully understood. Recent research has focused on IAA (indole-3-acetic acid) (Fig. 1.3), the natural auxin hormone, and its biosynthesis, transportation and the molecular basis of its action.

- **The IAA biosynthesis pathway**

There are two types of IAA biosynthesis pathways; tryptophan-dependent synthesis and tryptophan-independent synthesis (Fig. 1.3). The tryptophan-dependent synthesis pathway begins with tryptophan (Trp), which is converted to indole-3-acetaldoxime (IPOx) through cytochrome P450 or the tryptamine-FMO-YUCCA pathway, and then IPOx is converted to indole-3-S-alkylthiohydroximate or indole-3-acetonitrile or indole-3-acetaldehyde to finally form IAA. Another pathway which bypasses IPOx is from Trp to indole-3-pyruvic acid (IPA), to indole-3-acetaldehyde which then converts to IAA (Zhao et al., 2001; Zhao et al., 2002; Ljung et al., 2002). The tryptophan-independent synthesis pathway exists more widely in green plants than the tryptophan-dependent synthesis pathway (Sztein et al., 2000). It begins from indolic precursors through indole and is converted to IAA without Trp. Limited information on the genes or enzymes involved in this pathway is available (Ljung et al., 2002; Kelley and Riechers, 2007).

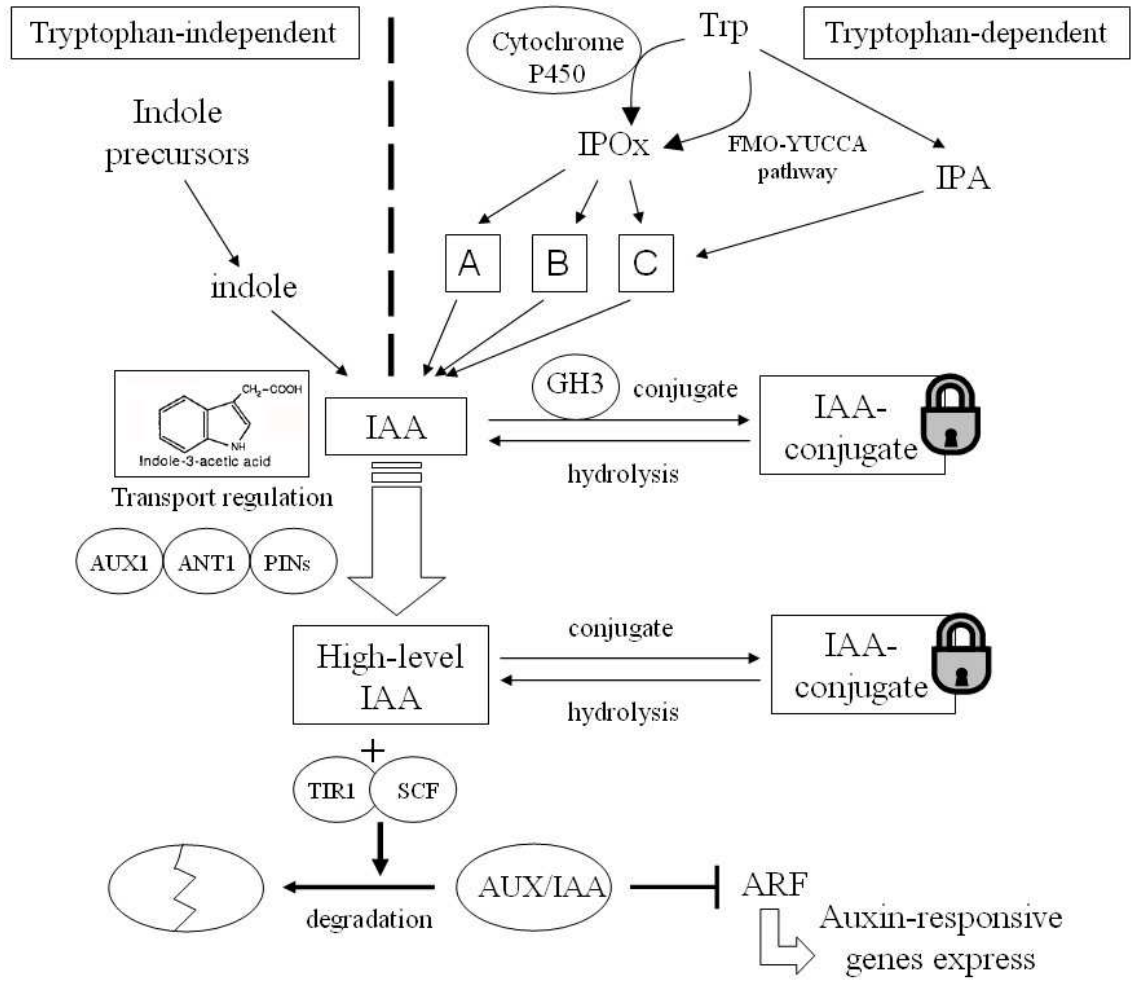


Figure 1.3 IAA biosynthesis, transportaion, and gene expression regulation. A: indole-3-S-alkylthiohydroximate, B: indole-3-acetonitrile, C: indole-3-acetaldehyde. Data obtained and modified from Ljung et al., 2002, Staswick et al., 2005, Blakeslee et al., 2005, and Callis, 2005. See text for abbreviations.

After synthesis, IAA can be inactivated by the formation of conjugates with sugars, peptides or amino acids. These structures may serve for auxin storage, and can release free auxin by hydrolysis (Woodward and Bartel, 2005). In higher plants, most IAA is stored as inactive conjugates (Sztein et al., 2000). These conjugate processes may be involved in IAA regulation e.g. in the removal of excess exogenous auxin (Fig. 1.3). GH3 was the first IAA amino acid conjugating enzyme identified, found in soybean (Hagen and Guilfoyle, 1985), and six enzymes with similar function were found in *Arabidopsis* which can conjugate IAA to amino acids *in vitro* (Staswick et al., 2005). All these enzymes can be induced in response to exogenous auxin (Staswick et al., 2005) and likely function to remove the excess auxin. GH3 enzymes can interact with many types of auxin including IAA and α-Naphthalene acetic acid (NAA), but importantly not for 2,4-Dichlorophenoxyacetic acid (2,4-D) (Staswick et al., 2005) and explains the common use of 2,4-D in tissue culture to obtain the stable and operatable auxin response.

- **Auxin transport**

All parts of young plants can synthesise IAA (Ljung et al., 2001), but the localized concentration of auxin is very important for organ development. This auxin localization can be produced by auxin transport in the phloem and between cells (Morris and Thomas, 1978). The long distance auxin transport from shoot to root helps form auxin gradients, and is related to the development of the shoot apical meristem (Benjamins et al., 2001), shoot bud branching (Bennett et al., 2006), and lateral root development (Reed et al., 1998).

Some genes related to auxin transport have been identified (Fig. 1.3). Key transporters include AUX1 (Bennett et al., 1996) and ANT1 (Chen et al., 2001), which are auxin uptake carriers in the membrane, for auxin influx; and PINs (Chen et al., 1998; Gälweiler et al., 1998; Luschnig et al., 1998), which are transmembrane proteins, for

auxin efflux. This influx and efflux complex in plant cells means auxin can transport against the concentration gradient to accumulate in specific locations (Friml and Palme 2002a). By investigating these genes, the auxin gradients formed by polar auxin transport in embryo development, shoot apex differentiation, lateral root formation and root growth show clear patterns that can be related to development (Friml et al., 2003; Heisler et al., 2005; Smet et al., 2007; Grieneisen et al., 2007).

- **Auxin and the regulation of gene expression.**

One model provides an explanation for how auxin can regulate gene response by degradation of a transcription repressor (Fig. 1.3). The Aux/IAA proteins are transcription repressor proteins, and repress the auxin response factor (ARF) family of transcription factors (Ulmasov et al., 1999). Auxin can degrade the Aux/IAA proteins (Liscum and Reed, 2002) through the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) protein and the SCF complex (named by its subunits SKP1, cullin and F-Box protein) (Gray et al., 2001; Tan et al., 2007). When auxin directs binding to TIR1, the SCF-TIR1 complex is modified and then degrades the Aux/IAA. The degradation of the repressor protein allows these auxin responsive genes to express (Kepinski and Leyser, 2005).

- **Auxin and somatic embryo induction**

Auxins, such as IAA, 2,4-D and NAA, are required for the induction of somatic embryogenesis in many species (Ammirato, 1983). Endogenous IAA increases in embryogenic callus but not in non-embryogenic callus (Jiménez and Bangerth, 2001). On the other hand, anti-auxins inhibit SE and the early stages of embryo development (Fujimura and Komamine, 1979). Additionally, the concentration of endogenous IAA increases and accumulates in this induction stage in both zygotic embryogenesis (Ribnicky et al., 2002) and SE (Thomas et al., 2002). This shows the importance of the endogenous auxin in the somatic embryo induction stage which is a key event in cell fate determination (Fehér et al., 2003). The exogenous auxin 2,4-D used in culture also causes endogenous IAA levels to increase but it may not directly stimulate IAA synthesis. It might cause IAA to accumulate by disturbing IAA metabolism (Michalczuk et al., 1992).

The most common protocols to induce SEs involve treatment with auxin for a period of time then removal of the exogenous auxin in the culture. After this induction stage, no or low levels of exogenous auxin are required for embryo development (Ammirato, 1983). High level of exogenous auxin may lead to abnormal development or another cycle of embryogenic cell formation (Halperin, 1966; Merkle et al., 1995). However, this does not mean that auxin is not important at this SE development stage. At this later stage, the most important event is the establishment of polarity, which is required in both zygotic embryogenesis (Russell, 1993), and SE (Dijak and Simmonds, 1988). The auxin transport gene *PIN1* expression pattern changes in the Arabidopsis *gnom* mutant and causes abnormal asymmetric division of zygotes (Steinmann et al., 1999). The root meristem formation in both zygotic and somatic embryos also requires auxin accumulation at the root pole (Friml et al., 2003; Bassuner et al., 2007). This type of evidence indicates that the localization and accumulation of endogenous auxin is still important for the morphological differentiation of embryos following induction (Fehér et al., 2003).

- **Cytokinin**

- **Cytokinin regulation of plant function**

Cytokinins have been investigated for more than 50 years (Miller et al., 1955). The major function of cytokinins in plant cells is the regulation of cell division and differentiation (Sakakibara, 2006). Cytokinins have also been implicated in developmental processes such as the delay of leaf and flower senescence, the control of apical dominance (Dun et al., 2006) and the regulation of the shoot meristem. Investigation of cytokinin receptor mutants in *Arabidopsis* has greatly facilitated the understanding of the role of cytokinins in plant growth and development (Higuchi et al., 2004; Riefler et al., 2006).

In classic tissue culture, cytokinin promotes shoot induction as opposed to auxin which promotes root induction (Skoog and Miller, 1957). Recently cytokinin - related genes like *ISOPENTYL TRANSFERASE* (*IPT*), *Arabidopsis* response regulators *ARR5*, *ARR7* (Jasinski et al., 2005; Yanai et al., 2005; Leibfried et al., 2005), and *LONELY GUY* (*LOG*) (Kurakawa et al., 2007) have been implicated in the regulation of the shoot

apical meristem. Cytokinin also plays an important role in SE in *Medicago* (Nolan et al., 1989; Dudits et al., 1991; Nolan and Rose 1998) and is critical to our understanding of this process.

- **The cytokinin biosynthesis pathways**

There are two groups of cytokinins: isoprenoid and aromatic (Fig. 1.4). Common active forms of isoprenoid cytokinins are *N*6-(Δ2-isopentenyl)-adenine (iP), trans-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DZ). The tZ and iP types of cytokinin with their sugar conjugates are the major form in plants (Sakakibara, 2006). However importantly, in maize and rice, the cZ-type is the major cytokinin (Veach et al., 2003; Izumi et al., 1988). Only some plant species have the aromatic cytokinins, *ortho*-topolin (oT), *meta*-topolin (mT), *ortho*-methoxytopolin (MeoT), *meta*-methoxytopolin (MemT) and benzyladenine (BA) (Strnad, 1997).

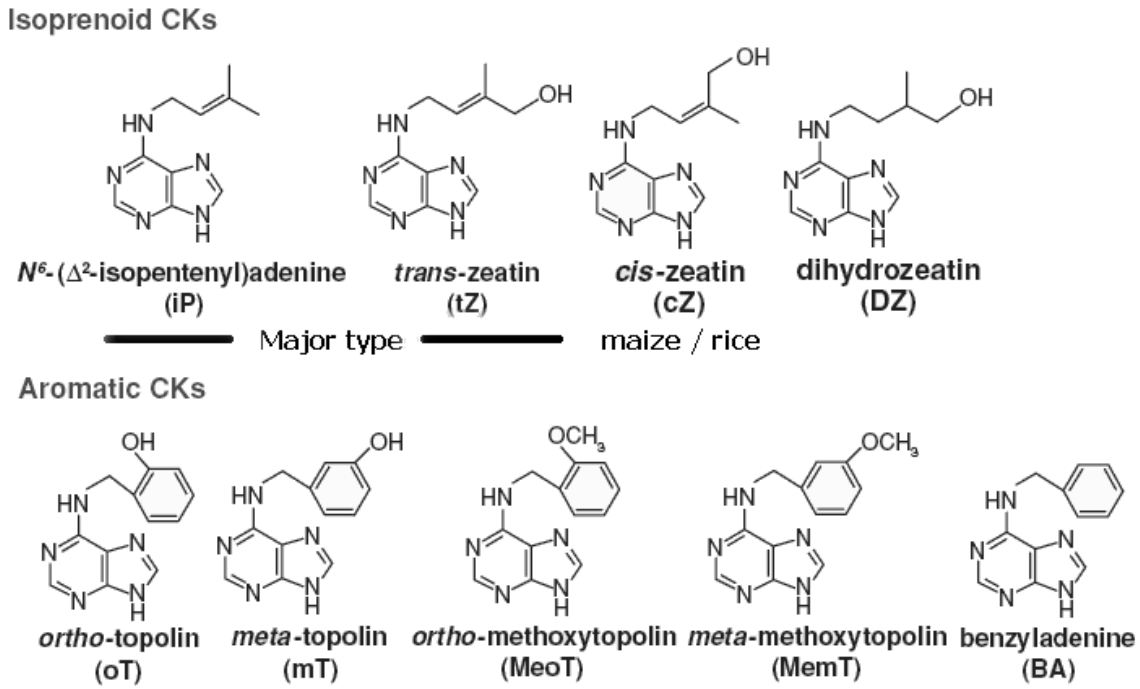


Figure 1.4 Isoprenoid type and aromatic type cytokinin structures. Figure modified from Sakakibara, 2006; Veach et al., 2003; Izumi et al., 1988; Strnad, 1997. See text for abbreviations.

For isoprenoid type cytokinin biosynthesis, iP and tZ, precursors are from the methylerythritol phosphate (MEP) pathway, through hydroxymethylbutenyl diphosphate (HMBDP) and dimethylallyl diphosphate (DMAPP) (Kasahara et al., 2004)

(Fig. 1.5). There is conjugation with ATP, ADP or AMP by IPT (adenosine phosphate-isopentenyltransferase) to form the iP riboside 5'-triphosphate (iPRTP), iP riboside 5'-diphosphate (iPRDP) or iP riboside 5'-monophosphate (iPRMP), and can convert to the major active product iPRMP (Kakimoto, 2001). The iPRMP metabolic pool can convert to iP, and also other free-base isoprenoid cytokinins like tZ through tZRMP. The tZ precursor can also convert from iPRTP and iPRDP through tZRTP and then tZRDP to convert to tZRMP then tZ (Sakakibara, 2006). In Arabidopsis, several IPT genes have been identified (Takei et al., 2001) and the *LOG* gene converts iPRMP and tZRMP directly to iP and tZ through phosphoribohydrolase activity (Kurakawa et al., 2007). The cZ can be synthesized from iP and tZ precursors, but also has another synthesis pathway from DMAPP and tRNA (Sakakibara, 2006). For aromatic cytokinins, the biosynthesis and degradation pathways are still unclear, but may use the same pathways as isoprenoid type cytokinins to regulate their active levels (Mok and Mok, 2001; Mok et al., 2005).

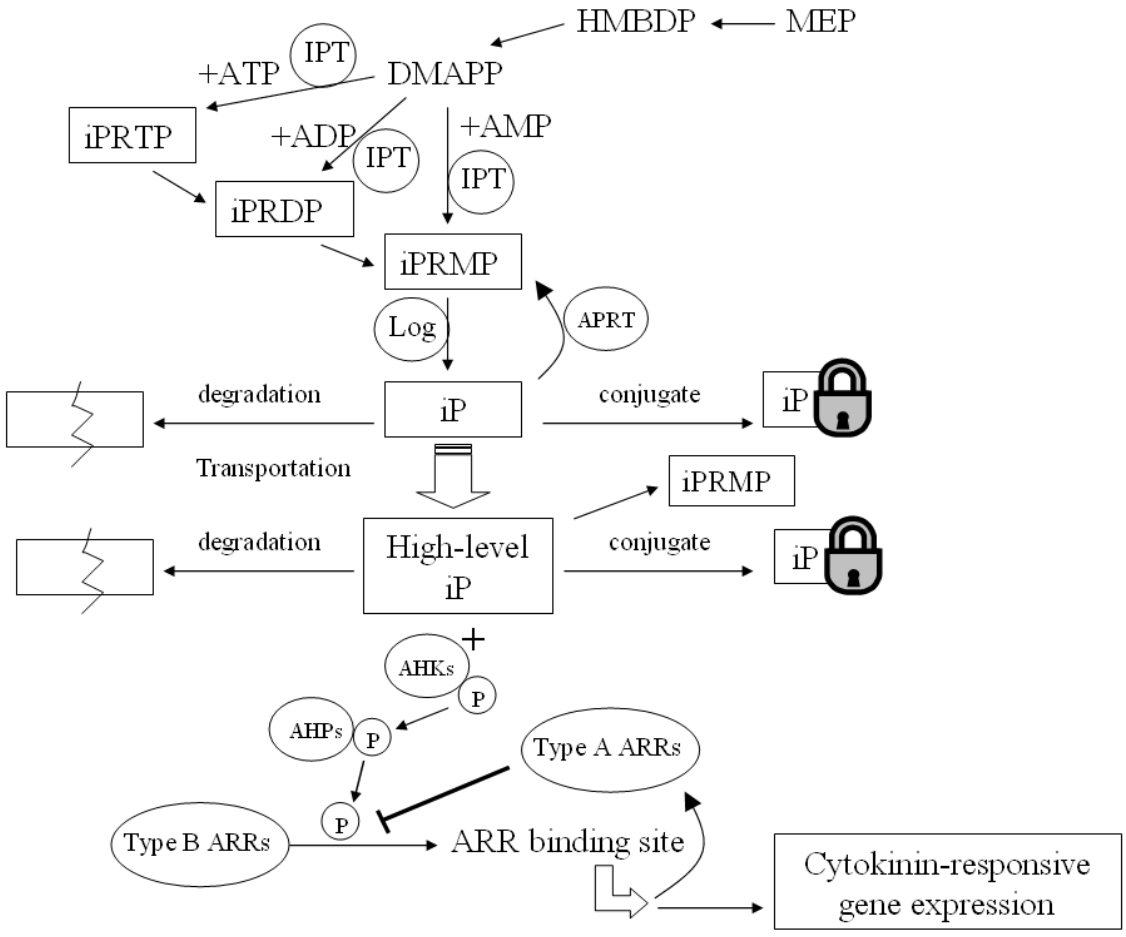


Figure 1.5 Cytokinin biosynthesis, transport and gene expression regulation. Figure modified from Sakakibara, 2006; Ferreira and Kieber, 2005; Müller and Sheen, 2007. See text for abbreviations.

Cytokinin nucleobases are the active forms and can induce target gene expression (Schmitz and Skoog, 1972) (Fig. 1.5). The level of active cytokinin can be regulated by inactivating conjugation, such as glycosylation (Brzobohatý et al., 1993); conversion by phosphoribosylation of cytokinin nucleobases to iPRMP by adenine phosphoribosyltransferase (APRT) (Allen et al., 2002); or degradation by cytokinin oxidase/dehydrogenase (CKX) (Schmülling et al., 2003).

- **Cytokinin transport**

Cytokinin like auxin can be synthesized in whole plants, and be transported to specific locations to induce gene expression. Cytokinins are transported between roots and shoots through xylem as tZ derivatives and through phloem as iP derivatives. Both of these transport processes are influenced by the nitrogen concentration in the root (Sakakibara, 2006). In axillary bud outgrowth, long distant cytokinin transport from the root is required, but cytokinins also act as local signals and cytokinin biosynthesis can be inhibited by auxin (Tanaka et al., 2006; Dun et al., 2006).

- **Cytokinin and the regulation of gene expression**

Three groups of genes are involved in the cytokinin regulation pathway, *Arabidopsis* HISTIDINE KINASES (AHKs) as the signal receptors, *Arabidopsis* histidine-phosphotransfer proteins (AHPs) as signal carriers, and Arabidopsis Response Regulators (ARRs) as activators and feedback regulators (Fig. 1.5). The cytokinin molecular signal is perceived by the "cyclases/histidine-kinases-associated sensory extracellular" (CHASE) domain (Heyl and Schmülling, 2003) of the AHK receptor kinases on the plasma membrane (Riefler et al., 2006). The phosphorelay in the cytoplasm involves AHP proteins which shuttle into the nucleus regulating the ARR response regulators. The *ARRs* genes encode the major response regulators (Fig. 1.5) and can be divided into two groups: type-A and type-B. Type-A *ARR* genes can respond to exogenous cytokinin and are considered the response genes (D'Agostino et al., 2000; Rashotte et al., 2003). Inside the nucleus, the phosphate carried by AHPs proteins can activate the type-B ARR proteins (ARR 1, 2, 10) which can bind to the ARR binding sites ((G/A)GGAT(T/C) at the promoter region of many cytokinin response genes and which can then also start the transcription of type-A *ARR* genes (*ARR 4, 5, 6, 7*). Single

mutants of any type-A *ARR* gene do not change plant morphology, but multiple mutants of type-A *ARRs* increase the sensitivity of cytokinin (To et al., 2004). This indicates the type-A *ARR* genes have highly overlapping functions and some of these genes might act as negative regulators of the pathway (Hwang and Sheen, 2001; Ferreira and Kieber, 2005; Müller and Sheen, 2007).

- **Cytokinin and somatic embryo induction**

For the somatic embryo induction stage, cytokinin unlike exogenous auxin is not essential for species such as carrot (Jiménez and Bangerth, 2001), and may even inhibit somatic embryo formation in some species (Merkle et al., 1995). However, for some species, like *M. truncatula* 2HA, cytokinin is required to be used with auxin in culture for forming callus and embryogenic cells (Nolan and Rose, 1998).

Some reports indicate that cytokinin alone can stimulate somatic cells into the embryogenesis process but in most species, exogenous cytokinin is not required for somatic embryo induction (Maheswaran and Williams, 1985). The level of endogenous cytokinin (iP) in cotton somatic embryo culture decreases through the induction stage, but increases once the somatic embryo has formed (Zeng et al., 2007) indicating that the endogenous cytokinin is important in somatic embryo development. The cytokinin level needs very sensitive regulation to form normal embryos.

- **Gibberellin (GA)**

Gibberellic acid (GA_3) has multiple functions related to plant morphology. It acts as an inhibitor of embryo development and lateral root induction, and as a stimulator of shoot and root elongation (Richards et al., 2001; Busov et al., 2008). GA_3 can also inhibit the induction of somatic embryogenesis and organogenesis *in vitro* (Flick et al., 1983).

GA (gibberellin) is synthesized by conversion of geranylgeranyl diphosphate to 19-carbon GAs and by the subsequent action of GA 20-oxidase (GA20ox) and 3β-hydoxylase (GA3ox). GA can be deactivated by 2β-hydroxylase (GA2ox) (Bethke and Jones, 1998; Fleet and Sun, 2005). Feedback regulation of GA synthesis by inhibiting the GA20ox and GA3ox expression slowly reduces bioactive GA synthesis

(Fleet and Sun, 2005). *STM*, which is essential for shoot apical meristem development, also represses GA20ox expression to reduce active GA (Hay et al., 2002). *FUSCA3* (*FUS3*) which is essential for SE can repress GA3ox expression and decrease bioactive GA_3 synthesis (Gazzarrini et al., 2004). *AGAMOUS-like 15* (*AGL15*) can enhance SE development, and also regulates the GA2ox6 in deactivating bioactive GA (Wang et al., 2004). GA appears to act as a repressor of embryo and shoot apical meristem formation and can prevent abnormal organ induction.

Based on the morphology of many GA related mutants, GA is thought to promote plant growth by repressing inhibitory genes (Bethke and Jones, 1998). The key inhibition genes down-regulated by GA are the *DELLA* (Richards et al, 2001; Olszewski et al, 2002). GA suppresses DELLA proteins through inducing *SLY1/SNE/GID2* gene expression or suppressing the *SPINDLY* (*SPY*) gene which may modify and activate the DELLA proteins (Richards et al, 2001; Olszewski et al, 2002; Thomas and Sun, 2004). *PICKLE* (*PKL*) is one of the genes regulated by DELLA. *PKL* inhibits embryonic cell fate during post-embryonic development to prevent abnormal embryogenesis in plant tissue. The *PKL* gene inhibits *LEC1*, *LEC2* and *FUS3* gene expression. DELLA proteins suppress *PKL* expression to allow embryo development. Therefore in normal tissue, GA suppresses DELLA and allows the *PKL* expression to inhibit embryo development; but in embryogenic tissue, low levels of GA allow DELLA to suppress *PKL* and embryo cell fate can be determined. *FUS3* expression also keeps the GA levels low (Fleet and Sun, 2005) (Fig. 1.6). GA_3 suppression of shoot and root initiation in cultures may also use a similar regulation pathway.

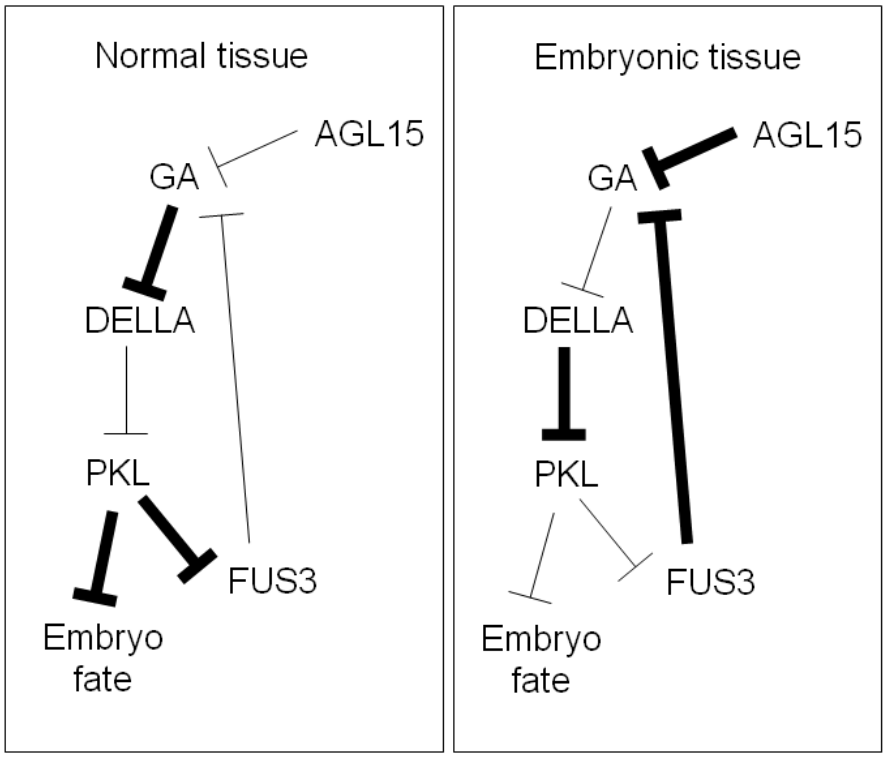


Figure 1.6 Embryo fate may be regulated by GA through DELLA and *PKL*. A wide line indicates strong activity and a narrow line indicates weak activity. Data obtained and modified from Fleet and Sun, 2005.

1.4.2 Stress

Stress factors, including osmotic pressure, heavy metals, low or high temperature, starvation, mechanical wounding of explants and high 2,4-D levels, can stimulate somatic cells to acquire embryogenic competence, and the induction of SE (Fehér et al., 2003). The importance of stress for somatic embryo induction is emphasized in some reports as SEs can be induced by stressors alone without exogenous hormones being supplied (Kiyosue et al., 1989; Touraev et al., 1997).

In tissue culture protocols, wounding occurs in both explant culture and protoplast culture. Wounding of cells is a drastic environmental change and causes a significant stress effect, and the switching on of some stress defence genes (Fehér et al., 2003). The genes induced by wounding in *Arabidopsis* include signal transduction and regulatory factors, followed by metabolic genes and lignin pathway related genes (Delessert et al., 2004). Wounding also induces reactive oxygen species (ROS) (Orozco-Cardenas and Ryan, 1999) and in the non-culture situation represses the auxin function first (Cheong

et al., 2002; Delessert et al., 2004), and then induces ethylene and ABA responsive stress genes later (Delessert et al., 2004). In the *M. truncatula* culture system, wounding is the major stress in the culture process and many proteins related to biotic and abiotic stress are produced in the early stage of culture (Imin et al., 2004, 2005). Ethylene biosynthesis genes are also activated (Mantiri et al., 2008a).

- **Reactive oxygen species (ROS)**

ROS include superoxide anions, hydroxyl radicals, and hydrogen peroxide, and can be induced by stresses such as ozone (Conklin et al., 1996), UV-B (Surplus et al., 1998) and wounding (Orozco-Cardenas and Ryan, 1999). ROS cause damage to cellular organelles, but can also function as signalling molecules (Levine et al., 1994; Noctor, 2006) to regulate some ABA and ethylene stress response genes (Fujita et al., 2006). The linkage with ROS and somatic embryo induction is still not clear. The flavoprotein inhibitor diphenyleneiodonium sulfate (DPI), which inhibits ROS production, also inhibits callus production and SE in *M. truncatula* 2HA (Rose and Nolan, 2006) indicating the possibility of a linkage.

- **Abscisic acid (ABA)**

Abscisic acid (ABA) is the major phyto-hormone responsive to abiotic stresses such as drought, low temperature, and osmotic stress, and together with other phytohormones like salicylic acid (SA), jasmonic acid (JA) and ethylene responds to stresses such as pathogens and wounding (Fujita et al., 2006).

ABA biosynthesis in higher plants requires the cleavage of carotenoids (Schwartz et al., 2003). Active ABA can be down regulated by degradation or conjugation processes. In degradation processes, ABA degradation is catalyzed by the *CYP707* genes where expression is regulated by stress or development (Umezawa et al., 2006; Okamoto et al., 2006). For the conjugation process, glucose is conjugated with ABA by ABA glycosyltransferase forming an inactive ABA glucosyl ester (Xu et al., 2002). Conjugated ABA can release ABA through some β-glucosidases, such as AtBG1 in Arabidopsis, to increase intra- and extracellular ABA levels and induce stress-responsive gene expression (Lee et al., 2006).

The ABA regulation signalling pathway is complex and acts independently at multiple sites in the cell to induce different functions (Hirayama and Shinozaki, 2007). Three putative ABA receptors have been described: the flowering-time control protein FCA in the nucleus which regulates mRNA stability and flower induction (Razem et al., 2006), the Mg-chelatase H subunit ABAR (ABSCISIC ACID RECEPTOR) in plastids involved in the synthesis of chlorophyll (Shen et al., 2006), and a G protein coupled receptor GCR2 in the plasma membrane which presumably transduces the signal through the GPA1 (G protein *Arabidopsis* α subunit) gene which plays an important role in secondary messenger production (Liu et al., 2007; Hirayama and Shinozaki, 2007). Secondary messengers such as Ca^{2+} and ROS are important in ABA signal transduction. Intercellular Ca^{2+} increase is important for guard-cell movement which responds to ABA, and ROS which is induced by ABA through NAPDH oxidases (Pei et al., 2000). ROS such as H_2O_2 have also been reported to reduce the activity of ABA INSENSITIVE 1 (ABI1) protein and the ABA INSENSITIVE 2 (ABI2) protein which can negatively regulate the ABA response in *Arabidopsis* (Meinhard and Grill, 2001; Meinhard et al., 2002).

Exogenous ABA does not usually induce somatic embryos, but it does in some cases (Nishiwaki et al., 2000). However, it is used in many protocols to improve the number and quality of embryos (Ammirato, 1983; Nolan and Rose, 1998). It might influence embryogenesis by interacting with endogenous IAA through some other factors (Nishiwaki et al., 2000; Fehér et al., 2003). ABA also contributes to the accumulation of the storage proteins in cotyledons and assists mature zygotic embryos into dormancy.

- **Ethylene**

Ethylene is involved in many aspects of plant development and is synthesized in response to biotic and abiotic stress, including wounding, hypoxia, ozone, chilling, or freezing (Wang et al., 2002). Ethylene biosynthesis can also be stimulated by wounding directly and through ROS and JA (Watanabe and Sakai, 1998). In SE, ethylene is not commonly used in culture and may even inhibit somatic embryo development (Tisserat and Murashige, 1977). Endogenous ethylene influences bud induction arising from cultured tobacco cotyledons (Evertt, 1982). Endogenous ethylene is also required for SE

in *M. truncatula* as ethylene biosynthesis inhibitors can dramatically decrease the somatic embryo numbers in *M. truncatula* 2HA (Mantiri et al., 2008a). Ethylene can stimulate auxin biosynthesis and auxin transport in the root elongation zone (Růžička et al., 2007; Swarup et al., 2007). In SE, ethylene can be induced by wounding in the induction process and is involved in the regulation of hormone or developmental genes during the induction and development of SEs (Mantiri et al., 2008a).

1.4.3 Developmental Genes

Some specific genes have been implicated in somatic embryogenesis, including SE stimulation genes and SE inhibition genes. These genes provide a basis for investigating the molecular mechanism of somatic embryo induction.

Overexpression of SE stimulation genes will induce somatic embryos from vegetative tissue. Most of these genes are transcription factors including *WUSCHEL (WUS*) , *BABY BOOM (BBM), AGAMOUS-like 15 (AGL15), LEAFY COTYLEDON1 (LEC1)* and *LEAFY COTYLEDON2 (LEC2)* (Zuo et al., 2002; Boutilier et al., 2002; Harding et al., 2003; Lotan et al., 1998; Stone et al., 2001). One SE stimulator is the receptor kinase *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1)* (Hecht et al., 2001).

The *WUS* gene has a homeobox domain called the WUSCHEL-related homeobox (WOX) domain and expresses in the OC (organizing centre) of the embryo and shoot meristem, and also the floral meristem. Its function is to maintain undifferentiated stem cells (Laux et al., 1996). Overexpression of *WUS* can induce SEs from whole seedlings (Zuo et al., 2002). Gallois et al., (2004) described how *WUS* expression could induce shoot stem cell activity and embryos in roots if treated with extra auxin. *WUS* expression combined with high levels of auxin might be the condition required to switch somatic cells into SE.

BBM is a transcription factor of the AP2/ERF family and expresses in developing embryos, seeds, and root meristems (Boutilier et al., 2002; Imin et al., 2007). Its function may be related to promoting cell proliferation and morphogenesis during embryogenesis. Ectopic *BBM* led to spontaneous SE from seedlings, and influenced the leaf and flower morphology (Boutilier et al., 2002).

SOMATIC EMBRYO RELATED FACTOR1 (SERF1) is another transcription factor of the AP2/ERF family and is essential for SE in *M. truncatula*. It is an ethylene responsive gene and expresses primarily in somatic embryos and the globular stage of zygotic embryos. Inhibition of *SERF1* expression by RNAi also suppresses somatic embryo formation but not callus formation indicating it is essential and specific for embryo development (Mantiri et al., 2008a).

AGL15 is a transcription factor with a MADS domain (found in MCM1, AGAMOUS, DEFICIENS and SRF) and many genes with this MADS box function in developmental regulation (Riechmann and Meyerowitz, 1997). *AGL15* expresses mainly in whole embryos including the suspensor from the 8 cell stage to the maturation stage, in the endosperm and shoot apex of young seedlings; and may support development in an embryonic mode (Perry et al., 1996; Harding et al., 2003). It also enhances GA deactivation (Wang et al., 2004). Ectopic expression of *AGL15* can enhance production of secondary embryos from cultured zygotic embryos. Secondary embryo induction can be maintained without exogenous hormones, but for vegetative tissue 2,4-D in liquid media is required to promote SEs from the shoot apical meristem (Harding et al., 2003). Overexpression of the ortholog gene in soybean (*GmAGL15*) also enhances somatic embryo development (Thakare et al., 2008).

LEC1 is a HAP3 subunit of a CCAAT-binding transcription factor (Lee et al., 2003). It only expresses in embryo and endosperm tissue and its expression may be involved in cotyledon identity and late zygotic embryogenesis (West et al., 1994). *LEC1* was the first gene shown to induce SE from seedling tissue by gene overexpression, but the frequency is quite low (Lotan et al., 1998). The *LEC2* gene contains a B3 DNA-binding motif. Its RNA accumulates during seed development and expression is required for maintenance of suspensor morphology, specification of cotyledon identify, progression through the maturation phase, and suppression of premature germination (Stone et al., 2001). Ectopic *LEC2* also induces SEs and callus from adaxial cotyledons (Stone et al., 2001). The *FUSCA3* (*FUS3*) gene which also has a B3 domain expresses in the early and maturation phases of embryogenesis (Harada, 2001) and suppresses GA synthesis (Gazzarrini et al., 2004). Its function may be partially redundant with *LEC2* in the stimulation of seed storage protein genes (Kroj et al., 2003). These *LEAFY*

COTYLEDON-like genes *LEC1*, *LEC2* and *FUS3* are all essential for somatic embryo induction in *Arabidopsis* (Gaj et al., 2005).

The *SERK1* gene is a leucine-rich repeat (LRR) receptor-like kinase (Albrecht et al., 2005) first identified from carrot somatic embryo suspension cultures and can be used as a SE marker for tracking single cells which will produce SEs (Schmidt et al., 1997). *SERK* expression is not limited to the embryo but is also expressed in male and female reproductive tissue, leaf primordia and developing roots (Hecht et al., 2001; Nolan et al., 2003; Kwaaitaal et al., 2005). *SERK1* and its family members may be involved in signal transduction chain through forming complexes with other receptors or transcription factors, such as *AGL15* (Karlova et al., 2006). *SERK* family genes may be also involved in other functions such as SE regulation and pathogen defence (Hu et al., 2005).When overexpressed the *SERK* gene also enhances the SE from seedlings when combined with 2,4-D (Hecht et al., 2001).

One SE inhibition genes is known - *PICKLE* (*PKL*). The *pkl* mutant in hormone free root tissue culture can produce SEs from callus (Ogas et al., 1997). The *PKL* gene produces a CHD3 protein which may function by regulating chromatin architecture and act as a repressor of transcription (Ogas et al., 1999). It has been discussed in Section 1.4.1 and its regulation of embryo fate is schematised in Fig. 1.4.

These development genes which have been described are summarized in Table 1.1.

Gene Name	Gene type	Domain	Expression location	*In vivo* functions	SEs induced by gene:		Reference about SE
					How	Where	
WUS	TF	WOX Homeobox	Shoot and floral meristem, OC of SAM and embryo	Maintains undifferentiated stem cells in SAM	Ectopic	Seedling	Zuo et al., 2002.
BBM	TF	AP2/ERF domain	Embryo	Promotes cell proliferation and morphogenesis in the embryo root	Ectopic	Seedling	Boutilier et al., 2002.
SERF1	TF	AP2/ERF domain	Embryo	Ethylene responsive gene, essential for SE	Unknown	Unknown	Mantiri et al., 2008a
AGL15	TF	MADS domain	Embryo, endosperm, shoot apex of young seedling	Supports development in an embryonic mode, enhances deactivation of GA	Ectopic + 2,4-D	SAM of seedling	Harding et al., 2003.
LEC1	TF	HAP3 subunit	Embryo, endosperm	Cotyledon identify and late zygotic embryogenesis	Ectopic	Leaf	Lotan et al., 1998.
LEC2	TF	B3 domain	Embryo	Maintains suspensor morphology, cotyledon identity, suppresses premature germination	Ectopic	Adaxial cotyledon	Stone et al., 2001.
FUS3	TF	B3 domain	Embryo	Regulates seed storage protein genes and GA synthesis, essential for SEs induction	Unknown	Unknown	Gaj et al., 2005
SERK1	Receptor	LRR domain	Embryo, gametophyte, leaf primordium, lateral root	SE regulation, pathogen defense	Ectopic + 2,4-D	Callus	Hecht et al., 2001.
PKL	Repressor	CHD3 domain	Throughout plant	Regulates chromatin architecture, represses transcription, prevents SE	Mutant (*pkl*)	Callus from root	Ogas et al., 1997

Table 1.1 Developmental genes related to SE induction. TF: transcription factor.

1.4.4 The *Medicago truncatula* Somatic Embryogenesis System

In *M. truncatula* cv Jemalong 2HA, cytokinin and auxin are essential for somatic embryo induction (Fig. 1.7). Auxin treatment alone will not induce SEs but will induce different types of callus morphology and roots in both Jemalong and 2HA. Explants cultured on cytokinin alone medium will only induce a little callus and will induce some SEs in 2HA. The maximum number of SEs that can be induced is with a ratio of auxin to cytokinin of 10:4 and the same ratio is maintained until the SEs have matured. Detached SEs can be cultured on solid medium without exogenous hormone and germinate to form roots. ABA added with auxin and cytokinin in the late stages of embryo induction is used to improve embryo quality and quantity.

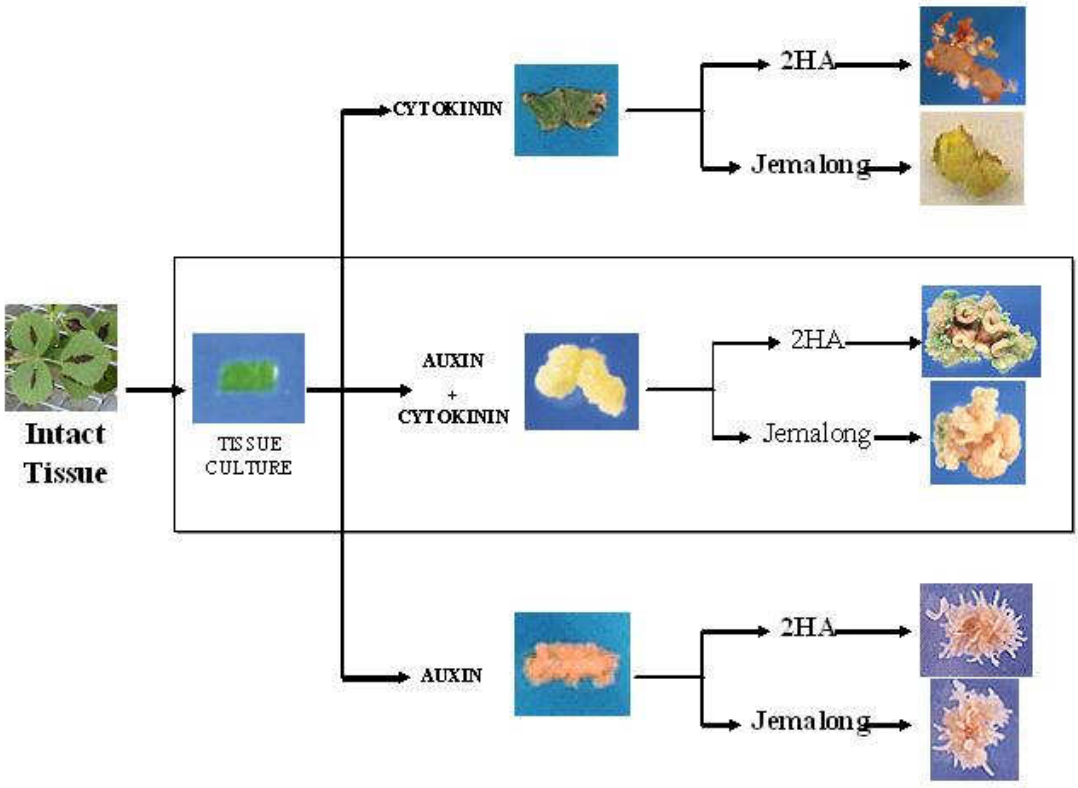


Figure 1.7 Experimental system used in the study of *in vitro* embryo and organ formation in *M. trucatula* with Jemalong and 2HA explants. Diagram obtained from Dibley, 2003.

1.5 *De novo* Root Induction and Development

In *M. truncatula,* somatic embryogenesis produces a bipolar embryo following auxin plus cytokinin treatment. Explants treated with high concentration of auxin (10 μM) alone can produce a mass of callus and roots. These *de novo* roots proliferate from the vascular tissue of explants and are suppressed by ethylene (Rose et al., 2006). These cultured roots are also used to investigate the genes related to root development (Imin et al., 2007). The mechanism of the *de novo* root formation and how it is related to normal primary root development or lateral root induction is still unclear. It was the intent in this study to contrast the induction of a bipolar embryo from totipotent cells with the development of a unipolar meristem from pluripotent cells.

1.5.1 The Root Meristem

Depending on the source cells, plant roots can be classified as primary roots, lateral roots, or adventitious roots. The primary root forms from the embryo during seed germination; lateral roots arise from other roots; and adventitious roots form from uncommon locations such as stems or leaves. The primary taproot and lateral root branching structure are characteristic of most dicotyledon plants like *Arabidopsis* and *Medicago*. In monocots such as wheat and rice, primary roots also form after seed germination, but the majority of roots are adventitious roots arising from the end of the stem, and then lateral roots branch from them. This type of root system in monocots is referenced to as a fibrous root system. However, after the roots have matured, the structures of roots are similar.

The structure of a mature root (Fig. 1.8) can be separated into the root tip, elongation zone and the mature zone. The root tip can be divide into the root cap (including the columella cells in the centre surrounded by the lateral root cap), root meristem (including the quiescent centre (QC) cells surrounded by a couple of layers of stem cells), and the elongation zone developing from the meristem (with stele, ground tissue and epidermis). In the mature zone, the stele is differentiated into vascular tissue and pericycle. The ground tissue differentiates into the endodermis and cortex layer, covered by the epidermis (Bäurle and Laux, 2003; Jiang and Feldman, 2005).

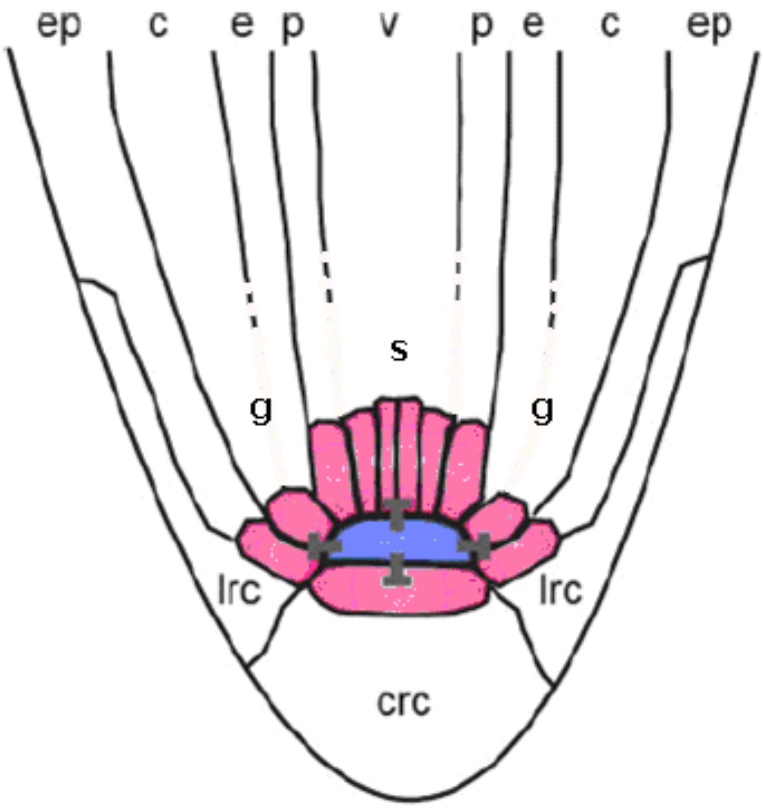


Figure 1.8 Diagram of the *Arabidopsis* root tip in longitudinal section. From the centre to the periphery: stele (s), ground tissue (g), vascular tissue (v), pericycle (p), endodermis (e), cortex (c), epidermis (ep) and lateral root cap (lrc). The central root cap (crc) is located at the very tip of the root. The QC (blue) inhibits differentiation of the neighbouring stem cells (pink). The diagram is modified from Bäurle and Laux, 2003. Blue indicates the quiescent centre. Pink indicates stem cells.

- **Primary root formation**

Primary roots developing from the embryo root pole grow underground and mature after germination. All the cells differentiate from the stem cells in the root meristem surrounding the QC, and the QC maintains the surrounding stem cells in an undifferentiated state (Jiang and Feldman, 2005). The stages of QC formation in the embryo were discussed in Section 2. The QC is essential for root growth, and the size of the QC can be influenced by environmental factors and can change the root meristem architecture (Feldman and Torrey, 1975). The effect of environmental factors on the QC might be due to the influence of the adjacent stem cells. When the QC is damaged, the remaining root meristem tissue will reorganise to form a new QC and a new meristem will re-form after that (Feldman 1976).

- **Lateral and adventitious root formation**

Lateral roots in *Arabidopsis* were shown to form from the pericycle of the mature zone (Casimiro et al., 2001). The initiation of lateral roots starts with the nucleus of two adjacent pericycle cells moving closer and is primed by auxin to go through the first asymmetric cell division (Smet et al., 2007). The asymmetric cells continue dividing to form a mass of cells which differentiate to form a root meristem and the lateral root. After the lateral root has elongated, the new vascular tissue will link with the vascular tissue of the primary root (Smet et al., 2007).

In monocots, adventitious roots (or crown roots) form from the stem and have been investigated over several decades. In rice, the primordia of adventitious roots are initiated from the pericycle layer of cells that are adjacent to the peripheral vascular cylinder in the stem (Kaufman, 1959). Periclinal divisions produce two cell layers. The outer cell layer develops into the epidermis, endodermis, cortex, and root cap, and the inner layer forms the tissue of the central cylinder. The vascular system forms after columella root cap formation and at this stage the root primordium contains all the tissues of the root (Kawata and Harada, 1975). The OsWOX9 (called the QHB, QUIESCENT-CENTER-SPECIFIC HOMEOBOX) gene, which expresses in the QC of the rice root tip, indicates that the QC-like activity starts early in the outer layer of cells at the two layer stage, and becomes more localized during primordium development. After all root structures have formed, the OsWOX9 gene is expressed in the QC, the same as in the matured root tip (Kamiya et al., 2003).

- ***In vitro* root formation**

Roots induced *in vitro* from leaf explants by treatment with high concentrations of exogenous auxin have been reported in many species (Skoog and Miller, 1957; Rose et al., 2006b). The root primordium induces from the vascular bundle cells near the wounding boundary of explants, and forms new roots from the primordium. After maturation, the structures of these *de novo* roots are similar to normal roots (Rose et al., 2006b). The *de novo* roots, lateral roots and adventitious roots are initiated from the cells adjacent to the vascular tissue; therefore they might have similar induction processes.

1.5.2 Hormone-Regulated Root Formation

Auxin has an important role in root induction and root architecture. The localized auxin accumulation influences polar root formation in the embryo, and also root meristem maintenance in the mature root. In root systems, the establishment of the auxin gradient depends on two sources of auxin. One source is auxin synthesis in the root tissue, especially in the root meristem; the other and major source is that transported from the shoot. Auxin from the shoot can be phloem-mediated or by polar transport in parenchyma cells (Ljung et al., 2005). The dynamic auxin flow has also been investigated by Grieneisen et al. (2007). They showed that auxin was transported downstream from the shoot to the root tip through the vascular tissues and accumulates in the root meristem, and then it is transported out to epidermal cells and root cap. Auxin can stimulate cell division or cell expansion depending on the concentration *in vitro* and the physiological status of the tissue (Skoog and Miller, 1957). The auxin gradient can also be correlated with cell division in the root meristem and cell elongation in the elongation zone, and can also potentially explain lateral root initiation (Grieneisen et al., 2007). The location and process of lateral root initiation is regulated by local auxin transport regulated by a group of genes such as ARF7, ARF19 (Okushima et al., 2007, Smet et al., 2007) and long-distance auxin transport from the shoot (Reed et al., 1998). The regulation of adventitious root initiation from the stem may be similar to lateral root formation (Kamiya et al., 2003).

Cytokinin inhibits root induction and growth in culture (Skoog and Miller, 1957). Cytokinin also inhibits the lateral root induction through the arrest of the cell cycle of pericycle founder cells (Li et al., 2006).

In vitro root induction is inhibited by ethylene and root formation is enhanced in the ethylene-insensitive mutant *skl* or treatment with the ethylene inhibitor aminoethoxyvinylglycine (AVG) (Simms, 2006; Rose et al., 2006b). After induction, root growth depends on two processes, one is cell division in the root meristem, and the other is cell elongation after division (Scheres et al., 2002). Ethylene has been reported to influence root growth by affecting cell elongation through regulating auxin biosynthesis (Růžička et al., 2007) and modulates stem cell division in *Arabidopsis* (Ortega-Martinez et al., 2007).

GA tends to suppress root initiation in culture (Flick et al., 1983). GA is also involved in the suppression of adventitious root induction as exogenous GA can reduce the adventitious root number and GA insensitive mutants produce more roots (Busov et al., 2006). On the other hand, GA influences root growth and development by (1), by regulating the maturation of the ground tissue which controls the root diameter (Paquette and Benfey, 2005), and by (2), inducing longitudinal cell expansion in roots in parallel with auxin derived from the shoot (Fu and Harberd, 2003).

1.6 Stem Cells in Relation to *in vitro* Somatic Embryogenesis and Organogenesis

Stem cells are undifferentiated cells whose daughters can either remain stem cells or undergo differentiation (Laux, 2003). By this definition, stem cells in normal plants are only located in the shoot, root and lateral meristems, and the early embryo. However, for example, stem cells can be obtained by de-differentiation *in vivo* in lateral root meristem formation, which arises from differentiated pericycle cells in the main root (Malamy and Benfey, 1997), or nodule formation which arises from inner cortical and surrounding cells of legume roots (Beveridge et al., 2007). This indicates the ability of plant cells to induce stem cells from differentiated cells.

Some *in vitro* tissues such as callus may also have stem cell potential. Many organs like the shoot, root, and somatic embryo can be formed *de novo* from callus indicating the pluripotency and totipotency of callus cells. The stem cells in the shoot meristem are small, undifferentiated, and with small central vacuoles (Laux, 2003). Most callus cells are large cells with large vacuoles, but there are also clusters of cells which are smaller, have a larger nucleus, and small vacuole (Na et al., 2007) which match the morphology of stem cells.

The fate determination of stem-like cells in callus is influenced by hormones, especially cytokinin and auxin, and may lead to the formation of (1) shoots, (2) roots, or (3) somatic embryos (Skoog and Miller, 1957; Steward et al., 1958).

The question arises, when stem cells form *in vitro*, are the genes involved in stem cell formation *in vivo* also involved in the *in vitro* stem cell formation.

(1) Cytokinin: Cytokinin influences shoot cell fate determination in *Arabidopsis in vitro*. *WUS* expression occurs in the callus after a high level of cytokinin is applied, and becomes more localized when shoot meristem structures start to form. *CLV3* expression occurs after *WUS* expression and is localized in the OC of the new shoot meristem (Gordon et al., 2007). *WUS*, but not *CLV3*, expression has been detected in one SE system using *Arabidopsis* roots (Gallois et al., 2004).

(2) Auxin: In *M. truncatula* 2HA culture, one week of high auxin treatment is enough to

determine the root cell fate and may activate procambial-like cells to form pluripotent cells (Rose et al., 2006b; Imin et al., 2007). The *WOX5* gene which expresses in the QC of the root meristem has a similar function to the *WUS* gene (Sarkar et al., 2007) suggesting that the *WOX5* gene may be involved in the determination of root cell fate in callus cells.

(3) Cytokinin and auxin: Cytokinin and auxin are required for somatic embryo induction in *M. truncatula* 2HA (Nolan and Rose, 1998). The *WUS* gene may also be involved in somatic embryo fate determination as shown by overexpression and the correlation of expression with auxin-induced SE in roots (Zuo et al., 2002; Gallois et al., 2004). *CLAVATA* family genes involved in *WUS* regulation can enhance SE ability in loss-of-function mutants (Mordhorst et al., 1998) which also suggests a relationship of *WUS* with somatic embryo induction.

In the *M. truncatula* system there is a need to fully understand the role of stem-cell related genes discussed above i.e. *WUS*, *WOX5*, and *CLAVATA* genes. In particular, how do these genes link to *SERK1* and *SERF1* studied in some detail in this system.

1.7 Specific Objectives of Thesis

It is the objective of this thesis to investigate the formation of stem cells in relation to the induction of embryos and root meristems *in vitro* (Fig. 1.9). The highly embryogenic mutant Jemalong 2HA and its wild-type progenitor represent a valuable system to study these processes. This investigation aims to link earlier studies on *M. truncatula SERK1* and *SERF1* to a fuller understanding of the developmental biology of SE.

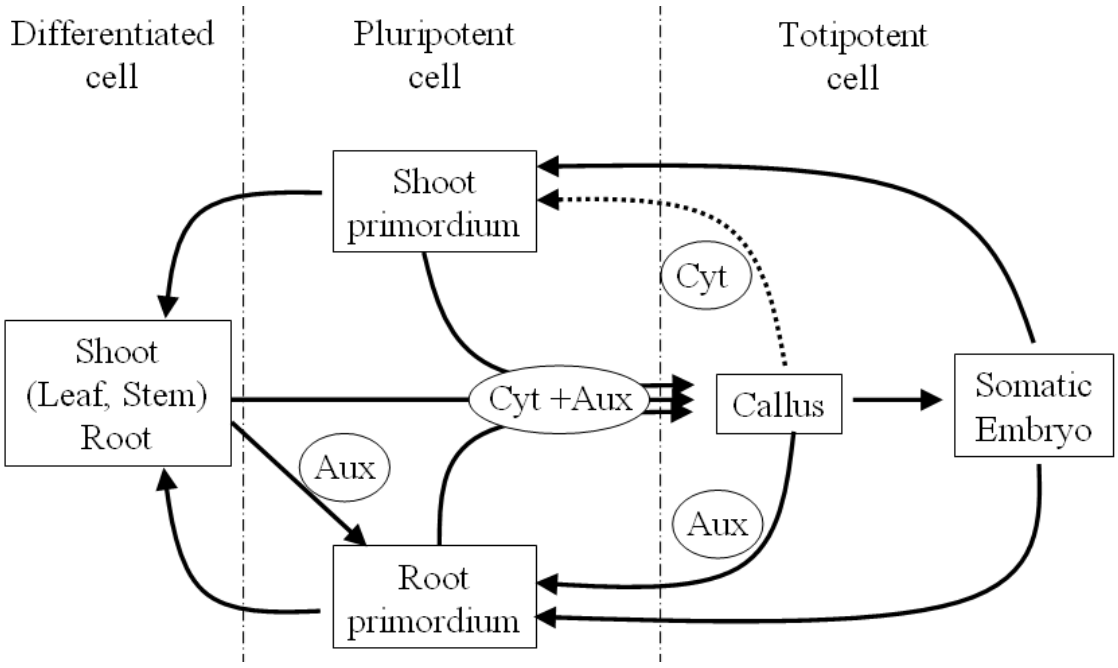


Figure 1.9 *De novo* primordium and embryo formation in tissue culture. The diagram indicates how both totipotent stem cells and pluripotent stem cells are likely to form in the induction of primordia and somatic embryos. The diagram is based on the information from *Arabidopsis* and *M. truncatula* 2HA. The pathway from callus to shoot primordium (dashed line) occurs in *Arabidopsis*, but not in *Medicago* (Beveridge et al., 2007; Gordon et al., 2007).

The thesis chapters investigating the mechanisms of SE and root primordia development *in vitro* are set out in the following way:

- In Chapter 2 and 3 genetic and physiological experiments with *M. truncatula* Jemalong and 2HA will be described.

- In Chapter 4 bioinformatics data on the *WUS, WOX5,* and *CLAVATA* family genes will be presented.

- In Chapter 5 *MtWUS* and *MtCLAVATA* family genes in relation to totipotent stem cells in SE induction are investigated.

- Chapter 6 focuses on *MtWOX5* gene expression in relation to pluripotent stem cells and root primordium formation.

- In the final Chapter there is a discussion of Chapters 2-6 and their significance in relation to SE and root primordia induction *in vitro*.

CHAPTER 2

Investigation of the Genetic Requirements for Somatic Embryogenesis in *Medicago truncatula*

2.1 INTRODUCTION

This chapter focuses on the genetics of somatic embryogenesis in Jemalong and 2HA. The *M. truncatula* 2HA mutant was first identified in 1989 and was selected from Jemalong via a cycle of tissue culture, and subsequently the 2HA seed line was derived by selection over four generations (Rose et al., 1999). There has been research on the mechanism of somatic embryogenesis research using 2HA, but the karyotype and genetics of 2HA have not been investigated.

The karyotype data of *Medicago* species were investigated in *M. sativa* subsp. *coerulea* by C-banding (Bauchan and Hossain, 1997) and in *M. truncatula* Jemalong J5 and *M. truncatula* R-108-1 by fluorescent *in situ* hybridization (FISH) (Cerbah et al., 1999). There are 16 chromosomes in diploid *M. truncatula* (2n=2x=16), with 2 pairs of metacentric chromosomes and 6 pairs of submetacentric chromosomes. Four pairs of these submetacentric chromosomes are difficult to identify because of their similar chromosome length and arm ratio (Cerbah et al., 1999).

In the 2HA genotype, it was shown that the phenotype of somatic-embryogenic capacity could be inherited in the progeny and this phenotype was probably dominant (Rose et al., 1999). It is useful to have back-cross data for 2HA with its progenitor Jemalong to obtain more information on the nature of the mutation (e.g. is it inherited as a dominant character).

There is no morphological marker for 2HA other than somatic-embryogenesis. The floral morphology of *M. truncatula* A17 has been investigated in detail and can be used as a reference to identify homeotic mutations (Benlloch et al., 2003). Therefore the floral morphologies were also investigated in this chapter.

In the first part of this chapter the chromosome karyotype in Jemalong, 2HA and the reference genotype A17 is investigated to assess if in the 2HA mutant major deletions or translocations had occurred. The second part of the chapter investigates the segregation of the 2HA somatic embryogenesis phenotype in 2HA X Jemalong crosses. The final part of the chapter investigates the floral morphology of Jemalong and 2HA.

2.2 MATERIALS AND METHODS

2.2.1 Chromosome Karyotypes

The protocol is modified from Singh (1993) and Chen (2001) using *M. truncatula* 2HA, Jemalong and A17 seed. The 2HA seed was collected in Feb. 2005, Jemalong seed was collected in Nov. 2005 and A17 seed was collected in Apr. 2005.

Collections of roots:

About 40 seeds were placed on moistened filter papers in a Petri dish and incubated in the dark at room temperature for 2 d. The roots should remain in contact with the moistened filter papers, otherwise the mitotic index will be very low. Actively growing root tips about 1-2 cm long were collected and the pre-treatment procedure initiated.

Pre-treatment of roots:

Pre-treatment serves several purposes: it stops the formation of spindles, increases the number of metaphase cells by arresting the chromosomes at the metaphase plate, contracts the chromosome length, increases the viscosity of the cytoplasm, and facilitates rapid penetration of fixative. Roots were transferred to cold water in vials, and the vials placed in an ice chest and covered with a thick layer of ice. The container was kept at 4 ºC for 22 h. Longer treatment will cause excessive shortening of the chromosomes.

Fixation:

Propionic acid alcohol was used for fixation. It is very good for plants with small chromosomes. It provides clear cytoplasm and optimal staining for chromosomes.

After pre-treatment, roots were fixed for 3 d in a 3:1 mixture of ethanol: propionic acid (propionic acid: ICN cat no. 151955, purity: ≥99%), in which a small amount of ferric chloride (ferric chloride hexahydrate, $FeCl_2 \cdot 6H_2O$, BDH cat. no. 10110) had been dissolved as a mordant (0.2 g/100mL).

Staining:

After fixation for 3 d, the root tips were hydrolysed in 1N HCl at 60 ºC for 6 min. The hydrolysed root tips were stained in aceto-orcein for 5 d. Aceto-orcein at 1% was prepared by pouring boiling 45 mL acetic acid over 1 g of orcein powder (orcein, Sigma cat. No. 7505), cooling and adding 55 mL distilled water and then filtered. These processes were handled in a fume hood.

Softening of roots:

After sufficient staining, the meristematic tissue was removed with a razor blade. The meristematic region (1 mm long) was treated with 1% pectinase at 25 ºC for 30 min. Pectinase at 1% (pectinase, Sigma cat. No. P-4625) was prepared by dissolving in distilled water.

Chromosome preparation:

The meristematic region was transferred to 45% acetic acid for 5 min to clear the cytoplasm. Each slide contained two root tips. The meristematic region was squashed in 45% acetic acid with a T-bar and the debris removed with a needle under a 10 X objective of the microscope. A cover slip was applied with one drop of 45% acetic acid and the slide turned upside down. The slide was squashed evenly at the back of the slide on several layers of filter paper. The slides were checked under the light microscope and good slides sealed with paraffin wax.

Examination of the chromosomes:

For each plant, intact chromosomes were viewed under a 100 X objective with bright field using a Zeiss Axiophot microscope. Digital images were captured by a digital camera (Zeiss AxioCam HRc) and the software (Zeiss AxioVision 4.0) provided by this system.

Chromosome alignment was carried out from the digital image using the software ACD FotoCanvas 3.0. Each chromosome was dissected from the same cell and aligned according to the length and arm ratio with the longer chromosomes displayed first.

2.2.2 Plant Growth Condition and Crossing Methods

Plants were grown 1~2 months after transplanting, under glasshouse conditions with partially controlled temperatures, between 13 and 25 ºC with a 14 h photoperiod. The crossing experiments were usually carried out in the morning when the flowers were partially opened with the staminal tube still covered by keels. By pushing the keel of the receptor flower the staminal tube flips up and the pollen can be removed. The donor pollen was then used to fertilize the stigma of the receptor flowers to complete the crossing process. Pods were collected after becoming dark grey and were then stored at 4 ºC for 3 months after thorough drying.

2.2.3 Tissue Culture of *M. truncatula* 2HA and Jemalong

Explant material was taken from the youngest expanded trifoliate leaves. The leaves were sterilised for 30 sec in 70% ethanol, 10 min in 25% White King bleach solution, then washed two times in sterile distilled water. Explants were obtained by trimming off the edges of the foliole to form a rectangular piece of tissue with the midvein in the centre. A cut was made perpendicular to the midvein to obtain two or three pieces of tissue measuring approximately 4 mm x 9 mm.

Explants were cultured 6 pieces per plate, abaxial side down in a 9 cm plastic Petri dish containing approximately 25 ml of P4 or P40 media, based on Gamborg's B5 basal media (Gamborg et al, 1968; Thomas et al., 1990). The media was supplemented with 10 μM 1-Naphthalene Acetic Acid (NAA) and 4 μM 6-Benzylaminopurine (BAP) (designated P4 (10:4) media), 10 μM NAA (P4 (10NAA) media), 4 μM BAP (P4 (4BAP) media), or 10 μM NAA plus 4 μM BAP and 1μM ABA (P4 (10:4:1) media).

Plates were incubated in the dark at 27 °C, and subcultured every 3-4 weeks onto fresh media with 4-5 explants per plate, depending on the size of the callus. For somatic embryo inducing cultures, plates were incubated in the light, 6-7 weeks after set up. Somatic embryos usually can be seen on the callus surface after 4 weeks. In this chapter, the recording of somatic embryos was carried out after 10 to 15 weeks of culture.

2.3 RESULTS

2.3.1 Karyotypes of *Medicago truncatula* 2HA, Jemalong and A17

Dividing cells were obtained from the root apex of seedlings and the chromosomes stained by aceto-orcein as described above. Investigation of chromosome morphologies in 2HA, Jemalong and A17 showed that all have 16 chromosomes with similar arm ratios and locations of heterochromatin (Figs. 2.1A, B and C, Fig. 2.2). There is no significant translocation or arm deletion in 2HA. These results suggest that 2HA may have gene mutations or epigenetic changes compared to Jemalong, rather than large scale rearrangements.

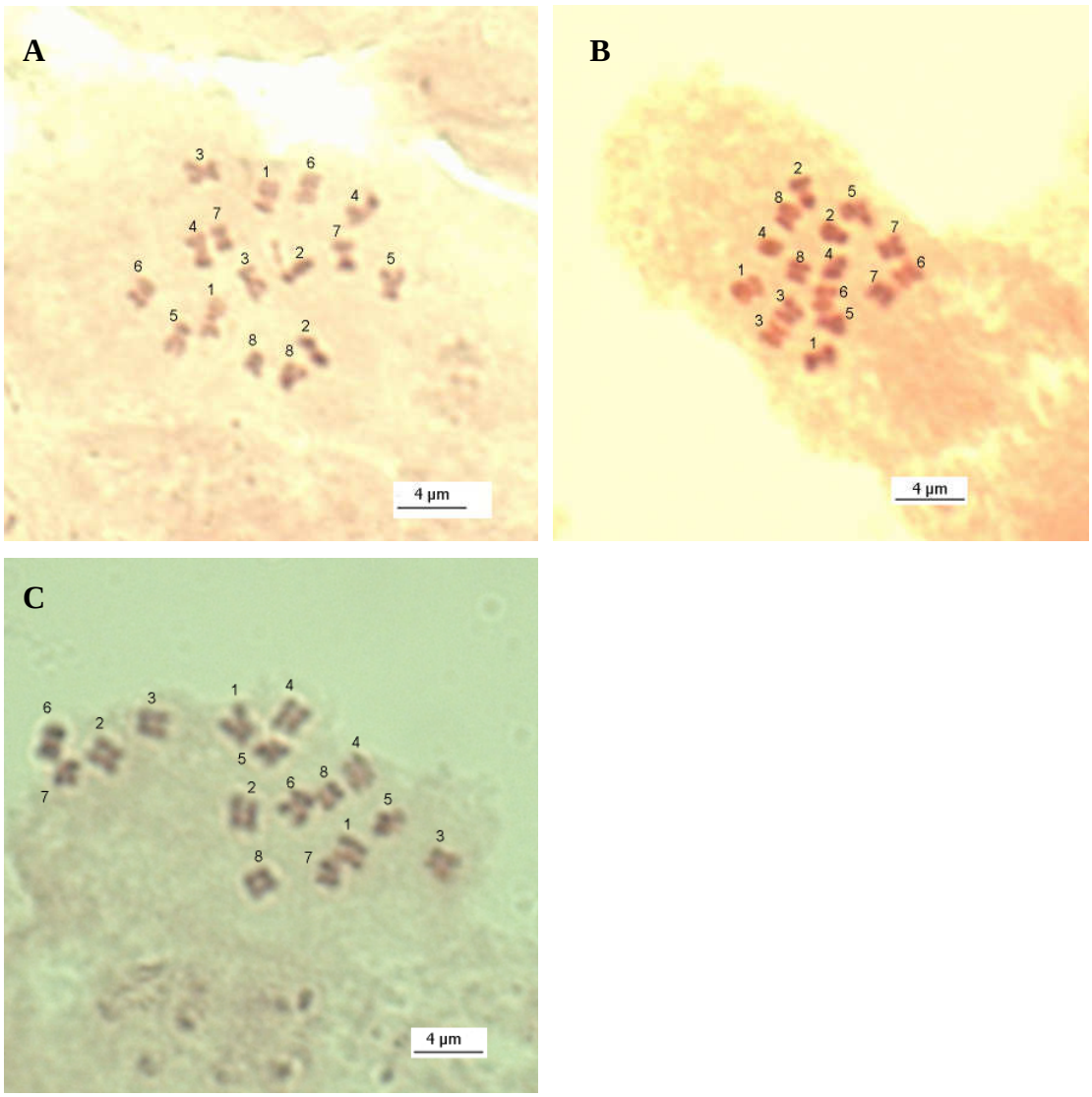


Figure 2.1 Karyotypes of 2HA (A), Jemalong (B) and A17 (C). 16 chromosomes can be identified in each species. Bar = 4 µm.

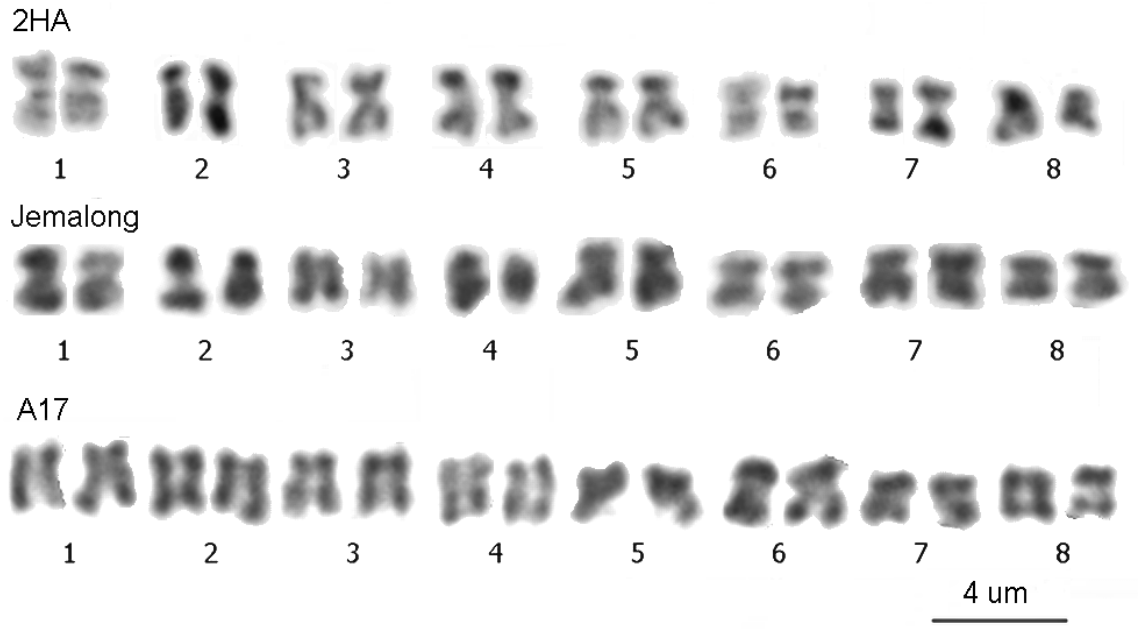


Figure 2.2 2HA, Jemalong and A17 chromosome showing aligned karyotypes. The chromosome forms were obtained from Figs. 2.1 A, B and C, and aligned by the chromosome lengths and arm ratios. Bar = 4 μm.

2.3.2 2HA (Male) X Jemalong (Female) Crossing Experiments

In order to investigate the genetics of the 2HA phenotype, we designed a crossing experiment with Jemalong and 2HA. Because the *M. truncatula* is self-fertile, 2HA pollen was used to fertilize Jemalong female flowers. We could be reasonably sure that the transfer of any SE was a result of successful hybridisation given the rarity of SE in Jemalong. Several F1 plants were obtained with the SE phenotype, consistent with the dominance of this phenotype. The segregation of the SE and non-SE type was followed in the F2 generation.

Data obtained for the F2 generation showed 25 SE and 7 non-SE plants, approximating a 3:1 ratio (Fig. 2.3). A similar ratio was also obtained in a repeat experiment where there were 30 SE and 10 non-SE plants. In this later experiment the amount of SE was ascertained, using 6 classes from low to high. These later data (Fig. 2.4) showed that SE was very variable and categories 1 and 2 had very low numbers. The data suggest that a single dominant gene will give some SE penetrance but that other, possibly additive, effects are required for maximum SE.

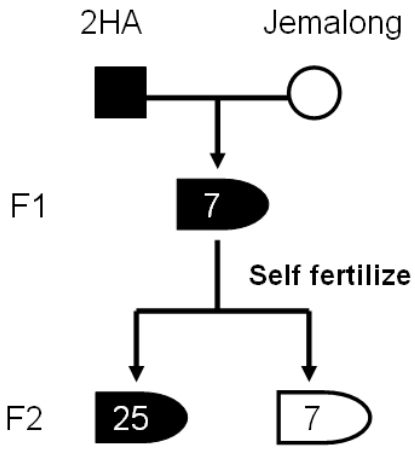


Figure 2.3 Crossing results for 2HA X Jemalong. ■ indicates pollen from 2HA and ○ indicates the egg from Jemalong. ⬬ indicates plant can produce somatic embryos (SE type), and ⬭ is non-SE type. Numbers indicate the plants numbers.

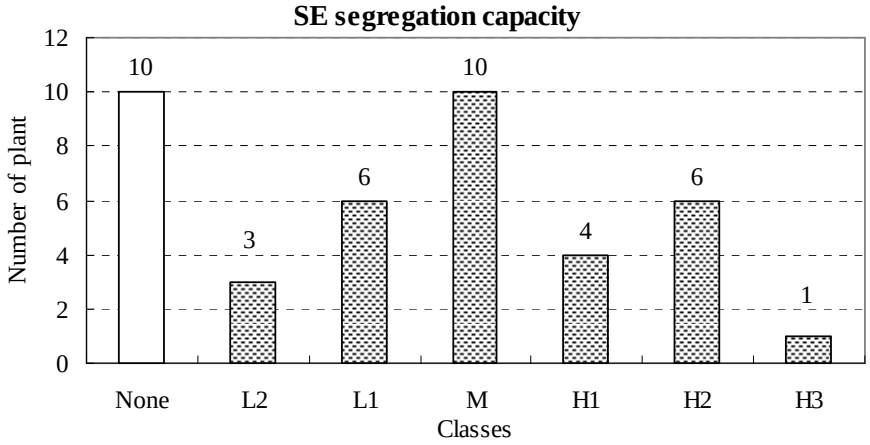


Figure 2.4. SE segregation capacity for F2 plants in 2HA X Jemalong experiment. Open column indicates non-SE type, where no embryos form in callus (None class). Filled columns indicate SE type and are divided into 6 classes: low SE capacity level 2 (L2), <20% calli forming embryos, average embryo number per callus <0.5; low SE capacity level 1 (L1), <20% calli forming embryos, average embryo number per callus is 0.5~1; medium SE capacity (M), 20% ~ 80% calli forming embryos, average embryo number per callus is 1~3; high SE capacity level 1 (H1), >80% calli forming embryos, average embryo number per callus is 1~3; high SE capacity level 2 (H2), >80% calli forming embryos, average embryo number per callus is 3~5; high SE capacity level 3 (H3), 100% calli forming embryos, average embryo number per callus >5. The classification is modified from Rose et al., 1999.

The morphology of plants was also investigated in the crossing experiments. The morphology of leaves, pods, and plant size did not show significant differences between 2HA and Jemalong, but some differences were noted in flower numbers and floral morphology.

2.3.3 Flower Numbers in 2HA and Jemalong

The number of flowers in 2HA and Jemalong were different. The numbers were obtained by examining ten inflorescences from each of 10 plants of Jemalong and 2HA of similar age and grown under the same conditions. The average flower numbers per inflorescence in Jemalong is 2.63, higher than the 1.1 per inflorescence in 2HA. Jemalong can produce more flowers in an inflorescence than 2HA (Table 2.1).

Flower numbers in single inflorescence				**Average / inflorescence**
Jemalong (100 inflorescences)	3	31	66	**2.63 ± 0.2**
2HA (100 inflorescences)	90	10	0	**1.1 ± 0.1**

Table 2.1 Flower numbers in Jemalong and 2HA inflorescences. Three types of flowers were noted in the inflorescences. Ten inflorescences were randomly selected from each of 10 plants in Jemalong and 10 plants in 2HA. All plants were grown in the glasshouse for 2.5 months.

Flower numbers were also investigated in the crossing experiments described above. A total of 11 SE type plants and 3 non-SE type plants, and 6 to10 inflorescences were selected for each plant. The average number of flowers in a non-SE type plant was 1.87 per inflorescence, higher than the 1.16 in SE type plants (Table 2.2). The flower numbers of the SE type plants were similar to 2HA. This indicated that the number of flowers per inflorescence is associated with the somatic-embryogenic phenotype.

Plant type	**Explants forming embryos (%)**	**Average no. flowers / inflorescence**	**Total Average**
SE type (11)	100	1.0 ± 0.0	**1.16 ± 0.28**
	80	1.3 ± 0.5	
	100	1.6 ± 0.5	
	80	1.0 ± 0.0	
	60	1.8 ± 0.4	
	20	1.0 ± 0.0	
	100	1.0 ± 0.0	
	100	1.0 ± 0.0	
	100	1.0 ± 0.0	
	100	1.0 ± 0.0	
	100	1.1 ± 0.3	
non-SE type (3)	0	1.9 ± 0.3	**1.87 ± 0.06**
	0	1.8 ± 0.4	
	0	1.9 ± 0.3	

Table 2.2 Flower number analysis in inflorescence of 2HA X Jemalong experiment related to SE type.

2.3.4 Floral Morphology Changes in 2HA Compared to Jemalong

The normal *M. truncatula* flower has 5 petals including one vexillum, 2 alae and 2 keels (Benlloch et al., 2003). The staminal tube is covered by keels before the flower is fully opened (Figs. 2.5A and B). In 2HA, most flowers have normal morphology, but some distinctive floral phenotypes occurred. These were deficient in part of the petal structures (Figs. 2.5C-F). The flowering process also showed some differences. In Jemalong, flowers usually opened for one day between 10 am and 4 pm, and then the petals closed and faded followed by pod formation. In 2HA, most flowers followed these stages, but some flowers opened longer than one day without closing or fading, even after the pods had formed (Fig. 2.5E white arrow).

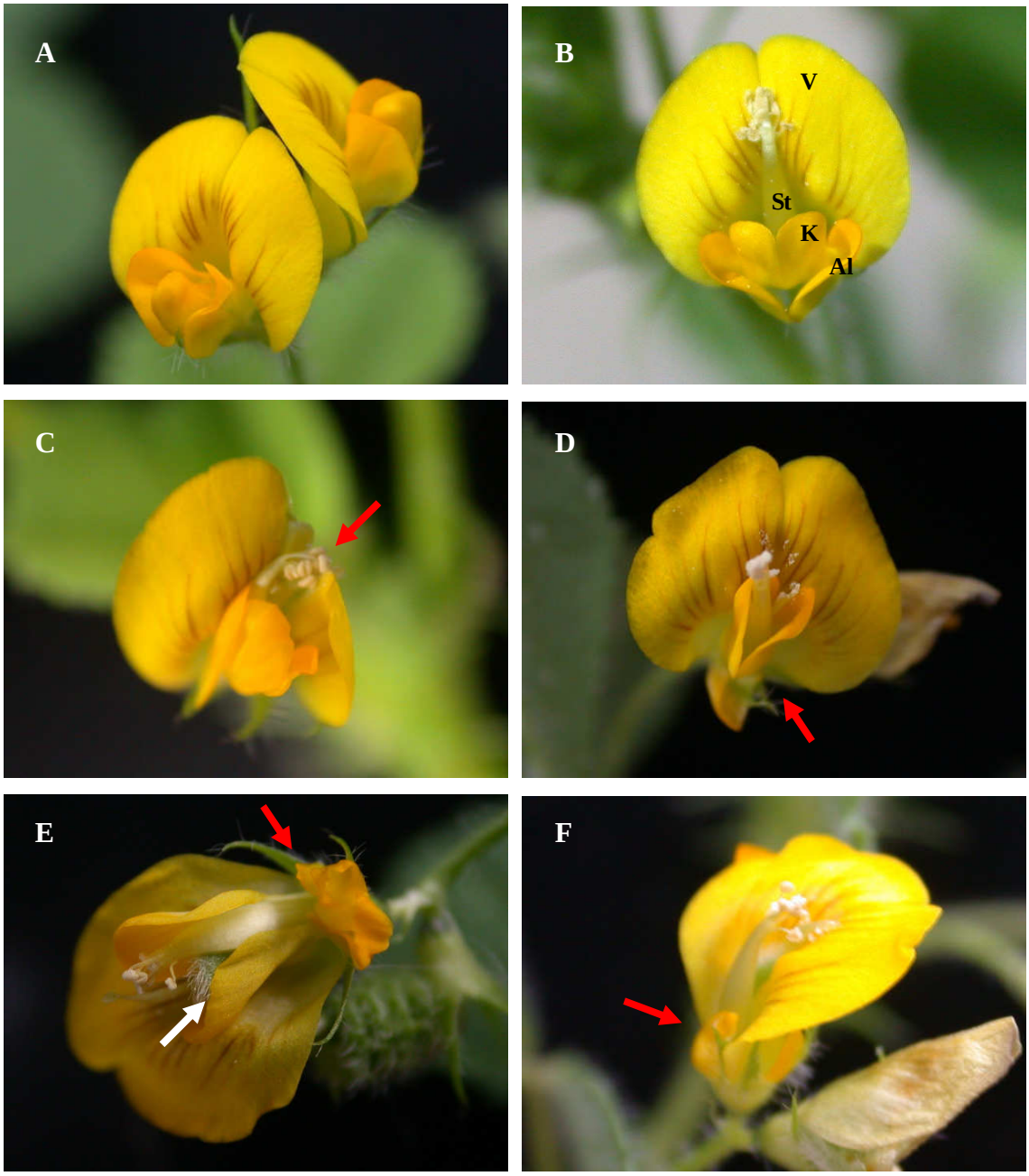


Figure 2.5 Floral morphology phenotypes in *Medicago truncatula* 2HA. Normal flower morphology in Jemalong and 2HA is shown in A and B. There are 5 petals with one vexillum (V), 2 alae (Al) and 2 keels (K), and the staminal tube (St) covered in keels before the flower opens (A). There are some petal phenotypes seen occasionally in 2HA but not Jemalong. The changes might appear in the vexillum (C), alae (D, E) or keels (F). Some pods had started development while the flower was still open (E, white arrow) which did not occur in Jemalong.

2.4 DISCUSSION

M. truncatula 2HA was obtained after a cycle of tissue culture (Nolan et al., 1989; Rose et al., 1999). This type of procedure was also carried out in two other *Medicago* somatic embryogenesis lines *M. truncatula* R-108 (Hoffmann et al., 1997) and *M. truncatula* M9-10a (Araújo et al., 2004). Further, in *M. truncatula* cv. Jemalong a cycle of tissue culture is always enough to enhance regenerability (Nolan et al., 1989). These data suggest that enhanced regenerability via SE in *M. truncatula* is an epigenetic effect as the mutation frequency is too high for a consistent DNA sequence change. Other reports indicate that epigenetic mutants may occur through tissue culture procedures (Bednarek et al., 2007). Information in this chapter was obtained to provide more information on the genetic nature of the greatly enhanced SE in 2HA.

The karyotype of 2HA was investigated in this chapter. Compared to the chromosome morphology of Jemalong and A17, no large scale change was found in chromosome number and structure. There was clearly no change in ploidy or large translocations or deletions. However, the aceto-orcein staining method cannot clearly identify each chromosome, especially for those submetacentric chromosomes. Some other techniques like C-banding or FISH (fluorescent *in situ* hybridisation) would be required for more detailed investigation.

The experiments involving analysis of the F2 population from 2HA and Jemalong crosses provide some additional insight. If phenotypes are characterised as SE or non-SE there is a 3:1 ratio SE:non-SE. This suggests a single dominant gene is involved. However there is a large variation in SEs per plant and a single dominant gene only guarantees a few embryos. To obtain high embryogenicity other genes must come in via some type of additive or enhancing effect. This interpretation fits with the way in which 2HA was developed.

Fig. 2.6 shows the 2HA selection process. The initial experiment was carried out in 1989 by Nolan et al. on regenerated plants from Jemalong. One of the regenerated plants called R3 produced large number of SEs in tissue culture and was named Jemalong 2HA. The high frequency SE ability passes to the next generation through seed, but there were some low SE frequency plants in the progeny. In 1999, Rose et al.,

classified SE into three groups: “High” which indicated 80% of calli produced embryos and 30% of calli had more than 10 embryos; “Low” indicated less than 20% of calli produced embryos; and “Moderate” was the remainder of plants that were not “high” or “low”. Fifty seeds of the R3 progeny were germinated, tested for SE ability and classified into the three groups. The seeds of several plants belonging to the high frequency group were selected for the next tests. The selection procedures were repeated with the “high” SE group for three generations. In each generation test, the percentage of the high frequency group was always over 50% till the last generation where 100% of the progeny were classified in the “high” frequency group. These final high frequency somatic-embryogenic plants suggested that 2HA was homologous for SE and could produces 500 times more somatic embryos than its wild type progenitor Jemalong (Rose et al., 1999). These data on segregation rate suggested that a number of genes are involved in obtaining the high frequency somatic-embryogenic phenotype. The ability of different genes to initiate or stimulate SE (*WUS*, *LEC1*, *LEC2*, *SERK*, and *CLAVATAs*) is also consistent with this.

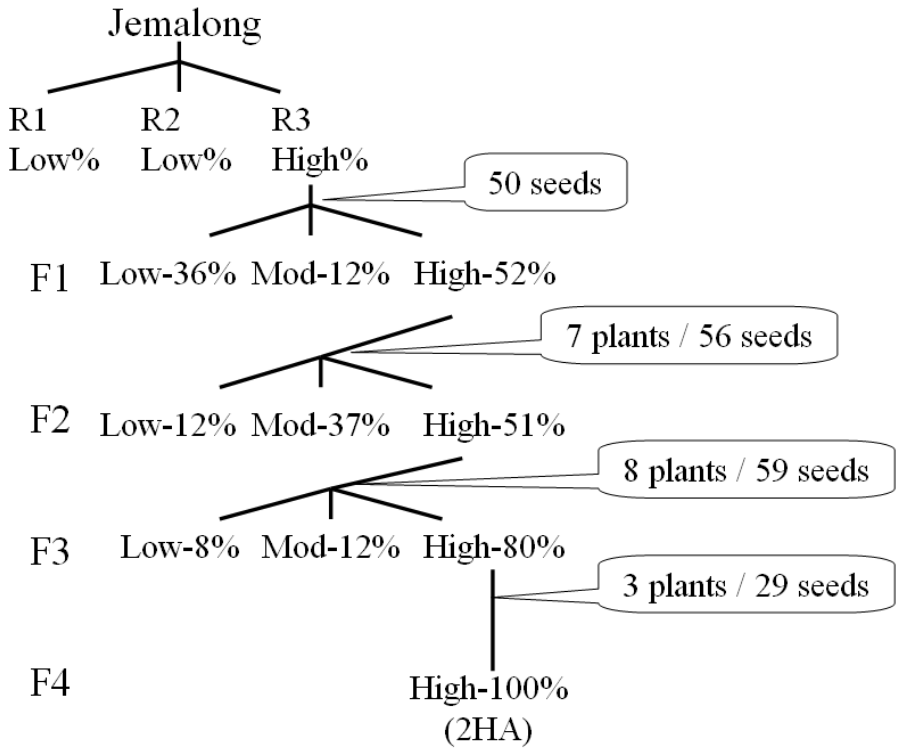


Figure 2.6 *Medicago truncatula* Jemalong 2HA selection process (modified from Rose et al., 1999). **“**High (frequency)” indicates more than 80% calli produce embryos and 30% of them contain more than 10 embryos. “Low (Frequency)” indicates less than 20% calli produce embryos. “Mod (frequency)” indicated the frequency was between high and low.

In searching for mutations or epigenetic changes in 2HA, the AFLP (amplified fragment length polymorphism) and AMP (amplified methylation polymorphisms) was investigated in Jemalong and 2HA and revealed similar AFLP patterns (Irwanto, 2004), but different methylation patterns as assessed by AMP. These data provide some support for epigenetic change being related to SE. Chromosome remodelling and methylation change may proceed during the induction of somatic embryos (Merkle et al., 1995). It is possible that an epigenetic change enables SE potential but to maximise this potential a number of genes are involved.

The 2HA genotype is not only characterised by high SE, but has fewer flowers per inflorescence. This characteristic segregates with high SE. Similarly changes in flower morphology have been noted in 2HA. Of particular interest is the delayed senescence of petals in some 2HA flowers (Fig. 2.5E) which has also been noted in Brassicas with high SE (Malik et al., 2008).

Data in this Chapter does not unequivocally define the genetics of 2HA but does provide some guide to potential genetic mechanisms. 2HA may have a number of modified genes but probably only one of them opens up the somatic embryo induction ability. This could be, for example, a gene influencing epigenetic change such as one influencing methylation. In the following chapter the hormone requirements for somatic embryogenesis in 2HA will be investigated further and the information may help to provide more information on key genes.

CHAPTER 3

Investigation of the Timing of the Hormone Requirements for the Induction of Somatic Embryogenesis and Organogenesis in *M. truncatula*

3.1 INTRODUCTION

This chapter focuses on the hormone requirements for somatic embryogenesis and organogenesis in Jemalong and 2HA. Exogenous auxin is required for somatic embryo induction but only for a short exposure time in many species (Ammirato, 1983). Standard protocols for SE in *M. truncatula* 2HA from leaf explants and protoplast cultures have been developed (Nolan and Rose, 1998; Rose et al., 1999), where both auxin and cytokinin are essential for SE. Auxin and cytokinin are not only used in the induction on the semi-solid agar medium, however, but are also used in the following subculture medium at the same concentration. ABA is used in a second subculture medium with auxin and cytokinin and enhances the frequency of regenerated plants (Nolan and Rose, 1998).

GA has received much less attention than auxin and cytokinin on *in vitro* development studies. There is good reason for this as GA does not usually help somatic embryo induction (Ammirato, 1983). There is evidence that GA acts as an active repressor of SE and is important in preventing unexpected somatic embryo formation in a growing plant. The *PICKLE* gene, which is regulated by GA through DELLA proteins, can suppress somatic embryogenesis and there is a reduction in biologically active GA (See Chapter 1 Fig. 1.4). However GA can help the germination of SEs (Ammirato, 1983) and can stimulate root growth (Fu and Harberd, 2003).

This chapter will focus on the induction stage of SE to more critically investigate the time required for auxin, cytokinin and ABA to induce SEs in the *M. truncatula* 2HA system. These data will enable a better understanding of molecular mechanisms, investigated in later chapters. The chapter also includes an investigation of GA given the evidence that it appears to have a role in repressing SE in the growing plant.

Root organogenesis can be induced *in vitro* by culture of the leaf explants of Jemalong and 2HA in high levels of auxin alone (Nolan et al., 2003). The *de novo* root formation from leaf explants can be divided into three stages: stage 1, root primordia induction from procambial-like cells of explants; stage 2, root differentiation from root primordia; and stage 3, the root structure is formed and elongation follows (Rose et al., 2006b). Imin et al., (2007) indicated the stem cell fate for *de novo* root induction was

determined after 1 week of culture, however, detailed investigations have not been carried out on the time requirements for stem cell fate determination. These investigations have also been carried out in this chapter and in addition GA examined given its potential role in suppression of meristem formation as has been reported in SE and discussed above.

3.2 MATERIALS AND METHODS

3.2.1 Tissue Culture Methods for Hormone Timing Requirements (TR)

The sterilisation and culture processes were described in 2.2.3. Special subculture cycles and media were used in the different treatments. All the explants were initially cultured in the dark and transferred to the light after 5 weeks. Somatic embryo formation was assessed in week 6 or week 8.

For the standard somatic embryo induction procedure in *M. truncatula* cv. Jemalong 2HA (Nolan and Rose, 1998), 2HA explants are cultured in agar medium (P4 medium) with 10 µM NAA and 4 µM BAP [called P4 (10:4) medium] for 3 weeks, and subcultured to agar medium with 10 µM NAA, 4 µM BAP and 1 µM ABA [called P4 (10:4:1) medium] every 4 weeks until somatic embryos can been seen. Experiments to investigate the minimum time for treatment with auxin, cytokinin and ABA were modified from the standard procedure.

Type (i) Hormone Time Requirement experiments (Hormones TR)

Experiments can be designed to investigate the time requirements (TR) of the hormones auxin and cytokinin for somatic embryo induction. The following treatments are described in Fig. 3.1.

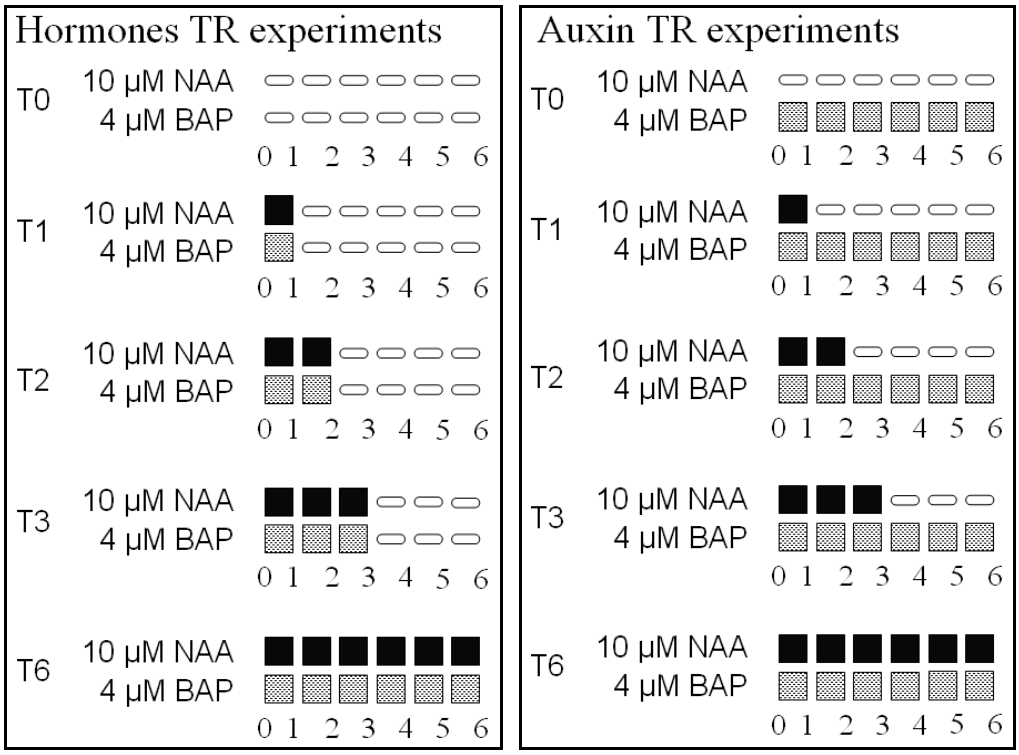


Figure 3.1 Schematic of hormone time requirements (Hormones TR) and auxin time requirements (Auxin TR). ■ indicates auxin (10 µM NAA), ▩ indicates cytokinin (4 µM BAP), and ⬭ indicates no hormone. There were five types of treatments in Hormones TR experiments: 0 week of auxin and cytokinin (T0), 1 week of auxin and cytokinin (T1), 2 weeks of auxin and cytokinin (T2), 3 weeks of auxin and cytokinin (T3), and 6 weeks of auxin and cytokinin (T6) as the control. The difference in Auxin TR experiments is 6 weeks of cytokinin is supplied for all types of treatments.

Type (ii) Auxin Time Requirement experiments (Auxin TR)

These experiments were modified from type (i) above, and focused on the time requirements for auxin. The following treatments were carried out: 2HA leaf explants were cultured in P4 (10:4) medium for different lengths of time (0, 1, 2, or 3 weeks) and subcultured into a 2nd P4 medium containing 4 µM BAP [called P4 (4BAP) medium]. The auxin was supplied for set periods of time and the cytokinin was supplied continually (Fig. 3.1). The results were assessed at week 6.

The above auxin TR experiment was carried out and another experiment including 4 weeks of 10 µM NAA with cytokinin continually supplied. In this latter experiment, the fresh weight of somatic embryos and callus was obtained at week 8.

One set of Auxin TR experiments were also carried out in Jemalong to investigate the

influences of different auxin exposure times on callus formation in Jemalong compared to 2HA.

Type (iii) ABA-auxin Time Requirement experiments (ABA-auxin TR)

These treatments were similar to Type (ii) with ABA supplied together with auxin to investigate the influence of ABA on the somatic embryo induction. The following procedures were carried out: 2HA leaf explants were cultured initially in P4 medium containing 10 µM NAA, 4 µM BAP and 1 µM ABA [called P4 (10:4:1) medium] for different lengths of time (0, 1, 2, or 3 weeks) and subcultured in P4 (4BAP) medium. The auxin and ABA were only supplied for set periods of time and cytokinin was supplied continually (Fig. 3.2). The results were assessed at week 6.

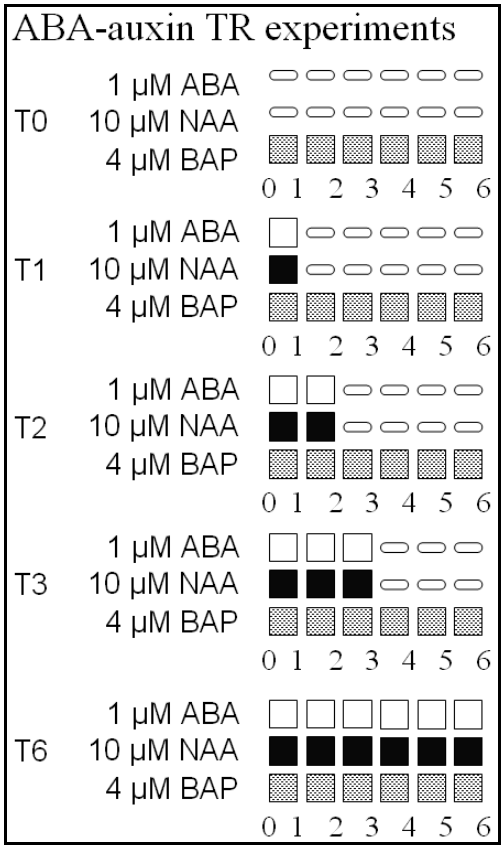


Figure 3.2 Schematic of ABA-auxin time requirement (ABA-auxin TR) experiment. ■ indicates auxin (10 µM NAA), ▩ indicates cytokinin (4 µM BAP), □ indicates 1 µM ABA, and ⬭ indicates no hormone. There were five types of treatments including 0 week of auxin and ABA and 6 weeks of cytokinin (T0), 1 week of auxin and ABA and 6 weeks of cytokinin (T1), 2 weeks of auxin and ABA and 6 weeks of cytokinin (T2), 3 weeks of auxin and ABA and 6 weeks of cytokinin (T3), and 6 weeks of auxin, ABA and cytokinin (T6) as the control.

3.2.2 GA Experiments on Somatic Embryogenesis Induction

The sterilisation and culture processes are described in 2.2.3. 2HA explants were cultured in P4 (10:4) medium plus 5, 10, or 50 µM GA_3 and subcultured every 3 weeks. Explants were also cultured in P4 medium containing 4 µM BAP plus 10 µM GA_3. Explants cultured in P4 (10:4) medium were the controls. Explants were initially cultured in the dark and transferred to the light after 5 weeks. SEs were assessed after 6 weeks.

3.2.3 Auxin-GA Experiments on *de novo* Root Formation

The culture process has been described in 2.2.3. The standard procedure for *de novo* root induction using *M. truncatula* leaf explants is the culture in 10 µM NAA P4 medium for 3 to 4 weeks in the dark. Callus and roots form around the edge of the explants. To investigate the response of auxin and GA in the *de novo* root induction process, three types of experiments were carried out.

(i) Auxin, GA, and primordia induction

These experiments were designed to investigate the influence of different concentrations of GA and auxin on root primordia induction. Four different concentrations of GA_3 (0 µM, 0.1 µM, 1 µM, and 10 µM) were mixed with 3 different concentration of NAA (0 µM, 1 µM, 10 µM) in P4 medium (Table 3.1). Six leafexplants from 2HA were cultured in each of these different media in the dark and root formation assessed after 21 days.

NAA/ GA_3 (µM)	0	0.1	1	10
0	No hormone	0.1 GA_3	1 GA_3	10 GA_3
1	1 NAA	1 NAA + 0.1 GA_3	1 NAA + 1 GA_3	1 NAA + 10 GA_3
10	10 NAA	10 NAA + 0.1 GA_3	10 NAA + 1 GA_3	10 NAA +10 GA_3

Table 3.1 GA_3 and NAA components in GA experiments.

(ii) Auxin, GA, and primordia development

These experiments were designed to investigate primordia development after the root

primordia had been induced. The concentrations of GA_3 and NAA tested were the same as in experiment (i) (Table 3.1). The 2HA explants that had been cultured in 10 µM NAA for 28 days and had obtained callus or some roots were then cultured in different media in the dark. Root formation results were subsequently assessed after 21 days further culture.

(iii) Time requirements for *de novo* root induction in Jemalong and 2HA with auxin and GA treatments

Information obtained from (i) and (ii) showed that GA_3-NAA concentrations for suppression of primordia induction but stimulation of primordia development is 10 µM GA_3 or 10 µM NAA plus 0.1 µM GA_3. Leaf explants from Jemalong and 2HA were cultured in 10 µM NAA-P4 medium and subcultured to 10 µM GA_3 or 10 µM NAA plus 0.1 µM GA_3 medium 3 d and 7 d after set up (Fig. 3.3). Controls were set up in 10 µM NAA together with the 10 µM GA_3 or 10 µM NAA plus 0.1 µM GA_3 treatments. Root formation was assessed at 21 days.

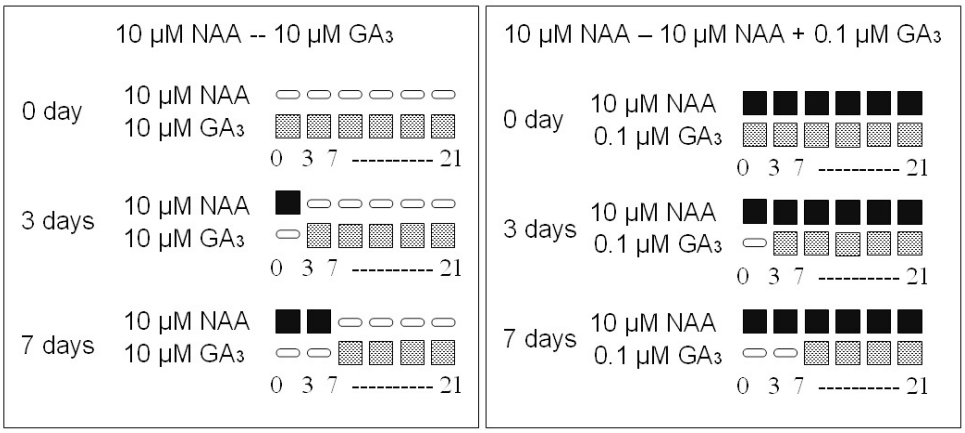


Figure 3.3 Schematic of time requirement for *de novo* root induction. ■ indicates auxin (10 µM NAA), ▩ indicates GA (10 µM or 0.1 µM GA_3), and ⬭ indicates no hormone. There were two GA concentrations with GA starting at 0 day, 3 days of auxin then transferred to GA, 7 days of auxin then transferred to GA.

3.2.4 Histology

Tissue fixation, subsequent embedding in LR white resin, cutting of 0.5-1 µm sections and photography, has been described in Rose et al. (2006).

3.3 RESULTS

3.3.1 The Time Requirement for Hormones in Somatic Embryo Induction

The time requirements for hormone induction of somatic embryos were carried out in three types of experiments described in 3.2.1 (Fig. 3.4). The somatic embryo induction rates, size of explant images, and the weights of somatic embryos and callus were investigated.

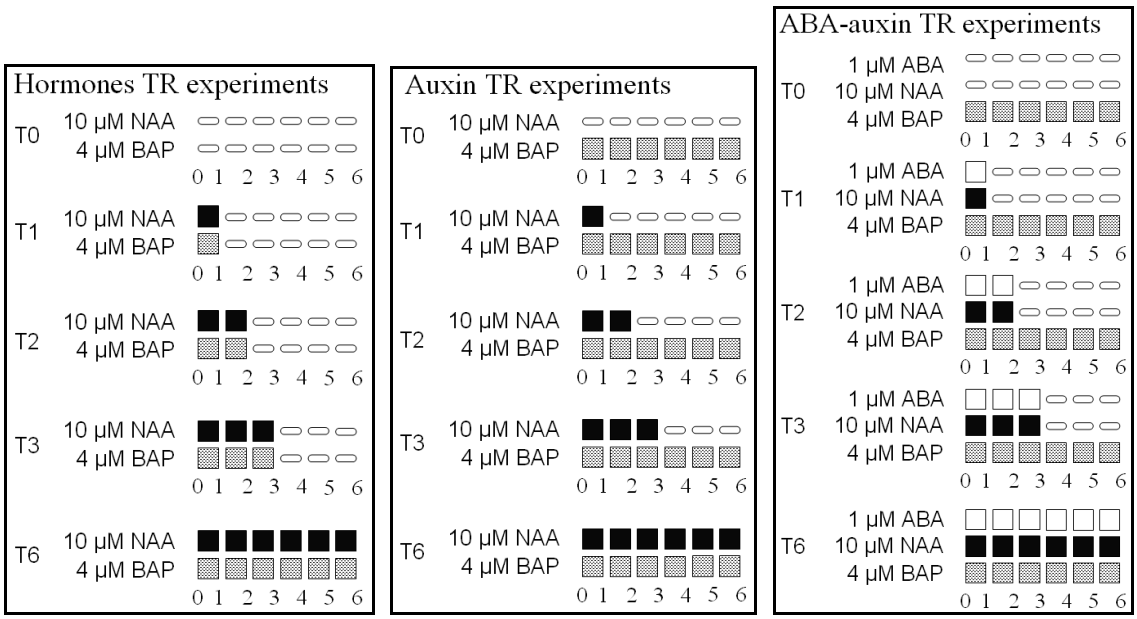


Figure 3.4 Summary of schematic of time requirement experiments.

3.3.1.1 Somatic embryo induction rates

The somatic embryo induction rates for three types of experiments are listed in Table 3.2. In Hormones TR experiments, the T2 sample shows only a 16.7% somatic embryo induction rate, and T3 and T6 control samples show a 100% induction rate indicating at least 3 weeks of exogenous hormone supply is required for maximum somatic embryo induction. In Auxin TR experiments, the induction rate in T1 is 33.3%, and in T2 and T3 samples it is 83.3% indicating exogenous auxin is required for at least 2 weeks to approach a 50% induction rate. Comparing these two experiments also indicates that cytokinin is required for 3 weeks for maximum induction.

Name \ treat weeks	T0	T1	T2	T3	T6 (Control)
Hormones TR	0/6 = 0%	0/6 = 0%	1/6 = 16.7%	6/6 = 100%	6/6 = 100%
Auxin TR	0/6 = 0%	2/6 = 33.3%	5/6 = 83.3%	5/6 = 83.3%	6/6 = 100%
ABA-auxin TR	0/6 = 0%	3/6 = 50%	6/6 = 100%	6/6 = 100%	4/6 = 66.7%

Table 3.2 Somatic embryo induction at week 6 in three types of experiments. The results show the number of explants producing somatic embryos / total number of explants and the percentage of explants producing somatic embryos at week 6.

In ABA-auxin TR experiments, the T1 sample had already reached the 50% somatic embryo induction rate, and in T2 and T3 had a 100% induction rate. These results indicate that ABA present from the beginning of culture enhances somatic embryo induction.

Because of the small sample sizes these results must be interpreted with some caution. However, what is clear is that the inductive hormones auxin and cytokinin are only required for 2-3 weeks. There is also a clear indication that ABA given at zero time will enhance SE.

3.3.1.2 Explant growth results

The results of explant growth for the three treatments are listed in Table 3.3. In general maximum callus size requires continuous hormone treatment. However it appears that ABA will inhibit callus growth, even through there are high rates of somatic embryo formation.

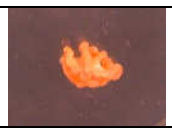
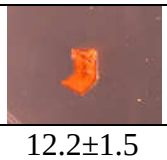
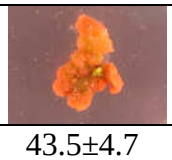
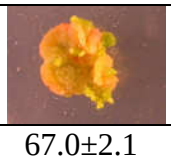
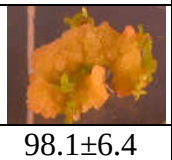
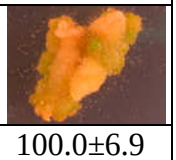
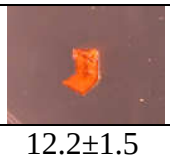
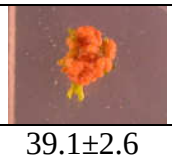
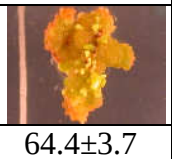
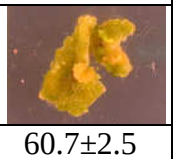
Name \ treat weeks	T0	T1	T2	T3	T6 (Control)
Hormones TR					
	15.0±1.4	19.3±1.1	44.7±3.8	74.4±4.5	100.0±6.9
Auxin TR					
	12.2±1.5	43.5±4.7	67.0±2.1	98.1±6.4	100.0±6.9
ABA-auxin TR					
	12.2±1.5	39.1±2.6	67.0±8.6	64.4±3.7	60.7±2.5

Table 3.3 Explant growth results displayed with explants images at week 6 and relative explant image sizes. The image sizes were related to the week 6 explants of the hormones TR control set at 100%. Image size was determined using the Image J program.

The explants size comparisons for Auxin TR experiments for 2HA and Jemalong are shown in Table 3.4. This explant growth rate is similar in Jemalong and 2HA, although Jemalong produced more callus than 2HA for every treatment. This suggests that as Jemalong does not form embryos, it will produce slightly more callus.

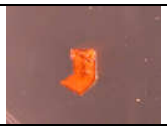
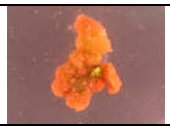
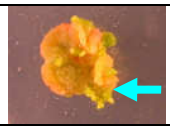
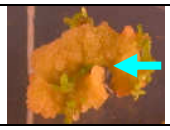
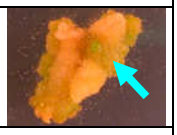
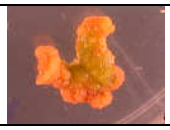
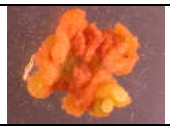
Name \ treat weeks	T0	T1	T2	T3	T6 (Control)
2HA auxin TR					
	12.2±1.47	43.5±4.71	67.0±2.07	98.1±6.35	100.0±6.92
Jemalong auxin TR					
	18.0±2.61	50.2±3.25	75.7±6.52	100.2±8.11	124.2±9.25

Table 3.4 Explants growth comparisons between Jemalong and 2HA. The image sizes are relative to the week 6 explants image sizes of the 2HA hormones TR control set at 100%. Blue arrow indicates the somatic embryos occur in 2HA callus. Image size was determined using the Image J program.

3.3.1.3 Somatic embryo and callus growth in auxin TR experiments

Somatic embryos (SEs) and callus weights were investigated in one auxin TR experiment where auxin was subcultured for 1, 2, 3 and 4 weeks with 8 weeks cytokinin, and weights were obtained at week 8 (Fig. 3.5). The maximum total weight was obtained after the T3 treatment. However, the components of T3 and T4 are different in that T4 has more callus than T3. These results indicate the 3 weeks auxin treatments can obtain maximum somatic embryo weight, and longer treatments of auxin increases callus formation relative to embryogenesis. The data are consistent with those in Table 3.2 and 3.3.

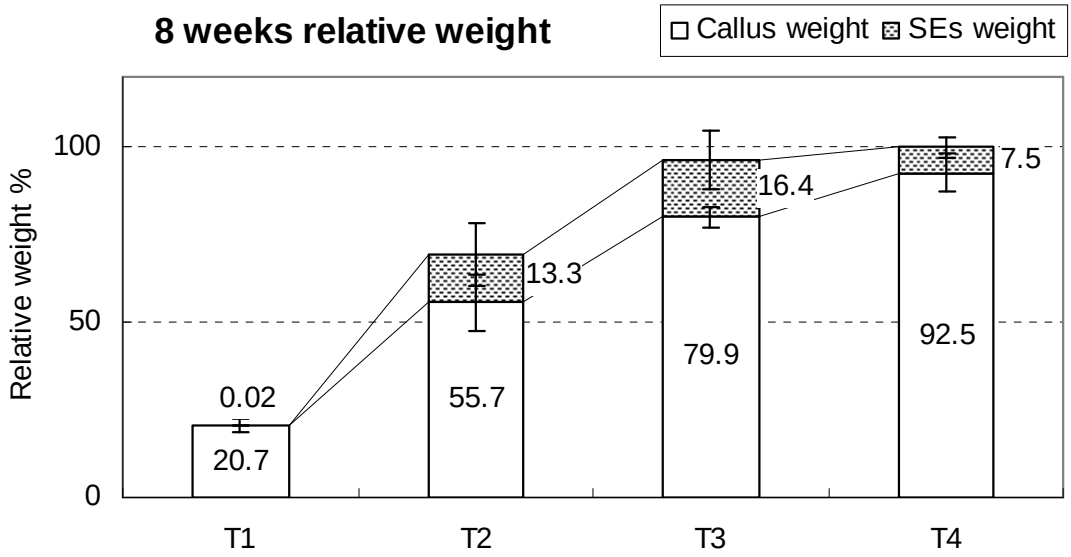


Figure 3.5 Relative weight of callus and SEs for different treatments in auxin TR experiment. The data were obtained after 8 weeks culture and the weights were relative to total weight (SEs plus callus) with the 8 weeks T4 sample as 100%. Somatic embryo weight shown in filled dots, and callus weight shown in white.

3.3.2 GA and Somatic Embryogenesis

Three concentration of GA was added with P4 (10:4) medium to investigate the influence of GA on SE in 2HA (Table 3.5). The somatic embryo induction rate and somatic embryo numbers per explant decreased in 10 µM GA_3 treatment but increases slightly in lower and higher concentration (Table 3.5). Overall this suggests minimal influence of GA. GA added with cytokinin did not produce somatic embryo indicating that the GA could not substitute for auxin to induce the somatic embryo formation.

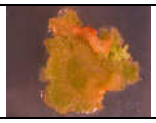
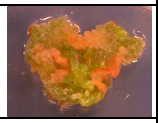
2HA	Control	Control + 5 µM GA_3	Control + 10 µM GA_3	Control + 50 µM GA_3	4 µM BAP + 10 µM GA_3
Morphology					
% Explants Forming SEs	100 %	100 %	66.7 %	100 %	0 %
Average no. SEs/ explant	4.7 ± 0.71	7.0 ± 1.29	4.2 ± 1.60	7.5 ± 1.45	0

Table 3.5 Somatic embryo induction results in different GA treatments. The results show the percentage of explants forming somatic embryos and the average numbers of somatic embryo per explant. The data obtained after 6 weeks. Control medium is P4 medium with 10 µM NAA plus 4 µM BAP.

3.3.3 Auxin, GA, and *de novo* Root Formation Experiments

3.3.3.1 Auxin, GA, and primordia induction

For the root primordia induction phase, different concentration of auxin and GA described in 3.2.3 (i) were investigated. After 21 days, only explants in 10 µM NAA alone produced primordia tissue from the edge of explants and some roots formed from the primordia tissue (Table 3.6). No primordia formed in other conditions. 10 µM NAA was required for root primordia induction, and even 0.1 µM GA is enough to inhibit the process. High concentration of GA alone also did not induce root primordia and root formation.

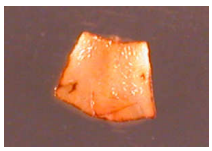
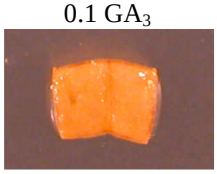
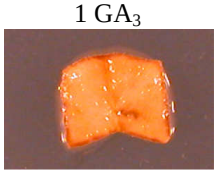
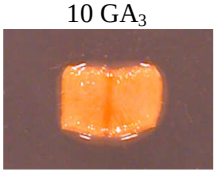
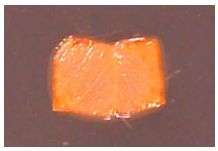
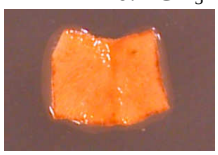
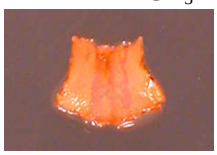
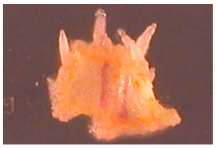
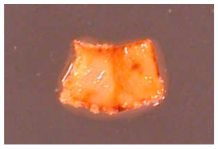
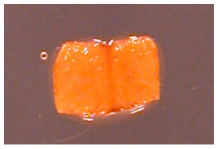
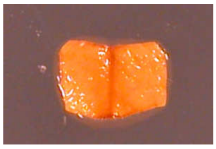
NAA / GA_3 (µM)	0	0.1	1	10 (GA_3)
0	No hormone  0/6 = 0%	0.1 GA_3  0/6 = 0%	1 GA_3  0/6 = 0%	10 GA_3  0/6 = 0%
1	1 NAA  0/6 = 0%	1 NAA + 0.1 GA_3  0/6 = 0%	1 NAA + 1 GA_3  0/6 = 0%	1 NAA + 10 GA_3  0/6 = 0%
10 (NAA)	10 NAA  6/6 = 100%	10 NAA + 0.1 GA_3  0/6 = 0%	10 NAA + 1 GA_3  0/6 = 0%	10 NAA + 10 GA_3  0/6 = 0%

Table 3.6 Explant morphology and percentage of explants with *de novo* root formation in different auxin and GA treatments. The photograph and percentage was assessed after 21 days incubation.

3.3.3.2 Auxin, GA, and primordia development

Auxin and GA might have different roles after the root primordium has been induced. Different concentration of auxin and GA described in 3.2.3 (ii) were investigated in these experiments. Leaf explants that had been treated with 10 µM NAA for 28 days were used. Tissue regions selected had callused but root primordia had developed only

with 0~2 visible roots. Twenty-one days after an auxin induction treatment, auxin alone treatments did not produce roots, but association with low concentrations of GA significantly helped (Table 3.7). The best ratio of NAA and GA_3 for root formation was 10 µM GA_3, 10 µM NAA plus 0.1µM GA_3, and 1 µM NAA plus 0.1µM GA_3. GA but not auxin can enhanced the primordia development, but the responses of GA were enhanced by auxin.

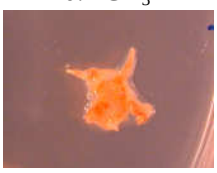
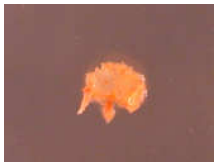
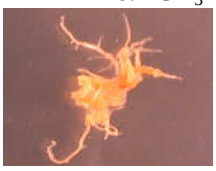
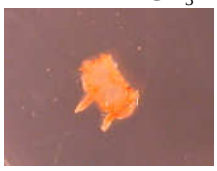
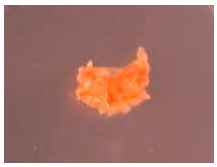
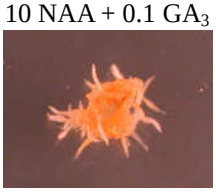
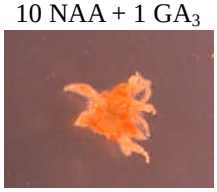
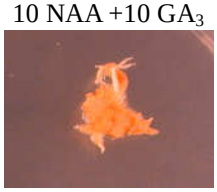
NAA / GA_3 (µM)	0	0.1	1	10 (GA_3)
0	No hormone  0/5 = 0%	0.1 GA_3  0/5 = 0%	1 GA_3  2/4 = 50%	10 GA_3  5/5 = 100%
1	1 NAA  1/4 = 25%	1 NAA + 0.1 GA_3  3/5 = 60%	1 NAA + 1 GA_3  0/5 = 0%	1 NAA + 10 GA_3  0/5 = 0%
10 (NAA)	10 NAA  0/4 = 0%	10 NAA + 0.1 GA_3  4/5 = 80%	10 NAA + 1 GA_3  1/5 = 20%	10 NAA +10 GA_3  2/5 = 40%

Table 3.7 Explant morphology and percentage of explants with *de novo* root formation in different auxin and GA treatments after root primordia formed. The photographs and the percentage of explants forming roots were assessed after 21 days.

3.3.3.3 The timing requirement for root stem cell activation in Jemalong and 2HA studied using auxin and GA

The responses to GA were different before and after root primordium formation. Suitable treatments of auxin and GA can help to determine the timing of root primordium formation. The procedure for investigation of the time requirement for root primordia induction in auxin culture was described in 3.2.3 (iii). After a 3 day treatment in 10 µM NAA Jemalong had visible roots and after a 7 day treatment both Jemalong and 2HA had visible roots, after transfer to 10 µM GA_3 (Table 3.8). Auxin was only

required for a short period of time to induce root primordia. In a similar experiment, but with leaf explants transferred to 10 µM NAA plus 0.1 µM GA_3 after auxin treatment (Table 3.9) similar results were obtained, but there was a greater density of roots per explant.

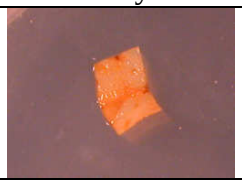
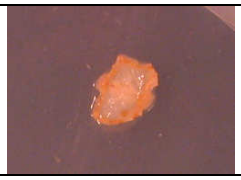
10 µM GA_3 after auxin treatment	Days treatment with 10 µM NAA		
	0 days	3 days	7 days
Jemalong			
	0/3 = 0%	3/3 = 100%	3/3 = 100%
2HA			
	0/3 = 0%	0/3 = 0%	3/3 = 100%

Table 3.8 Induction time required for auxin root induction in Jemalong and 2HA. The 1st medium is 10 µM NAA and the 2nd medium is 10 µM GA_3. Root results are shown with callus morphology and percentage of explants with root formation after 21 days culture.

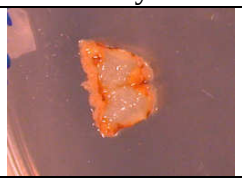
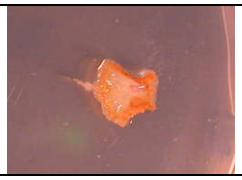
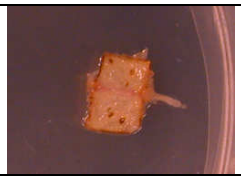
10 µM NAA + 0.1 µM GA_3 after auxin treatment	Days treatment with 10 µM NAA		
	0 days	3 days	7 days
Jemalong			
	1/3 = 33.33%	3/3 = 100%	3/3 = 100%
2HA			
	1/3 = 33.33%	1/3 = 33.33%	3/3 = 100%

Table 3.9 Induction time required for auxin root induction in Jemalong and 2HA. The 1st medium is 10 µM NAA and the 2nd medium is 10 µM NAA plus 0.1 µM GA_3. Root results are shown with callus morphology and percentage of explants with root formation after 21 days culture.

Data obtained in the three different types of experiments (sections 3.3.3.1, 3.3.3.2, 3.3.3.3) were consistent with auxin inducing primordia and GA being inhibitory at this stage, but then GA assisting in primordia development particularly if some auxin is present.

3.4 DISCUSSION

Somatic embryo induction

Examination of the experiments labelled as Hormones TR and Auxin TR, confirms what is well known for this system that auxin and cytokinin are essential for SE induction in 2HA. However there is a requirement for auxin and cytokinin for just 2-3 weeks (a "pulse"). The longer treatment with auxin can help to enhance the callus formation, but does not enhance the somatic embryo induction or formation. On the other hand, the longer cytokinin treatment enhances both the somatic embryo induction and callus formation. These data are consistent with a large body of work (section 3.3.1) showing that the exogenous auxin is only required for the induction stage of SE and not for embryo development. However, for *M. truncatula*, cytokinin is also necessary, but again is only required for an inductive period. It should also be noted that cytokinin suppresses root formation. These data also indicate that the first 2-3 weeks of culture is the key stage for callus, stem cell and SE induction. The expression of the important genes related to the SE induction procedure must occur in this period (see Chapter 5 Results).

ABA from the beginning of culture can enhance the somatic embryo induction rate. Though ABA induces more somatic embryos, less callus tissue is formed. These observations are of interest as the standard lab procedure was based on ABA addition at 3 weeks to enhance embryo quality even though embryo numbers were reduced (Nolan and Rose, 1998). ABA addition from the beginning assists the histology as embryo development can be followed more readily in tissue that does not callus so much. As can be seen in Fig. 3.6 at least some embryos develop near the edge of the explant from dedifferentiated mesophyll cells. This system is now being used for more extensive histology studies.

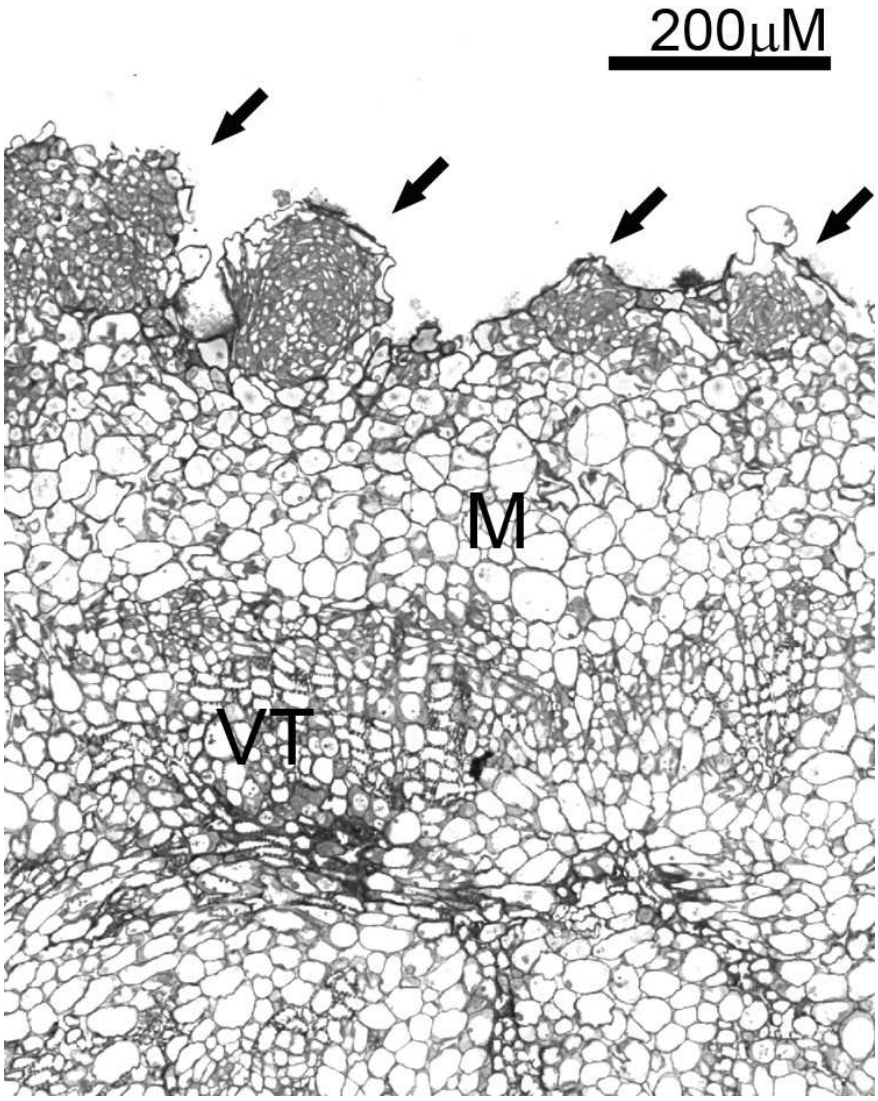


Figure 3.6 Section showing somatic embryos developing in leaf explants. M, mesophyll cells; VT, vascular tissue. Arrows indicate the somatic embryos at the edge of the explants. Explants grown in P4 (10:4:1) medium from the beginning of culture.

2HA can form SEs from leaf explants, but Jemalong cannot respond to cytokinin in this way. Auxin and cytokinin can produce callus in both 2HA and Jemalong, with callus production being slightly enhanced in Jemalong.

GA did not suppress SE as hypothesised and essentially appeared to have no effect. This work needs further experimentation, particularly in relation to the timing of the application. We note that in alfalfa GA can stimulate SE (Ruduś et al., 2000). This is discussed more fully in Chapter 7 in relation to the nature of the genotype that enables SE induction.

Root primordia induction

Suitable concentrations of GA and auxin for root primordia induction and *de novo* root formation in 2HA were investigated. It confirms what is known for this system that 10 µM NAA is a suitable concentration for root primordia induction. However once the primordia are produced their development requires hormone changes. This can be in the form of a lowering of the auxin concentration and the addition of 10 µM GA_3, or surprisingly the maintenance of the auxin concentration at 10 µM NAA but plus 0.1 µM GA_3. This indicates two functions of GA, the ability to suppress stem cell induction and primordia formation, but to assist primordia development. This supports the view that GA predominantly acts to stimulate growth rather than induce new organs (Fleet and Sun, 2005).

The data in this chapter also shows that Jemalong and 2HA only require 3 ~ 7 days to form the root primordium which is consistent with the conclusions of Imin et al., 2007. Jemalong appears to respond to auxin faster than 2HA in the root induction process where all explants can produce roots after 3 days auxin treatment.

The *de novo* root formation culture system is a suitable tool for investigating root primordia induction and development, especially for transgenic experiments, where transformation can be carried out without regenerating plants (see Chapter 6).

Meristem production *in vitro*

SE induction involves the formation of two primary meristems and for the root a single primary meristem. In the *M. truncatula* systems investigated it is clear that the induction phase of SE and root primordia occurs early in the culture process and then the hormonal requirements change. In the case of SE the developing embryo probably starts to self regulate its hormone requirements and hormone additions may do more harm than good in producing normal embryos. As there is a primary focus in this thesis on the induction phase the timing of the hormonal treatments is important.

The data obtained in this Chapter suggest that Jemalong may more readily respond to auxin in producing roots. This is consistent with the ability of 2HA to have the greater

capacity to respond to cytokinin in producing SEs.

GA is usually associated with rapid plant growth by cell enlargement (Richards et al., 2001) and there is evidence that it suppresses SE (Section 3.3.2). In the data here GA does suppress root primordia induction but an SE effect was not demonstrated. The SE area requires more work but may be connected to the special genotypes like 2HA that are able to be induced to form SEs. For example, microarray studies show upregulation of GA 2-oxidase in SE induction in 2HA (Mantiri et al., 2008a) so that GA may be inactivated in the SE studies with 2HA.

CHAPTER 3 Investigation of the Timing of the Hormone Requirements for the Induction of Somatic Embryogenesis and Organogenesis in *M. truncatula*

CHAPTER 4

Bioinformatics of the *WUSCHEL*, *WOX5* and *CLAVATA* Family Genes

4.1 INTRODUCTION

Of critical importance in somatic embryo and meristem induction is understanding how totipotent and pluripotent cells are produced. This chapter focuses on genes which may be involved in the induction of somatic embryogenesis. A clue as to the potential mechanism of the formation of totipotent stem cells has come from the studies of Zuo et al., (2002) who overexpressed *WUS* in Arabidopsis which initiated somatic embryo formation. The *AtWUS* gene was identified in 1996 (Laux et al. 1996) and is expressed in the shoot apical meristem. It enables the stem cells of the nearby central zone to remain in an undifferentiated state and is required for the maintenance of the stem cell cluster as a source of pluripotent cells for shoot development (Laux et al., 1996; Mayer et al., 1998).

In the Arabidopsis shoot apical meristem *AtWUS* expression is regulated through *CLAVATA* (*CLV*) family genes. There are three *CLAVATA* genes: *AtCLAVATA1* (*AtCLV1*), *AtCLAVATA2* (*AtCLV2*) and *AtCLAVATA3* (*AtCLV3*). AtCLV1 and AtCLV2 are receptors but AtCLV2 lacks an intracellar kinase domain. Expression of both *AtCLV1* and *AtCLV3* can be detected in the shoot apex (Clark et al., 1997; Jeong et al., 1999). AtCLV3 is a signal peptide which is expressed in the stem cells of the shoot and floral apex (Fletcher et al., 1999). All the CLV family genes are involved in the WUSCHEL/CLAVATA feedback loop.

The WUSCHEL/CLAVATA feedback loop is described as a regulation pathway in shoot and floral meristems that maintains the population size of pluripotent stem cells in the meristem (Brand et al., 2000; Fiers et al., 2007) (Fig. 4.1). *AtWUS* expresses in the OC which is several layers below the epidermis (in the L3 layer) and induces the *AtCLV3* gene expression through unknown chain reactions in the central zone of the L1, L2, and L3 layers of the shoot apical meristem (Fig. 4.1). The AtCLV3 signal peptide in the L2 and L3 layer cells binds to AtCLV1 and AtCLV2 and triggers signal transduction pathways to inhibit the *AtWUS* expression of the OC. Therefore the expression level of *AtCLV3* and *AtWUS* regulate the size of OC and stem cell numbers (Schoof et al., 2000).

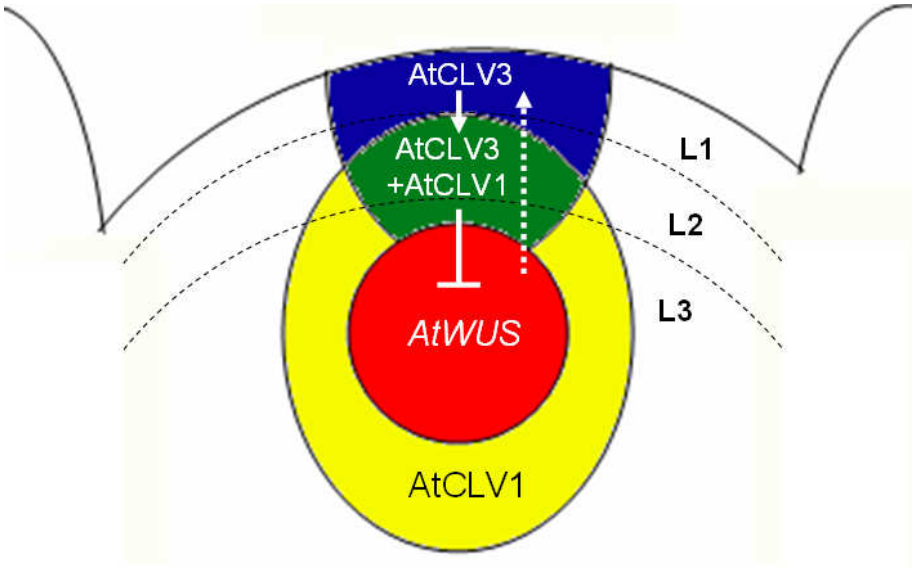


Figure 4.1 Diagram of the WUSCHEL/CLAVATA feedback pathway in the shoot apical meristem of *Arabidopsis*. *AtWUS* gene expression is shown in red and locates in the OC. *AtCLV1* gene expression is shown in yellow/green and locates inside and around the OC (L2 and L3 layers). *AtCLV3* gene expression is shown in blue/green, and locates in the L1, L2, and L3 layers of the central zone. *AtWUS* induces *AtCLV3* expression in the central zone, and the AtCLV3 peptide signal binds with the AtCLV1 protein to inhibit *AtWUS* gene expression and limit the OC size. The AtCLV2 protein is also involved in the pathway, but the protein is widely expressed and does not show in the diagram. Diagram modified from Fiers et al., 2007.

In addition, data from Mordhorst et al., (1998) has shown that CLAVATA mutants enhance somatic embryo formation which may also be caused by *AtWUS* expression increasing through the feedback loop. This emphasises the importance of investigating WUS and CLV genes in relation to somatic embryogenesis in *M. truncatula*.

It is possible that in the root there is a similar stem cell regulatory system. *WUS* and *WOX5* are members of the WOX family of genes first studied by Haecker et al., (2004) in *Arabidopsis*. Some of the genes expressed in different stages of embryo development indicate the importance of WOX genes for embryogenesis. *AtWOX5* expression was importantly detected in the quiescent centre (QC) of the embryo root pole (Haecker et al., 2004) and in the QC of primary or lateral roots (Blilou et al., 2005). It also has a similar function to *AtWUS* in stem cell maintenance (Sarkar et al., 2007) suggesting that *WOX5* may also be important in totipotent or pluripotent stem cell formation. It is therefore also valuable to investigate this gene in root stem cell formation.

In order to follow the expression of these genes in *M. truncatula*, the first step is to identify the orthologs. This was carried out by amino sequence alignments and dendrogram analysis, to select the putative *M. truncatula* candidates from the NCBI database.

WUS and *WOX5* are members of the WOX family of genes studied by Haecker et al., (2004) in *Arabidopsis*. The WOX family of genes share 62% to 87% amino acid identity in the WUS homeodomain and some of them are expressed in the embryo but at different stages of development. It is necessary to include all the *Arabidopsis WOX* genes in amino acid alignments to help identify the orthologs of *WUS* and *WOX5* in *M. truncatula*.

The CLAVATA family genes CLV1, CLV2 and CLV3 have not been identified in *M. truncatula*, but it seems likely that the CLV system might be more complex than in *Arabidopsis*. Several CLV1-like genes in legumes, including *GmCLV1A* and *GmNARK* (*GLYCINE MAX NODULE AUTOREGULATION RECEPTOR KINASE*) in soybean (Yamamoto et al., 2000) and *SUNN* in *M. truncatula* (Penmetsa et al., 2003), have similar amino acid sequence to *Arabidopsis* CLV1 and can regulate nodulation in roots, but do not influence shoot apex structure in mutants (Searle et al., 2003; Penmetsa et al., 2003).

Several CLV1-like genes *AtBAM1* (*BARELY ANY MERISTEM 1*), *AtBAM2* and *AtBAM3*, which have high similarity in protein structure to AtCLV1 but with opposite functions to AtCLV1 in shoot apical meristems, were investigated by DeYoung et al., in 2006.

The expression pattern of a gene is commonly regulated by the promoter region. Many reports indicate that using different lengths and regions of the promoter will change the expression pattern of GUS (Bäurle and Laux, 2005) or GFP (Takada and Jürgens, 2007) and raises the question of how long the promoter length should be to obtain the correct expression pattern. This information is important when gene expression patterns are investigated by GUS or GFP promoter fusions. Promoter region investigations are presented in the second part of the chapter using elements associated with promoter regulation and published functional analysis of the *AtWUS* promoter (Bäurle and Laux, 2005).

4.2 MATERIALS AND METHODS

4.2.1 Sequence Analysis and Construction of Dendrograms

The identification of the WUS and WOX5 transcription factor gene sequences in *M. truncatula* was obtained by comparison with known gene sequences from other plant species using the NCBI and *M. truncatula* databases. Searches were carried out using different BLAST search methods in the NCBI database:

Nucleotide blast: Searching a **nucleotide** database using a **nucleotide** query
Protein blast: Searching a **protein** database using a **protein** query
BLASTx: Searching a **protein** database using a **translated nucleotide** query
tBLASTn: Searching a **translated nucleotide** database using a **protein** query
tBLASTx: Searching a **translated nucleotide** database using a **translated nucleotide** query
Align (Bl2seq): Alignments of two sequences

"Genescan" was used to predict mRNA and protein sequences from the genomic sequence. Multiple alignment analyses were performed with ClustalW using the Clustal 2.0.8 software in Clustal default colours. The dendrogram was drawn using TreeView (Win32) 1.6.0 software (Page, 1996). All these programs are also available through the Australian National Genomic Information Service (ANGIS, www.angis.org.au). Standard parameters were used.

The dendrogram analysis of the *WUS* gene was based on *Arabidopsis* and similar putative orthologs in some other species such as *Antirrhinum majus* (Kieffer et al., 2006), *Petunia* (Stuurman et al., 2002) and tomato (Pracros et al., 2006). *WOX5* has also been investigated in *Arabidopsis* (Haecker et al., 2004), rice (Kamiya et al., 2003) and also in *M. truncatula* (Imin et al., 2007). Arabidopsis WOX family genes were also involved in the analysis.

CLV1 and CLV2 have partially similar protein structures in *Arabidopsis*, but CLV3 is a peptide which is quit different to CLV1 and CLV2 proteins. Therefore the CLV3 was investigated separately.

4.2.2 Promoter Region Analysis

Bäurle and Laux (2005) investigated the *AtWUS* promoter region about 4 kb upstream by partial deletion and five regions with regulatory sequences have been identified. Each of them has different effects on the WUS expression in the OC, floral meristem or ovule. These data produced a clue to the meaning of these elements. However, the sequence structures of promoter regions are not as uniform as the coding regions between orthologous genes from different species.

Many special short sequences (or elements) in promoter regions which influence gene expression were found by the techniques of protein binding or by promoter partial deletion. These elements may be required for protein or signal binding regulated by other factors such as hormones, stress or environmental factors. These elements can be investigated by using the PLACE database (Plant Cis-acting Regulatory DNA elements) http://www.dna.affrc.go.jp/PLACE/ and obtaining the elements present in the promoter sequence. An objective of this chapter was to utilize the elements of the *AtWUS* promoter selected by PLACE and the regions described in Bäurle and Laux (2005) in a comparative analysis with the putative *M. truncatula* promoter. The *AtWUS* promoter region 4037 bp upstream of the ATG site was selected by PLACE. Only the elements located inside the five significant regulatory regions of the *AtWUS* promoter described by Bäurle and Laux, (2005) were selected.

(i) Region -2240 to -3434: Ovule Expression and Floral Expression (OE/FE) (Grey)

The region from -2240 to -3434 is related to expression in the ovules and floral meristem. Eleven unique elements belong to this region painted in grey, and two partial-unique elements belong to this region painted in white (Table 4.1).

Display colour	Related region(s)	Element code	Element full name
Grey (Unique)	OE/FE	ABREAT	ABREATCONSENSUS
		ABREOS	ABREOSRAB21
		ACGTAB	ACGTABREMOTIFA2OSEM
		ACGTTB	ACGTTBOX
		BOXIIP	BOXIIPCCHS
		CGCGBO	CGCGBOXAT
		E2FCON	E2FCONSENSUS
		GARE2O	GARE2OSREP1
		MARARS	MARARS
		PALBOX	PALBOXAPC
		SV40CO	SV40COREENHAN
White (Partial-unique)	OE/FE and MQE	ABRELA	ABRELATERD1
		ABRERA	ABRERATCAL

Table 4.1 List of the code and full name of elements in the OE/FE region.

(ii) Region -1753 to -2240: General Quantitative Element required for enhanced expression levels (GQE) (Yellow)

The region from -1753 to -2240 is related to elements required for enhanced general expression levels. Four unique elements belong to this region painted in yellow and six partial-unique elements located in GQE and MQE regions painted in green (Table 4.2).

Display colour	Related region(s)	Element code	Element full name
Yellow (Unique)	GQE	MYBPLA	MYBPLANT
		REBETA	REBETALGLHCB21
		SORLRE	SORLREP3AT
		XYLAT	XYLAT
Green (Partial-unique)	GQE and MQE	GAREAT	GAREAT
		MYB1LE	MYB1LEPR
		MYBGAH	MYBGAHV
		RBCSCO	RBCSCONSENSUS
		RHERPA	RHERPATEXPA7
		SREATM	SREATMSD

Table 4.2 List of the code and full name of elements in the GQE region.

(iii) Region -1486 to -1753: Meristem Quantitative Element required for enhanced expression levels (MQE) (Blue)

The region from -1486 to -1753 is related to the quantitative elements required for enhanced expression levels in the meristem. Three unique elements belong to this region painted in blue, six partial-unique elements located in GQE and MQE regions painted in green, and two partial-unique elements located in OE/FE and MQE regions painted in white (Table 4.3).

Display colour	Related region(s)	Element code	Element full name
Blue (Unique)	MQE	BP5OSW	BP5OSWX
		PROLAM	PROLAMINBOXOSGLUB1
		T/GBOX	T/GBOXATPIN2
Green (Partial-unique)	GQE and MQE	GAREAT	GAREAT
		MYB1LE	MYB1LEPR
		MYBGAH	MYBGAHV
		RBCSCO	RBCSCONSENSUS
		RHERPA	RHERPATEXPA7
		SREATM	SREATMSD
White (Partial-unique)	OE/FE and MQE	ABRELA	ABRELATERD1
		ABRERA	ABRERATCAL

Table 4.3 List of the code and full name of elements in the MQE region.

(iv) Region -655 to -712: Stem cell Niche Spatial Control (SNSC)(Red)

The region from -655 to -712 is related to the spatial control of expression in the stem cell niche, which is perhaps the most important function of *AtWUS*. The elements that belong to this region are painted in red and listed in Table 4.4.

The elements RE1 and RE2 were described as the region essential for promoter activity in the stem cell niche of the inflorescence meristem located in position -691 and -671. The RE1 is located between ATHB6C and ARFAT, and RE2 is located between ARFAT and AACACO, and do not overlap each other.

There are two elements, SEBFCO and SURECO, also located in this region but with less specific sequences. Therefore these two elements are not involved in the analysis.

Display colour	Related region(s)	Element code	Element full name
Red	SNSC	AACACO	AACACOREOSGLUB1
		ARFAT	ARFAT
		ATHB6C	ATHB6COREAT

Table 4.4 List of the code and full name of elements in the SNSC region.

(v) Region -595 to -624: Stem cell Niche Quantitative element required for Enhanced expression levels (SNQE)(Pink)

No elements were selected in this region.

More information on these elements can be obtained from the PLACE web site and the

list in Appendix 1.

To investigate the promoter regulation region in the *M. truncatula* genes, these twenty-nine were used as markers to locate similar regions to those of the *AtWUS* promoter. The elements are drawn as markers on a physical map of the gene and nearby elements belonging to the same region were grouped in a rectangle to indicate one of the five regions identified by Bäurle and Laux (2005). The details of the analysis procedure are described in Appendix 2. Fifteen genes were investigated, using similar strategies to those for the WUS comparison, with a 4 or 5 kb promoter sequence upstream prior to the ATG site (not including ATG) being used for each gene. A list of the genes analysed is given in Table 4.5.

Gene name	Accession no	Promoter length	Gene name	Accession no	Promoter length
AtWUS	AC006201	4037 bp	*AtBAM1*	AB010075	4037 bp
MtWUS	CT009654	5000 bp	*MtRLK1*	AC141862	4041 bp
AtWOX5	AC073395	4011 bp	*MtRLK2*	CR955004	4040 bp
MtWOX5	CU326389	5074 bp	*AtCLV2*	AC004512	4004 bp
AtWOX4	AC083835	4019 bp	*MtCLV2-1*	AC124218	4027 bp
MtWOX4	AC148486	5000 bp	*AtCLV3*	AC006233	4036 bp
AtCLV1	AC007396	4020 bp	*MtCLV3*	AC151522	4078 bp
SUNN	AY769943	4000 bp			

Table 4.5 Genes used for promoter region analysis. At, *Arabidopsis thaliana*. SUNN obtained from *Medicago truncatula* (Mt).

4.3 RESULTS

4.3.1 WOX Family Homeodomain Alignment and Dendrogram

Based on the methods described by Haecker et al. (2004), the alignment of the WOX homeodomain of WOX family genes of some species including *Arabidopsis* and the *M. truncatula* candidates are shown in Fig. 4.2 and Table 4.6. A dendrogram from the alignments are shown in Fig. 4.3.

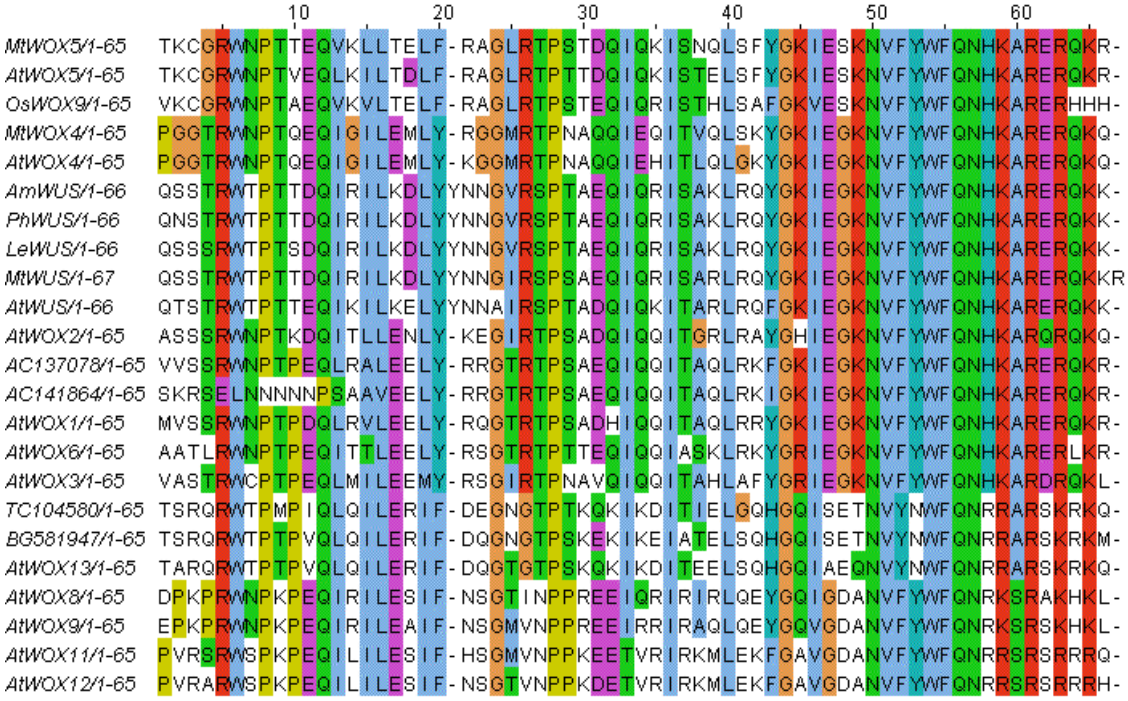


Figure 4.2 Alignments of the WOX homeodomain protein sequences. 23 peptide sequences included in the analysis of the genes listed in Table 4.6.

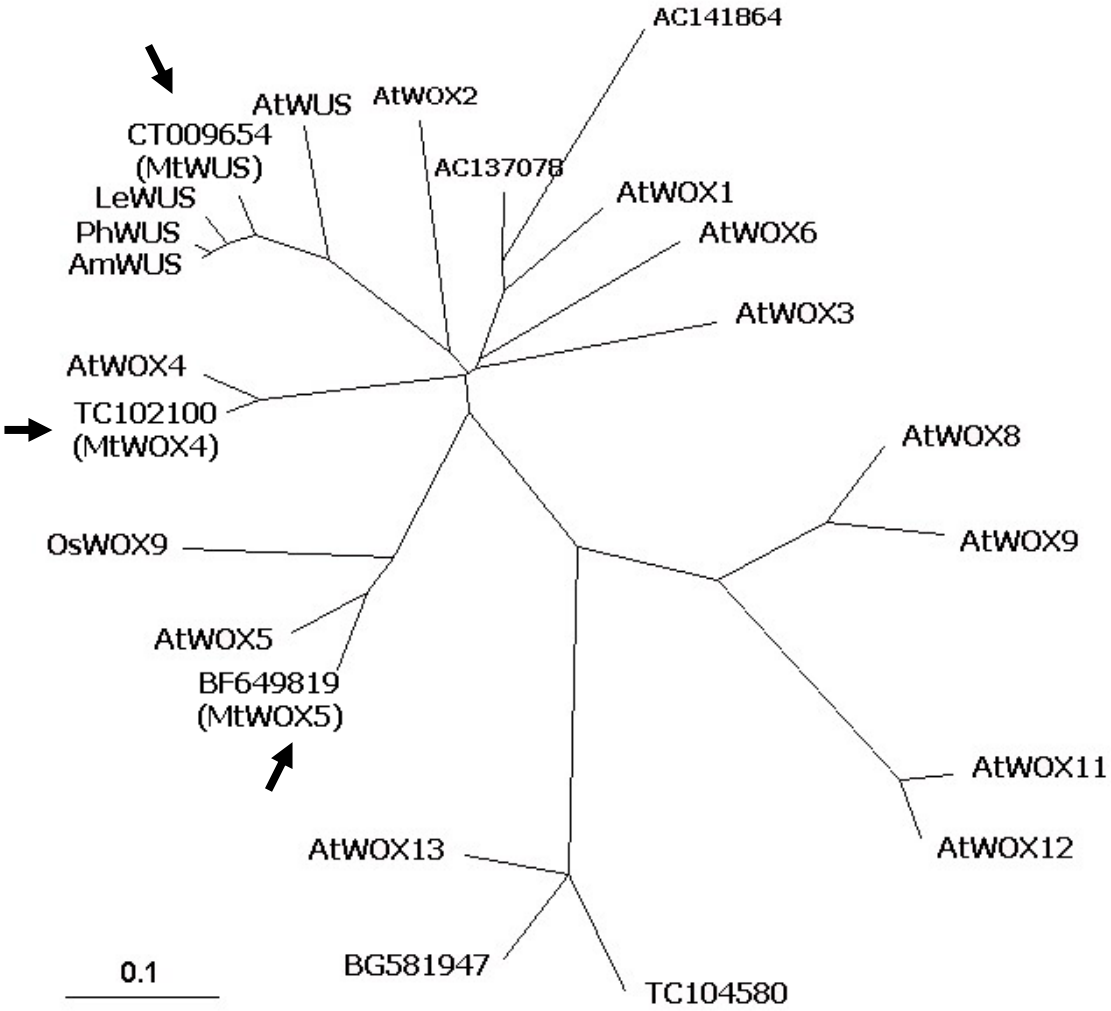


Figure 4.3 Dendrogram of WOX genes based on the sequence of the homeodomains. Black arrows indicate the location of the MtWUS, MtWOX4 and MtWOX5.

Gene name	Accession no.	Similarity			Reference	Amino acids
		MtWUS	MtWOX5	MtWOX4		
MtWUS	CT009654	-	27(61)	25(63)	This study	306(67)
MtWOX5	BF649819	27(61)	-	29(64)	Imin et al., 2007	184(65)
MtWOX4	TC102100	25(63)	29(64)	-	Imin et al., 2007	236(65)
AtWUS	At2g17950	23(84)	29(63)	25(64)	Mayer et al., 1998	292(66)
AmWUS	AY162209	47(95)	28(60)	23(63)	Kieffer et al., 2006	281(66)
LeWUS	LES538329	47(92)	25(58)	23(61)	Pracros et al., 2006	272(66)
PhWUS	AF481951	41(93)	26(60)	23(63)	Stuurman et al., 2002	307(66)
OsWOX9	Q8W0F1	27(56)	42(80)	19(56)	Kamiya et al., 2003	200(65)
AtWOX1	AY251394	24(69)	30(67)	34(69)	Haecker et al., 2004	349(65)
AtWOX2	AY251392	23(70)	28(61)	25(67)	Haecker et al., 2004	260(65)
AtWOX3	AY251397	21(64)	30(60)	23(69)	Matsumoto and Okada 2001	244(65)
AtWOX4	AY251396	22(63)	27(61)	55(93)	Haecker et al., 2004	251(65)
AtWOX5	AY251398	30(61)	51(89)	28(64)	Haecker et al., 2004	182(65)
AtWOX6	AY251399	23(61)	30(64)	25(67)	Haecker et al., 2004	271(65)
AtWOX8	AY251400	13(49)	16(43)	12(44)	Haecker et al., 2004	325(65)
AtWOX9	AY251401	14(46)	17(41)	22(46)	Haecker et al., 2004	378(65)
AtWOX11	AY251402	14(36)	15(33)	16(43)	Haecker et al., 2004	268(65)
AtWOX12	AY251403	15(36)	14(35)	11(40)	Haecker et al., 2004	268(65)
AtWOX13	AY251404	10(40)	17(40)	14(44)	Haecker et al., 2004	268(65)
-	TC104580	19(38)	24(35)	23(41)	This study	131(65)
-	BG581947	12(43)	17(40)	11(40)	This study	274(65)
-	AC137078	21(69)	30(67)	34(72)	This study	345(65)
-	AC141864	29(55)	26(58)	34(61)	This study	152(65)

Table 4.6 Accession information for the WOX family genes and the homeodomain. The information includes accession numbers for Genbank in NCBI for protein sequences or nucleotide sequences where there is no protein sequence available. The similarity (percent protein identity) for whole protein (or homeodomain only) of each gene with MtWUS or MtWOX5 or MtWOX4 are listed. The reference of the gene and length of whole protein (or homeodomain only) are also given. Am, *Antirrhinum majus*; At *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Os, *Oryza sativa*; Ph, *Petunia x hybrida*. TC104580, BG581947, AC137078 and AC141864 sequences obtained from *Medicago truncatula* (Mt).

Based on the alignment and dendrogram analysis with WOX genes of *Arabidopsis* and some different species, three genes from *M. truncatula* with the highest similarity to the target genes were chosen for investigation. *MtWUS* is predicted to be the genomic sequence CT009654, in which the WUS-homeodomin showed highest similarity of 84% amino acid identity with AtWUS (Table 4.6). *MtWOX5* is predicted to be the EST ID BF649819 and the Genomic ID CU326389, in which the homeodomain showed highest

similarity of 89% identity with AtWOX5, and *MtWOX4* is predicted to be the EST ID TC102100 and the Genomic ID AC148486, in which the homeodomain showed highest similarity of 93% identify with AtWOX4.

The full length proteins were also investigated in alignments for WUS, WOX5, and WOX4 genes in several species (Figs. 4.4, 4.5, and 4.6). The similarity scores are described in Table 4.6. Although MtWUS has around 80% to 90% similarity to the homeodomains of AtWUS and other species, the similarity of the full length protein is only 23% to AtWUS, but up to 47% for other species. MtWOX5 has high similarity with AtWOX5 in both the homeodomain (89%) and full length alignments (51%). MtWOX4 also has high similarity with AtWOX4 in both the homeodomain (93%) and full length alignments (55%).

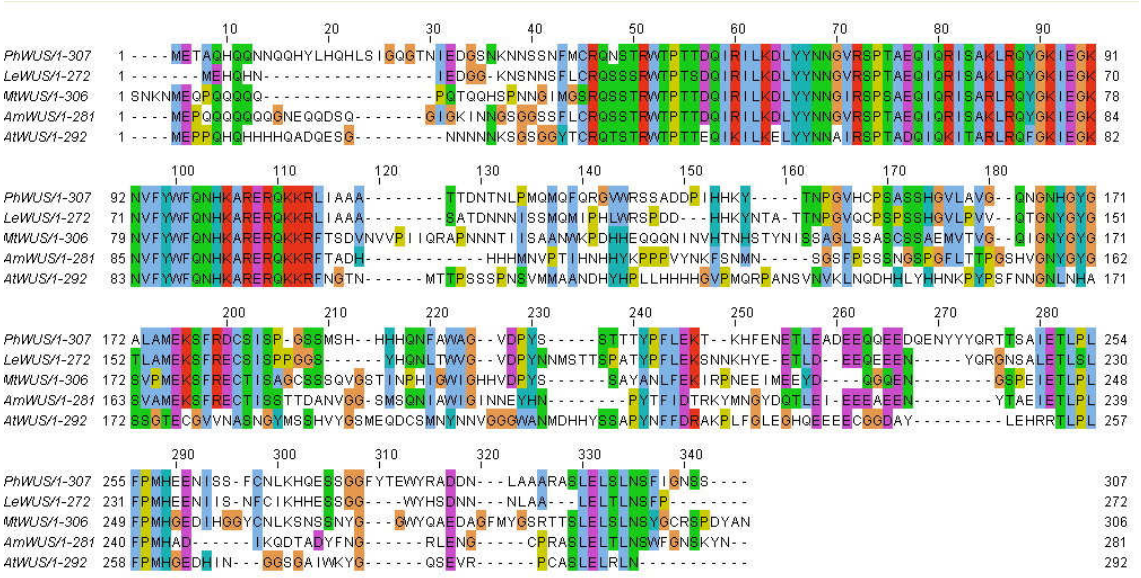


Figure 4.4 Alignments of full length protein sequences of AtWUS with its putative orthologs PhWUS, LeWUS, AmWUS, and MtWUS. MtWUS has 23% similarity with AtWUS, 41% with PhWUS, 47% with LeWUS, and 47% with AmWUS for the full length protein.

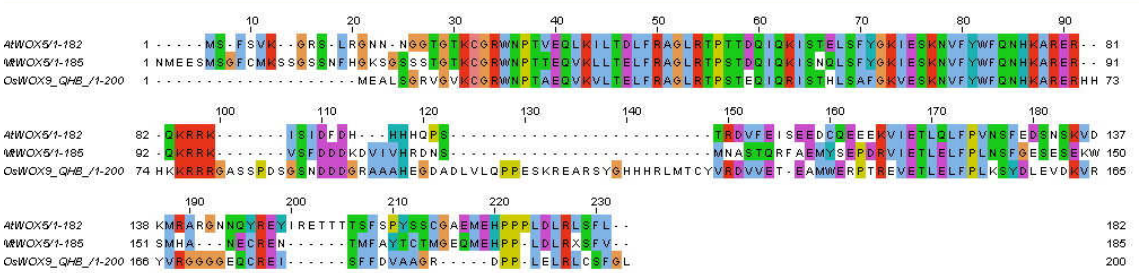


Figure 4.5 Alignments of full length protein sequences of AtWOX5 with its putative orthologs OsWOX9 and MtWOX5. MtWOX5 has 51% similarity with AtWOX5 and 42% similarity with OsWOX9 for the full length protein.

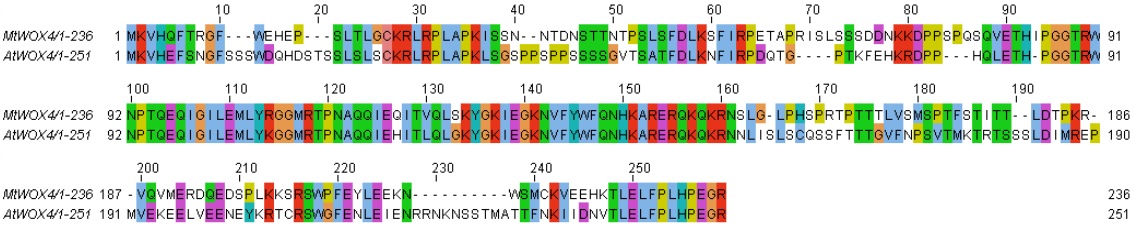
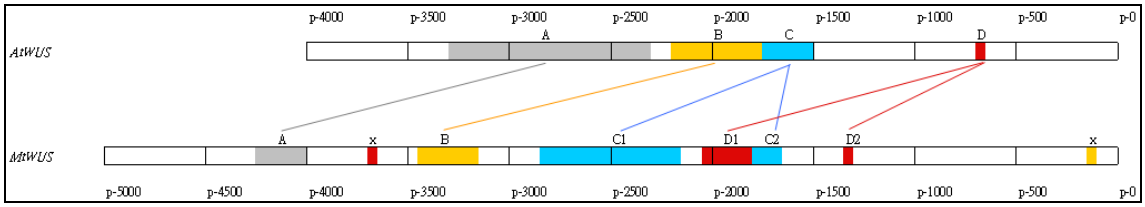


Figure 4.6 Alignments of full length protein sequences of AtWOX4 with its putative orthologs MtWOX4. MtWOX4 has 55% similarity with AtWOX4 for the full length protein.

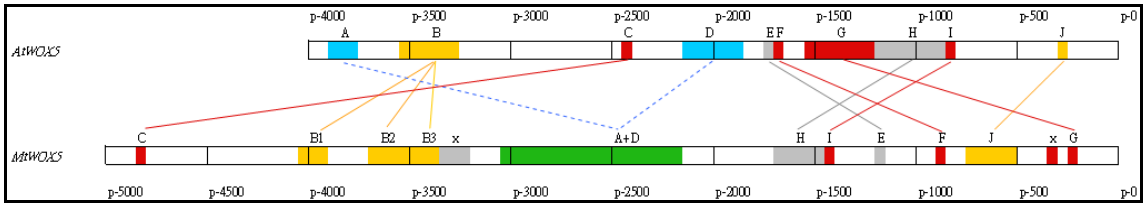
4.3.2 WOX Genes Promoter Region Analysis

The promoter region investigations were carried out for *WUS*, *WOX5* and *WOX4* in *A. thaliana* and *M. truncatula* to help gene identification (Fig. 4.7). *AtWUS* and *MtWUS* have four matched regions and the orders of these regions for the two species are quite similar. *AtWOX5* and *MtWOX5* have seven matched regions but the order in the two species has some differences. Eight matched regions were found between *AtWOX4* and *MtWOX4*, but the order of them in the two species are quite different. The patterns for the same gene have better correspondence between species than for different but related genes.

WUS



WOX5



WOX4

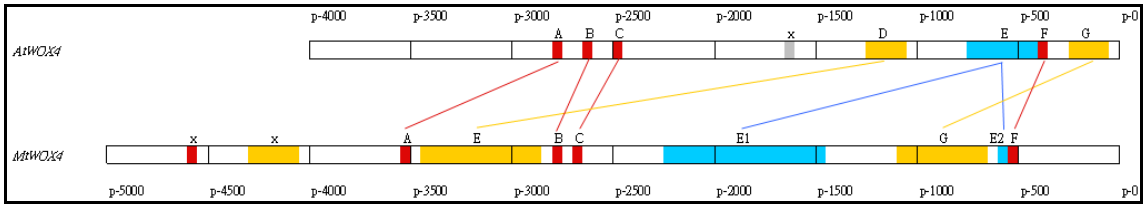


Figure 4.7 Promoter regulation region patterns for *WUS*, *WOX5*, and *WOX4*. The Grey colour indicates the OE/FE region; the yellow colour indicates the GQE region; the blue colour indicates the MQE region; the red colour indicates the SNSC regions; and the green colour indicates the partial similarity to GQE and MQE. The "p-number" indicates the distance upstream of the coding site. Matched regions were marked (from A to J), and the marker "x" indicates the region did not find the matched region in other species. The matched regions are linked by coloured lines, and dashed line in region A and D for the *WOX5* patterns indicates these regions have high similarity but less than the other matches.

4.3.3 CLV1 and CLV2 Alignments and Dendrogram

Based on the methods described by Searle et al. (2003), the dendrogram of the whole protein sequence of CLV1-like or CLV2-like genes of some species and the candidates for *M. truncatula* are shown in Fig. 4.8. The information for genes involved in the dendrogram analysis for CLV1 and CLV2 are listed in Table 4.7, Table 4.8 and Table 4.9.

There are several predicted CLV1-like genes in *M. truncatula*. SUNN obtained from *M. truncatula* has the highest similarity to AtCLV1 (61%). MtRLK1 and MtRLK2 have the second highest similarity score with AtCLV1 (53% for both, Table 4.7), but have a higher similarity to AtBAM1 (78% for both) and GmRLK1 (83% for MtRLK1 and 82%

for MtRLK2, Table 4.8). MtRLK2 has 81% similarity to MtRLK1 and are located on different chromosomes. SUNN and MtRLK1 were chosen for further investigation of gene expression. MtCLV2-1 is similar to AtCLV2 (Table 4.9) and has also been investigated.

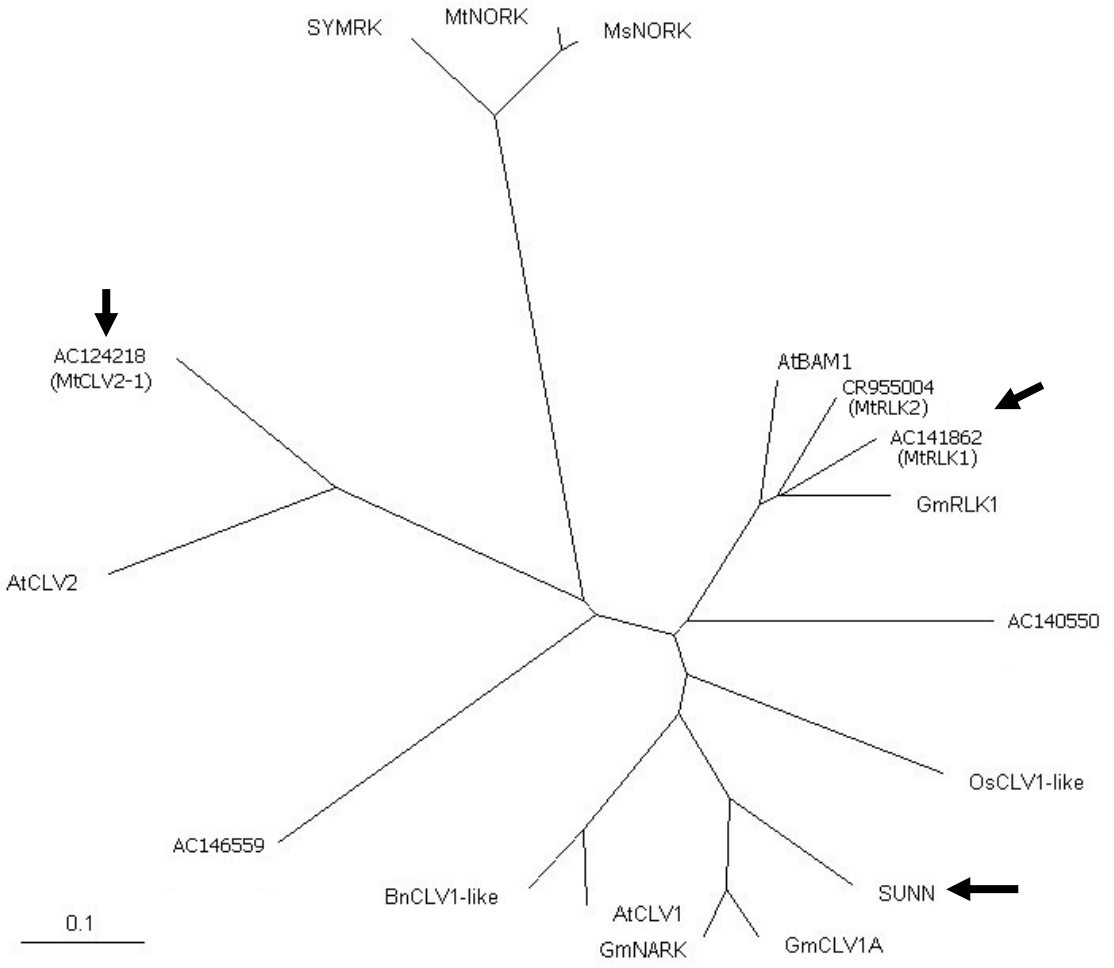


Figure 4.8 Dendrogram for CLV1 andCLV2 based on the full sequence. 17 genes were analysed and are detailed in Table 4.7, Table 4.8 and Table 4.9. Black arrows indicate the location of the SUNN, MtRLK1, MtRLK2 and MtCLV2-1 genes.

Gene name	Accession no.	Similarity AtCLV1	Reference	Amino acids
AtCLV1	Q9SYQ8	-	Clark et al., 1997	980
AtBAM1	AAP68249	54	DeYoung et al., 2006	1003
BnCLV1-like	AAP49010	87	Martynov et al., 2004	978
GmCLV1A	AAF59905	59	Yamamoto et al., 2000	981
GmNARK	AAN74865	61	Searle et al., 2003	987
GmRLK1	AAF91322	53	Yamamoto and Knap, 2001	1008
SUNN	AY769943	61	Penmetsa et al., 2003	974
MtRLK1	AC141862	53	This study	1005
MtRLK2	CR955004	53	This study	1014
-	AC140550	46	This study	985
-	AC146559	35	This study	1038
MsNORK	AJ418368	15	Endre et al., 2002	925
MtNORK	AJ418369	15	Endre et al., 2002	924
OsCLV1-like	BAD82812	56	Suzaki et al., 2004	994
SYMRK	AAM67418	16	Stracke et al., 2002	923

Table 4.7 Alignment information for CLV1-like genes. The information was obtained as in Table 8, and the similarity (percent protein identity) scores are related to AtCLV1. At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Gm, *Glycine max* (soybean); Ms, *Medicago sativa*; Os, *Oryza sativa*. SYMRK obtained from *Lotus japonicus*, and SUNN, AC140550, and AC146559 sequences obtained from *Medicago truncatula* (Mt).

Gene name	Similarity			Location in *M. truncatula* genome	Amino acids
	AtBAM1	GmRLK1	MtRLK1		
AtBAM1	-	77	78	-	1003
GmRLK1	77	-	83	-	1008
MtRLK1	78	83	-	Chromosome 8	1005
MtRLK2	78	82	81	Chromosome 5	1014

Table 4.8 Additional similarity information for RLK-like genes. The information includes the similarity scores (percent protein identity) related to AtBAM1, GmRLK1, and MtRLK1, the location of these genes in the *M. truncatula* genome, and the length of the amino acids.

Gene name	Accession no.	Similarity	Reference	Amino acids
MtCLV2-1	AC124218	-	This study	580
AtCLV2	AAF02654	63	Jeong et al., 1999	720

Table 4.9 Alignment information for genes similar to CLV2.

4.3.4 CLV3 Alignment and Dendrogram

Based on the methods described by Fiers et al. (2005, 2007), the alignment and dendrogram of the whole protein sequence of CLV3, CLEs of *Arabidopsis* and the CLV3 candidates for *M. truncatula* are shown in Fig. 4.9, Fig. 4.10, and Table 4.10. The gene with highest similarity to MtCLV3 is AtCLV3 (28%).

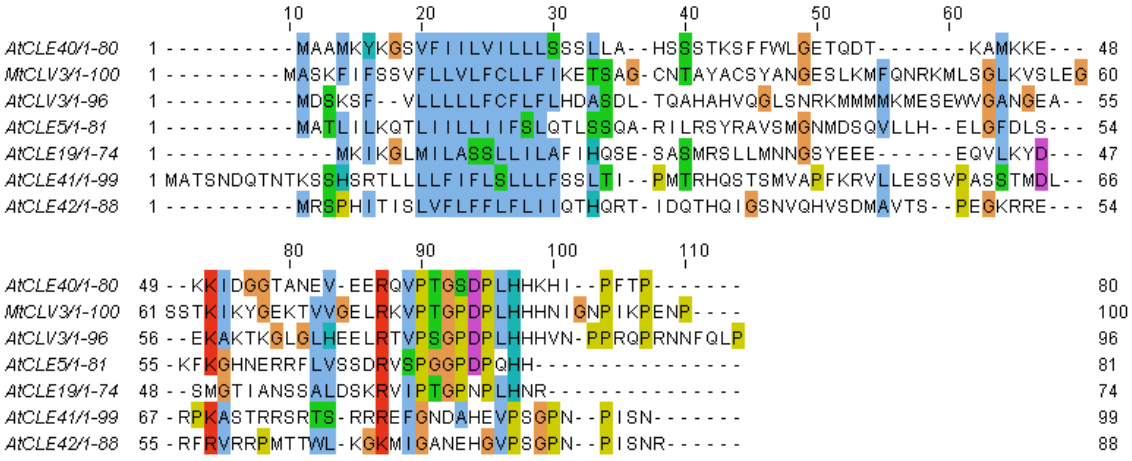


Figure 4.9 Protein sequence alignment of MtCLV3, AtCLV3 and AtCLEs.

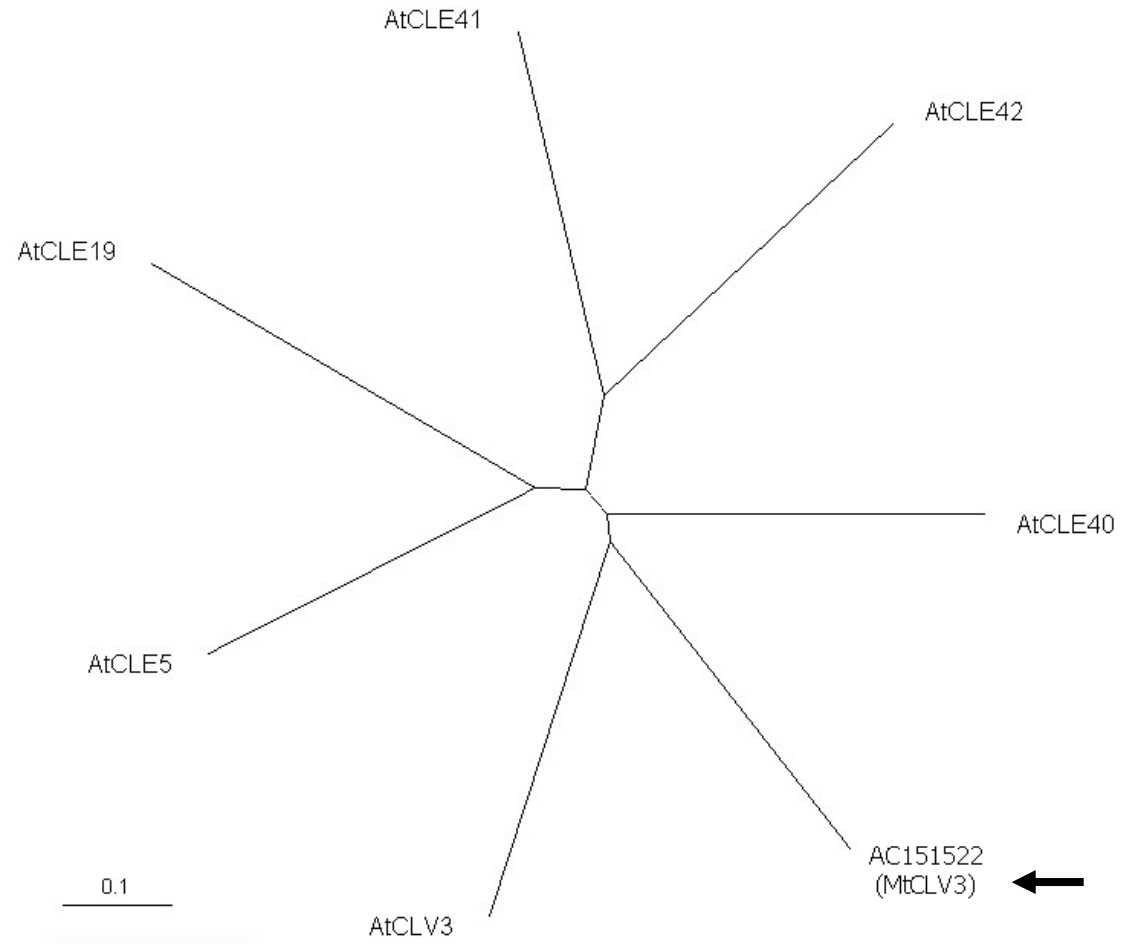


Figure 4.10 Dendrogram for CLV3 and CLEs based on the full peptide sequence. 7 genes were analysed and are detailed in Table 4.10. Black arrow indicates the location of the MtCLV3.

Gene name	Accession no.	Similarity MtCLV3	Reference	Amino acids
MtCLV3	AC151522	-	This study	100
AtCLV3	AA037219	28	Xiao et al., 2002	94
AtCLE5	NP_850159.2	23	Fiers et al., 2005	81
AtCLE19	NP_683589.1	10	Fiers et al., 2005	74
AtCLE40	NP_106803.1	26	Fiers et al., 2005	80
AtCLE41	NP_566754.1	14	Fiers et al., 2007	99
AtCLE42	NP_001078005.1	18	Fiers et al., 2007	88

Table 4.10 Alignment information for CLV3 similar genes.

MtCLV3 also has the CLE family sequence near the 3' end of "ELR*VP*GPDPLHHH". Based on the methods described by Oelkers et al. (2008), the alignment and phylogram of the CLE domain sequence of CLE members of Arabidopsis, CLV3 ortholog genes in

other species, and the CLV3 candidates for *M. truncatula* are shown in Fig. 4.11. The gene with highest similarity score to AtCLV3 is MtCLV3 (72%).

The MtCVL3 sequence information was supplied by Attila Kereszt of the CILR of University of Queensland. The MtCLV3 is predicted from the same genomic sequence (AC151522) as MtCLE68 but from different regions. This *MtCLV3* was also chosen for investigation, as all the bioinformations and expression data indicates it is the most likely ortholog.

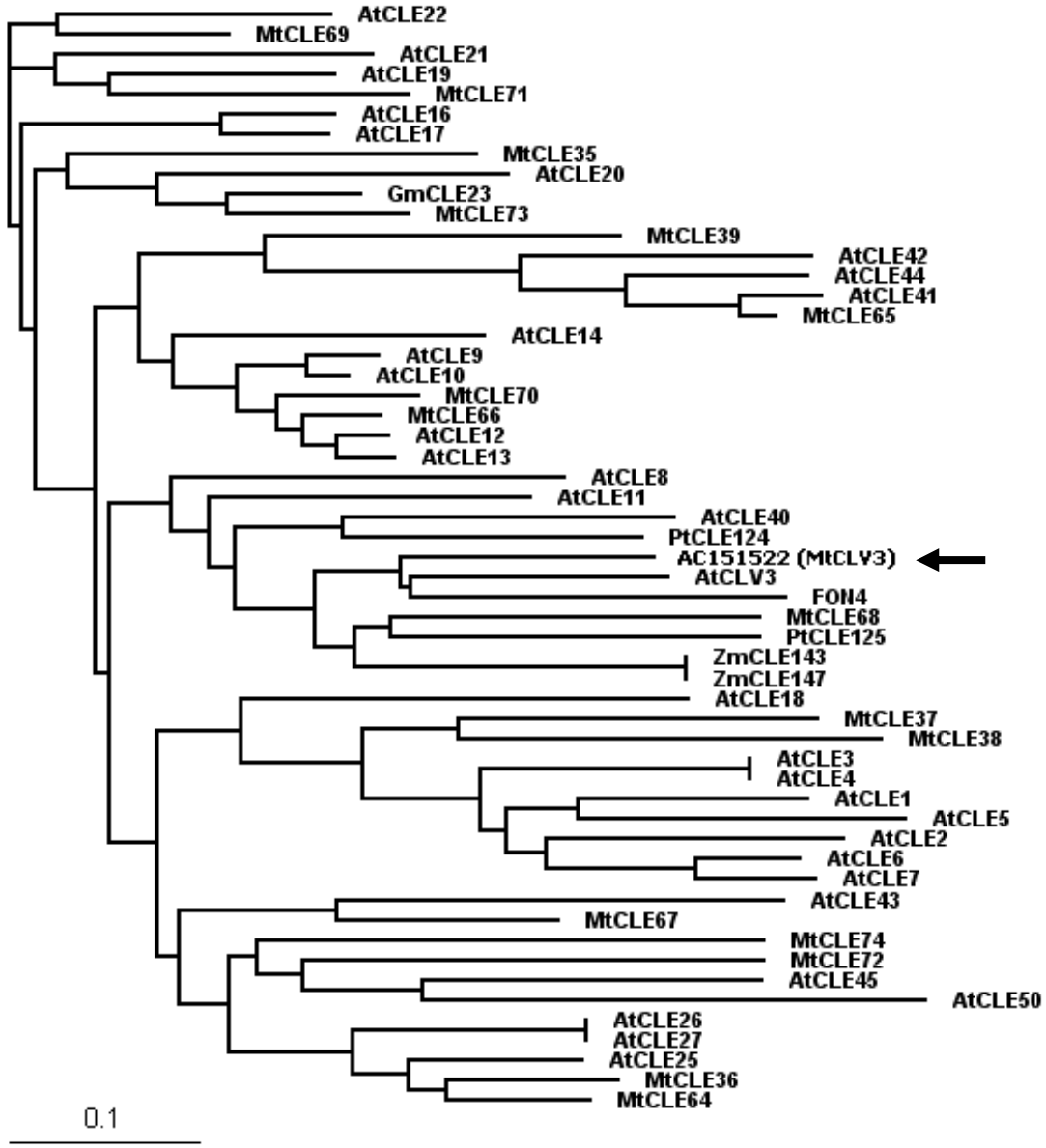
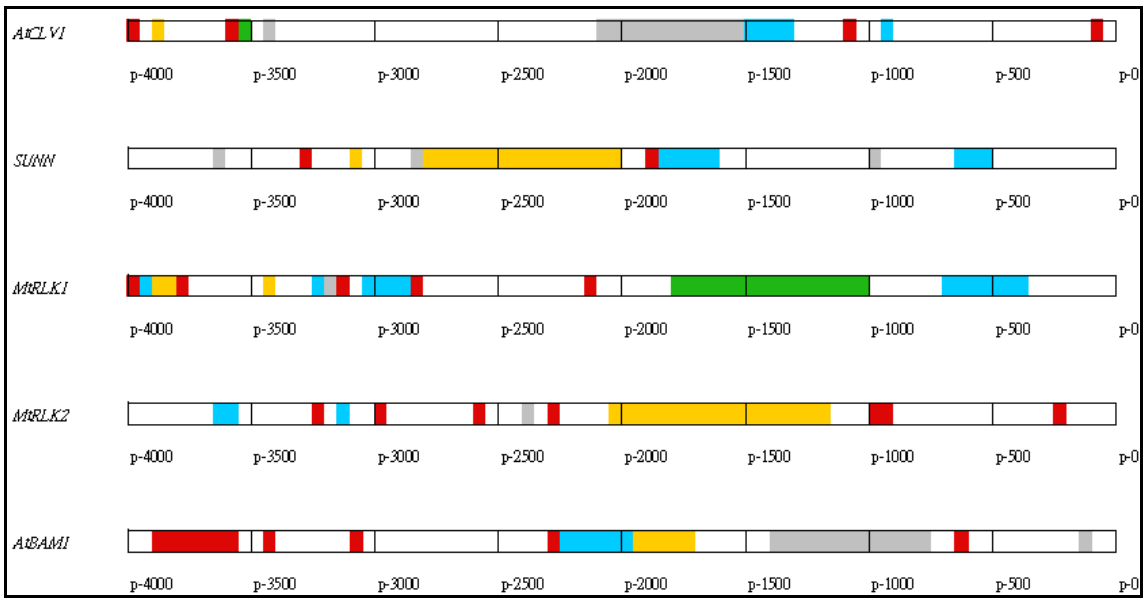


Figure 4.11 Phylogram for CLV3 and CLEs based on the CLE domain sequence. 55 genes were analysed and are detailed in Oelkers et al. (2008) [except the AC151522 (MtCLV3)]. Black arrow indicates the location of the MtCLV3.

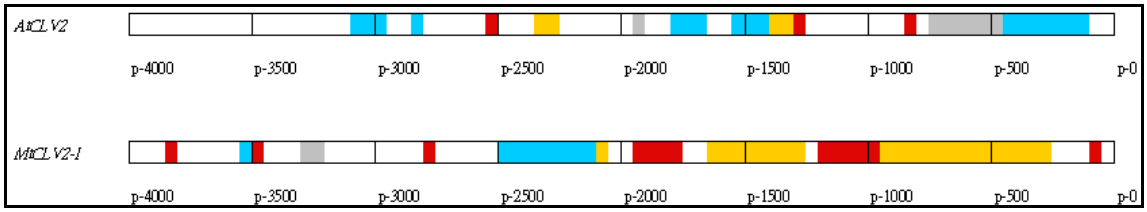
4.3.5 CLAVATA Gene Promoter Region Analysis

The promoter region analyses were also carried out for the CLAVATA genes. Even though these are different types of genes to the WOX family they have important developmental associations, the reason for their investigation here. The patterns for *AtCLV1*, *AtBAM1*, *SUNN*, *MtRLK1* and *MtRLK2* are compared in Fig. 4.12. The element components of CLV1-like genes and the two *CLV2* genes are quite different and therefore no matched regions were found. *AtCLV3* and *MtCLV3* in Fig. 4.12 have seven matched regions. The order of these matched regions in *AtCLV3* and *MtCLV3* are analogous.

CLV1



CLV2



CLV3

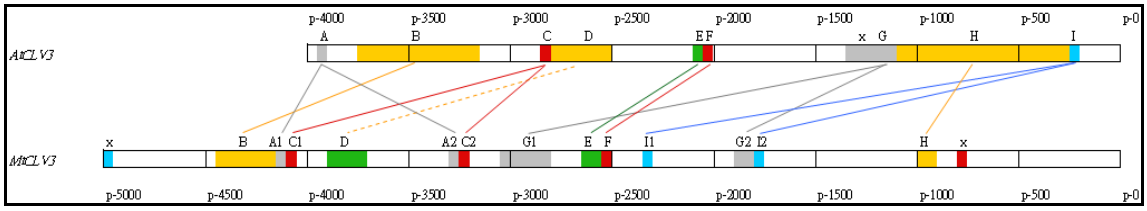


Figure 4.12 Promoter regulation region patterns for *CLV1, CLV2,* and *CVL3* similar genes. The Grey

colour indicates the OE/FE region; the yellow colour indicates the GQE region; the blue colour indicates the MQE region; the red colour indicates the SNSC regions; and the green colour indicates the partial similarity to GQE and MQE. The "p-number" indicates the distance upstream of the coding site. Matched regions were marked (A to I), and the marker "x" indicates the region did not find matched regions in the other species. The matched regions are linked by coloured lines, and dashed line in region D for the *CLV3* patterns indicates these regions have high similarity but less than the other matches. The patterns for *AtCLV1*, *SUNN*, *MtRLK1*, *MtRLK2* and *AtBAM1* are included in the *CLV1* comparison; *AtCLV2* and *MtCLV2-1* are included in the *CLV2* comparison; and *AtCLV3* and *MtCLV3* are included in the *CLV3* comparison.

4.4 DISCUSSION

Based on the amino acid alignment, phylogenetic analysis and the promoter regulation region analysis, MtWUS is the best candidate for an ortholog of AtWUS. It has the highest similarity for the homeodomain and similar promoter regulation region patterns. Although the similarity of the full length protein sequence for AtWUS is only 23%, even lower than MtWOX4 which is 25%, it has around 40-50% similarity with the WUS genes of other species. MtWOX5 is the best candidate for the ortholog of AtWOX5 with 89% similarity for the homeodomain, 51% similarity for the full length amino acid sequence, and similar promoter regulation region patterns as AtWOX5. As for WOX4, MtWOX4 is the best candidate for the ortholog of AtWOX4 with 93% similarity for the homeodomain and 55% for the full length amino acid sequence of AtWOX4.

In the WOX family gene promoter region analysis, putative ortholog genes do have similar patterns. The patterns for *AtWUS, AtWOX5* and *AtWOX4* are quite different and have their specific characteristics. The WUS-like gene *MtWUS* has similar patterns to *AtWUS*, but not *AtWOX5 or AtWOX4*. On the other hand, the WOX5-like gene *MtWOX5* has a similar pattern to *AtWOX5* but not to *AtWUS* and *AtWOX4*. *MtWOX4* also has the best match with *AtWOX4*. This differentiation gives more support for the genes that might be the right orthologs and suggests that this type of analysis is useful for transcription factor gene identification.

The order of regulation regions can influence the expression pattern. The *AtWOX5* promoter-GUS fusion construct has been expressed in *M. truncatula* and the expression pattern in roots was reported (Wan, 2007). An *MtWOX5* promoter-GUS fusion was investigated in Chapter 6 but shows some differences with *AtWOX5* expression in *M. truncatula*.

The promoter length can influence the expression pattern for promoter and GUS or GFP promoter fusions. In *AtWUS*, the requirement of promoter length for correct expression is 3434 bp. Using the regulation element pattern, *MtWUS* may require 4273 bp upstream. Compared to the ortholog gene patterns in *Arabidopsis*, the length of requirement for

MtWOX5 may be 4822 bp, *MtWOX4* may be 3550 bp, and *MtCLV3* may be 4450 bp. Further experiments such as promoter region partial deletion are needed to confirm the predictions.

For the CLAVATA family genes, the closest identity to AtCLV1 is SUNN, followed by MtRLK1 and MtRLK2. The SUNN gene is in the same clade as GmNARK and GmCLV1A. GmNARK does not have the same function as AtCLV1 in the shoot apical meristem (Searle et al., 2003). MtRLK1 and MtRLK2 are in the same clade as AtBAM1 and GmRLK1, and *AtBAM1* which shows a reduction of meristem size with the double mutants of the BAM family genes (DeYoung et al., 2006). They are not the same as the CLAVATA family mutants with an enlarged meristem. AtBAM2 is redundant with AtBAM1 in function and may explain the similarity with MtRLK1 and MtRLK2 which are possibly also redundant.

For the CLAVATA family promoter analysis, there are no similar patterns between *CLV1* and *CLV2* candidates. For *CLV3*, the patterns are more similar. The differences with these genes are that *CLV1* and *CLV2* are receptors and related genes in legumes are involved in both nodulation and in shoot meristems. *WUS* and *WOX5* are transcription factors with more conserved patterns between different species. The selection of the regulatory elements was also based on using the *WUS* promoter as a reference. This means it should be most useful in the *WOX* family. With other genes that have a function connected to *WUS* it might also be useful as expression may occur at similar locations or times *in planta*. This would apply to *CLV3*.

CHAPTER 5

Expression of the *MtWUSCHEL* and *CLAVATA* Family Genes in Relation to the Induction of Somatic Embryogenesis

5.1 INTRODUCTION

The *AtWUSCHEL* (*AtWUS*) gene and its sequence was first identified in 1996 by Laux et al., and the promoter region was also fully investigated in 2005 by Isabel Bäurle and Thomas Laux. *AtWUS* expression occurs in the organizing center (OC) of the shoot apex and inflorescence meristems, young floral meristems (Laux et al.,1996; Mayer et al., 1998), the early stage of the apical nucellus (Groß-Hardt et al., 2002; Sieber et al., 2004), and weakly in the stamens (Wellmer et al., 2004). During zygotic embryo development, *AtWUS* is initiated in the 16-cell embryo and localized in the centre of the shoot apical meristem, and remains a few cell layers below the top of the shoot apex throughout embryo development (Mayer et al., 1998).

AtWUS expression is also involved in *de novo* shoot meristem formation from callus induced by cytokinin treatment. The *AtWUS* expression increased 3 days after treatment of callus with high cytokinin, spread out in large domains of callus after 5 days, and then declined and localized after 10 days. This gradual localization of *AtWUS* expression may relate to the promotion of shoot meristem cell fate within callus tissue with *de novo* shoots regenerated from places in the callus with low *AtWUS* expression and surrounded by the cells where the *AtWUS* expressions is still high (Gordon et al., 2007). The ability of *AtWUS* to allow cells to remain in an undifferentiated state (Mayer et al., 1998) may explain this. *AtWUS* is required for the maintenance of stem cell numbers which are a source of pluripotent cells for shoot meristem development (Laux et al., 1996), but too many cells with *AtWUS* expression also causes abnormal development (Brand et al., 2000). The regulation of *AtWUS* expression levels in shoot meristems is essential.

CLAVATA family genes are important regulators of *AtWUS* expression in the Arabidopsis shoot apical meristem through the WUSCHEL/CLAVATA feedback loop described in Chapter 4 (Fig. 4.1). The focus of this chapter is to investigate these gene expression relationships with somatic embryogenesis. *AtCLV1* expresses in the shoot apex around the organizing centre (Clark et al., 1997). *AtCLV2* expression can be detected in many different tissues, including the shoot apical meristem and floral meristem (Jeong et al., 1999); and *AtCLV3* expresses in the stem cells of the shoot and floral apex (Fletcher et al., 1999). Loss of function of the CLAVATA family genes cause

enlarged shoot and floral meristems, stem overgrowth and the production of extra flowers and floral organs (Clark et al., 1993; Kayes and Clark, 1998; Clark et al., 1995). The At*CLV3* gene has a dynamic role in meristem development, restricting meristem size directly by feedback regulation with the *AtWUS* signals (Müller et al., 2006).

How the WUSCHEL/CLAVATA feedback pathway operates in somatic embryo induction is still unclear. Somatic embryos can be induced by overexpression of the *AtWUS* gene (Zuo et al, 2002), but mutants of other members of the feedback pathway *AtCLV1, AtCLV2* and *AtCLV3* which also can increase the *AtWUS* expression level in shoot apical meristems do not induce somatic embryos (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). However, the mutants of *AtCLV1*, *AtCLV2* and *AtCLV3* do enhance the frequency of the somatic embryo induction in a double mutant with the *primordial timing* (*pt*) line mutant which can form somatic embryos, and the enhancement may be caused through increased *AtWUS* expression levels (Mordhorst et al., 1998). These data emphasize a potential relationship with *WUS* and somatic embryo induction but its role in relation to SE remains unclear.

In *M. truncatula* the likely orthologs to *AtWUS*, *AtCLV2* and *AtCLV3* were identified by bioinformatics. The *AtCLV1* situation was less clear (see Chapter 3) and the genes designated *MtRLK1* and *SUNN* were investigated. The *Medicago AtCLV1* ortholog is probably not yet on the database, as sequencing of the *Medicago* genome is not yet complete. This Chapter investigates the hormone regulated expression of these genes in relation to the induction of somatic embryogenesis.

5.2 MATERIALS AND METHODS

5.2.1 Tissue Culture of *M. truncatula* 2HA and Jemalong (Regeneration)

The processes of sterilization, explant treatment and culture were described in the Materials and Methods of Chapter 2.2.3.

After the somatic embryo developed it was gently removed from the callus, and subcultured onto P40 media without supplementary hormones. Embryos were incubated in the dark at 27°C for a further 2 weeks, then transferred into the light (14 h photoperiod with light intensity of 10 $\mu mol.m^{-2}.s^{-1}$) and subcultured onto fresh media every 4-5 weeks.

For somatic embryos, re-culture in P4 (4BAP) media for 4-5 weeks can help to induce more secondary somatic embryos and develop new leaves. Re-culture in P4 (10NAA) or P4 (10:4) media for 4-5 weeks then a transfer back to P40 media can help to induce root development.

After roots and new leaves form on young regenerated plants, plants were transferred into soil. Newly regenerated plants were grown under glasshouse conditions, with high moisture conditions for 1-2 weeks.

5.2.2 Tissue Collection for RNA Extraction

Tissue was collected from each cultivar/hormone combination for each day for the first 3 days and every 1 or 2 weeks for 11 weeks, under sterile conditions. Somatic embryo samples were collected from a mixture of embryo stages. Cultured root tip tissue was collected from P4 (10NAA) cultured roots. Leaf tissue was also collected from both 2HA and Jemalong. The shoot apical meristem sample included some surrounding tissue from the shoot apex. The developing flowers were collected from the shoot apex before the flowers grew out. About 100mg of tissue was collected, flash frozen in liquid nitrogen and stored at -80°C until used for RNA isolation.

5.2.3 Plant Genomic DNA Extraction

The method was modified from Gawel and Jarret (1991). Approximately 1 g of sample tissue was weighed and snap frozen in liquid nitrogen. The samples were ground to a fine powder under liquid nitrogen with a mortar and pestle. To the ground tissue was added 3 mL CTAB lysis buffer (1% PVP, 1.2 M NaCl, 100 mM Tris, 20 mM EDTA, 1% CTAB, pH 8.0) and 60 μL β-mercaptoethanol. After vigorous vortexing, the mixture was shaken at 65ºC for 40 minutues. RNaseA (1 μL) was added to the mixture, which was shaken at 37 ºC for 20 minutes. The extract was then fully mixed with 5 mL chloroform, and centrifuged at 4 ºC, 3000 rpm for 10 min. The upper layer of clear liquid was carefully transferred to a 15 mL tube. Then 1.2 mL of 100% isopropanol was used to precipitate the DNA from the liquid followed by gentle inversion and kept at room temperature for 5 min. There was then a 10 min 3000 rpm centrifugation at room temperature and the liquid removed. To the pellet was added 1 mL of 1 M NaCl for solubilizing the DNA pellet. The solubilized DNA was then distributed into two 1.5 mL eppendorf tubes and mixed with 500 μL chloroform and vortexed. A 13000 rpm centrifugation at room temperature was then carried out for 8 min and the upper layer of liquid transferred to a new eppendorf tube. One mL of 100% ethanol was added to the eppendorf and gently mixed to precipitate DNA. The DNA pellet was collected following a 3000 rpm centrifugation for 5 min. The pellet was washed twice with 70% (v:v) ethanol, then dried to remove ethanol. The pellet was dissolved in 400 μL TE buffer (pH = 8.0) and stored at 4 ºC or at -20 ºC for long term storage.

5.2.4 Total RNA Isolation and cDNA Preparation

- **Total RNA isolation and on column DNase treatment**

RNA isolation and on column DNase treatment was carried out using the QIAGEN RNeasy Plant Mini Prep Kit, as per the manufacturer's instructions. All pipette tip racks and mortar and pestles were made RNase free by treatment with 0.1 M NaOH and 1 mM EDTA solution, rinsing with double distilled water and autoclaving. The mortar and pestles were additionally baked overnight at 240ºC, and before use were placed in a minus 20ºC freezer to cool. Plasticware used was provided from the manufacturer RNase free.

Approximately 100 mg of tissue were weighed and snap frozen in liquid nitrogen. The plant samples were disrupted by grinding to a fine powder under liquid nitrogen with a mortar and pestle, the addition of a lysis buffer and vigorous vortexing and heating to 56ºC. The lysate was then passed through a QIAshredder homogeniser column to remove the tissue residues. To the eluate was added 225 µL of 100% ethanol. Total RNA was isolated by passing the eluate through an RNeasy mini column where the RNA is bound to a silica gel membrane. Buffers were washed through the column to remove contaminants, followed by treatment with a DNase1 mixture (0.5M Tris-HCl (pH 7.5), 0.5 M $MgCl_2$, sterile water and RNase-free DNase1 (1 unit/µL)) and incubation at 37°C for 15 min. After washing again with RW buffer, the RNA was eluted with RNase free water. RNA samples were stored at -80°C to prevent degradation.

- **Evaluation of RNA quality**

The concentration and purity of the RNA samples was determined by spectrophotometry and by running on an agarose gel. For spectrophotometer analysis the samples were diluted 1 in 50 in double distilled water, then the optical density (OD) was determined at 260 and 280 nm for each sample, with double distilled water used as the standard.

The concentrations of the original RNA samples were determined using the formula:

Original RNA Concentration = 40 x OD260 x Dilution Factor

The amount of RNA degradation was evaluated by running the samples on a denaturing agarose/formaldehyde gel that removed secondary structure. The electrophoresis apparatus was soaked in 0.1 M NaOH and a 1 mM EDTA solution for ten minutes to remove any RNase contamination. Agarose was dissolved in double distilled water by heating in a microwave, and then cooled to 55ºC in a water bath. In the fume hood, 10 x MOPS buffer and 37% formaldehyde were added to the solution immediately before pouring. The samples were prepared (containing the RNA, 10 x MOPS buffer, 37% formaldehyde and formamide) and heated at 55ºC for 15 min, then loaded onto the gel

with loading buffer and ethidium bromide. The gels were run in 1 x MOPS buffer at 80 Volts. Electrophoresis was performed at 80-120 V (5.5-8 V/cm) for 0.5-1 h. Gels were examined under UV illumination and digital images captured using a Gel Documentation image analyser (BioRad).

- **cDNA synthesis: Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

RT-PCR was as per manufacturers instructions (Invitrogen SUPERSCRIPT First-Strand Synthesis System for RT-PCR). RNA/primer mixture was prepared to include up to 1 or 2 µg of total RNA, 10 mM dNTP mix, Oligo(dT)12-18 and DEPC-treated water, and was incubated at 65ºC for 5 min then in ice for at least 1 min. Reaction mixture (containing 10 x RT buffer, 25 mM $MgCl_2$, 0.1 M DTT and RNaseOUT Recombinant RNase Inhibitor) was added to each sample and incubated at 42ºC for 2 min. Then Reverse transcriptase enzyme Superscript II RT was added to each sample except non-RT controls, and all samples were incubated at 42ºC for 50 min. The reaction was terminated by heating to 70ºC for 15 min then placing on ice. RNaseH was added to each sample, and samples were incubated at 37ºC for 20 minutes. These cDNA products were stored at –20ºC.

5.2.5 Primer Design for Polymerase Chain Reaction (PCR)

Primers were designed using the primer design program "Primer3" (Rozen and Skaletsky, 2000). The primers were synthesised by the Sigma Proligo Company. The primers used in PCR and qRT-PCR (Quantitative Reverse Transcriptase Polymerase Chain Reaction) are listed in Table 5.1.

Gene name	Primer name	Product size	Sequence (5' → 3')
GAPDH	GAPDH For1	606 bp	TGGTCATCAAACCCTCAACA
	GAPDH Rev1		CCTCGTTCTTTCCGCTATCA
MtWUS	MtWUS-9654-For1	204 bp	CTTACAACATTTCATCTGCTGGGCT
	MtWUS-9654-Rev1		CGACATGATGACCAATCCATCCTAT
MtCLV3	MtCLV3UQ-Full-For1	303 bp	ATGGCTTCTAAGTTCATCTTTTCTT
	MtCLV3-3E-Rev1		TCAAGGGTTTTCAGGCTTAATAGGG
SUNN	CLV1_1-For1	206 bp	CCTACGGCTACATTGCTCCAGAAT
	CLV1_1-Rev1		TGCTGACACTAATGCTTTATCTGATGG
MtRLK1	CLV1_2-For1	242 bp	TTCTTATGGATACATAGCTCCAGAGTA
	CLV1_2-Rev1		CATGCATCACCTCATTAAGTGGAAC
MtCLV2-1	MtCLV2_1 For1	175 bp	AGTTCAGCTTGGTTATTGTTCATTC
	MtCLV2_1 Rev1		AGGCAAAGTACCTGTAAACTGATTG
MtWOX5	WOX5 For2	246 bp	CAAGCACTGATCAAATTCAGAAAAT
	WOX5 Rev2		GAAAAAGCTCAAGAGTCTCAATCAC
MtWOX4	WUSFor1	259 bp	TCACCACAAAGCCAGGTTGAAACG
	WUSRev1		GAGGACTATGAGGAAGGCCAAGACTG

Table 5.1 List of the primers for the genes used in qRT-PCR.

5.2.6 Standard Polymerase Chain Reaction

Reactions were 25 μL in volume, and were run in 0.2 mL thin-walled PCR tubes, with 1 to 2 μL of diluted DNA template using the components shown in Table 5.2. The PCR cycle program is given in Table 5.3.

	Stock	Running concentration
PCR Buffer	10 x	1 x
$MgCl_2$	25 mM	1.5 mM
dNTP	2 mM	0.12 mM
Forward Primer	10 μM	4 μM
Reverse Primer	10 μM	4 μM
Taq Polymerase	5.5 units/μL	1 unit
Double distilled water	-	-

Table 5.2 Components used for the standard PCR reaction

Temperature (ºC)	Time (minutes)	Action	Number of Cycles
94	5	Initial denature	1
94	0.5	Denature	35
X	0.5	Anneal	35
72	1	Extension	35
4	∞	Hold	1

Table 5.3 PCR cycle program. The annealing temperature (X) was set equal to the lowest TM value of the primer pair used in the reaction.

5.2.7 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Real time PCR using the DNA Engine Opticon System 3 and SYBR Green 1 fluorescent dye is a highly sensitive quantitative method for determining comparative gene expression. The real time PCR program is given in Table 5.4. Expression of each gene was determined by comparison of the expression levels of *MtGAPDH* (glyceraldehyde-3-phosphate dehydrogenase), a housekeeping gene, using a modification of the comparative threshold cycle (Ct) method and was calculated as $E^{-\Delta\Delta C(T)}$, where $-\Delta\Delta C(T) = (Ct_{target} - Ct_{GAPDH})_{Time\ x} - (Ct_{target} - Ct_{GAPDH})_{Calibrator}$ and E is the amplification efficiency, which was calculated using the linear regression method on the log (fluorescence) per cycle number data for each sample using the LinRegPCR software (Ramakers et al., 2003). PCR reactions were performed in triplicate in at last two biological repeats.

Step	Temperature (ºC)	Time	Action	Number of Cycles
1	50	2 min		1
2	95	2 min	Initial denature	1
3	95	15 sec	Denature	39 ~ 45
4	X	20 sec	Anneal	39 ~ 45
5	72	1 min	Extension	39 ~ 45
6	-	-	Signal read	39 ~ 45
7	50 ~ 90	1°C/2 sec	Melting Curve	1

Table 5.4 Real Time PCR Program. The annealing temperature (X) was set equal to the lowest TM value of the primer pair used in the reaction.

5.2.8 Agarose Gel Electrophoresis of DNA

All PCR products, plasmids, or digested DNAs were run on a 1.5 % (w/v) agarose gel in 1 x TAE running buffer. The DNA samples were mixed with 0.1 volumes of 10 x loading buffer and loaded onto the gel alongside the standard 100 bp or 1 kb ladder. Electrophoresis was performed at 80-120 V (5.5-8 V/cm) for 0.5-1 h. Ethidium bromide was added in the gels or stained after running. Gels were examined under UV illumination and digital images captured using a Gel Documentation image analyzer (BioRad).

5.2.9 Restriction Enzyme Digestion

Digestions were carried out to check for the presence and orientation of the insert. The following restriction enzymes were used during digestion assays:

BamHI, EcoRV, XbaI, Each tube contained the following:
1 μL of 10x buffer
0.25 μL of restriction enzyme
3-8 μL of plasmid
Add MilliQ water to 10 μL

The samples were incubated at 37°C for 3 hour to over-night, followed by a 65°C or 80°C incubation for 20 min. Agarose gel electrophoresis was carried out to check for bands.

5.2.10 Gene Transformation Procedures

- **Gene sequences for transformation**

For promoter region experiments, PCR primers were designed based on a suitable upstream sequence of 1 to 3 kb. The 3’ end was located around the predicted coding starting site (ATG). The promoter region was amplified using PCR and the PCR product purified for cloning into a TOPO vector. The primer sequences for these gene promoter regions are listed in Table 5.5.

Gene name	Primer name	Product size	Sequence (5' → 3')
MtWUS	MtWUS-9654Pro-For1	3182 bp	CTAACTTCCGTTATCCGAGAATCTT
	MtWUS-9654Pro-Rev1		TGTTCCATGTTTTTGTTGGACTGAA
	MtWUS-9654Pro-For2	2157 bp	CACGTTAAACGACGCCTTACTTTTG
	MtWUS-9654Pro-Rev1		TGTTCCATGTTTTTGTTGGACTGAA
MtCLV3	MtClv3P2k-For1	2014 bp	TCCATCCAAACATTCTAAATCCTTA
	MtClv3P-Rev1		AGCCATTATAAATATTGGAGATACG
MtRLK1	MtCLV1-2pr2k_For1	2145 bp	GAGAACAATAATGTCTCATCGGAAT
	MtCLV1-2pr4k_Rev1		TTTGAGGGAGAGAGGGAAGTGAGAT
MtWOX5	W5Prom_For1	1024 bp	TTCCCAACATAATTTGTAACCTCAT
	W5Prom_Rev1		CATGCTCTCTTCCATATTTCAATTC
MtWOX4	MtWusPr-For1	966 bp	CTGCTTTGATTGAGTTTGGGTTAT
	MtWusPr-Rev1		ATGAAGAAATGAATGACAGGGAAT

Table 5.5 List of the primers for each gene for promoter region analysis.

For RNAi experiments, the primers were designed in the whole coding region or regions close to the 3' end. The primers are listed in Table 5.6. For the empty vector control, 88 bp DNA was taken from multiple cloning site of the vector pASK-IBA44, (5'-CCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAGGCCTGA GAGGATCGCATCACCATCACCATCACTAATAAGCTT-3') (IBA, Götingen, germany), courtesy of Dr. Sergey Kurdyukov.

Gene name	Primer name	Product size	Sequence (5' → 3')
MtWUS	MtWUS-9654-For1	204 bp	CTTACAACATTTCATCTGCTGGGCT
	MtWUS-9654-Rev1		CGACATGATGACCAATCCATCCTAT
MtWOX5	W5-i3E-For3	178 bp	AAATGAATGCAGGGAAAATACAAT
	5Wr2		TCCTAAACATTTTTCATATTATGCT
MtRLK1	Clv1-2 For1	242 bp	TTCTTATGGATACATAGCTCCAGAGTA
	Clv1-2 Rev1		CATGCATCACCTCATTAAGTGGAAC

Table 5.6 List of the primers for each gene for RNAi analysis.

- **TOPO entry vector cloning reaction**

The following regents were setup in the order shown below:

PCR product with polyA in 3' end	2 μL
Dillute salt solution	1 μL
Sterile MiliQ water	2 μL
TOPO vector	1 μL

The contents were mixed gently and incubated for 5 min at room temperature after which the sample was placed on ice. 1 μL of solution was used by electroporation into *E coli*. The entry vector is pCR8/GW/TOPO (Invitrogen, Carlsbad, CA).

- **Electroporation of competent *E. coli,* AGL-1 and LBA4404**

Electroporation was carried out to transform plasmids into bacteria or *A. tumefaciens*. The electro competent *E. coli* strain used was ElectroMAX DH10B, and the electro competent *A. tumefaciens* strains used were AGL-1 and LBA4404. The electroporation took place using the BioRed Gene Pulsar. One to two μL of plasmid was added into 50 μL of electro competent cells that had been thawed on ice. The sample was then transferred to a pre-cooled electroporation cuvette (2 mm gap). The cuvette was then placed in the pulsar where it was shocked at 2.5 kv for approximately 5 microsec. After the electric pulse, the tube had 1 mL of LB media (for *E. coli*) or YEP media (for *A. tumefaciens*) added and pipette mixed with a pipette, transferred to a tube, and shaken at 37°C (for *E. coli*) or 26°C (for *A. tumefaciens*) for approximately 1 h. After the incubation aliquots of 20 to 50 μL of the culture with 60 or 30 μL LB or YEP media were spread onto plates containing suitable antibiotic and incubated overnight at 37°C (for *E. coli*) or over 2-3 nights at 26°C (for *A. tumefaciens*).

Antibiotic for each plasmid and *A. tumefaciens* lines:

pCR8/GW1/TOPO	100 μg/mL Spectinomycin
pBHS164/206	50 μg/mL Kanamycin for *E coli*; 3 μg/mL Basta for plant

pMDC164/206	50 μg/mL Kanamycin for *E coli*; 15 μg/mL Hygromycin for plant
pOpOff2-Hyg	100 μg/mL Spectinomycin for *E coli*; 15 μg/mL Hygromycin for plant
AGL1	100 μg/mL Ampicillin, 25 μg/mL Rifampincin
LBA4404	100 μg/mL Streptomycin, 25 μg/mL Rifampincin

- **Plasmid isolation**

Plasmid isolation was carried out using the Wizard *Plus* SV Minipreps DNA Purification system (PROMEGA) according to the manufacturer's instructions with the elution amount being changed to 20-50 μL instead of 100 μL.

- **Gateway LR recombination reaction for insert transfer from entry to destination vector**

The following components were mixed together

Destination vector	1 μL
Entry Vector containing insert	1 μL
Gateway LR Clonase II enzyme mix	1 μL

The destination vectors used to generate promoter::reporter constructs were either pMDC164 (GUS), pMDC206 (GFP), pBHS164 (GUS), pBHS206 (GFP).

After incubating at in 25°C for 1 hour to overnight, 0.5 μL Proteinase K was added, incubated at 37°C for 20 min and stored at 4°C.

- **Transformation from *A. tumefaciens* to *M. truncatula* explants**

A. tumefaciens clones containing the appropriate construct were cultured at 26°C in 20 mL YEP liquid medium with suitable antibiotic for 1 or 2 days. The bacteria were pelleted were to remove the medium, and 20 mL liquid P4 (10:4) medium was added and shaking continued at 26°C.

Leaf explants were prepared and sterilized as described in Chapter 2.2.2 and transferred to liquid P4 (10:4) medium. The explants were then transferred into Agrobacterium-P4 (10:4) mix suspension and incubated at room temperature for 10 min. After removing the unnecessary suspension liquid from explants, the leaf explants were co-cultured on agar medium and incubated in the dark at 26°C for 2-3 days.

After the Agrobacterium colony border around explants was 1 to 2 mm, explants were washed with sterilized water for 5 min and sterilized water with 500 mM Timentin for another 5 min to wash out the bacteria. After the wash procedure, explants were placed on P4 (10:4) solid medium with suitable antibiotics for somatic embryo induction on P4 (10 NAA) solid medium with suitable antibiotics for root formation.

5.2.11 GUS Staining, Embedding and Sectioning Procedure and RNA *in situ* Hybridisation

- **GUS staining procedure**

Staining for GUS enzyme activity is described below:

GUS staining solution (10 mL)	Stock solution	Volume added
50mM sodium phosphate buffer (pH 7.0)	200 mM (pH 7.0)	2.5 mL
1mM EDTA	500 mM	20 μL
0.1% Triton X-100	10% v/v	10 μL
1 mM X-Gluc (dissolved in DMF)	20 mM (10 mg/mL)	0.5 mL
5 mM potassium ferricyanide ($K_3Fe(CN)_6$)	50 mM	1 mL
5 mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$)	50 mM	1 mL
H_2O to 10 mL		

Stain tissue at 37°C for 16 h (overnight). Wash tissue in 70% ethanol at 37°C for a few times to remove chlorophyll. Store stained tissue in 70% ethanol at 4°C.

After staining, the sample was also embedded in Steedmans Wax (described later) for sectioning. After the sectioning and de-waxing, some sections were stained with 1%

safranin dissolved in water to produce a red colour in some GUS expression pictures.

- **Fixation and embedding**

The 4% formaldehyde fixative solution was prepared in 100 mL phosphate buffer saline (PBS) (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) and adjusted to pH to 11 using NaOH. The solution was heated to 60°C then 4 g paraformaldehyde was added and stirred for 1 to 2 min until dissolved. After cooling on ice the solution was adjusted to pH 7.0 using H_2SO_4.

Plant material was cut under fresh fixative and placed in ice-cold fixative at 4°C overnight. Fixative was replaced by ice cold 0.85% NaCl and kept on ice, and swirled occasionally, for 30 min. The solution was replaced by 50% ethanol containing 0.85% w/v NaCl for 90 min, and the process was repeated with 70% and 85% ethanol containing 0.85% w/v NaCl, followed by 95% ethanol and then 100% ethanol. The material was kept in fresh 100% ethanol at 4°C overnight.

After transfer to fresh 100% ethanol for 2 h at room temperature, 37°C ethanol was used for the embedding procedure in Steedmans Wax. Material was infiltrated at 2:1, 1:1, 1:2 ethanol:wax at 37°C over 2 h for each step, and in pure wax overnight. The material was transferred to warmed moulds with fresh wax, checking orientation and allowed to cool.

- **Sectioning and de-waxing**

The sample wax block was trimmed leaving 2 mm around the sample and attached to the object holder by melting some wax. The sample block was cut by microtome in sections 7-8 μm thick, and placed on the surface of 40 ~ 45°C distilled water. The sections were picked up by coated slides, and fixed on to the slides through heating on a 42°C plate for 24 to 48 h. The slides can be stored at room temperature or 4°C.

To remove the wax from the section in which the tissue is embedded, the slides were treated with 100% histoclear three times for five minutes or longer until all the wax was removed. Following this treatment slides were processed through each of the following solutions for two minutes: 1:1 histoclear: ethanol, 100% ethanol twice, 95%, 90%, 70%,

50%, and 30% ethanol, and water. After air drying, slides can be stored until used, but usually subsequent treatments were carried out the next day.

- **Preparation of the DIG-labelled RNA probes for *in situ* hybridisation**

RNA probes were prepared from plasmid or PCR products which have T7, SP6 or T3 sequences for RNA polymerase binding. The gene templates were full length or a partial coding sequence.

For *MtWUS*, the full length coding region was used as an RNA probe template. The T7 sequence at the 5' end and the T3 or SP6 sequence at the 3' end were added using PCR. The primer sequences shown in Table 4 are the three pairs of primers for the PCR procedure. The first set of primers were used to amplify the target sequence, and this PCR product was used as template in a 2nd PCR with a second set of primers to add partial T7 and T3 sequences at the ends. The 2nd PCR product was used as a template in a 3rd PCR with T7 and T3 or SP6 primers to amplify the final product which has the full sequence of T7 at the 5' end and a T3 or SP6 sequence at the 3' end. In the 3rd PCR process, the primer annealing temperature for the first cycle was set at 30°C to increase the annealing rate. The final 3rd PCR product can also link into the plasmid described in 5.2.8. The 3rd PCR products were purified by QIAGEN PCR purification kit spin columns.

The RNA probe synthesis procedure was performed in the following solution:

Plasmid DNA or 100-200 ng PCR product	1 μL
10 x transcription buffer (Roche)	2 μL
DIG-NTP labelling mix (Roche)	2 μL
T7, T3, or SP6 RNA polymerase	2 μL
RNase out	1 μL

Adjust volume to 20 μL with RNase-free H_2O

For the *MtWUS* RNA probe, T7 polymerase was used for sense probe preparation, and T3 or SP6 were used for anti-sense probe preparation. The primer sequences are given in Table 5.7.

PCR sequence	Primer name	Sequence (5' → 3')
1	MtW96-Full-For1	ATGGAACAGCCTCAACAACAACAA
	MtW96-Full-Rev1	GGTGACCTACAGCCGTAAGAGTTGA
2	MtW96-insi1-T7	GAGGCCGCGTATGGAACAGCCTCAACAACA
	MtW96-insi1-T3	ACCCGGGGCTGGTGACCTACAGCCGTAAGA
3	T7 primer	TTATGTAATACGACTCACTATAGGGAGGCCGCGT
	T3 primer	AATTAACCCTCACTAAAGGGAGACCCGGGGCT
	SP6 primer	CCAATTTAGGTGACACTATAGAAGTACCCGGGGCT

Table 5.7 Primer list for the *MtWUS* RNA probe preparation. Sequence underlining indicates the annealing regions for T7 primer or T3 and SP6 primer.

After incubating at 37°C for 2 h, 2 μL DNase1 (10 U/μL) was added to the mixture and incubated at 37°C for 15 min to remove the template DNA. Two μL 0.2 M EDTA (pH 8.0) was added to stop the DNase1 reaction and the probe was stored at -20°C for *in-situ* hybridisation.

- **RNA *in situ* hybridisation**

Before the process, the cover slips were dipped in 100% acetone three times, followed by treatment in acetone plus 1% surfasil, and drying in a vertical position.

Proteinase K digestion was used to remove the protein from the tissue. Slides were treated at 37°C in digestion buffer (50 mM EDTA, 100 mM Tris-HCl in DEPC water, pH 7.5) for 5 min, followed by treatment with digestion buffer plus proteinase K (1 μg/mL) at 37°C for 5 min, and then the slides were washed in 37°C digestion buffer for 3 to 5 min. Slides were then washed three times in DEPC H_2O for 5 min and kept in water.

Hybridisation solution components are shown in Table 5.8. Slides were pre-warmed at 70°C for 5 min. Then, 30 to 65 μL per slide of hybridisation solution was added and covered with a cover slip. The cover slip was sealed around the edge with rubber cement, and incubated overnight at 55°C in a humidified chamber for hybridisation.

Chemical name	Stock concentration	Amount (for 1000 µL)
50 % Formamide	100%	100 µL
10 % Dextran Sulphate	50%	36 µL
300 mM NaCl	5M	11.6 µL
TE buffer	100X	1.9 µL
1 X Denhard's reagent	50X	3.8 µL
Poly A (10 mg/ml)	20 mg/ml	2 µL
DIG-labelled probe	-	X µL (Final concentration 0.1-5 µg/mL)
DEPC water	0.01%	Adjust to 1000 µL
RNase inhibitor		0.5 µL

Table 5.8 Chemical components of the Hybridisation solution. The DIG-labelled probe, tRNA and poly A RNA were mixed together with DEPC water first and heated at 85°C for 2 min then immediately placed on ice, then added to hybridisation mixture before use.

The next day slides were washed in separate jars for antisense and sense slides. Slides were washed in 2x SSC at 37°C for 5 min to remove the cover slip, followed by incubating in 2x SSC at room temperature (R/T) for 5 min. Before the RNase A treatment, slides were treated in STE (500 mM NaCl, 20 mM tris-HCl, pH 7.5, 1 mM EDTA), at room temperature for 1 min. The RNase A in STE (3 mg in 75 mL STE) treatment was then carried out 37°C for 50 min.

The following wash process was carried out for slides after RNase A treatment.

Solution	Temperature °C	Time (Minutes)
2x SSC + 50% formamide	60	5
1x SSC	R/T	30
0.5x SSC	R/T	30
0.25x SSC	R/T	30
0.1x SSC	R/T	5

Slides were air dried overnight or the signal detection step carried out immediately.

For signal amplification and detection three antibodies were used to show the colour signal. The probes was DIG labelled, therefore the first antibody (Antibody 1) was the anti-DIG mouse antibody. The second antibody (Antibody 2) was anti-mouse antibody

conjugated to another DIG label. The third antibody (Antibody 3) was an anti-DIG antibody conjugated to the enzyme alkaline phosphatase (AP), used to act on a suitable substrate to form a colour signal. Antibody 1 and 2 were diluted 1:25 with a 1x blocking solution, and Antibody 3 was diluted 1:75 with a 1x blocking solution. The 1x blocking solution was diluted from a 10x stock with 1x PBS. The 10x PBS was prepared with 80 g NaCl and 14.4g Na_2HPO_4 in 1 L.

For the detection process procedure, 300 µL of a 1x blocking solution was added to the slide and incubated at room temperature for 30 min in humid chamber. The solution was washed off by water and 50 µL Antibody 1 solution was added. A cover slip was added and the slide incubated in a 37°C humid chamber for 1 h. For the washing process, the cover slip was floated off with a pre-warmed wash solution (1x PBS plus 0.2% Tween). The wash solution was then added to the slides and incubated in a 37°C humid chamber for 15 min. After pouring off the solution, 50 µL Antibody 2 solution was added and covered with a cover slip, and incubated in a 37°C humid chamber for 1 h. After another cycle of the washing procedure, 50 µL Antibody 3 solution was added and covered with a cover slip, and incubated in a 37°C humid chamber for 1 h.

The cover slip was floated off from the slides with the wash solution, Antibody 3 treatment was done and wash solution was added to slides and incubated at 37°C in a humid chamber for 5 min, and this process repeated three times. The slides were then washed in colour buffer (100 mM tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5).

For colour development, the substrate for the colour reaction was prepared with 200 µL NBT/BCIP and 60 µL Levamisolein in 10 mL PVA (Polyvinyl-alcohol) colour buffer. Fifty µL of colour reaction was added for each slide and the colour development was monitored continually until the probe displayed a pink/purple colour. Distilled water was used to wash out the solution and stop the colour development. The slide was checked under the microscope with or without a cover slip.

5.3 RESULTS

5.3.1 *MtWUS* and *MtCLV3* Expression Patterns *in planta* and *in vitro*

The expression of the genes we designated *MtWUS* and *MtCLV3* was determined in different tissues, that based on Arabidopsis studies would show different expression patterns. In the case of *MtWUS* there was an expression pattern (Fig. 5.1A) that is consistent with information available on *WUS* that it is expressed in the organiser centre of the apical meristem (Bäurle and Laux, 2005) and also in floral meristems (Müller et al., 2006). *WUS* is also expressed in the developing embryo (Mayer et al., 1998) and expression would be expected in the somatic embryo. There is no *WUS* expression in the leaf or the auxin-induced cultured roots. The expression pattern is similar in Jemalong (Fig. 5.1B).

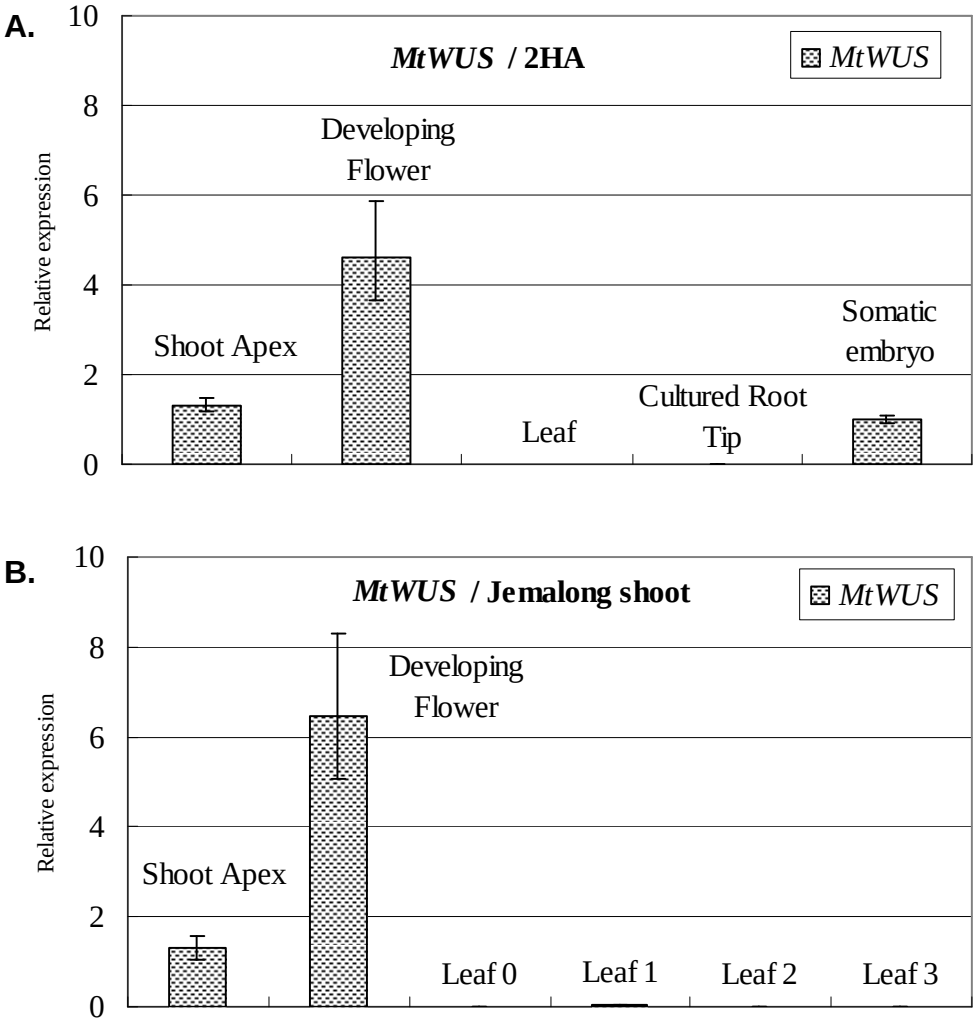


Figure 5.1 *MtWUS* expression in different tissues of 2HA and Jemalong. A: Expression of *MtWUS* in 2HA shoot apices, developing flowers, mature leaves, cultured root tips and somatic embryos. **B:** Expression of *MtWUS* in Jemalong shoot apices, developing flowers, and different stages of leaf development. Leaf 0 indicates unopened initiated leaf, and Leaf 1 to Leaf 3 indicates the fully opened leaf from tip to base. Expression, measured by qRT-PCR, is shown relative to the expression level of somatic embryos in (A). Error bars show SEM.

The location of *MtWUS* expression in plantlets was also investigated by using pr*MtWUS*::GUS transgenic tissue. In regenerated plantlets, *MtWUS* expresses highly in the shoot apex including the leaf primordium, also in the tip of the cotyledons and vascular tissue (Fig. 5.2A). There is no expression in well-formed leaf structures (Fig. 5.2B). The expression of *MtWUS* is broader than the organiser centre of the apical meristem as known for *AtWUS*, especially in the leaf primordium (including cotyledons) and vascular tissues in plantlets, suggesting differences between *Medicago* and *Arabidopsis*.

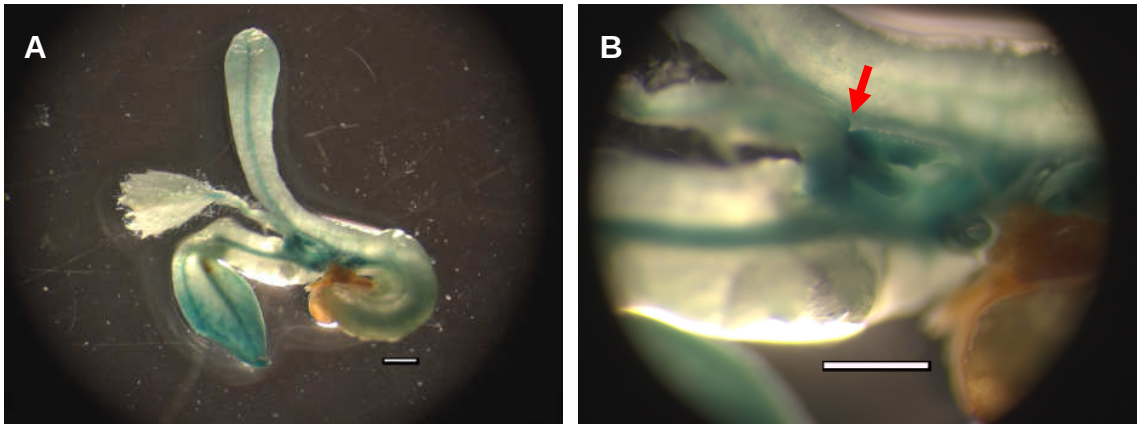


Figure 5.2 pr*MtWUS*::GUS expression in regenerated plantlet. The signals were investigated in germinated somatic embryos (A and B). Red arrow in (B) indicates the shoot and leaf primordium structure. Bar = 500 μm.

In the case of *MtCLV3* there was an expression pattern consistent with what is known of *CLV3* expression (Fig. 5.3). *CLV3* expresses in the stem cells of the shoot and floral apex (Fletcher et al., 1999), and *MtCLV3* expresses in the shoot apex of Jemalong and 2HA, consistent with *AtCLV3*, but not in developing flowers, leaves and auxin-induced cultured root tips. *AtCLV3* is also expressed after the heart stage embryo (Fletcher et al., 1999), and is also consistent with the *MtCLV3* expression in the somatic embryo of 2HA. The sequence data in Chapter 4 and the expression data are consistent with the *MtWUS*

and *MtCLV3* being orthologs of *AtWUS* and *AtCLV3*.

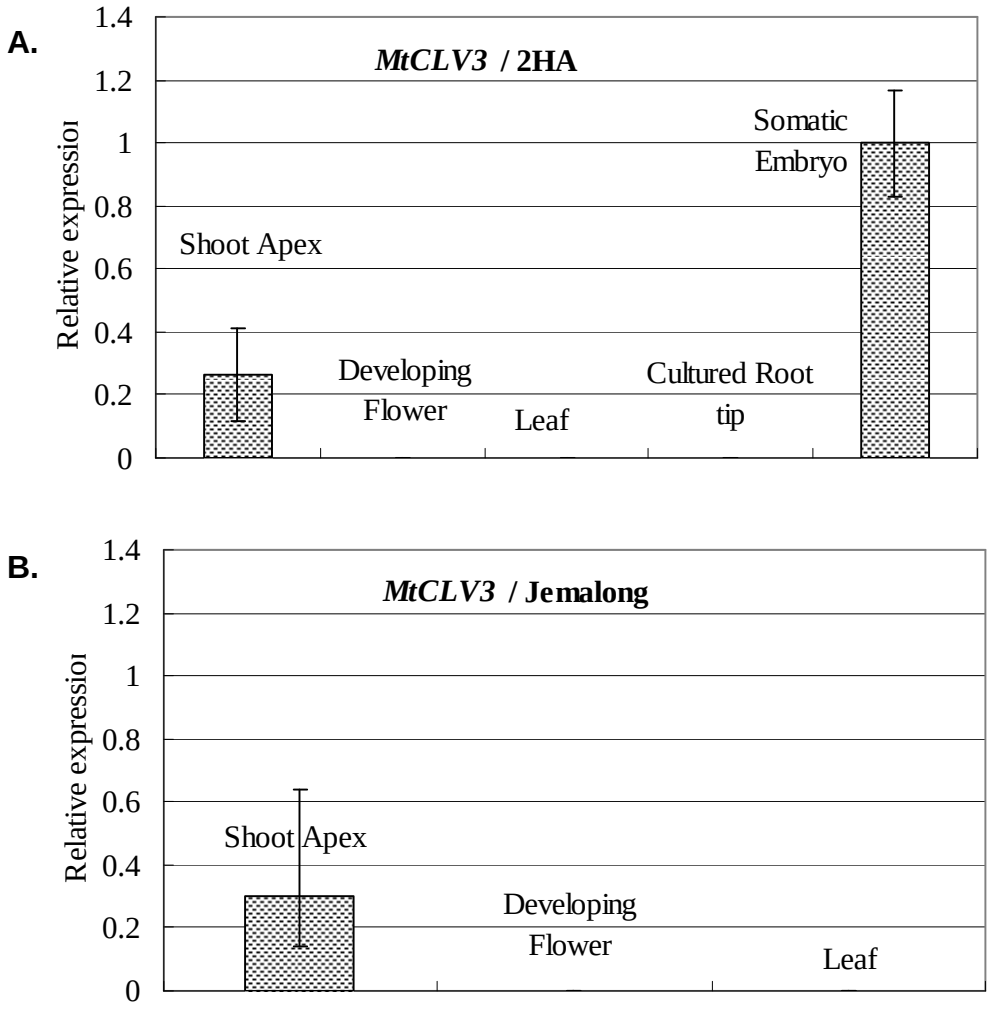


Figure 5.3 *MtCLV3* expression in different tissues of 2HA and Jemalong. A: Expression of *MtCLV3* in 2HA shoot apices, developing flowers, mature leaves, cultured root tips, and somatic embryos. **B:** Expression of *MtCLV3* in Jemalong shoot apices, developing flowers, and mature leaves. Expression, measured by qRT-PCR, is shown relative to the expression level of somatic embryos in (A). Error bars show SEM.

5.3.2 Expression Dynamics of *MtWUS* and *MtCLV3* in Relation to the Induction of Somatic Embryogenesis

Given that ectopic expression of *AtWUS* can induce somatic embryos (Zuo et al. 2002). It was important in understanding the mechanism of induction of SE in *M. truncatula* to know the pattern of *MtWUS* expression and the hormonal relationship to its induction. In Fig. 5.4 it can be seen that in the standard auxin plus cytokinin medium that *MtWUS*

expression is induced early in the culture process (consistently less than 48 h) and peaks after 7 d. The increased *MtWUS* expression is cytokinin dependent. Auxin alone does not induce *MtWUS* expression and is not associated with root formation.

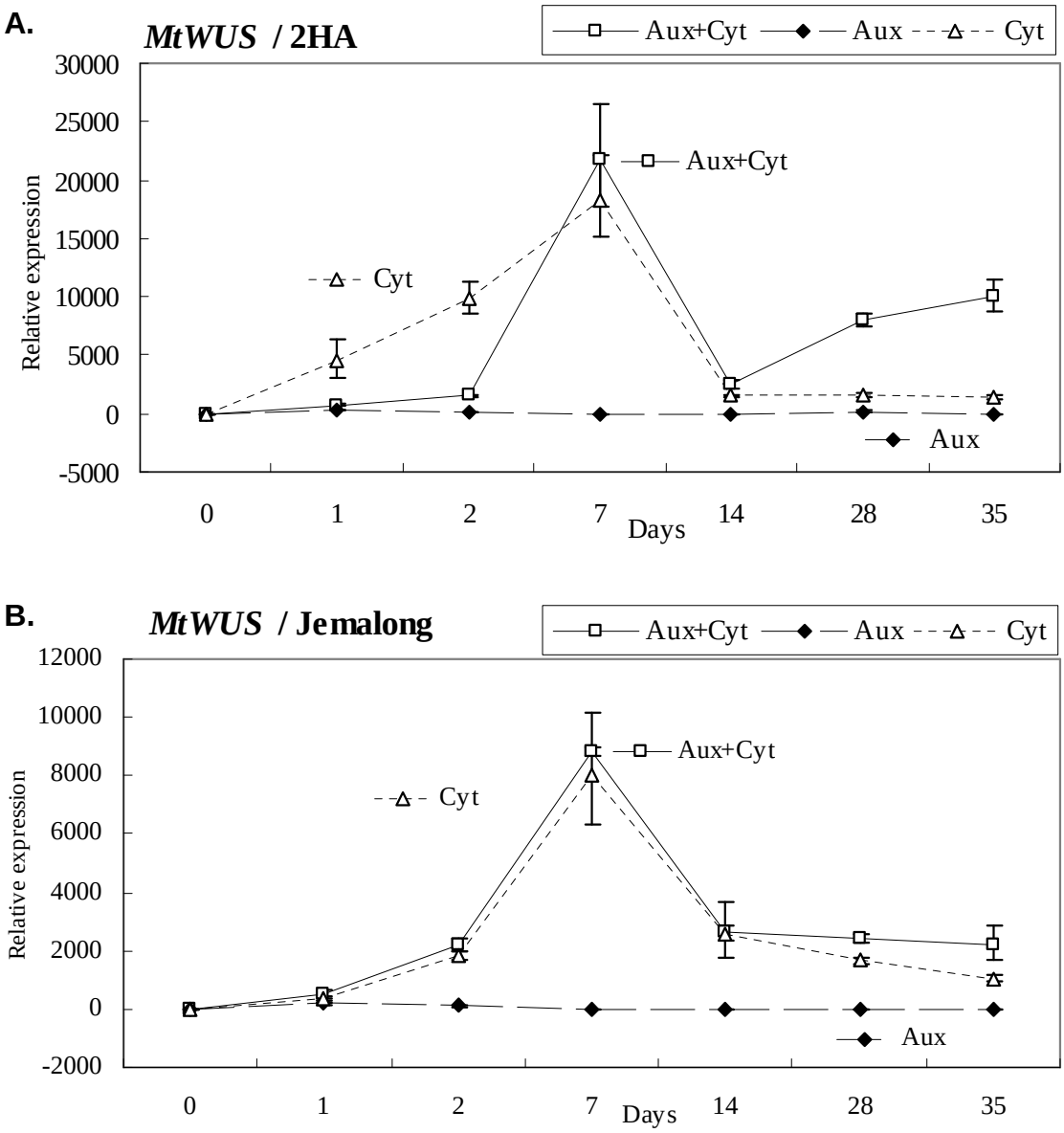


Figure 5.4 Differential timing of *MtWUS* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP) (Aux+Cyt, □, solid line), auxin (10 µM NAA) (Aux, ◆, dashed line), and cytokinin (4 µM BAP) (Cyt, △, dotted line). A: Expression of *MtWUS* in 2HA **B:** Expression of *MtWUS* in Jemalong. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d), and was followed for 35 d. Error bars show SEM.

In Fig. 5.5, *MtCLV3* can be detected after 28 d of standard auxin plus cytokinin culture and 35 d of cytokinin dependent culture in 2HA, but not in auxin culture and any

Jemalong cultures. The first somatic embryos are visible to the eye between 28 and 35 d in 2HA, but it also noticed that somatic embryos can induce in cytokinin dependent culture occasionally (Nolan et al., 2003). This result suggests that *MtCLV3* expression is associated with the development of the somatic embryo, rather than its induction.

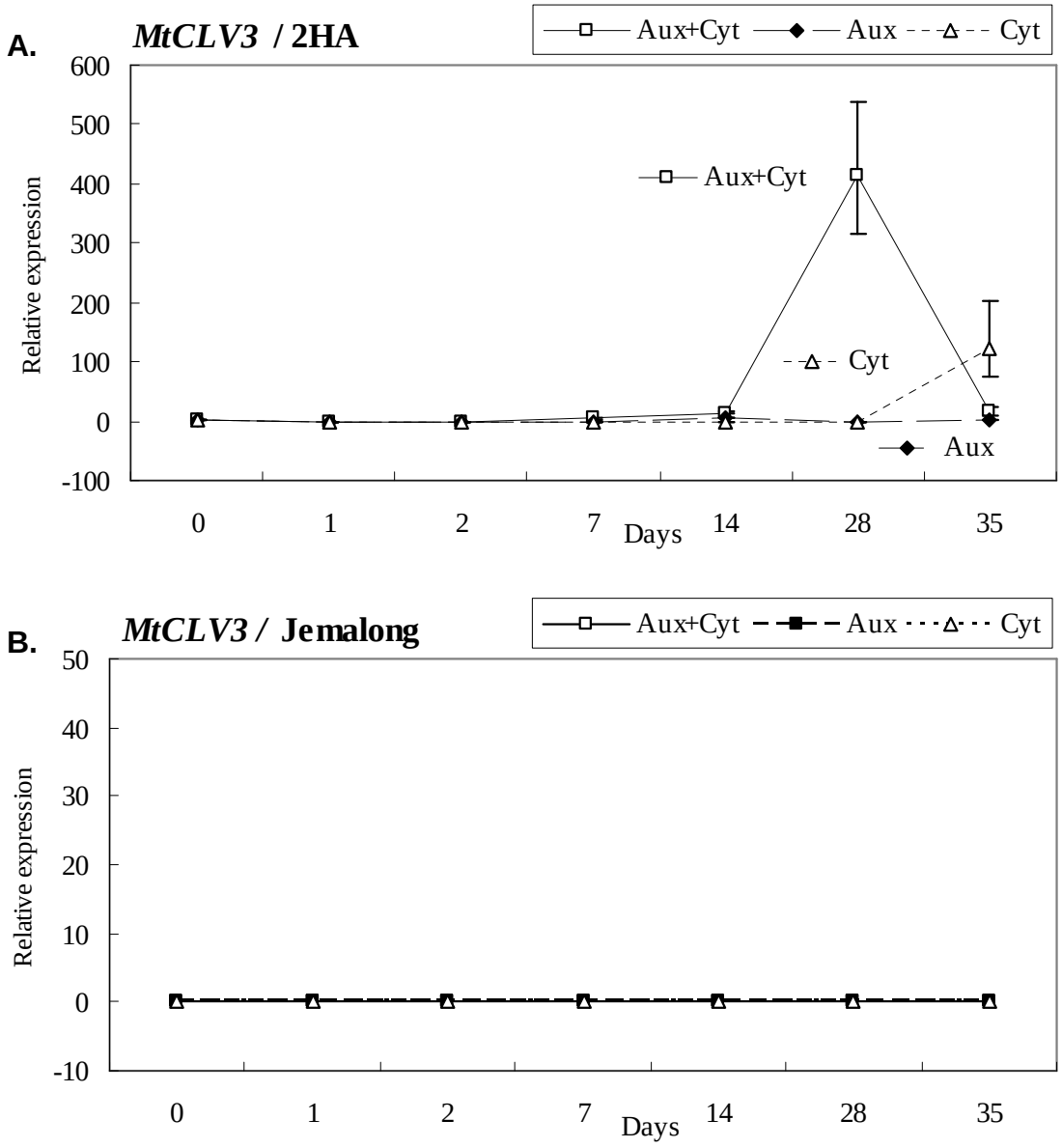
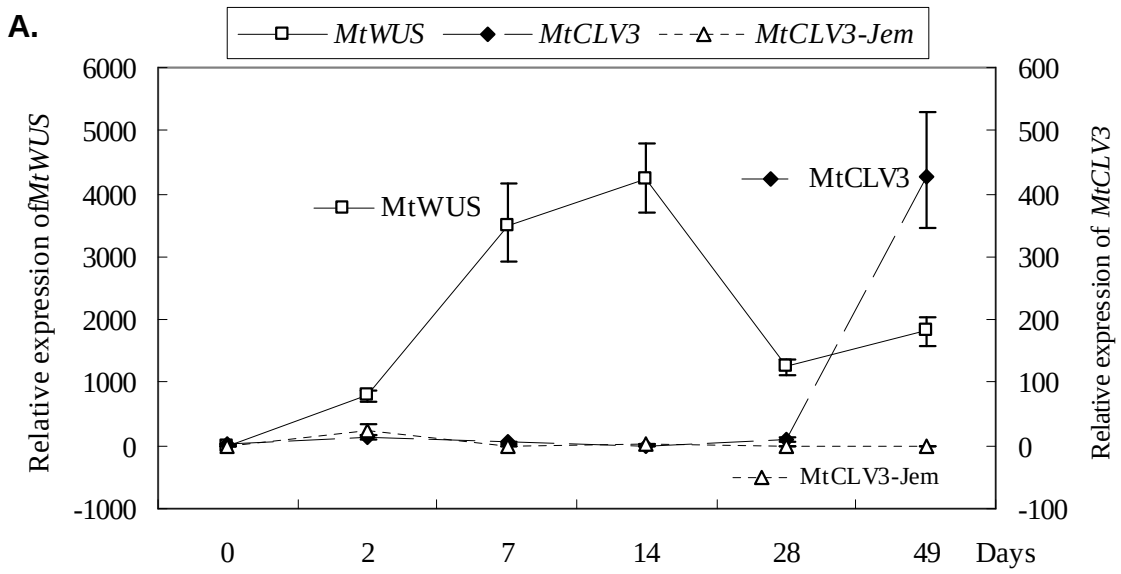


Figure 5.5 Differential timing of *MtCLV3* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP) (Aux+Cyt, □, solid line), auxin (10 µM NAA) (Aux, ◆, dashed line), and cytokinin (4 µM BAP) (Cyt, △, dotted line). A: Expression of *MtCLV3* in 2HA **B:** Expression of *MtCLV3* in Jemalong. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d), and was followed for 35 d. Error bars show SEM.

5.3.3 *MtWUS* and *MtCLV3* Expression and the Induction of Stem Cells

If *MtWUS* expression was associated with a process similar to that acting in the apical meristem then there would be expected to be a relationship with *CLV3* that could be interpreted in a similar way to that in the apical meristem. We determined the pattern of *MtCLV3* expression in relation to *MtWUS* expression. As we can see in Fig. 5.6 *MtCLV3* expression is not initiated until embryos begin to form i.e. until structures are differentiated. Importantly wild-type Jemalong that does not produce embryos does not show *MtCLV3* expression. This expression patterns also occurred in auxin plus cytokinin plus ABA (1 µM ABA) culture in Fig. 5.7 with longer investigating periods and less callus formation (see Chapter 3).



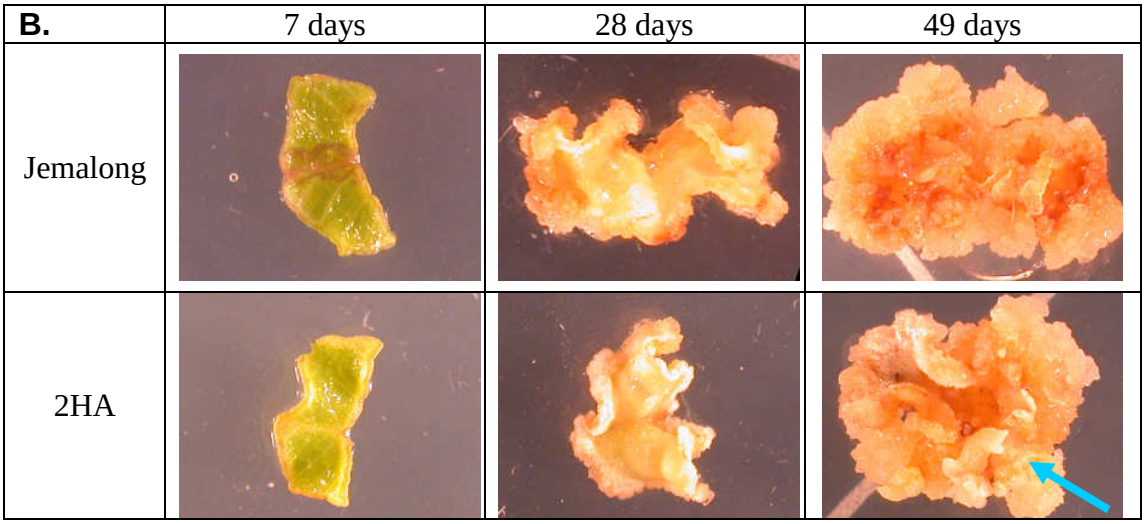


Figure 5.6 Differential timing of *MtWUS* and *MtCLV3* expression in tissue on P4 media containing auxin (10 µM NAA) and cytokinin (4 µM BAP). A: Expression of *MtWUS* in 2HA (□, solid line) and MtCLV3 in 2HA (Aux, ◆, dashed line) and Jemalong (△, dotted line) was followed for 49 d. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d). Error bars show SEM. **B:** Images show the development of calli over the life of the experiment. Blue arrow indicates somatic embryos.

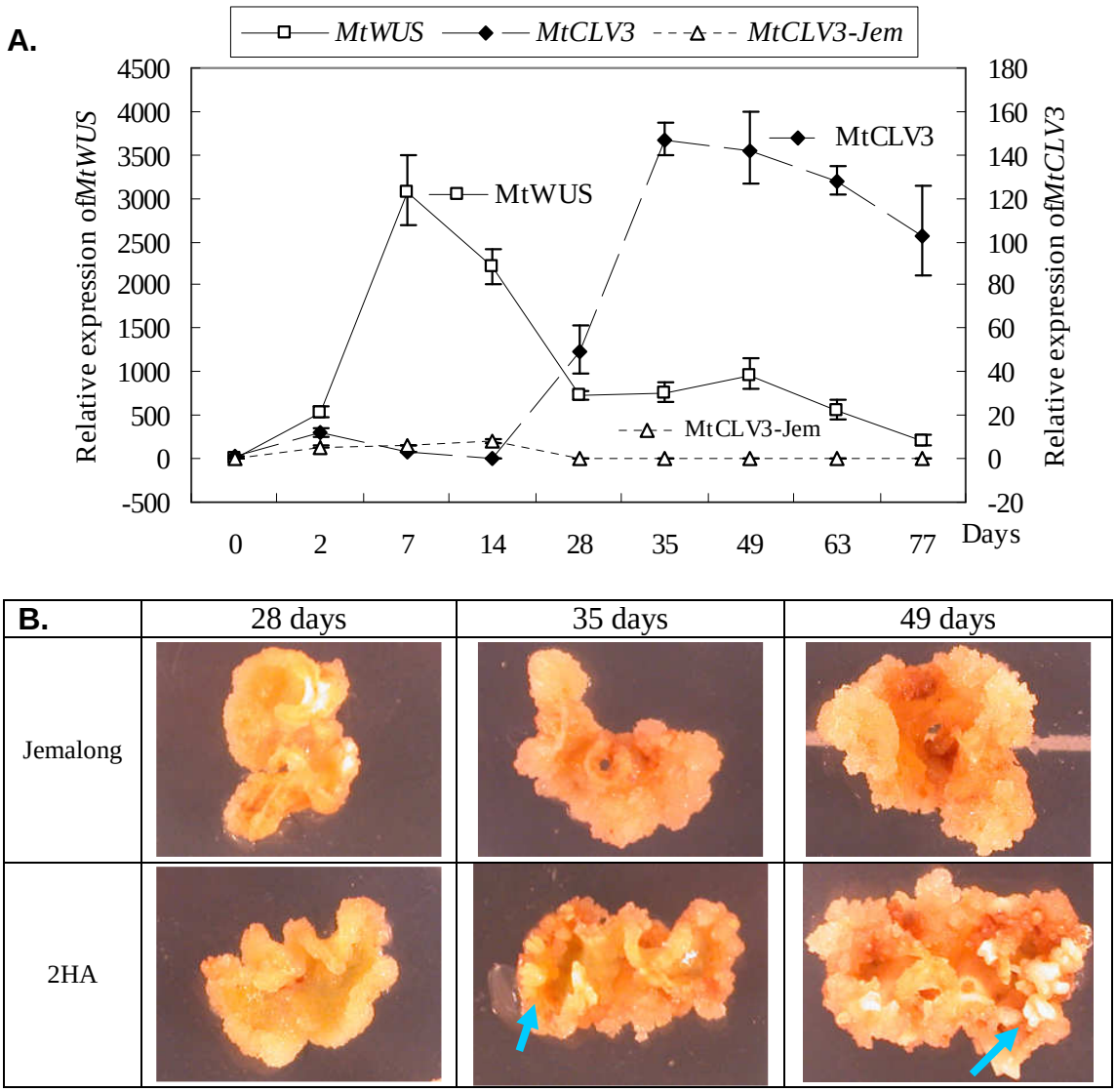


Figure 5.7 Differential timing of *MtWUS* and *MtCLV3* expression in tissue on P4 media containing auxin (10 µM NAA), cytokinin (4 µM BAP) and 1 µM ABA. A: Expression of *MtWUS* in 2HA (□, solid line) and MtCLV3 in 2HA (Aux, ◆, dashed line) and Jemalong (△, dotted line) was followed for 77 d. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d). Error bars show SEM. **B:** Images show the development of calli over the life of the experiment. Blue arrows indicate somatic embryos.

MtWUS appears to express in both callus and somatic embryos, but *MtCLV3* expression may locate specifically to somatic embryos. *MtWUS* has its first expression peak around 7 d, but *MtCLV3* only expresses after 28 d indicating the WUS-CLV3 feedback regulation of the shoot apical meristem may not operate in callus. However, *MtCLV3* reduces the *MtWUS* expression around 49 d in 2HA and correlates with the shoot apical meristem formation in somatic embryos.

5.3.4 *MtWUS* Expression Pattern in Callus Formation and Somatic Embryogenesis

MtWUS expression may be involved in both callus formation and somatic embryo induction. Through the use of promoter-GUS fusion transgenic plants and tissues, *MtWUS* expression in culture can be seen to start very early and expresses throughout the explant except at the cut edges (Fig. 5.8A). *MtWUS* expression then localises in clusters (Fig. 5.8B). Not all the clusters with signals necessarily initiate somatic embryos. The GUS signal located in a cluster of small cells around the newly forming vascular tissue (Fig. 5.8C) may indicate that some cell clusters are not somatic embryo specific but may also be related to vascular development in the callus. However procambium cells associated with the vascular tissue are likely to be the source of at least some somatic embryos (Rose et al., 2009). A strong signal detected in somatic embryos (white arrow in Fig. 5.8D) suggests that somatic embryos can be initiated from some of these clusters.

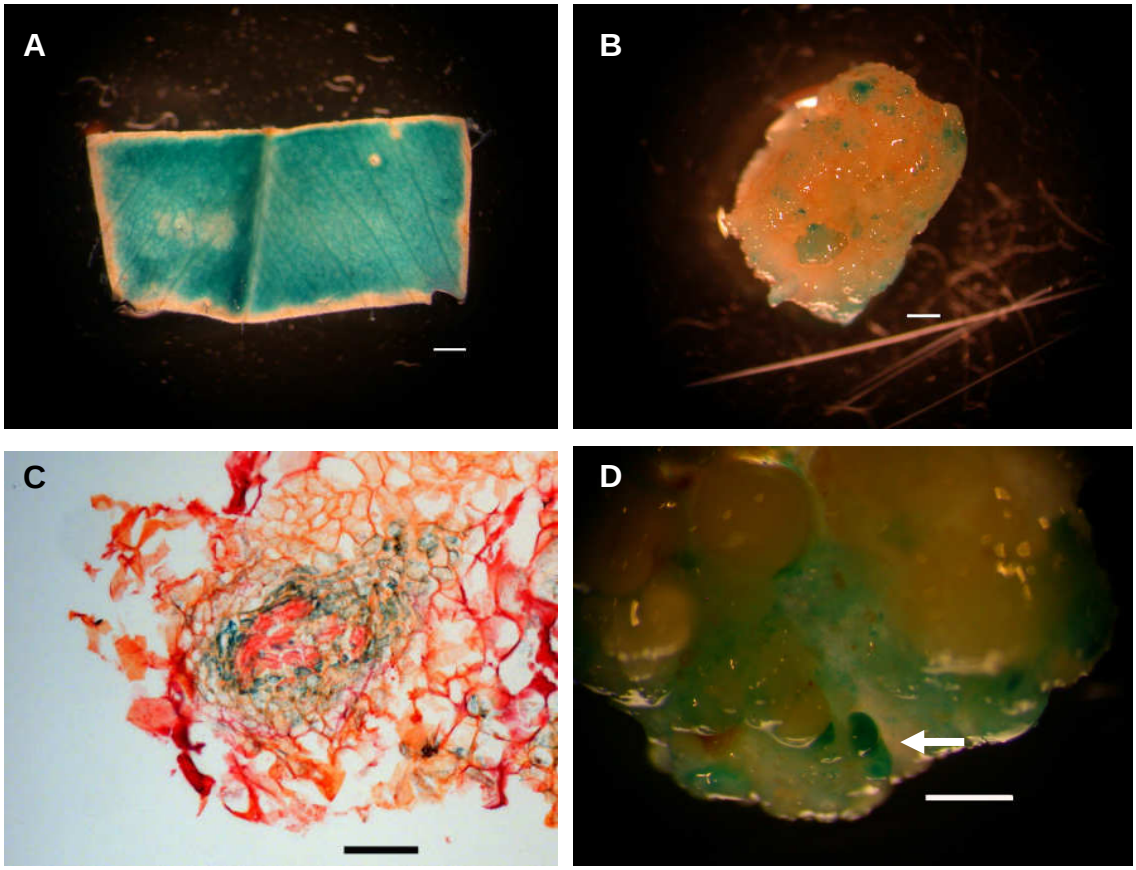


Figure 5.8 pr*MtWUS*::GUS expression in early stages of somatic embryo induction in callus. Blue-green indicates the GUS signal, and the red colour in C was obtained by staining with 1% safranine. The signals were investigated in 72 h cultured explants (A), 4 weeks callus (B), a callus section with the vascular tissue surrounded by a cluster of smaller cells (C), and callus (D) with somatic embryos (white arrow). White Bar = 500 µm, and black Bar = 80 µm.

MtWUS RNA *in situ* hybridisation in 2HA callus with somatic embryos shows that *MtWUS* expression can be visualised in whole globular somatic embryos (Fig. 5.9A) or in sectioned embryos in the callus (Fig. 5.9C). The suspensor-like structures were clearer when embryos were visualised in the callus (Fig. 5.9C). The *MtWUS* expression pattern at later stages of embryo development still requires further investigation.

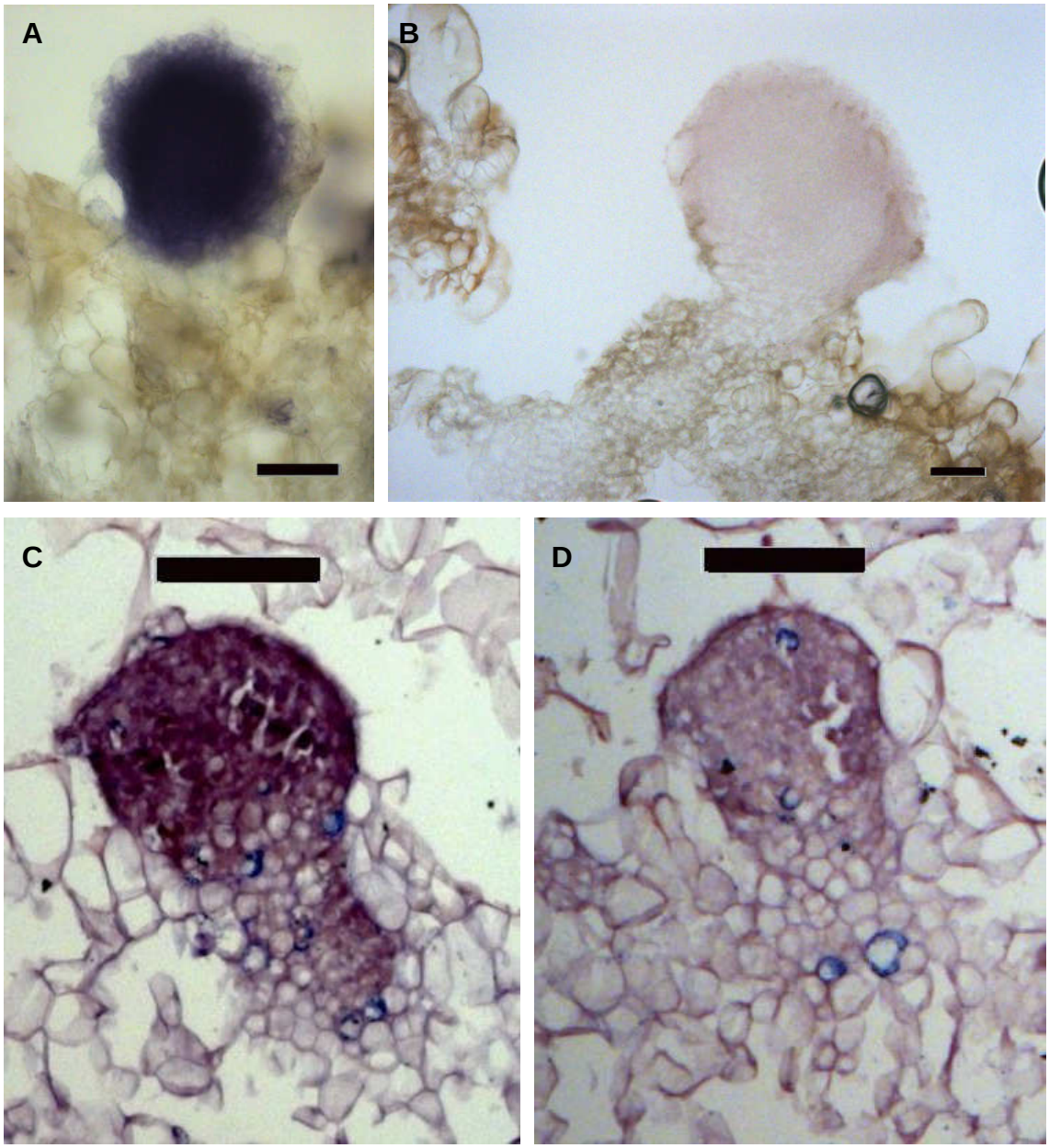


Figure 5.9 *MtWUS* RNA *in situ* hybridisation in somatic embryo induction in callus of 2HA. The signals were investigated in whole globular stage somatic embryos (A to D). A and C are with an anti-sense probe indicating the *MtWUS* signals, and B and D are with a sense for the controls. Bar = 80 µm.

5.3.5 Inducible *MtWUS* RNAi Experiments in Relation to Somatic Embryo Induction and Callus Formation

Dexamethasone-inducible RNAi for *MtWUS* was used to investigate *MtWUS* function in callus formation and somatic embryo induction. The *MtWUS* RNAi transgenic calli were set up in standard medium with dexamethasone (Dex) to trigger the RNAi system and inhibit the *MtWUS* expression. The empty vector transgenic callus described in 5.2

was used as control. The *MtWUS* RNAi transformed calli produced little callus growth with only 12.7% increase compared to 104.4% for the control after 28 d growth (Fig. 5.10). Somatic embryos form in control callus after 28 d (blue arrow in Fig. 5.10) but not in *MtWUS* RNAi callus. These results indicate that *MtWUS* is essential for both callus formation and somatic embryo induction.

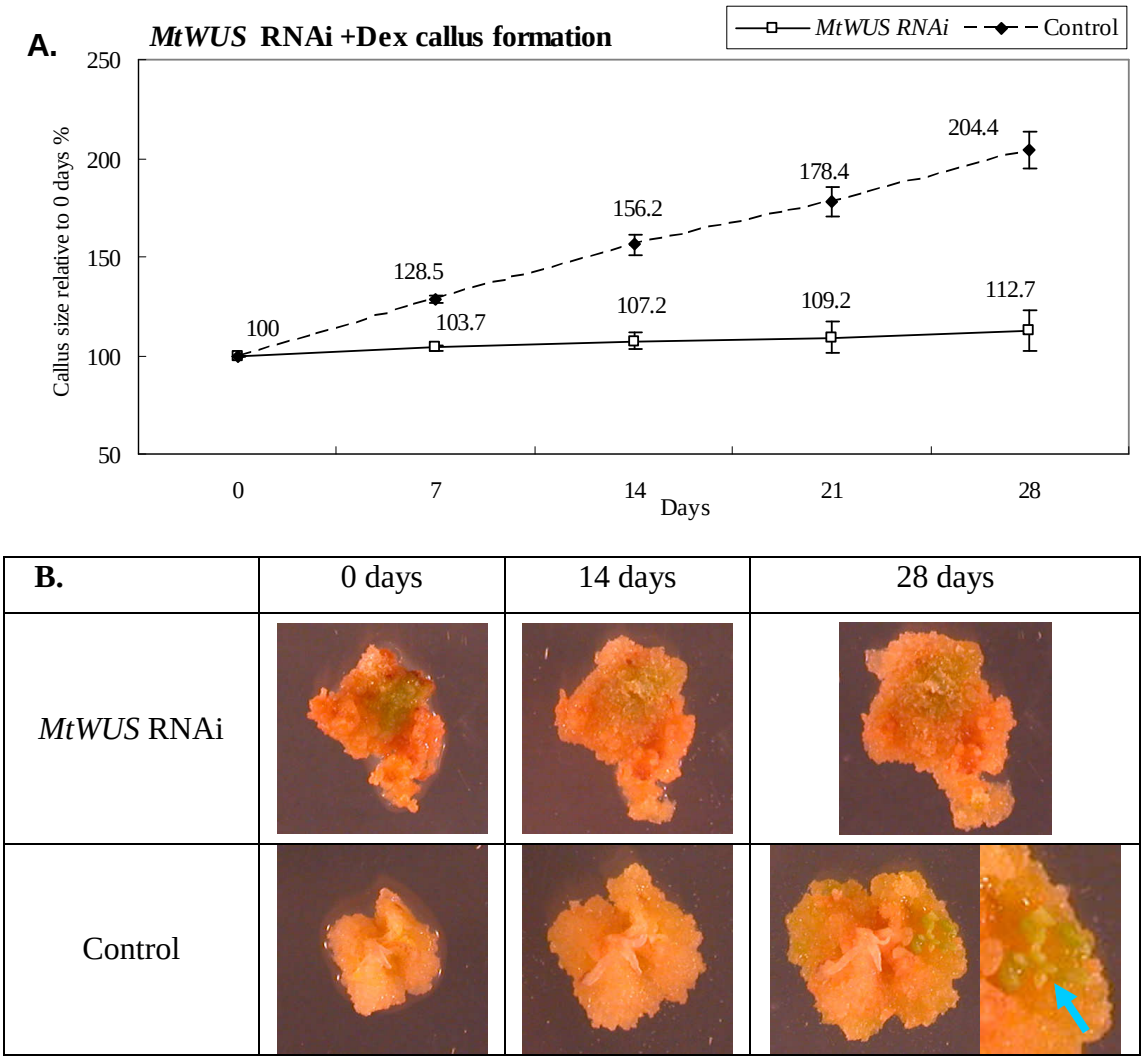


Figure 5.10 Differential timing of dexamethasone (Dex) -induced RNAi callus size of *MtWUS* and empty vector control in tissue on P4 media containing auxin (10 µM NAA), cytokinin (4 µM BAP) and 10 µM dexamethasone. A: Calli size of *MtWUS* (□, solid line) and empty vector control (◆, dashed line) was followed for 28 d. Calli size, measured from callus images by software Image J, is shown relative to that at the beginning of the experiment (0 d) set as 100. Error bars show SEM. **B:** Images show the development of calli over the life of the experiment. Blue arrow indicates somatic embryos.

5.3.6 *MtCLV3* Expression Pattern in Callus Formation and Somatic Embryogenesis

The *MtCLV3* expression in somatic embryos was investigated in promoter-GUS fusion transgenic tissues (Fig. 5.11) and showed no expression in early stage embryo and callus (white arrow in Fig. 5.11A) but in later stage somatic embryos (red arrow in Fig. 5.11A). After the cotyledon stage, the GUS signals accumulated around the shoot meristem (red arrow in Fig. 5.11B) and the base of the cotyledons (red arrow in Fig. 5.11C), and not in the root and young leaves. These results correlate with qRT-PCR data in 5.3.1 and 5.3.3 on the expression of *AtCLV3*.

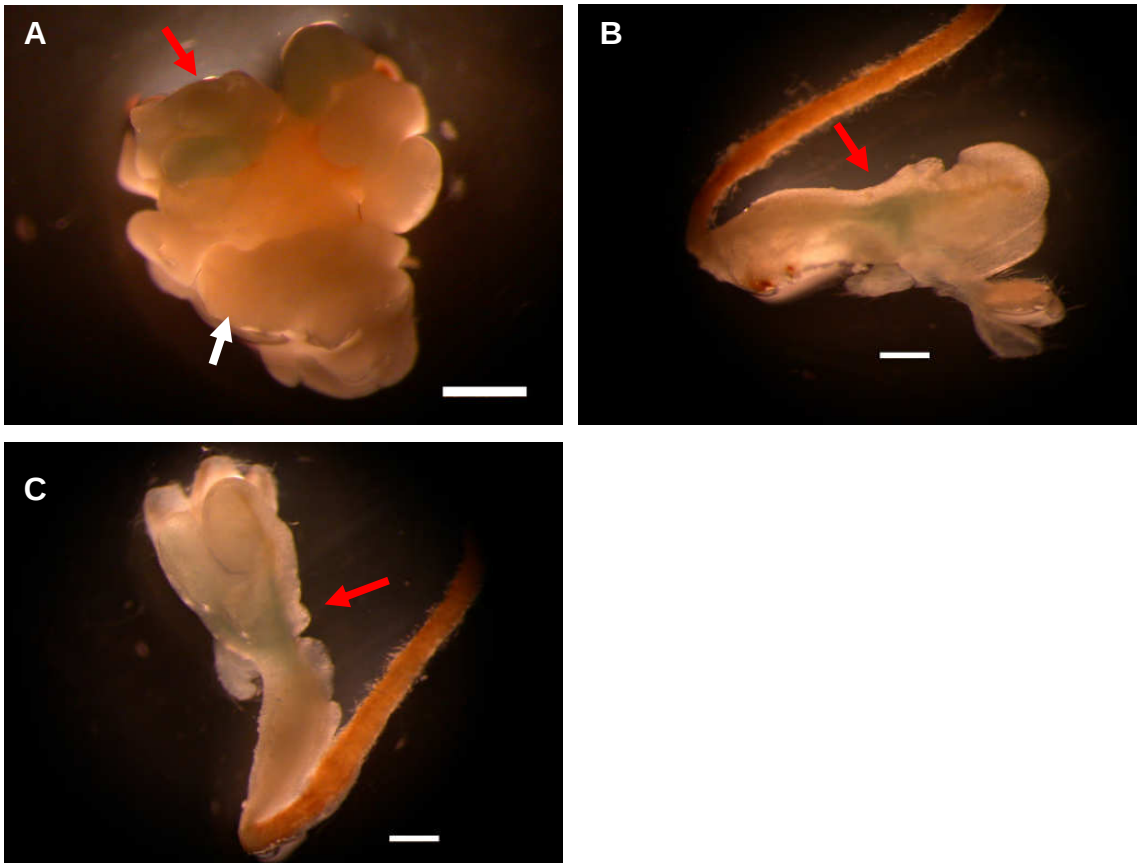


Figure 5.11 pr*MtCLV3*::GUS expression in somatic embryos and regenerated plantlet. The signals were investigated in callus with different stage somatic embryos (A, white arrow shows earlier stage, and red arrow shows later stage) and germinated plantlet (B and C). Red arrows indicates the expression region around the shoot meristem. Bar = 500 μm.

5.3.7 CLV1-like Gene *SUNN* Expression Pattern *in planta* and in Cultured Tissue

The *SUNN* gene was identified from the *sunn* mutant from the non-embryogenic A17 (Penmetsa et al., 2003) and is most similar to *AtCLV1*. *SUNN* expression in 2HA (Fig. 5.12) is higher in the leaves than the shoot apex, developing flower and somatic embryos. In the leaves, the expression increases after the leaf unfolds and matures (From L0 to L3 in Fig. 5.12B) and suggests a relationship to leaf function.

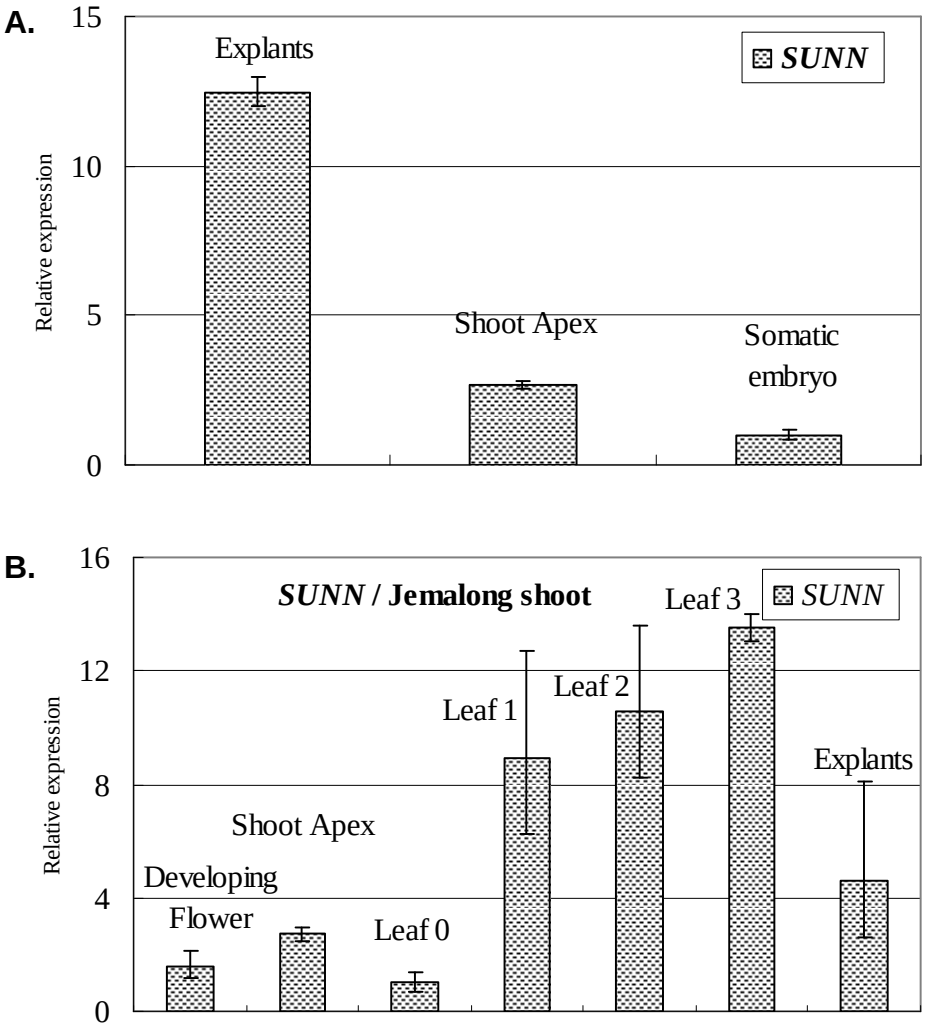


Figure 5.12 *SUNN* expression in different tissue of 2HA and Jemalong. A: Expression of *SUNN* in 2HA explants, shoot apices, and somatic embryos. **B:** Expression of *SUNN* in Jemalong shoot apices, developing flowers, different stages of leaf development, and explants. Leaf 0 indicates unopened initiated leaf, and Leaf 1 to Leaf 3 indicates the fully opened leaf from tip to base. Expression, measured by qRT-PCR, is shown relative to the expression level of somatic embryos (A) and the leaf 0 (B). Error bars show SEM.

Time course studies of *SUNN* expression in cultures incubated in auxin plus cytokinin in both Jemalong and 2HA show the expression dropping for 2 d but then expression starts to increase after 14 d as callus growth increases (Fig. 5.13). However during culture, expression does not increase above explant levels.

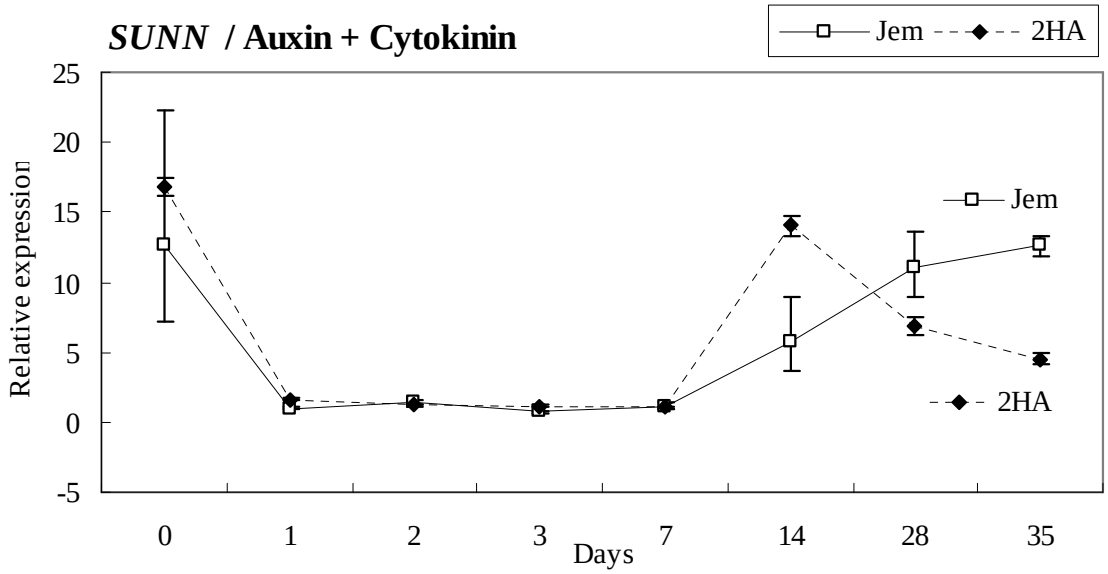


Figure 5.13 Differential timing of *SUNN* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP). Expression of *SUNN* in Jemalong (Jem, □, solid line) and 2HA (2HA, ◆, dashed line) was followed for 35 d. Expression, measured by qRT-PCR, is shown relative to the expression level of 1 d of 2HA in culture. Error bars show SEM.

5.3.8 CLV1-related Gene *MtRLK1* Expression Pattern *in planta* and in Cultured Tissue

MtRLK1 shows low expression in leaf explants, and higher expression in shoot apices and somatic embryos (Fig. 5.14). In time course studies in Jemalong and 2HA cultures incubated with auxin and cytokinin, *MtRLK1* expression increases after 2 d and approaches a high and stable level after 14 d and correlates with callus formation (Fig. 5.15). The expression patterns are similar in Jemalong and 2HA.

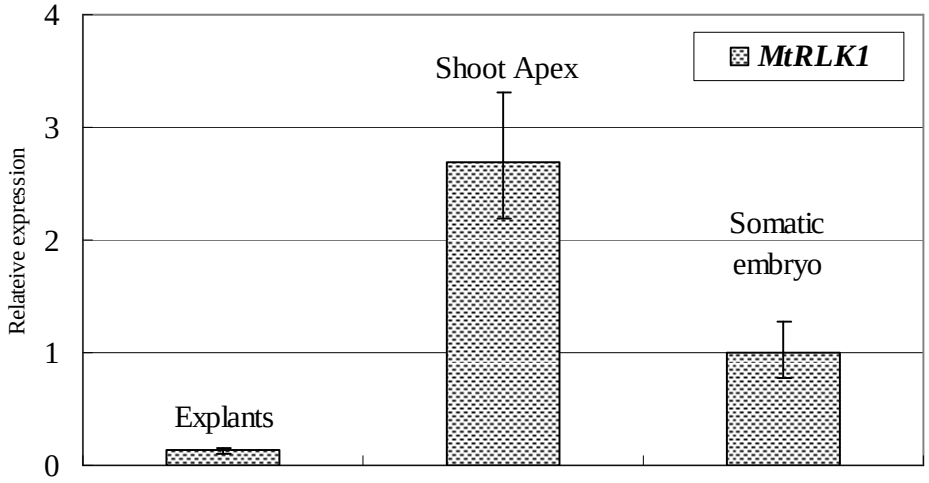


Figure 5.14 *MtRLK1* expression in different tissue of 2HA. Expression of *MtRLK1* in 2HA explants, shoot apices, and somatic embryos. Expression, measured by qRT-PCR, is shown relative to the expression level of somatic embryos. Error bars show SEM.

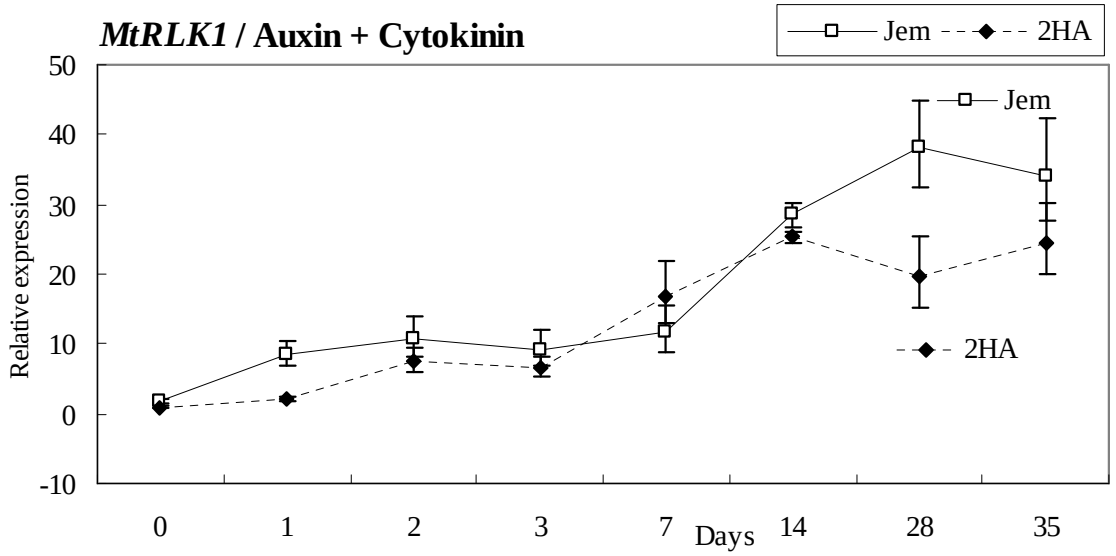


Figure 5.15 Differential timing of *MtRLK1* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP). Expression of *MtRLK1* in Jemalong (Jem, □, solid line) and 2HA (2HA, ◆, dashed line) was followed for 35 d. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d). Error bars show SEM.

5.3.9 *MtCLV2-1* Expression Analysis

We also investigated the expression of the CLV2-like gene *MtCLV2-1* in Jemalong and 2HA. The *MtCLV2-1* gene shows higher expression in the shoot apex than leaf explants, and expression is higher in callus in the presence of auxin plus cytokinin, whether or not

it is embryogenic (Fig. 5.16). In time course studies of culture in the presence of auxin plus cytokinin treatments, both Jemalong and 2HA had similar expression patterns for *MtCLV2*, which starts to express after 2 d and continues to increase until 49 d (Fig. 5.17).

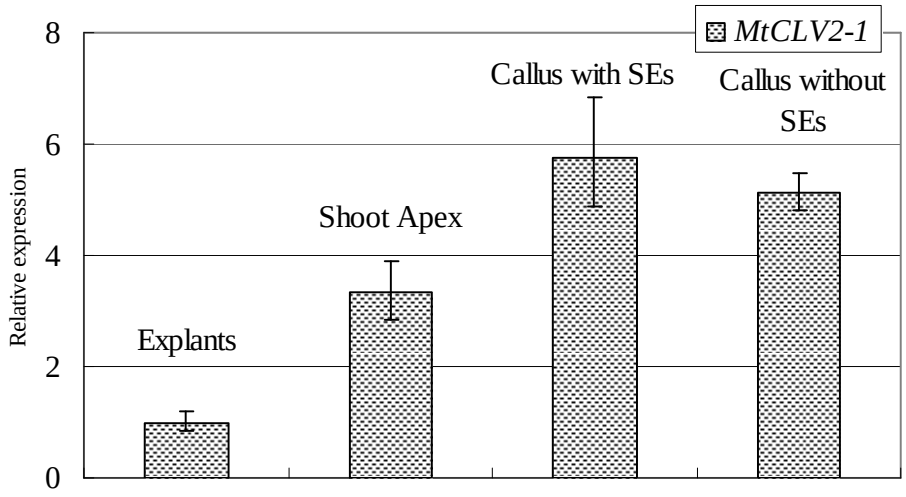


Figure 5.16 *MtCLV2-1* expression in different tissue of 2HA. Expression of *MtCLV2-1* in 2HA explants, shoot apices and callus plus somatic embryos (SEs) and callus without somatic embryos. Expression, measured by qRT-PCR, is shown relative to the expression level of explants. Error bars show SEM.

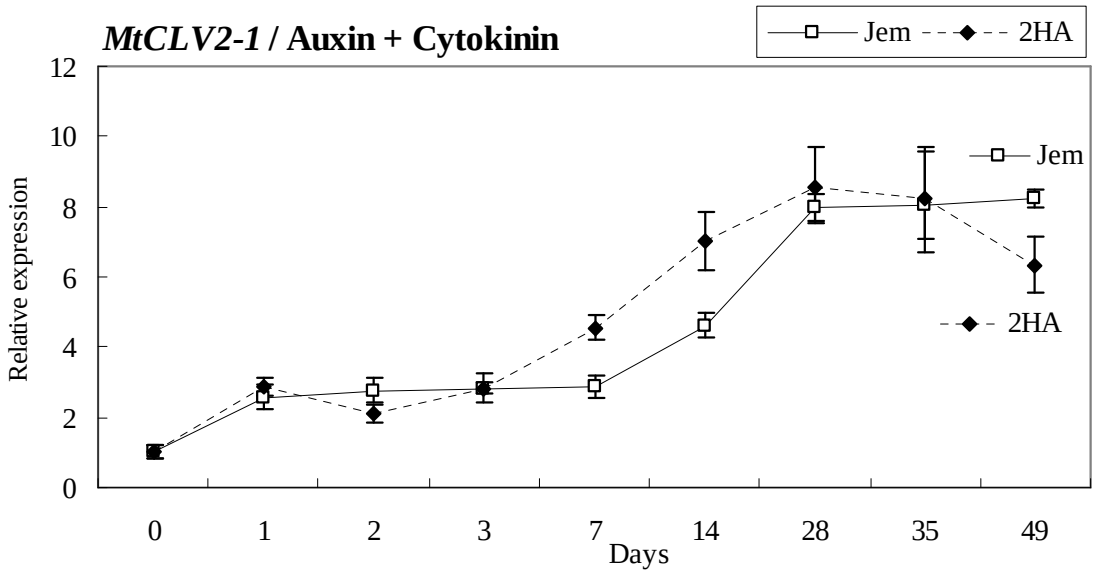


Figure 5.17 Differential timing of *MtCLV2-1* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP). Expression of *MtCLV2-1* in Jemalong (Jem, □, solid line) and 2HA (2HA, ◆, dashed line) was followed for 49 d. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d). Error bars show SEM.

5.4 DISCUSSION

The best candidate for the *M. truncatula* WUS gene was obtained by bioinformatics as described in Chapter 4. However to obtain supporting evidence that the putative *MtWUS* was an ortholog of *AtWUS* the expression pattern of *MtWUS* was initially examined in the intact plant prior to studying the expression in callus formation and somatic embryogenesis.

In the intact plant, *MtWUS* was expressed in the shoot apex (meristem and leaf primordium) and the early stages of flower initiation, but it was not expressed in leaves or roots. The expression pattern is similar to *AtWUS* in the shoot meristem and the flower primordium. The promoter GUS studies also indicate that there is strong expression in the shoot apex but there appears to be some expression in leaf primordia, unlike *AtWUS*.

However, it seems highly likely that *MtWUS* is the *AtWUS* ortholog and subsequent data supports this interpretation, particularly given the recent study by Gordon et al. (2007) on *in vitro* shoot formation.

In the *M. truncatula* SE system *MtWUS* expression is induced in the presence of auxin plus cytokinin and also cytokinin alone, but not by auxin alone. This is an important result consistent with what is known for *WUS* and cytokinin relationships in the regulation of *WUS* in the Arabidopsis meristem (Leibfried et al., 2005). Further, Gordon et al. (2007) have shown cytokinin-induced *AtWUS* expression in shoot induction in *in vitro* cultures.

What is suprising is the rapid onset of MtWUS expression visualised as GUS staining across the whole leaf explant (Fig. 5.8A). Recently however, whole explant studies in our laboratory with explants cleared and stained with fuchsin has clearly revealed cell proliferation all over the explant, emanating from near the leaf veins (Appendix 8).

During callus formation, groups of small cells with *MtWUS* expression cluster together, and are scattered around the callus. These clusters of cells are likely the source of cells that form embryos but some will probably form vascular cells or callus cells, and

MtWUS expression is linked to these processes. However as the embryogenic callus develops the *MtWUS* expression is confined to the somatic embryos. These results indicate that *MtWUS* expresses in both undifferentiated cells in the callus and in the somatic embryo. This pattern is similar to *AtWUS* where it is expressed in callus induced by cytokinin, and the expression increasingly localizes in the differentiating shoot. The RNAi data also supports a *MtWUS* requirement for callus formation and somatic embryo induction, which suggests that it also has a function to maintain undifferentiated stem cells like *AtWUS*.

In the globular stage somatic embryo, *MtWUS* expression occurred throughout the whole embryo which is not found in Arabidopsis zygotic embryos (Mayer et al., 1998). However there are two points to note here: the hormonal environment is quite different in the somatic embryo developing in embryogenic callus and the *M. truncatula* embryo is not likely to be identical to Arabidopsis in its developmental strategy. As can be seen in Fig. 5.9 as the somatic embryo develops *MtWUS* tends to localise towards the apical pole.

The data obtained in the *M. truncatula* SE system gives a broader perspective to *WUS* function. Identifying the ortholog of *M. truncatula* will also facilitate an understanding future work on embryo and shoot development in *Medicago*.

The gene we have designated *MtCLV3* is similar to *AtCLV3* in peptide structure and in expression patterns. *MtCLV3* also expresses in the shoot apex but not in flowers or leaves. It does not express in callus but is expressed in the shoot regions of later stage somatic embryos. We suggest that *MtCLV3* is the ortholog of the *AtCLV3* gene in *M. truncatula*.

From the bioinformatics and expression data, the promoter-GUS studies, and the RNAi studies it would seem that the WUS gene is “hijacked” in the callus system as a component of the induction of totipotent stem cells that serve as the progenitors of the embryos. The CLV3 expression does not commence until the WUS-CLAVATA feedback loop starts to operate in a similar way to that characteristic of Arabidopsis. A schematic of how the *WUS* and *CLV3* appear to act *in vitro* is shown diagrammatically in Fig. 5.18.

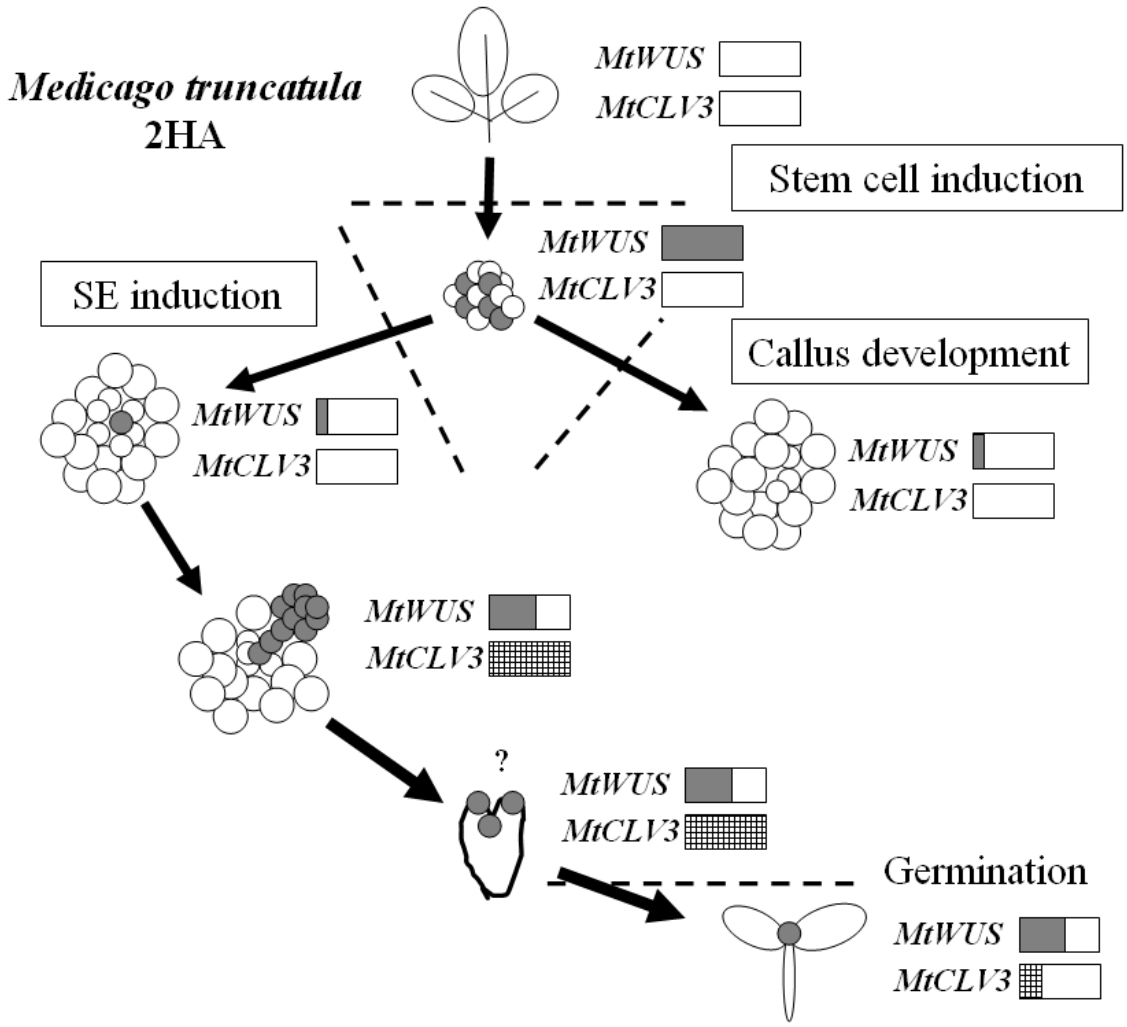


Figure 5.18 Diagram of the expression levels and patterns of *MtWUS* expression and expression levels of *MtCLV3* in somatic embryogenesis *in vitro*. Gray colour indicates the relative expression of *MtWUS* and nets indicates the relative expression of *MtCLV3*. Question mark indicates the region of *MtWUS* expression at this stage is still unclear.

Because of what is known of the WUS-CLAVATA feedback loop (Chapter 4) the expression of CLV1- and CLV2- like genes were investigated. Through the bioinformatics in Chapter4, *SUNN* and *MtRLK1* were selected for investigation.

The *SUNN* gene has a similar protein structure to *AtCLV1* and *GmNARK*, and its function is similar to *GmNARK* (Searle et al., 2003), influencing nodulation but not acting like *AtCLV1* to increase shoot meristem size (Penmetsa et al., 2003). *SUNN* has high expression in leaves, especially in mature leaves, and some expressions in callus but little in somatic embryos. The expression patterns show no difference between

somatic embryogenic and non-somatic embryogenic callus indicating this gene is not a key regulator of somatic embryo induction. This is consistent with the current understanding of auto-regulation in legumes. There is little evidence to support a role for *SUNN* in SE.

The CLV2-like gene *MtCLV2-1* was investigated and is expressed in the shoot apex and callus in both Jemalong and 2HA. The *MtRLK1* gene has similar expression patterns and levels in both Jemalong and 2HA cultures indicating this gene is not related to the enhanced somatic embryo induction in 2HA. Nevertheless it is important to note that both genes are induced by auxin plus cytokinin and expression continues to increase in the culture period. This means such genes are expressed in the period where SEs are present. However the key gene in the SE induction phenomenon appears to be the cytokinin-induced *WUS*.

The significance of the *WUS* data is reinforced from what is known of the *MtSERF1* transcription factor (Appendix 6). It has WUS binding sites in its promoter and cytokinin-induced *WUS* is likely essential for *MtSERF1* expression.

CHAPTER 5 Expression of the *MtWUSCHEL* and *CLAVATA* Family Genes in Relation to Induction of Somatic Embryogenesis

CHAPTER 6

Investigation of *WOX* Family Gene Expression in *M. truncatula* in Relation to Root Formation and Somatic Embryo Formation

6.1 INTRODUCTION

Development in somatic embryogenesis and zygotic embryogenesis is similar from the globular stage but there may be some differences in the early segmentation pattern (Williams and Maheswaran, 1986). Therefore gene expression patterns in the two processes would be expected to have some similarity. The *WOX* (*WUSCHEL* related homeobox) gene family members reveal early embryonic patterning events in *Arabidopsis* (Haecker et al., 2004). Therefore it is of interest to investigate these genes in the somatic embryogenesis and root formation process as both markers and as potential contributors to meristem formation *in vitro*.

The *AtWOX5* sequence was identified in 2004, and expression was detected in the embryo root quiescent centre from the early globular stage. Expression was also detected in the vascular primordium in the heart stage embryonic cotyledon and decreased after the late heart stage (Haecker et al., 2004). *AtWOX5* expression has also been detected in the root quiescent centre using the *AtWOX5* promoter region fused with reporter genes (GUS or GFP) (Blilou et al., 2005; Xu et al., 2006).

AtWOX5 is related to auxin regulation mechanisms in the root meristem, and plays a similar role to *WUSCHEL* in the OC in maintaining cells in an undifferentiated state. It has been shown that *AtWOX5* is essential for maintaining the root QC and the undifferentiated state of the distal stem cells (Gonzali et al., 2005; Sarkar et al., 2007). There is also some evidence that the *AtWOX5* gene can also maintain the stem cell niche in the shoot apical meristem as *wus* mutant studies indicated *AtWOX5* and *AtWUS* could supply similar signals for stem cell niche regulation (Sarkar et al., 2007).

A similar gene to *AtWOX5* called *OsWOX9* is also found in rice, which has similar protein structure and also expresses in the QC of the root apical meristem (Kamiya et al., 2003). *OsWOX9* also expresses very early during adventitious root initiation. Although *OsWOX9* expression occurs in roots, overexpression of *OsWOX9* does not induce more adventitious root formation, instead sometimes it can even suppress adventitious root initiation. It causes multiple shoot phenotype, the same phenotype as produced by *AtWUS* overexpression in rice (Kamiya et al., 2003).

The *WOX5* gene in *M. truncatula* has been investigated in roots initiated in culture (Chen et al., 2006; Imin et al., 2007). It shows a higher expression in the root tip than the elongation zone. The *MtWOX5* expression levels are higher in auxin-induced root cultures or auxin plus cytokinin cultured callus than in normal roots. The detailed expression patterns in root and somatic embryogenesis has not been investigated.

There have been preliminary investigations of *WOX* family genes in *M. truncatula*. One gene TC102100 (*MtWOX4*) was investigated in *M. truncatula* in 2003 by Dibley. It shows a similar protein structure to *AtWUS*, but is most similar to the *AtWOX4* gene. The *AtWOX4* gene was identified in 2004 by Haecker et al., but its expression patterns were not investigated. *MtWOX4* was also investigated by Imin et al., (2007) and showed that *MtWOX4* is highly expressed in the elongation zone compared to the root tip.

In *M. truncatula* the likely orthologs to *AtWOX4* and *AtWOX5* were identified by bioinformatics. This Chapter investigates the hormone regulated expression of these genes in relation to the root organogenesis and the induction of somatic embryogenesis. These studies complement those on *MtWUS*.

6.2 MATERIALS AND METHODS

The culture processes were described in Chapter 2. Other processes such as tissue collection, cDNA preparation, qRT-PCR analysis, transgenic plant preparation, GUS staining, tissue section, RNA *in situ* hybridisation were described in Chapter 5.

6.2.1 Influences of Auxin and GA on pr*MtWOX5*::GUS Transgenic Roots

These experiments were designed to investigate the influence of auxin and GA on the *MtWOX5* expression pattern in cultured roots. 15 weeks old pr*MtWOX5*::GUS transgenic tissues from10 µM NAA cultures (which included roots and root primordia) were cultured in P4 medium with different concentration of NAA (0, 1, 5, and 10 µM) or GA_3 (0, 0.1, 1, 5, and 10 µM). The root morphologies were assessed after 7 days and the GUS signals were obtained through the GUS staining process (described in Chapter 5).

6.2.2 Preparation of the DIG-Labelled RNA Probes for *in situ* Hybridisation

The preparation procedure was described in 5.2.11. The primer sequences used for *MtWOX5* RNA probes preparation are given in Table 6.1.

PCR sequence	Primer name	Sequence (5' → 3')
1	5Wf1	GTAAAAACATCTAGAATTGAAATATGG
	5Wr2	TCCTAAACATTTTTCATATTATGCT
2	insitu1WOX5T7	GAGGCCGCGTCATCTAGAATTGAAATATGG
	insitu11WOX5T3	ACCCGGGGCTAAACATTTTTCATATTATGCT
3	T7 primer	TTATGTAATACGACTCACTATAGGGAGGCCGCGT
	T3 primer	AATTAACCCTCACTAAAGGGAGACCCGGGGCT
	SP6 primer	CCAATTTAGGTGACACTATAGAAGTACCCGGGGCT

Table 6.1 Primer list for *MtWOX5* RNA probe preparation. Sequence underlining indicates sequences for T7 primer or T3 and SP6 primer.

6.3 RESULTS

6.3.1 *MtWOX5* Expression Pattern *in planta* and *in vitro*

AtWOX5 is expressed in the quiescent centre of the root meristem (Sarkar et al., 2007) and in the developing embryo (Haecker et al., 2004). *MtWOX5* was expressed in cultured roots and in the somatic embryo. There is little if any expression of *MtWOX5* in the shoot apex, developing flower or leaf (Fig. 6.1). The expression data are consistent with the *AtWOX5* expression patterns.

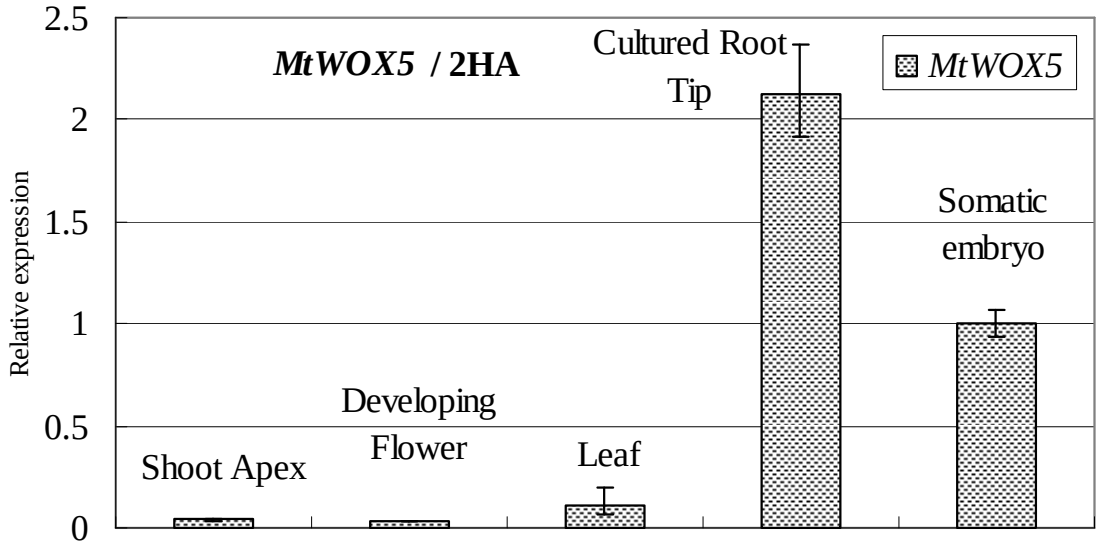


Figure 6.1 *MtWOX5* expression in different tissue of 2HA. Expression of *MtWOX5* in 2HA shoot apices, developing flowers, mature leaves, cultured root tips, and somatic embryos. Expression, measured by qRT-PCR, is shown relative to the expression level of somatic embryos. Error bars show SEM.

6.3.2 *MtWOX5* Expression in Cultured Tissue

It is known that *MtWOX*5 is expressed in culture (Chen et al., 2006; Imin et al., 2007) but it is important to understand its time course of expression to ascertain its potential relationship to root induction from procambial cells that we have previously described (Rose et al., 2006b). *MtWOX5* expression is induced by 2 d and is clearly auxin dependent (Fig. 6.2). The expression increases more when roots are visible to the naked eye. There was a small amount of expression in the presence of auxin plus cytokinin and no expression in the presence of cytokinin, indicating cytokinin inhibits expression.

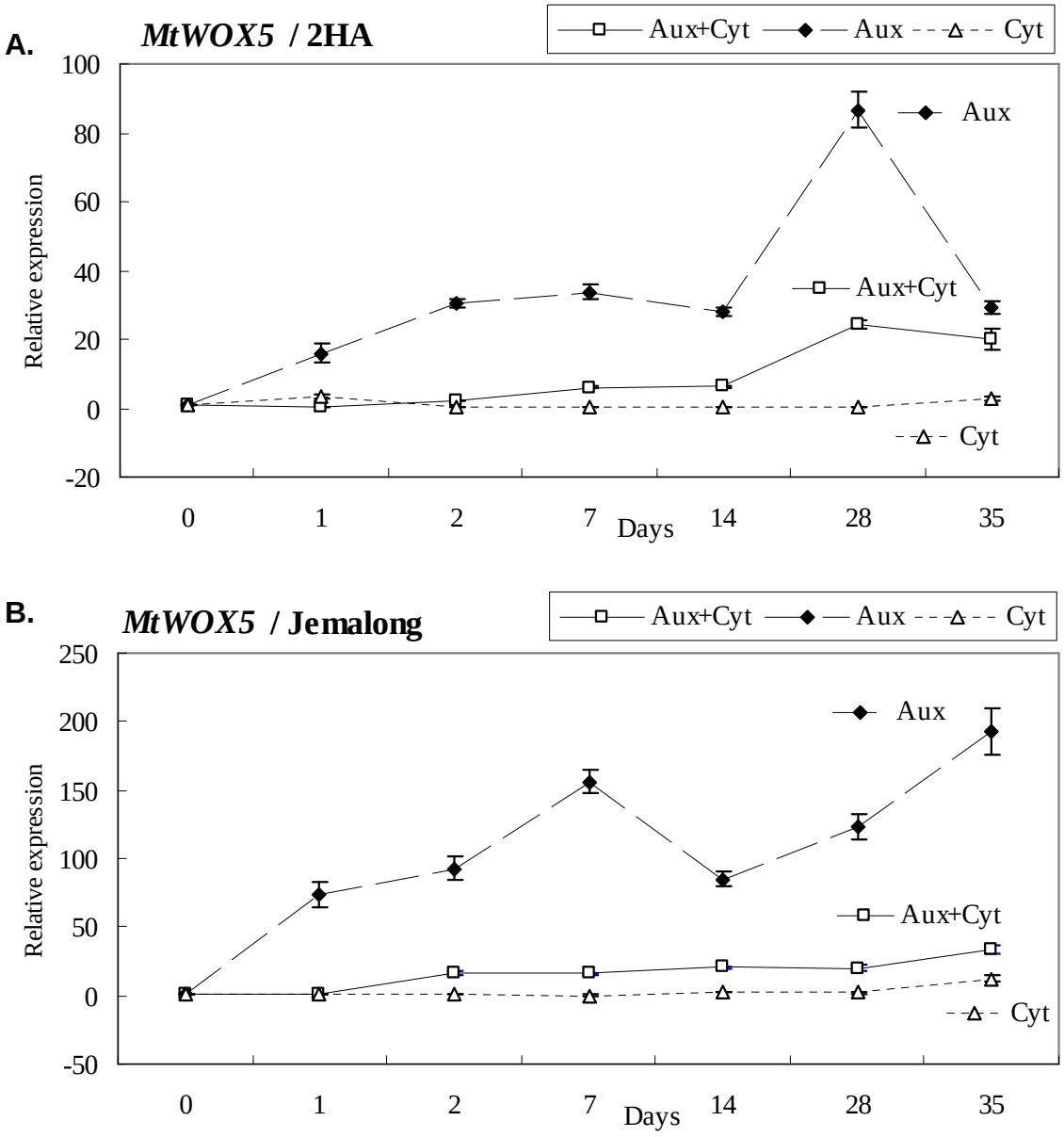


Figure 6.2 Differential timing of *MtWOX5* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP) (Aux+Cyt, □, solid line), auxin (10 µM NAA) (Aux, ◆, dashed line), and cytokinin (4 µM BAP) (Cyt, △, dotted line). A: Expression of *MtWOX5* in 2HA **B:** Expression of *MtWOX5* in Jemalong. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d), and was followed for 35 d. Error bars show SEM.

6.3.3 *MtWOX5* Expression Pattern in *de novo* Root Development

The *MtWOX5* RNA *in situ* hybridisation data in relation to the formation of root primordia formation is shown in Fig. 6.3. The arrow labelled 1 shows centres of expression in vein derived cells that emanate from the procambial cells. The arrow labelled 2 is the root primordia. The arrow labelled 3 in Fig. 6.3E is the root meristem

and the arrow labelled 4 is vascular tissue.

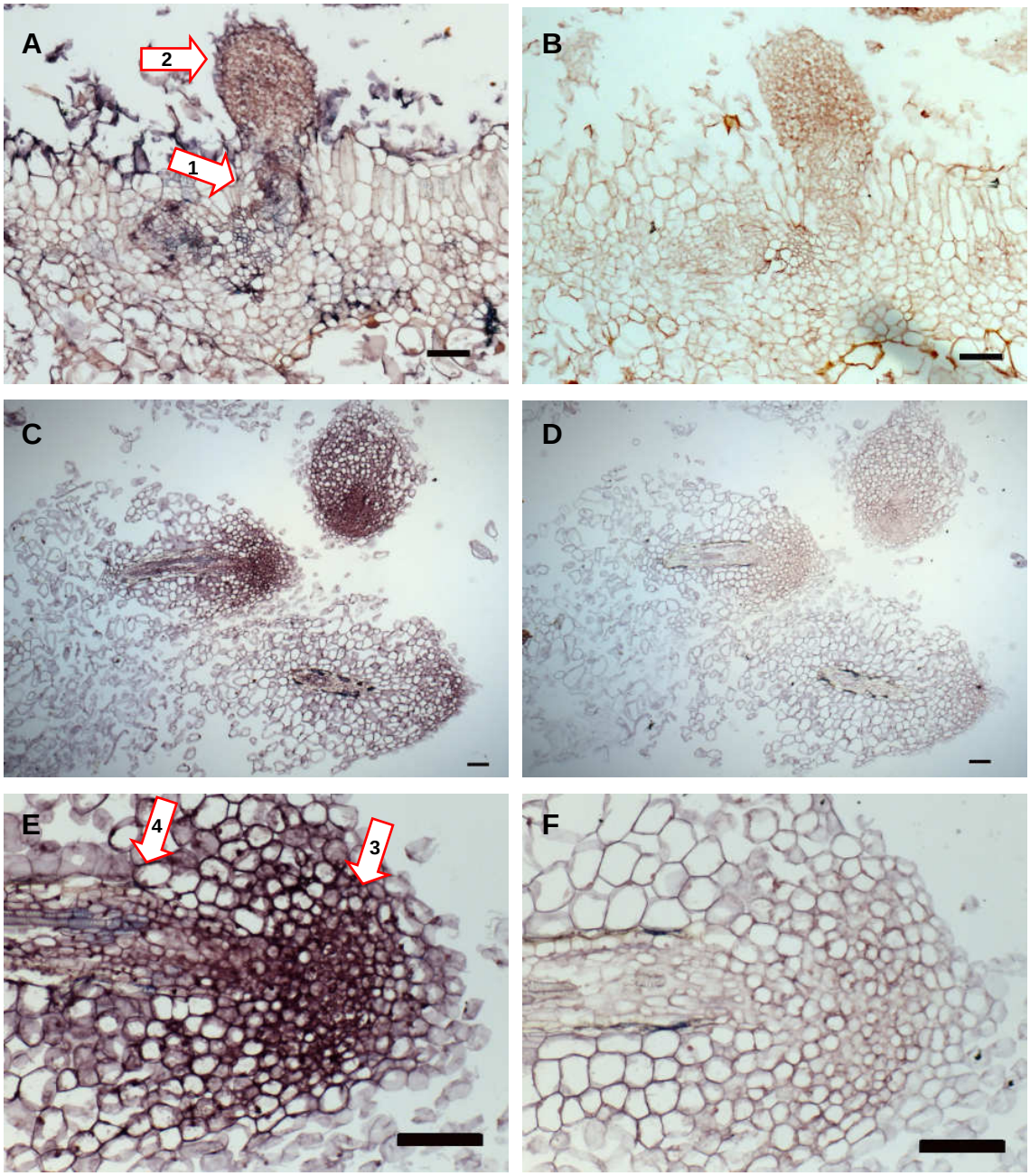


Figure 6.3 *MtWOX5* RNA *in situ* hybridisation in auxin-induced *de novo* root formation. (A), (C) and (E) use an anti-sense probe show the location of the RNA expression, and (B), (D) and (F) are with a sense probe for the controls. Bar = 80 μm.

Further investigation by pr*MtWOX5*::GUS in the root apex after longer auxin culture (Fig. 6.4A) shows two expression regions around the meristem (Fig. 6.4B, black and red arrows) and can be identified more clearly in the mature root apex (Fig. 6.4C). The *MtWOX5* expresses more strongly in stem cells (red arrow in Fig. 6.4C) and columella cells (black arrow in Fig. 6.4C) of the root meristem. The longitudinal section of the

root indicates most of the *MtWOX5* expression is in the stele of the maturation zone in pericycle cells, but there is also some expression in the vascular tissue (Fig. 6.4D).

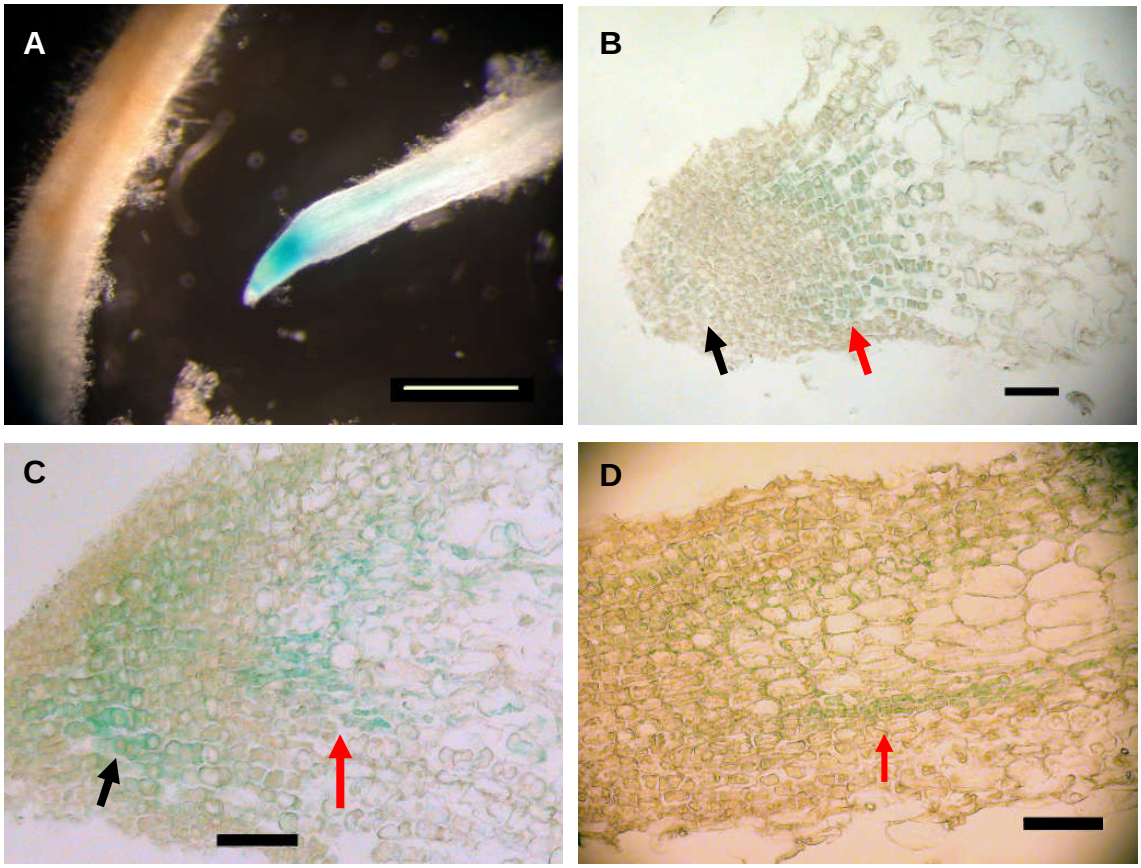


Figure 6.4 pr*MtWOX5*::GUS expression in cultured roots in auxin medium after 15 weeks. The GUS signals were investigated in the cultured root before section (A) and the longitudinal sections of different stages of root tip development (B, C) and root maturation zone (D). The red and black arrows indicate the GUS signals. Black bar = 80 μm, white bar = 500 μm.

A connection between auxin and *WOX5* expression in *de novo* root formation was likely, given the involvement of auxin in *AtWOX5* regulation in roots *in vivo* (Gonzali et al., 2005). The relationship between auxin concentration and *MtWOX5* expression in *de novo* root formation was investigated using pr*MtWOX5*::GUS transgenic root calli with root primordium described in 6.2.1. The GUS signals were examined in the root apex (including stem cells and columella cells) and upper zone (including elongation and mature zones) in each treatment (Table 6.2). In the root apex, the signal is stronger with higher auxin treatment and is localised to both stem cells and columella cells with treatments higher than 1 μM NAA. An increasing signal in the upper zone also occurred with much higher concentrations of auxin (10 μM NAA). These indicate that the

MtWOX5 expression level is enhanced by auxin. Root lengths were decreased by auxin, especially at the higher concentrations, but the root numbers increased (Table 6.2).

0 (Control)	0.1 μM NAA	1 μM NAA	5 μM NAA	10 μM NAA
				
				
0.1 μM GA_3	1 μM GA_3	5 μM GA_3	10 μM GA_3	
				
				

Table 6.2 pr*MtWOX5*::GUS expression patterns in cultured roots after 7 d in different auxin and GA treatments. Roots obtained from 15 weeks 10 μM NAA transgenic cultures after transfer to each of the hormone treatments for 7 d. Bar = 500 μm.

GA investigated in Chapter 3 influences *de novo* root development and the relationship between GA and *MtWOX5* expression was investigated in the GA culture system by pr*MtWOX5*::GUS transgenic cultured root calli with root primordium described in 6.2.1 and shown in Table 6.2. The GUS signals in the root apex are weaker when GA_3 is low,

but the signals in the upper zone are increased at concentrations greater than 1 µM GA_3. These data indicate that GA can change the *MtWOX5* expression patterns in different regions of the root which may influence the root architecture and root growth.

6.3.4 *MtWOX5* Expression During Somatic Embryogenesis

AtWOX5 expresses during zygotic embryo development (Haecker et al., 2004) and *MtWOX5* would also be expected to express during somatic embryogenesis (Fig. 6.3). The *MtWOX5* expression in auxin plus cytokinin culture which produces callus and somatic embryos in 2HA increases after 2 d in both Jemalong and 2HA, and the expression becomes higher in 2HA after 14 d (Fig. 6.5). The increase in 2HA may be related to somatic embryo formation.

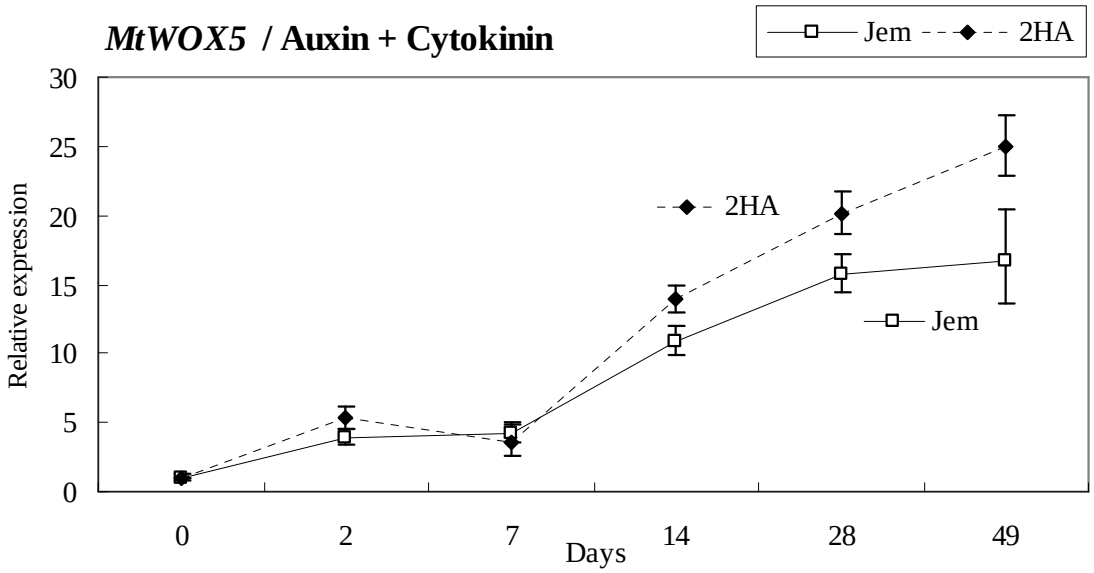


Figure 6.5 Differential timing of *MtWOX5* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP). Expression of *MtWOX5* in Jemalong (Jem, □, solid line) and 2HA (2HA, ◆, dashed line) was followed for 49 d. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d). Error bars show SEM.

The pattern of *MtWOX5* expression in somatic embryogenesis was investigated further in pr*MtWOX5*::GUS transgenic tissue. The 21 d callus has the *MtWOX5* GUS signal (Fig. 6.6A). Prior to 21 d, in the callus initiation phase, there is little GUS staining. This contrasts with *MtWUS* (Fig. 5.8A). In somatic embryos, the *MtWOX5* expression is localized in the central part of embryo (Fig. 6.6B). After the heart stage, the signal

accumulates in the cotyledon and root apex (Fig. 6.6C), consistent with the *AtWOX5* pattern in embryogenesis (Haecker et al., 2004).

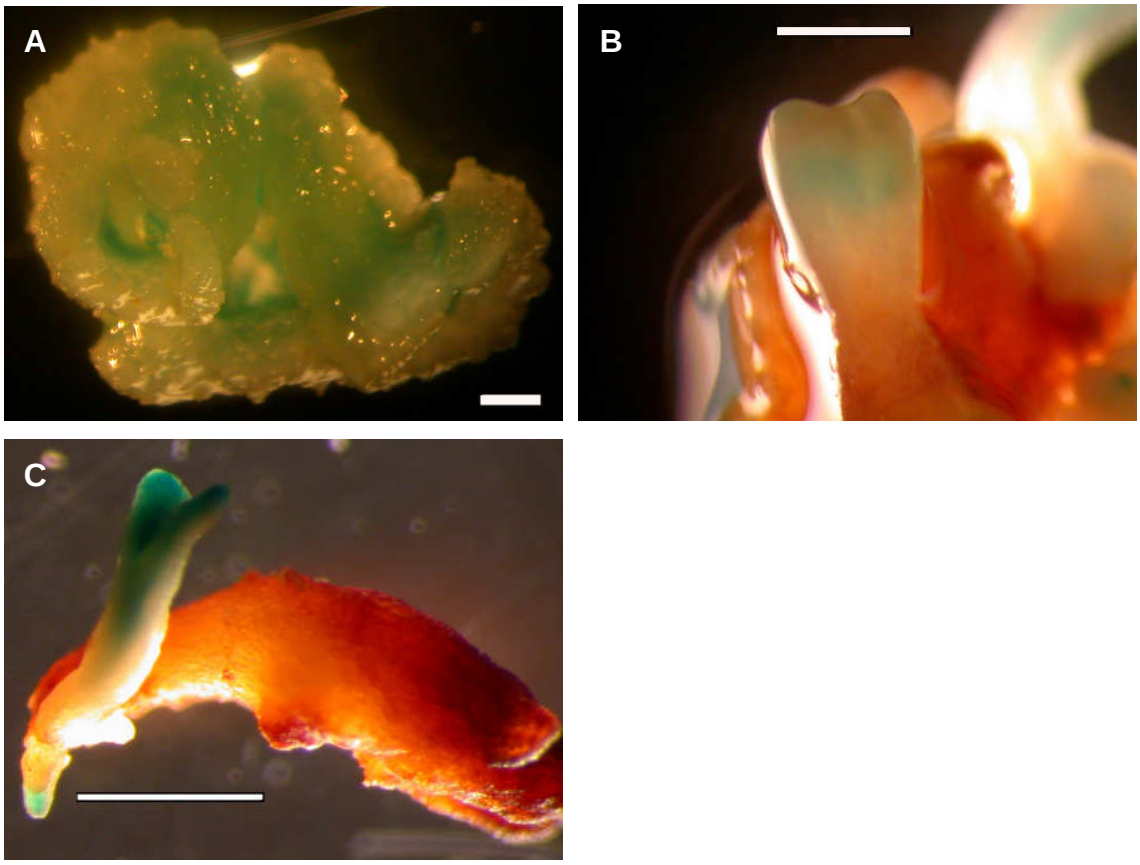


Figure 6.6 pr*MtWOX5*::GUS expression in callus and somatic embryos in auxin plus cytokinin culture. A, 21 d callus. Heart stage (B) and torpedo stage (C) somatic embryos. White Bar = 500 μm and black bar = 80 μm.

6.3.5 Inducible *MtWOX5* RNAi Experiments in Callus Development and Somatic Embryo Induction

To investigate *MtWOX5* function in callus formation and somatic embryo induction, dexamethasone-inducible RNAi was carried out using the *MtWOX5* gene. After the *MtWOX5* RNAi transgenic callus formed, the calli were set up in standard medium with dexamethasone (Dex) to trigger the RNAi system and inhibit the *MtWOX5* expression. The empty vector transgenic callus described in 5.2 was used as control.

MtWOX5 RNAi callus image size was increased only 12.3% after 28 d culture with dexamethasone where the control callus increased 104.4% (Fig. 6.7). Some somatic

embryos were also present in the control callus but not in *MtWOX5* RNAi callus (Table 6.3). These data suggest that *MtWOX5* is required for callus formation and somatic embryo induction. These data are similar to the *MtWUS* data, even though they have different expression patterns and different hormone dependences, although both are expressed in somatic embryos in the presence of auxin and cytokinin and both are important in embryogenesis in Arabidopsis (Haecker et al., 2004). In another experiment to directly compare the effect of RNAi on both *MtWUS* and *MtWOX5* on callus growth, *MtWUS* RNAi was more effective, virtually eliminating it (Fig. 6.8). In this experiment *MtRLK1* was a reference control gene, outside the *WOX* family of genes. It should also be noted that *WOX5* has been reported to be involved in the regulation of active auxin levels (Gonzali et al., 2005) as well as being auxin induced (Fig. 6.2) whereas *MtWUS* is rapidly induced by cytokinin following explant excision (Fig. 5.8). Callus growth requires auxin plus cytokinin.

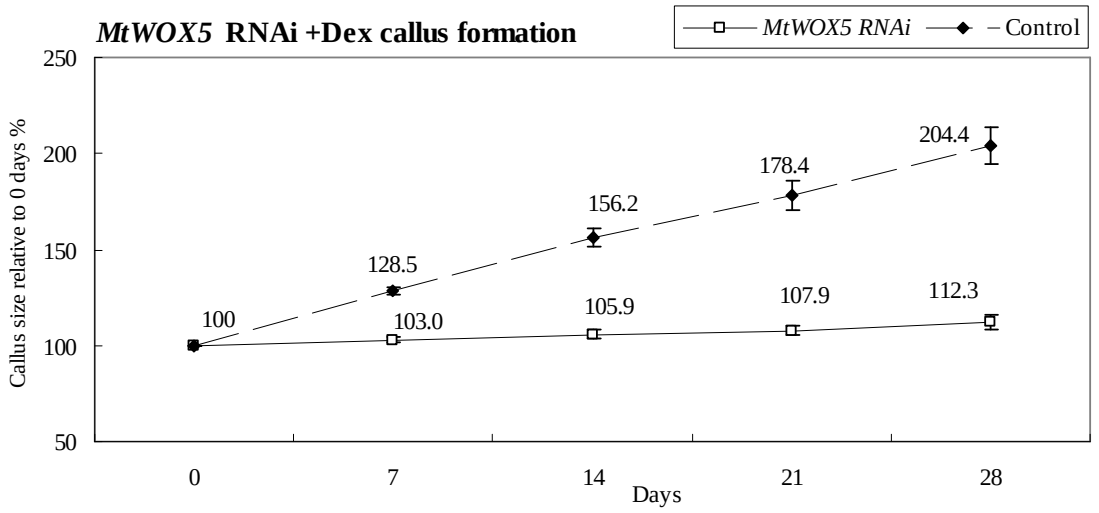


Figure 6.7 Differential timing of dexamethasone (Dex) -induced RNAi callus size of *MtWOX5* and empty vector control in tissue on P4 media containing auxin (10 µM NAA), cytokinin (4 µM BAP) and 10 µM dexamethasone. Calli size of *MtWUS* (□, solid line) and empty vector control (◆, dashed line) was followed for 28 d. Calli size, measured from callus images by software Image J, is shown relative to that at the beginning of the experiment (0 d) set as 100. Error bars show SEM.

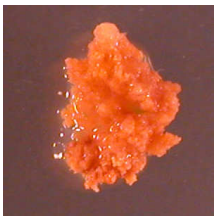
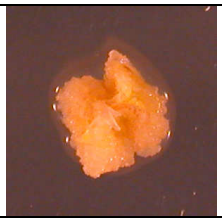
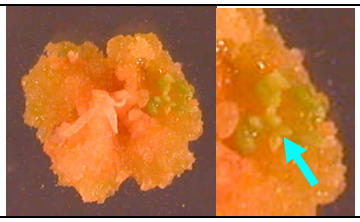
+ Dex	0 days	14 days	28 days
MtWOX5 RNAi			
Vector control			

Table 6.3 Differential timing of dexamethasone (Dex) -induced RNAi callus size of *MtWUS* and empty vector control in tissue on P4 media containing auxin (10 µM NAA), cytokinin (4 µM BAP) and 10 µM dexamethasone. Images show the development of calli over the life of the experiment for 28 d. Blue arrow indicates somatic embryos.

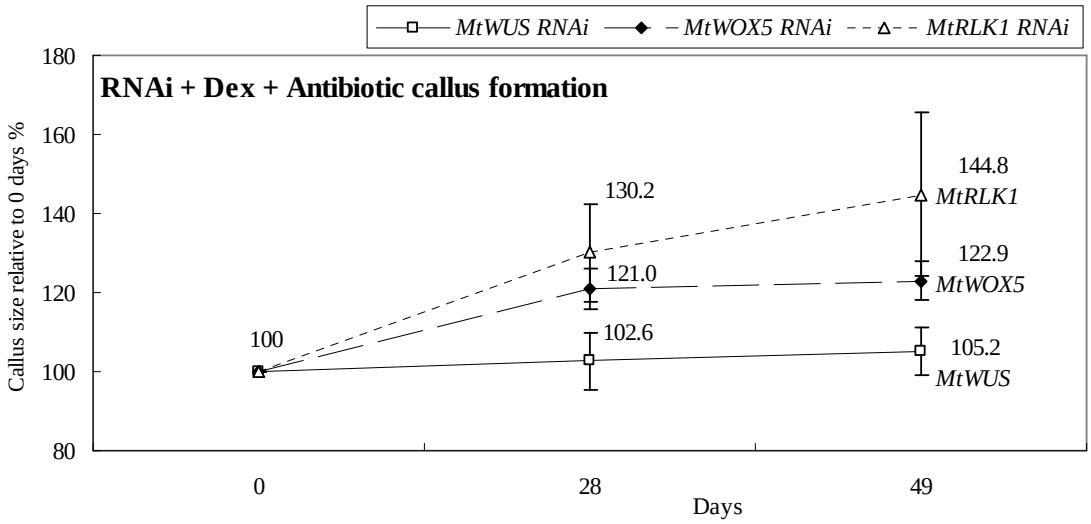


Figure 6.8 Differential timing of dexamethasone (Dex) -induced RNAi callus size of MtWUS, *MtWOX5* and MtRLK1 in tissue on P4 media containing auxin (10 µM NAA), cytokinin (4 µM BAP) and 10 µM dexamethasone. Calli size of *MtWUS* (□, solid line), *MtWOX5* (◆, dashed line), and *MtRLK1* (△, dotted line) was followed for 49 d. Calli size, measured from callus images by software Image J, is shown relative to that at the beginning of the experiment (0 d) set as 100. Error bars show SEM.

6.3.6 *MtWOX4* Expression Analysis

MtWOX4 was investigated by qRT-PCR in different tissues given its homology to

AtWOX4 and AtWUS (Chapter 4). The qRT-PCR expression results displayed in Fig. 6.9 show the expression of *MtWOX4* is highest in the shoot apex, then developing flower, cultured root tip, leaf, and lowest in the somatic embryo. This indicates this gene might function in the shoot apical meristem, and is of less importance in the somatic embryo and root apex. *MtWOX4* expression in leaf development was investigated by taking leaves from the shoot apex from the folded stage to fully opened leaves. The results show that during leaf development from the shoot meristem cells, that *MtWOX4* expression decreased as the leaf developed.

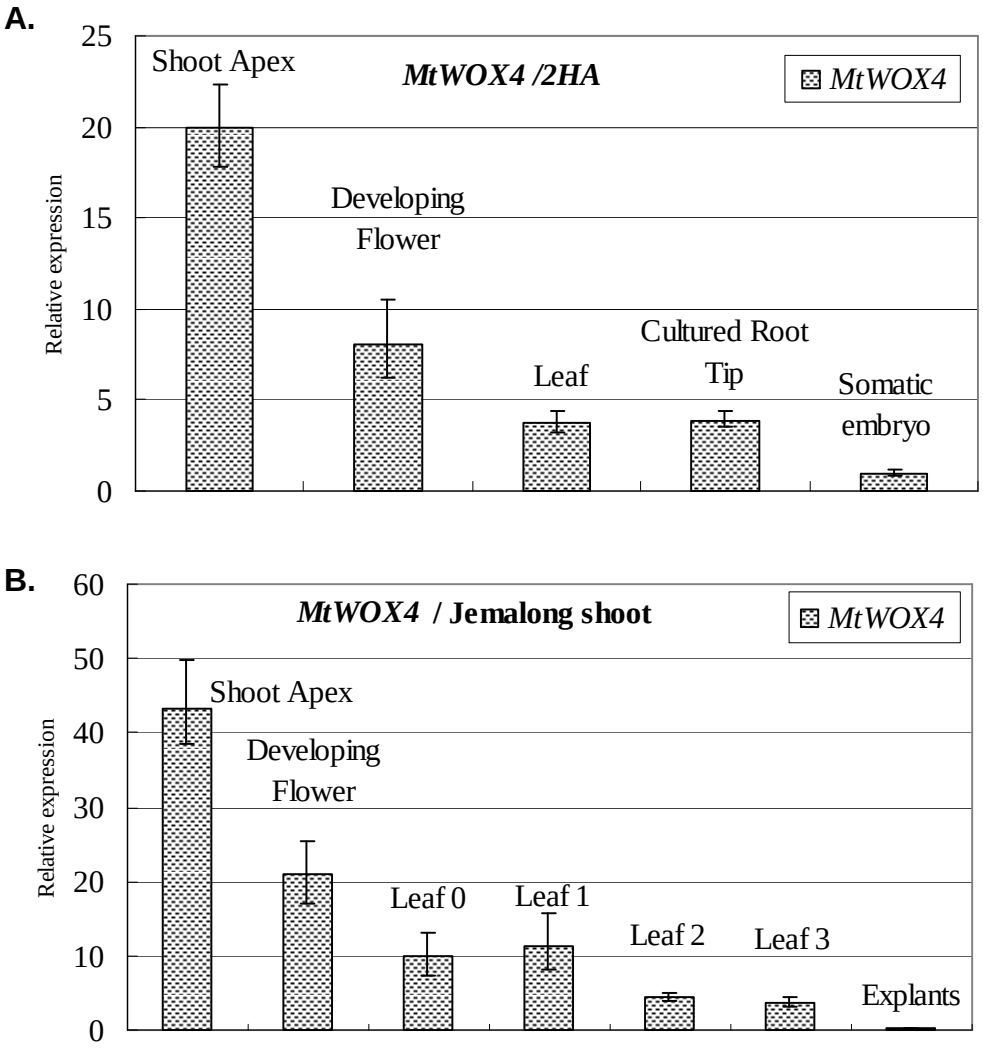


Figure 6.9 *MtWOX4* expression in different tissue of 2HA and Jemalong. A: Expression of *MtWOX4* in 2HA shoot apices, developing flowers, mature leaves, cultured root tips and somatic embryos. **B:** Expression of *MtWOX4* in Jemalong shoot apices, developing flowers, and different stages of leaf development. Leaf 0 indicates unopened initiated leaf, and Leaf 1 to Leaf 3 indicates the fully opened leaf from tip to base. Expression, measured by qRT-PCR, is shown relative to the expression level of somatic embryos in (A). Error bars show SEM.

MtWOX4 expression increased during callus growth (Fig. 6.10). The highest expression was in the auxin plus cytokinin treatment. In the auxin alone treatment a peak of expression occurred at 2 d. The expression patterns are similar in Jemalong and 2HA. What is of particular interest here is the different hormone requirements for this homeotic gene. *WUS* is induced by cytokinin, *WOX5* by auxin, while *WOX4* requires auxin plus cytokinin for maximum expression in the culture period.

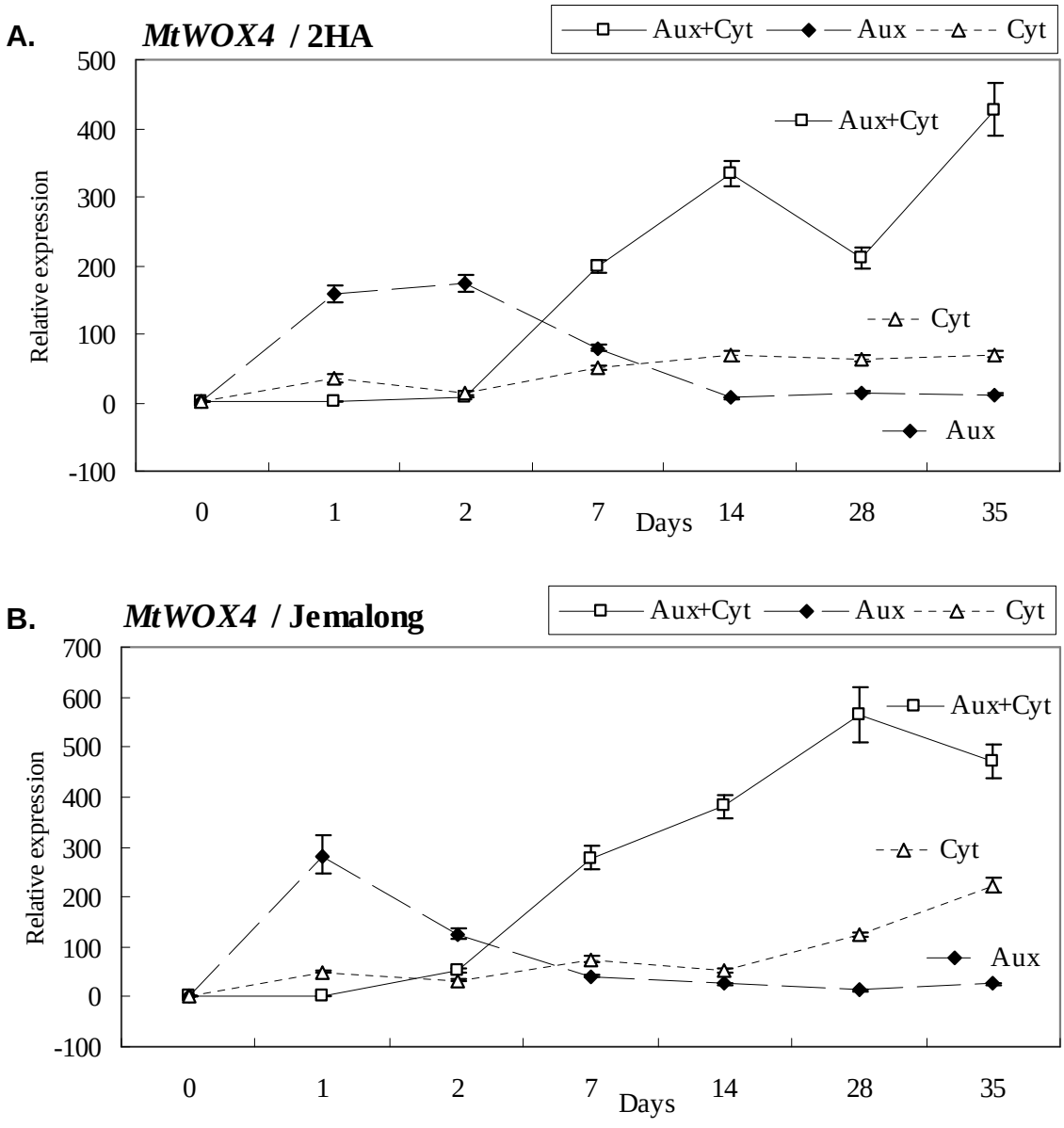


Figure 6.10 Differential timing of *MtWOX4* expression in tissue on P4 media containing auxin (10 µM NAA) plus cytokinin (4 µM BAP) (Aux+Cyt, □, solid line), auxin (10 µM NAA) (Aux, ◆, dashed line), and cytokinin (4 µM BAP) (Cyt, △, dotted line). A: Expression of *MtWOX4* in 2HA **B:** Expression of *MtWOX4* in Jemalong. Expression, measured by qRT-PCR, is shown relative to that at the beginning of the experiment (0 d), and was followed for 35 d. Error bars show SEM.

6.4 DISCUSSION

MtWOX5, based on the bioinformatics analysis in Chapter 4, is the putative homologue of AtWOX5. The expression data presented here is consistent with this and confirms other work recently reported (Imin et al., 2007). In this chapter its expression has been particularly investigated in relation to *de novo* root initiation, but also somatic embryogenesis. The putative ortholog to AtWOX4 has also been investigated.

MtWOX5 Expression in Relation to *de novo* Root Formation

In situ hybridisation has suggested that *MtWOX5* is expressed in the procambium cells and is associated with the induction of root primordia. It has been shown previously in our laboratory that root primordia are derived from these cells (Rose et al., 2006). In this it appears to have a somewhat similar role to WUS involvement in SE stem cell formation. After the root primordia formed the meristem showed strong *MtWOX5* expression, in what is likely the stem cell area that is the source of the root cells and root cap cells. The data in Table 6.2 clearly illustrates the auxin dependency of *MtWOX5* as does the qRT-PCR data in Fig. 6.2. Given the GA suppression of meristem formation it is not surprising that *MtWOX5* expression in the root meristem is inhibited by GA_3. That GA inhibits *MtWOX5* expression and root primordia formation strongly argues for a role for *MtWOX5* in root primordia initiation. A summary of the *MtWOX5* hormone relationships are shown in Fig. 6.11.

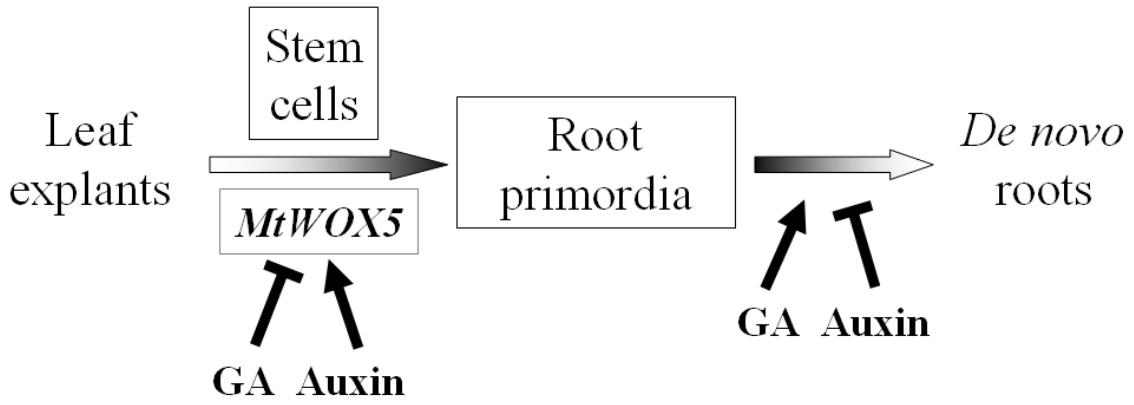


Figure 6.11 Summary of *MtWOX5* expression in the *de novo* root induction process and hormone regulation. T-bar indicates the inhibition, and black arrow indicates enhancement.

In the intact plant, Imin et al., 2007 showed that there is low *MtWOX5* expression in the root tip compared to root forming calli. This difference requires further examination and suggests a strong auxin response *in vitro*, and expression outside the quiescent centre. The *M. truncatula* root tip architecture is different to that of Arabidopsis and rice (Heimsch and Seago, 2008). Arabidopsis has a closed meristem, whereas *Medicago* has an open meristem (Heimsch and Seago, 2008). This study also indicated that there is *MtWOX5* expression in the pericycle and vascular tissue of mature roots. It is feasible that this expression is related to a capacity for lateral root formation *in planta*. This expression is not reported for Arabidopsis *WOX5*. The low expression in the root tip differs from *AtWOX5*, and is different to *BBM* (Baby Boom) and *PLT1* (Plethora 1) which express strongly in *M. truncatula* root-forming calli and root tips (Imin et al., 2007). These data are discussed further in Chapter 7.

The *MtWOX5* expression in the vascular tissue of the mature zone can be enhanced by GA. GA induces thin roots, which may link to the forming of the fibrous root type. This fibrous root type system can be seen in *M. truncatula*, but not in Arabidopsis. The *MtWOX5* expression patterns may have some close relationship to the root type that is being formed.

MtWOX5 and *MtWOX4* Expression in Relation to Somatic Embryogenesis

With explants treated with auxin plus cytokinin, *MtWOX5* shows increasing expression in culture in both Jemalong and 2HA. There is extra expression after SE formation in 2HA and *MtWOX5* also expresses in somatic embryos. The *MtWOX5* expression continues during somatic embryo induction and its expression pattern in the embryo is different to *MtWUS*. The RNAi data indicate that *MtWOX5* may have some involvement in callus formation and SE. *MtWOX5* expresses in the root pole and in cotyledons, but not in the apical meristem, previously shown in Arabidopsis. It should be noted that it is not induced by cytokinin.

MtWOX4 has different responses to auxin and cytokinin compared to *MtWUS* and *MtWOX5* and emphasises the hormone specificity of *MtWUS* and *MtWOX5* expression. *MtWOX4* requires auxin plus cytokinin for maximum expression. *MtWOX4* maybe involved in different developmental processes during *in vitro* culture e.g. vascular tissue

differentiation that occurs in callus. This could explain the increasing *MtWOX4* expression in callus and an early auxin response when new vascular tissues are induced in these tissues (Irwanto, 2004; Imin et al., 2007).

CHAPTER 7

General Discussion

● Characteristics of Jemalong 2HA

Somatic embryogenesis is a process unique to plants and it does not occur naturally in *M. truncatula*. Even in tissue culture, only special genotypes can produce embryos from explants. 2HA, derived from Jemalong (Rose et al., 1999), is one of the genotypes that can produce SE in *M. truncatula*. What is known about the differences between 2HA and Jemalong is listed in Table 7.1.

- **Differences between 2HA and Jemalong:**	**Reference**
1. SE capacity is 500 times higher in 2HA.	Nolan et al., 1989
2. SE depends on 2HA response to cytokinin, Jemalong cannot respond to cytokinin in this way.	Nolan and Rose, 1998
3. Auxin-induced root growth is slightly higher in Jemalong.	Chen, Chapter 3
4. Greater number of root nodules in 2HA.	Mathesius (unpub, collaboration RR lab) APPENDIX 7
5. *MtEIN3* expression level is very low in 2HA.	Kurdyukov (unpub, RR Lab)
6. DNA methylation patterns are different.	Irwanto, 2004
7. Flower number per inflorescence is less in 2HA.	Chen, Chapter 2
8. Some differences in flower morphology.	Chen, Chapter 2
9. Delayed senescence in some 2HA flowers.	Chen, Chapter 2
- **Similarities between 2HA and Jemalong:**	
1. Karyotype.	Chen, Chapter 2
2. RFLP patterns.	Irwanto, 2004
3. Plant morphology (e.g. plant size, leaf size and morphology, branching type).	Chen, Chapter 2
4. Inhibition of root growth by cytokinin *in vitro* and *in vivo*.	Nolan and Rose, 1998; Liu (unpub, RR lab)

Table 7.1 List of the differences and similarities between 2HA and Jemalong.

- **Hormone Requirements for Somatic Embryogenesis and *in vitro* Root Formation**

(i) Somatic Embryogenesis

Auxin and cytokinin are required for SE in 2HA. Wild-type Jemalong will only form callus in the presence of auxin and cytokinin and both Jemalong and 2HA will form roots in response to auxin alone (Nolan and Rose, 1998). Effectively this means that the auxin-induced root growth in Jemalong and 2HA is inhibited by cytokinin, but development ultimately goes in different directions in 2HA (embryogenic callus) and Jemalong (callus) in response to auxin plus cytokinin. In Chapter 3 it is apparent that the auxin and cytokinin are not required continuously in the culture for SE. Induction is complete within 2-3 weeks and the first embryos are visible to the naked eye after 4-5 weeks. It appears that once the embryos are formed they can then regulate their own hormone requirements. ABA in the standard medium used in our laboratory is added at 3 weeks to aid embryo maturation (Nolan and Rose, 1998). However ABA increases somatic embryo number if given at the beginning of the culture period. This appears to be related to integrating the stress response with the auxin and cytokinin in the medium to induce SE (Nolan et al., 2006; Rose and Nolan, 2006).

What is clear from the *MtWUS* studies is that *WUS* induction has a requirement for cytokinin and it is expressed in both Jemalong and 2HA. Initially Jemalong and 2HA respond similarly to the *in vitro* conditions and *WUS* continues to be expressed in the embryos induced in 2HA where *CLV3* then starts to be expressed (Figs. 5.3 and 5.6). As discussed further below it suggests that *WUS* is involved in stem cell formation in the callus as well as stem cell formation and maintenance in the embryo. In Jemalong the callus cells do not develop into embryos and *CLV3* is not expressed.

It is downstream from *WUS* induction that another hormone becomes important in embryo induction. This is the stress hormone ethylene. Ethylene is essential for SE and affects SE more than callus formation (Mantiri et al., 2008a). The transcription factor *MtSERF1* is dependent for its induction on ethylene as well as auxin plus cytokinin, and is essential for SE (Mantiri et al., 2008a). Ethylene is induced as a result of the culture process and is not added to the medium (Mantiri et al., 2008a).

What now is clear in the SE induction is that cytokinin and ethylene signalling in 2HA are important. The delayed flower senescence in 2HA also links to these two hormones which are closely related to senescence (Gan and Amasino, 1995; Johnson and Ecker, 1998). Cytokinin can delay senescence, while ethylene can speed up the process. An enhancement of cytokinin action (as in the SE increase in 2HA) and a mutation related to ethylene signalling could cause the delay. We now know (Kurdyukov, unpublished) that *MtEIN3*, important in ethylene signalling (Johnson and Ecker, 1998), is down-regulated in all tested 2HA tissues and 2HA like the *sickle* mutant (thought to be an *MtEIN2* mutant – Prayitno et al., 2006) has increased nodulation. This suggests that ethylene and cytokinin signalling is different in 2HA. It is also of interest that in Brassicas with high SE flower senescence is delayed (Malik et al., 2008).

The change in the number of root nodules in 2HA supports the link to cytokinin and ethylene. Cytokinin plays a positive role in nodulation (Beveridge et al., 2007), and ethylene is a negative regulator as shown in the *SICKLE* investigation (Prayitno et al., 2006). It should also be noted that the ERF sub-family transcription factor ERN is involved in the nodulation signalling pathway (Middleton et al., 2007).

GA was suggested to be involved in somatic embryo induction after the discovery that the *PKL* gene suppressed SE and was regulated by GA (Ogas et al., 1999; Henderson et al., 2004). *AGL15* investigations (Thakare et al., 2008) reveal a GA-modulated pathway that represses embryonic identity. It is essential to prevent inappropriate embryo formation as germination and subsequent plant development occurs. *LEC1* and *FUS3*, which can be repressed by GA through DELLA and *PKL* (Ogas et al., 1999; Rider et al., 2003; Henderson et al., 2004), are expressed in the leaf margins where the mature SEs form in *Kalanchoë daigremontiana* (Garcês et al., 2007) which supports the idea that GA suppresses SEs in nature. In Chapter 3, the GA treatments did not inhibit the SE in culture suggesting that the GA pathway suppression for SE induction was blocked in 2HA. This requires further investigation with both 2HA and Jemalong.

(ii) *In vitro* Root Formation

The *in vitro* root formation studies in Chapter 3, revealed interesting contrasts with the SE induction in terms of hormonal control. As already discussed auxin is essential and

cytokinin is inhibitory. The data in Chapter 3 suggests that auxin is essential for root meristem formation and after induction a lowering of auxin is necessary. Previous work in our laboratory (Rose et al., 2006b) has shown that ethylene is inhibitory to root formation *in vitro*. Once the primordia are induced then further root development is enhanced by the lowering of the auxin concentration (Fig. 3.7 and Table 6.2). The GA data were quite interesting in that GA inhibited root meristem formation consistent with the theme developed from investigation of SE with bipolar meristems that GA inhibits meristem initiation, but once meristems form GA enhances growth and seedling development.

MtWOX5 expression is up-regulated by auxin and is associated with meristem formation. GA inhibits *MtWOX5* expression. This provides support for the involvement of *MtWOX5* in directing pluripotent stem cell formation into root primordia induction. This is discussed further below.

In the *Medicago* system ABA inhibits *de novo* root induction as does ethylene (Simms, 2006), but both promote SE where there is a strong stress requirement. It is feasible that stress effects promote reproduction at the expense of root growth.

- **Genetic Regulation of Somatic Embryogenesis and Organogenesis**

(i) Somatic Embryogenesis

(a) *MtWUS*

The data in Chapter 4 and 5 are consistent with the view that *MtWUS* is the ortholog of *AtWUS*. The data supports the work of Zuo et al. (2002) where overexpression of *WUS* can induce SE in Arabidopsis. It appears that *in vitro* SE *WUS* is utilised to form the totipotent stem cells that will produce the embryos. This helps explain the very rapid *WUS* induction which is linked to callus proliferation but becomes restricted to small groups of cells from which the embryo develops (Figs. 5.3 and 5.7). *WUS* is expressed throughout the globular stage embryo and becomes more localised to the apical region as the embryo develops. In Arabidopsis tissue incubated *in vitro* with cytokinin *AtWUS* expression appears to involve pluripotent stem cells that produce shoots (Gordon et al.,

2007). This is a different *in vitro* system but has parallels with the cytokinin requiring 2HA system. Importantly RNAi studies (Fig. 5.9 and Table 5.9) support the postulated *WUS* role, by reducing both cell proliferation as well as embryo formation. It should also be noted that without cytokinin, and in the presence of auxin only cells would be directed into pluripotency and root meristem formation.

(b) Relationship to other genes in SE induction

WUS is induced in 24-48 h but the question remains as to how this expression relates to the overall process of SE induction. A model is presented in Fig. 7.1. Other work (Saeed 2004; Sheahan 2007; Sheahan et al., 2008) has shown that the production of ROS occurs very quickly (within minutes) and inhibiting ROS blocks callus and SE development. ROS is a contributor to the stress response which is a key part of SE induction. ROS modulated in appropriate ways has a signalling function (Fujita et al., 2006). It is also known that ethylene is rapidly induced in the culture (by 24 h) and a requirement for ROS is likely. It is feasible that both ROS and auxin contribute to the induction of ethylene biosynthesis genes. ROS has been reported to promote auxin-induced ethylene production in mung bean hypocotyls (Song et al., 2007). Further there is preliminary data in our laboratory supporting a requirement for ROS for ethylene production (Mantiri, unpublished). *MtWUS* appears to be an important early player requiring cytokinin. *WUS*, which has been suggested to be an embryo organiser (Zuo et al., 2002), appears to be associated with the production of totipotent stem cells, similar to the way it is involved in stem cell formation and maintenance *in planta*. Previous work has shown that *MtSERK1* is expressed 48 h after the beginning of culture and just after *MtWUS* expression and is associated with developmental change and appears to mark cells as they change into a new developmental pathway (Nolan and Rose, 2008). *MtSERF1* expression is evident after about 10 days of culture and is dependent on ethylene as well as auxin and cytokinin. It appears to act as a nexus between the stress of excision and culture and the developmental hormones auxin and cytokinin driving cells into somatic embryogenesis. Importantly there is some evidence that *WUS* may be necessary for *MtSERF1* expression as binding sites of *WUS* are present in the *MtSERF1* promoter region (Fig. A6.1). It appears that *MtSERF1*, possibly in conjunction with *WUS*, is involved in regulating downstream genes required for SE (Mantiri et al., 2008b). *MtSERF1* expression commences earlier than *MtCLV3*. *MtCLV3*

likely expresses when stem cells start to be regulated (Fig. 5.6). *MtWUS* expression is likely down regulated by *MtCLV3* after the shoot meristem structure has formed in somatic embryos as in zygotic embryogenesis in Arabidopsis (Brand et al., 2000).

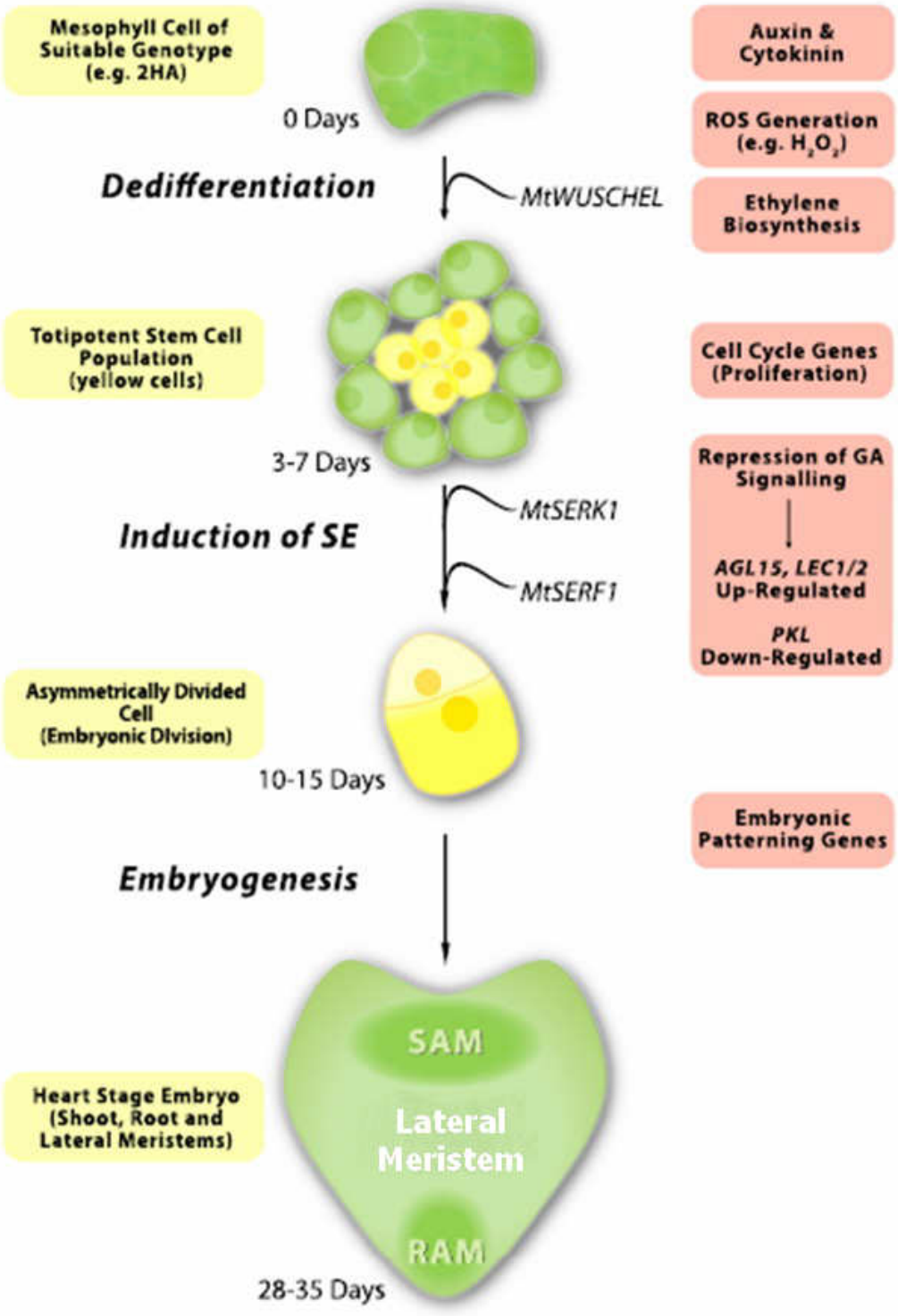


Figure 7.1 Model for the induction of somatic embryogenesis in *Medicago truncatula*. Diagram from Rose RJ, Mantiri FR, Kurdyukov S, Chen S-K, Wang X-D, Nolan KE and Sheahan MB, 2008b.

The GA involvement in the model is based only on Arabidopsis and soybean work and further studies are required in *Medicago* (Thakare et al., 2008).

M. truncatula requires the correct genotype for SE to be induced. The genetic studies in Chapter 2 suggest that a single gene may open the way to SE but additional genes are required to maximise the process. An initial epigenetic change may be key as has been previously discussed (Rose, 2008a). There are no large scale chromosome changes (Chapter 2) and methylation changes have been detected (Irwanto, 2004).

The major genes and hormones investigated in this thesis and some relevant to SE are diagrammed in Fig. 7.2. It shows the process of somatic embryogenesis and root organogenesis and the related genes and hormones for each stage. In the stem cell induction stage, *ROS*, *SERK1*, *WUS* and *WOX5* are the key genes.

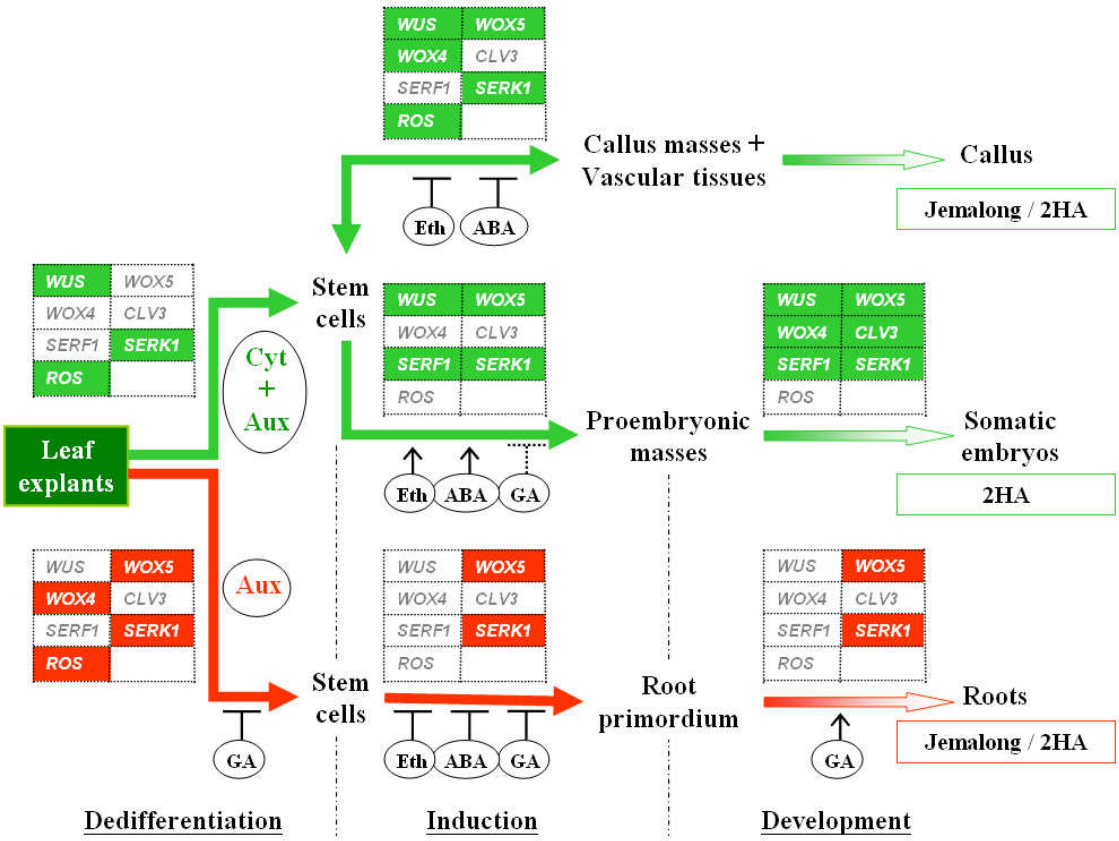


Figure 7.2 Diagram of processes of somatic embryogenesis and organogenesis including hormone influences and expressed genes in *Medicago truncatula*. Green arrows indicate the requirements for exogenous cytokinin (Cyt) and auxin (Aux), red arrows indicate the requirements for exogenous auxin, and variant coloured arrows indicate the requirement for exogenous hormone is decreased. Open arrows for ethylene (Eth), ABA and GA indicate enhancement of the process, and T bar indicates inhibition;

square dotted line indicates that these influences are predicted. The genes related to these processes are shown in blocks, and shown in colour when expressed and dark grey when not expressed. Some information obtained from Nolan and Rose, 1998; Saeed, 2004; Rose et al., 2006b; Simms, 2006; Imin et al., 2007; Sheahan, 2007; Mantiri et al., 2008a; Nolan and Rose, 2008 and Sheahan et al., 2008.

(ii) Root Formation

(a) *MtWOX5*

MtWOX5 expression is very closely associated with root meristem formation and appears to express in the stem cell areas of both the emerging primordium and in the cultured roots. The expression in the intact root may be confined to the quiescent centre as in Arabidopsis (Blilou et al., 2005). This may well be due to the close regulation of auxin which is different to the culture system. However *MtWOX5* is again serving the role as a stem cell signal and is in both cases intimately involved in maintenance of the stem cell systems. The GA studies support a role for *MtWOX5* in meristem formation as GA which blocks meristem formation inhibits *MtWOX5* expression (Table 6.2). *MtWOX5* is associated with the embryo development and as shown in a recent study there is some overlap with *WUS* in their developmental roles (Sarkar et al., 2007).

(b) Relationship to other genes in root meristem induction

Changes prior to *WOX5* expression have not been well documented, but *ROS* production would be an initial event and the regulation of redox is an important consideration in setting up a root meristem (Imin et al 2007). Other studies in *M. truncatula* also indicate induction of *PLETHORA* and *BABY BOOM* (Imin et al 2007) known to be key players in stem cell maintenance in the Arabidopsis primary meristem (Galinha et al., 2007). However we do not know the time course of their transcription in relation to *WOX5*.

(iii) Relationship Between Hormones and Gene Regulation in SE and Root Formation

In Fig. 7.2 the relationship between hormones, activation of gene transcription and the

developmental outcome is schematised.

As an overview it is apparent that the production of the SEs with their bipolar meristems and the production of the unipolar root meristem have different requirements for the key developmental and stress hormones. Through regulation of specific genes the hormones are able to exert their regulatory influence. In both cases the culture process has "hijacked" key developmental genes to drive the induction of the *in vitro* processes. Though these processes are not usual in the *M. truncatula* life cycle they do occur in nature in other species. It appears that *in vitro* it is a question of unlocking the expression of these genes. The GA inclusion has been linked to the need to turn off meristem processes when not required and to facilitate plant growth, but in *Medicago*, though there is some support for this GA effect, more work is required.

- ***MtWUS* and *MtWOX5* may have a Similar Function in Relation to Stem Cell Induction *in vitro***

MtWUS and *MtWOX5* may have similar functions in stem cell initiation. *WUS* and *WOX5* have been reported to have related roles in maintaining stem cells (Sarkar et al., 2007), and also have similar roles in stem cell induction in *Medicago*. *WUS* may induce stem cells for somatic embryos with cytokinin being essential, and *WOX5* which is partially suppressed by cytokinin may induce the stem cells for root primordia with auxin being essential. The requirement for cytokinin and auxin in the regulation of the key genes is of course dependent on the species, genotype and explant type as well as the culture process. However in this thesis a connection to the induction of stem cells is the suggested operating principle.

- **Future Research**

Future research needs to be directed at the connections between cytokinin and ethylene signalling. It is already known that some overlap can occur in the signalling pathways (e.g. ARR2 response regulators, Hass et al., 2004). More specifically, with *WUSCHEL*, its involvement in the regulation of *MtSERF1*, requires further investigation. Given the data obtained with GA here and the data with other systems on the repression of SE, GA involvement in SE in *M. truncatula* also requires further investigation.

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APPENDIX 1

Element information

The following information for these 29 elements can be obtained from the web site http://www.dna.affrc.go.jp/PLACE/. There is a display with the call name, full name, related sequence, serial number, a short description and the references in Table A1.1 to A1.4.

Elements in OE/FE region				
Name	**Full Name**	**Sequence**	**Description**	**Reference**
ABREAT	ABRE.AT.CONSENSUS (S000406)	YACGTGGC, Y=C/T	ABA-responsive elements (ABREs) found in the promoter of ABA and/or stress-regulated genes.	Choi et al., 2000
ABREOS	ABRE.OS.RAB21 (S000012)	ACGTSSSC, S=C/G	"ABA responsive element (ABRE)" of wheat Em and rice (O.s.) rab21 genes.	Marcotte et al., 1989
ACGTAB	ACGT.ABRE.MOTIFA2.OS.EM (S000394)	ACGTGKC, K=G/T	Experimentally determined sequence requirement of ACGT-core of motif A in ABRE of the rice gene, OsEM.	Hattori et al., 2002
ACGTTB	ACGT.TBOX (S000132)	AACGTT	"T-box" according to the nomenclature of ACGT elements. One of ACGT elements.	Foster et al., 1994
BOXIIP	BOXII.PC.CHS (S000229)	ACGTGGC	Core of "Box II/G box" found in the parsley (P.c.) CHS genes; Essential for light regulation.	Block et al., 1990
CGCGBO	CGCGBOX.AT (S000501)	VCGCGB, V=A/C/G; B=G/T/C.	"CGCG box" recognized by AtSR1-6; Multiple CGCG elements are found in promoters of many genes; Ca++/calmodulin binds to all AtSRs.	Yang and Poovaiah, 2002
E2FCON	E2F.CONSENSUS (S000476)	WTTSSCSS, W=A/T; S=C/G.	"E2F consensus sequence" of all different E2F-DP-binding motifs that were experimentally verified in plants.	Vandepoele et al., 2005
GARE2O	GARE2.OS.REP1 (S000420)	TAACGTA	"Gibberellin-responsive element (GARE)" found in the promoter region of a cystein proteinase (REP-1) gene in rice.	Sutoh and Yamauchi, 2003
MARARS	MAR.ARS (S000064)	WTTTATRTTTW, W=A/T;	"ARS element"; Motif found in SAR (MAR).	Gasser et al., 1989
PALBOX	PAL.BOXA.PC (S000137)	CCGTCC	Box A; Consensus; One of three putative cis-acting elements (boxes P, A, and L) of phenylalanine ammonia-lyase (PAL) genes in parsley (P.c.).	Logemann et al., 1995
SV40CO	SV40.CORE.ENHAN (S000123)	GTGGWWHG, W=A/T.	"SV40 core enhancer"; Similar sequences found in rbcS genes.	Weiher et al., 1983

Table A1.1 Elements list of OE/FE region.

Elements in GQE region				
Name	**Full Name**	**Sequence**	**Description**	**Reference**
MYBPLA	MYB.PLANT (S000167)	MACCWAMC, M=A/C; W=A/T	Plant MYB binding site; Consensus sequence related to box P in promoters of phenylpropanoid biosynthetic genes.	Sablowski et al., 1994
REBETA	REBETA.LG.LHCB21 (S000363)	CGGATA	"REbeta" found in *Lemna gibba* Lhcb21 gene promoter. Required for phytochrome regulation.	Degenhardt and Tobin, 1996
SORLRE	SORLREP3.AT (S000488)	TGTATATAT	One of "Sequences Over-Represented in Light-Repressed Promoters (SORLREPs) in Arabidopsis; Computationally identified phyA-repressed motifs.	Hudson and Quail, 2003
XYLAT	XYL.AT (S000510)	ACAAAGAA	cis-element identified among the promoters of the "core xylem gene set".	Ko et al., 2006
Elements in MQE region				
BP5OSW	BP5.OS.WX (S000436)	CAACGTG	OsBP-5 (a MYC protein) binding site in Wx promoter.	Zhu et al., 2003
PROLAM	PROLAMIN.BOX.OS.GLUB1 (S000354)	TGCAAAG	"Prolamine box" found in the rice (O.s.) GluB-1 gene promoter; Involved in quantitative regulation of the GluB-1 gene.	Wu et al., 2000
T/GBOX	T/G.BOX.AT.PIN2 (S000458)	AACGTG	"T/G-box" found in tomato proteinase inhibitor II (pin2) and leucine aminopeptidase (LAP) genes; Involved in jasmonate (JA) induction of these genes.	Boter et al., 2004

Table A1.2 Elements list of GQE and MQE regions.

Elements in both GQE and MQE regions				
Name	**Full Name**	**Sequence**	**Description**	**Reference**
GAREAT	GARE.AT (S000439)	TAACAAR	GARE (GA-responsive element); Occurrence of GARE in GA-inducible, GA-responsible, and GA-nonresponsive genes in Arabidopsis seed germination was 20, 18, and 12%, respectively..	Ogawa et al., 2003
MYB1LE	MYB1.LE.PR (S000443)	GTTAGTT	Tomato Pti4(ERF) regulates defence-related gene expression via GCC box and non-GCC box cis elements (Myb1(GTTAGTT), G box (CACGTG)).	Chakravarthy et al., 2003
MYBGAH	MYB.GA.HV (S000181)	TAACAAA	Central element of gibberellin (GA) response complex (GARC) in high-pI alpha-amylase gene in barley (H.v.).	Gubler et al., 1995
RBCSCO	RBCS.CONSENSUS (S000127)	AATCCAA	rbcS general consensus sequence.	Manzara and Grussem, 1988
RHERPA	RHE.RP.AT.EXPA7 (S000512)	KCACGW, K=G/T; W=T/A	"Right part of RHEs (Root Hair-specific cis-Elements)" conserved among the *Arabidopsis thaliana* A7 (AtEXPA7) orthologous (and paralogous) genes.	Kim et al., 2006
SREATM	SRE.AT.MSD (S000470)	TTATCC	"sugar-repressive element (SRE)" found in 272 of the 1592 down-regulated genes after main stem decapitation in Arabidopsis.	Tatematsu et al., 2005
Elements in both OE/FE and MQE regions				
ABRELA	ABREL.AT.ERD1 (S000414)	ACGTG	ABRE-like sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis.	Simpson et al., 2003
ABRERA	ABRER.AT.CAL (S000507)	MACGYGB, M=C/A; Y=T/C; B=T/C/G.	"ABRE-related sequence" or "Repeated sequence motifs" identified in the upstream regions of 162 Ca (2+) -responsive upregulated genes.	Kaplan et al., 2006

Table A1.3 Elements list of GQE+MQE and OE/FE+MQE regions.

Elements in SnSC region				
Name	**Full Name**	**Sequence**	**Description**	**Reference**
AACACO	AACA.CORE.OS.GLUB1 (S000353)	AACAAAC	Core of AACA motifs found in rice (O.s.) glutelin genes, involved in controlling the endosperm-specific expression	Wu et al., 2000
ARFAT	ARF.AT (S000270)	TGTCTC	ARF (auxin response factor) binding site found in the promoters of primary/early auxin response genes of *Arabidopsis thaliana* (A.t.)	Ulmasov et al., 1999
ATHB6C	ATHB6.CORE.AT (S000399)	CAATTATTA	Consensus binding sequence for Arabidopsis homeodomain-leucine zipper protein, ATHB6, which is a target of the protein phosphatase ABI1 and regulates hormone responses.	Himmelbach et al., 2002

Table A1.4 Elements list of SNSC region.

APPENDIX1 Element information

APPENDIX 2

Promoter regulation regions analysis procedures

The promoter region analysis was carried out in the following steps:

a. Assigning the regulatory elements to promoter regions

This has been carried out for each promoter region as specified in 4.2.2 and obtains the physical maps for the elements.

b. Assigning the coloured rectangles as funtional regions

Grey / Yellow / Blue:

At **least two** elements which belong to the same region (including unique or partial-unique elements) and are located nearby are grouped to a region and drawn within a rectangle. The type and colour of the region was determined by the **unique** elements. The edge of the region is indicated where it joins different coloured elements.

Red:

The red elements are the most important regulatory elements according to Bäurle and Laux (2005), so the presence of a **single** or **more than one** red element was assigned to a red region.

Green:

The green elements are only grouped when there are many clustered green elements but without yellow or blue elements.

The physical plus rectangles maps of *WUS* and *WOX5* are given in Fig. A2.1 and Fig. A2.2 as examples.

APPENDIX 2 Promoter regulation regions analysis procedures

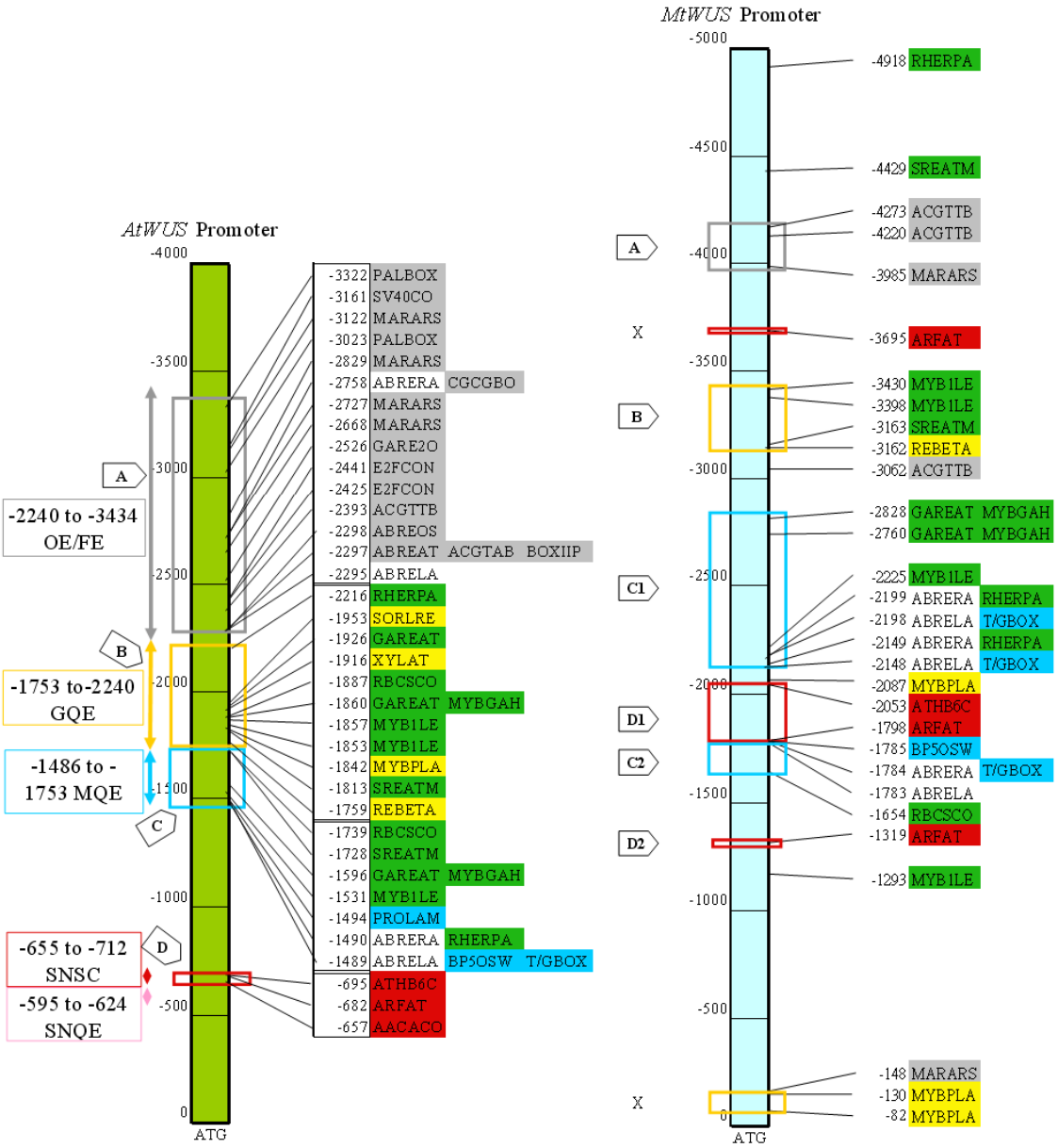

Figure A2.1 Physical map of *AtWUS* and *MtWUS* promoter regulation regions analysis.

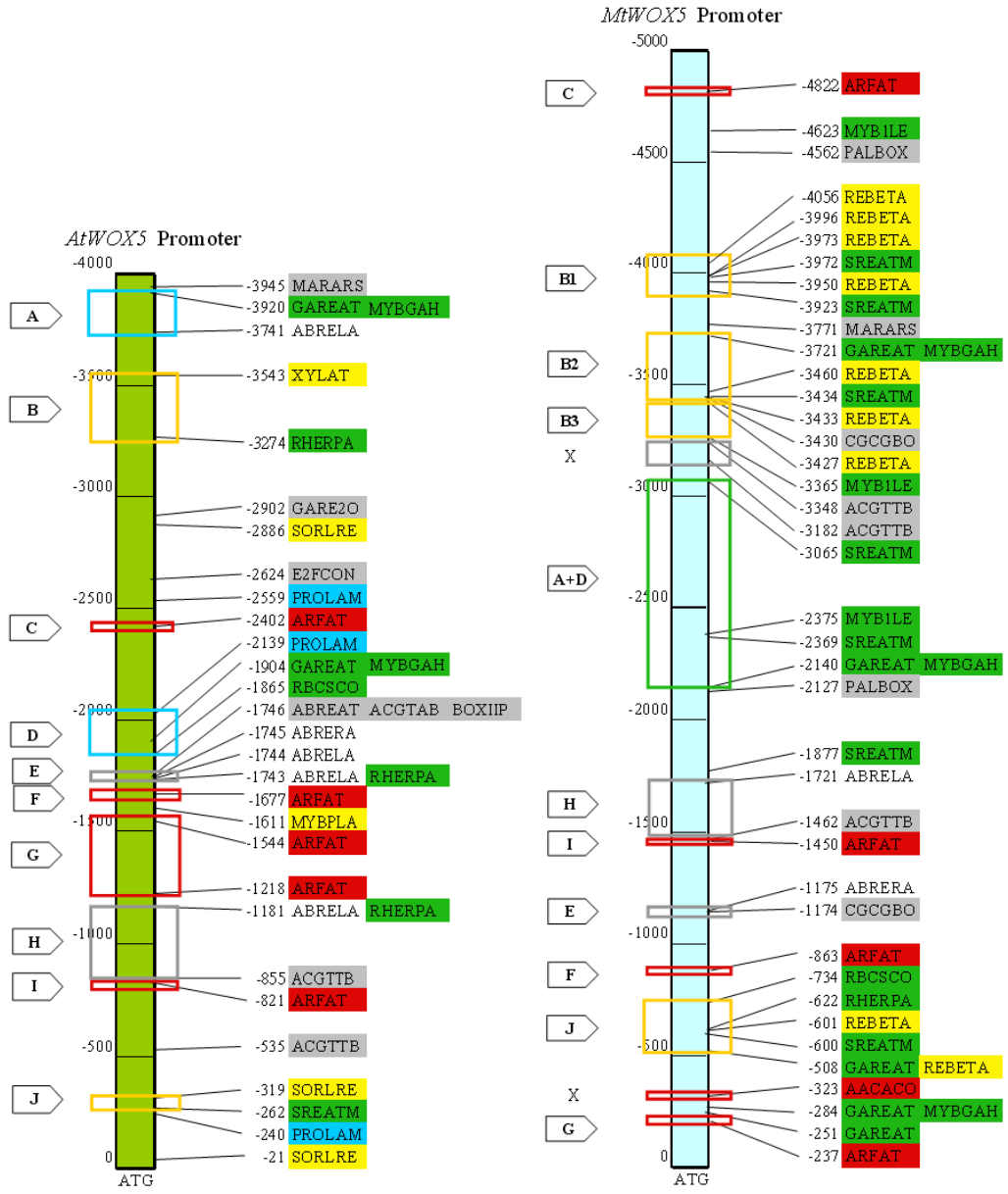

Figure A2.2 Physical maps of *AtWOX5* and *MtWOX5* promoter regulation regions analysis.

c. Identification of the matched regions and their order

The matched regions between species are identified by colours and location of the region, and **specific element components**. Because *AtWUS* is the reference gene, the matching for *WUS* is easier and the final result is shown in Fig. A2.3. For other genes e.g. *WOX5*, the matching is more complicated. The region B of *WOX5* in Fig. A2.2 is based on colour and location. The region H identification is a case which depends on a mix of element components. *AtWOX5*, region H, which contains grey elements ACGTTB, white elements ABRELA, and the green element RHERPA, matches best with the grey region of *MtWOX5* from -1462 to -1721 which also contains elements ACGTTB and ABRELA. The region A and D of *AtWOX5* is blue and the region A+D of

MtWOX5 is green. However, because the green element is a partial-unique element which is included in the blue region, and located at similar positions of the promoter region, these two regions also link but with a dashed line to indicate the partial similarity. After the colour and elements are compared, the region which may have specific elements and can not match a region with the same element and suitable location in another species, an "x" marker is attached to the region to indicate that no matched region was found in another species (Fig. A2.3 and A2.4).

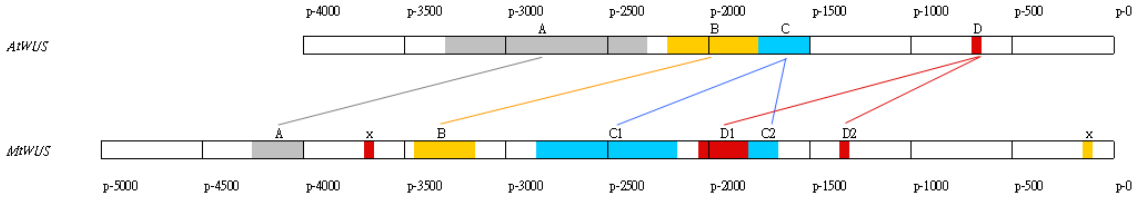

Figure A2.3 Final patterns of promoter region analysis for *AtWUS* and *MtWUS*. The Grey colour indicates the OE/FE region; yellow colour indicates the GQE region; blue colour indicates the MQE region; and red colour indicates the SNSC regions. The "p-number" indicates the distance upstream of the coding site. Matched regions were marked (from A to D), and the marker "x" indicates the region did not find the matched region in other species. The matched regions are linked by coloured lines.

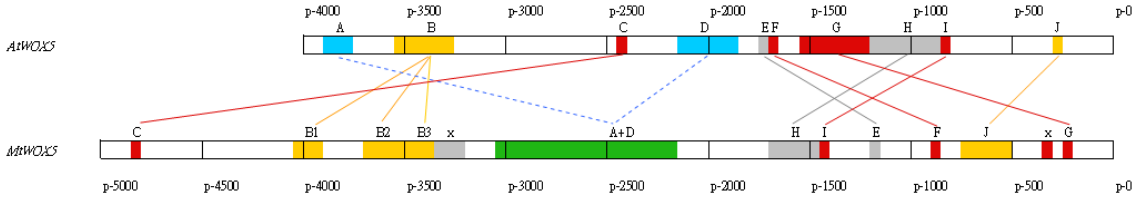

Figure A2.4 Final patterns of promoter region analysis for *AtWOX5* and *MtWOX5*. Display information was described in Figure A2.3. Green colour indicates QE region. Dashed line between regions indicates that these two regions are not the same but with highly similarity.

APPENDIX 3

Medicago truncatula WOX genes - DNA and protein sequences

MtWUS

cDNA: predicted from CT009654.

```
   1  TCCAACAAAAACATGGAACAGCCTCAACAACAACAACAACCACAGACACAACAACATTCA
  61  CCAAACAATGGGATTATGGGAAGCAGACAAAGTAGTACAAGGTGGACACCAACAACAGAT
 121  CAGATAAGAATATTGAAGGATCTTTACTACAACAATGGAATTAGATCACCAAGTGCAGAA
 181  CAGATTCAGAGAATCTCTGCTAGGTTAAGACAGTATGGTAAGATTGAAGGCAAAAATGTC
 241  TTTTATTGGTTTCAAAATCACAAAGCTAGAGAAAGACAGAAGAAAAGGTTCACTTCTGAT
 301  GTTAATGTTGTTCCCATCATTCAAAGAGCACCAAACAACAATACTATTATTTCTGCTGCT
 361  AATTGGAAACCTGATCATCATGAACAACAACAAAACATTAATGTTCATACCAACCATTCT
 421  ACTTACAACATTTCATCTGCTG
      -Intron-
 444  GGCTTTCTTCTGCTTCATGTTCTTCTGCTGAGATGGTT
 481  ACTGTAGGCCAGATTGGTAACTATGGATATGGATCTGTCCCCATGGAAAAGAGTTTTAGG
      -Intron-
 541  GAGTGTACAATTTCAGCTGGATGTAGCAGCAGTCAAGTTGGAAGTACCATAAACCCTCAC
 601  ATAGGATGGATTGGTCATCATGTCGATCCATATTCTTCAGCTTATGCCAACTTATTTGAA
 661  AAAATAAGACCAAATGAAGAAATCATGGAAGAATATGATCAAGGACAAGAAAATGGTTCA
 721  CCTGAAATTGAAACCCTCCCTTTATTCCCTATGCATGGTGAAGACATTCATGGTGGCTAT
 781  TGTAACCTCAAATCAAATTCATCTAACTATGGTGGTTGGTATCAAGCTGAAGATGCTGGA
 841  TTCATGTATGGTTCTCGTACTACTTCCTTGGAACTTAGCCTCAACTCTTACGGCTGTAGG
 901  TCACCAGATTATGCTAATTAA
```

Protein: 306 amino acids. Underline indicates WOX homeobox region.

```
   1  SNKNMEQPQQQQQPQTQQHSPNNGIMGSRQSSTRWTPTTDQIRILKDLYYNNGIRSPSAE
  61  QIQRISARLRQYGKIEGKNVFYWFQNHKARERQKKRFTSDVNVVPIIQRAPNNNTIISAA
 121  NWKPDHHEQQQNINVHTNHSTYNISSAGLSSASCSSAEMVTVGQIGNYGYGSVPMEKSFR
 181  ECTISAGCSSSQVGSTINPHIGWIGHHVDPYSSAYANLFEKIRPNEEIMEEYDQGQENGS
 241  PEIETLPLFPMHGEDIHGGYCNLKSNSSNYGGWYQAEDAGFMYGSRTTSLELSLNSYGCR
 301  SPDYAN
```

Promoter sequence: 5000 base pair upstream from coding site (Bold type)

```
-5000  TTTTTATACCATGAAAATTCACATGAACACCGTTTCAATAATATCAATTCATTTACTGTG
-4940  TTTGGTATCTTGTGGGTATGTTCGTGATTCTGATATACCTACTCTAAAACTAAACATAAG
-4880  GTTAATATATGTTTGATTATACTTTGAGAAAAATTGAGTTTGAGTCAATTAATTCCAATT
-4820  AAAAATGAGTCGTAGATAAAATAGTTTGTGTTTTGATATATTAATACAAGGGTAAGTTGA
-4760  AAAATAATTTTTTGCTAGAATCAACTCCACAATCAAGAACTATAAATAATAACTTCAAGT
-4700  AAATTATGTTAGAATTACTTTTAGGTGTTGGCTATAGTAAGTGTCCTTAATGGGTCTTTT
-4640  ATTGTAGGAGACTCAATTTATTTATTTATTTTTTGATGAAAATTGATTGTTAGCCTTTGG
```

```
-4580  ATATAGTAAGTAGCTATTGAATGGCATGGATCTCTTGACAAGTTGACCCATTAAAATTGT
-4520  ATATTTTAAATTTAATCATTAAAGTTGATCTTTTAAAAAGTTACTGATTTTGAAATGAAA
-4460  TTGTAATAAAGGGTGTTTTTGTCAAAGATGGGATAACTGTAAAAATCCCAGCAACTATTT
-4400  ATTTAGCTTATGTTCTCCTACATTTAATATTTTCTGTCATTAACCAACTAAACGAGCTTG
-4340  TAAATTCTTAAAAAAGCAATCCTTTAATTTTTGTTCATTGAATCTAAGGTTGGGCATGCT
-4280  TGATAAAACGTTCAGTAATTTTAAAATTCGTCGCATGCCTTTTGTATTATACTGAGTGTA
-4220  ACGTTCATTTTATTTAAAAAAACGGTTTCCACATAAATAGGTTTTTTTAAATATTATTTG
-4160  ATTTTTTTTAGTTTGATTTTATTTAGAATTTTGATATTATGTGGTATTTTACTCTCATAT
-4100  TTAAAATTGAAACAATAAATCACCGTTTATTGAAACTAAAAAGGTTAAATTTTATAACGA
-4040  TATACCTATTTTTATGAGAATGATAAAATAGTGGACAATAGTACAACTATACTTAAAATA
-3980  TAAAAACACTATGAATAATTAATAATATAATCTTAAAAATATATCTAATTAATTGATTGT
-3920  CAAAAAAAAAAAATCTAATTAATAAGAGTTCAAATTAACTTTAGTTTATCGGTCATTCAA
-3860  TATATTAAACAGGTATGATTACCTCTGTTTTAATCTACCACTATGAATAAAGTTATTACT
-3800  TTTTTTTTATTTAATCACAATACTATTTCGGATTTCAATTATAAAAAAAAAAATAAAAAA
-3740  AATCTAACTTCAAAAATTTACTTAAAGTATCATCTATTGGAACTTGTCTCACAGAGGTGC
-3680  TCGGAGCAAACTGAACATGTTCAAACGAACAGTAACGGTCTATGAAAATCTTTCAAAGAC
-3620  TAACTTATGGTATATGAAAAATTTCTAAATACTAACTTCCGTTCTATGAGAATCTTTCAA
-3560  ATACTAACTTTTGTTTTATGATAAACTTTCAAAGACTAACTTTCGTTCTATGAGAATCTT
-3500  TCAAAGACTAACTTTTGTTCTATAAGAAACTTCTATAGACTAACTTTTGTTCTATGAGAA
-3440  ACTTCTAAAAACTAACTTCTGTTGTATGAGAATCTTTCAAAAACTAACTTATGTTCTATG
-3380  AGAAACTTCTATAGACTAATTTCCGTTCTATGAGAATATTTTAAAGACTAACTCTTGTTT
-3320  AATGAGAAACTTCTAAAGACGGACTTTTGTTCTATGAGAAACTTCTAAAGACTAACTTTC
-3260  GTTCTATTAGAAACTTCGAAAGACTAACTTTTGTTCTATGAGAAACTTCCAAAGACTAAC
-3200  TTTATCTTGTGAAATTCTTCTAAAGACTAACTTCCGTTATCCGAGAATCTTCCAAAGACA
-3140  AACTATCATTATATATATGAGAATTTTCCAAGGATTGAAATGTTTGTATAATTTTTTTTA
-3080  ATTAAAATTTAGTTGTAAACGTTTATAATTTTTTGGGTTAAATATGTTTTTGGTCCCCAT
-3020  AAATATGTCAACTTTTCATTTTAGTCCCAATACAAAATTTGTTCAACTTTTAGTCTCTCA
-2960  AAAATTTTTCATATTCACTTTTGGTCCCTCCTTTAAAGTGAACTCATACGTAGATTCATA
-2900  TTTTTGAATAAAATTTTGCAGAAAAATTCATAATATAATAAGAATCTCTCCTAAAAAAAG
-2840  TTAGATTTTTTTAACAAAACATGAATTTAGTATGAATTTTTAAATGTTTTAAGGTTAAAA
-2780  ATTCATATTTAATTTCTGTTTTGTTAAAAAATTCTAGTTTTTTTTGGGATTTTTTTTAGA
-2720  ATATTTAACACATTTTTGCACAATTTTATTAAAAAATACAAAAATTAAATTAAAAATAGG
-2660  TACCGAAAGTAGTGATTGAAAATTTTAGAGGGATTAAAACGAAAAATTGATATATTTATA
-2600  AGGACCAAAAATATATTTAACCTTAATTTTTTTATTAGAAATTATGTGTTCAATTAATGT
-2540  ACTATTTTCCATATGTTATTCTATCGTAATAATTTATTAAGATTGATGGTGTTAAATGAT
-2480  TAATAATCAAATAAATATAATAAAAACACTACCAAAAAAGAAATATAATAAAATAAAAAG
-2420  GGAGGGATTAACTTTTAAAAATAAACAATGTTAATTAAGGCGTGTATTAAACCGGGAGGA
-2360  ATGGATATTCTCTTGTTTCAGGGTAAAGGACAATAAAAGAGAGGAAAGGTAGTGAATTTG
-2300  AAGTACCTTAAATGCCCTTTGCCTTCAATTTGAGAATATAATTCTTAAAATTAAAATTAC
-2240  ACACAAAATCAGGTAACTAACAGTAGATTTACCTACAGTTGCACGTTATAGCACCAATGT
-2180  TAAATTCTTTTTTTAGTAAAAATGCATCTCGCACGTTAAACGACGCCTTACTTTTGGTAC
-2120  TTGAAAAACACTTCTAAGATATATAAAAGTGAAACCAAACATATTTCTAACATTAATCAA
-2060  TCAAGTTAATAATTGTTTGCATCAATAAAAGTGACTTGATAATAGGTGACTCAGAAAATT
-2000  TTAGATAGAGTGCAATATCATTTTAAATTTTGAGCCGAGTCTAATTCAATAATTTTAACA
-1940  TCAATTTGTAAGTTGATGATTTTCCTCATTTATAAACACATATGTTTAATCCATATTTAA
-1880  TCAACGTAAGATACTGTAACCATATGTGCAAGCAATAAAAGATTACAATTTTTGTAAACT
-1820  AGCCATTGAGCTAGTTTGTACTGTCTCGCTTTCTCAACGTGTTAAACGAGACGAGAAAAC
-1760  TTGAAAGTTTTATGTTGAAAATCTCTACTGATCTTACCTAAAACGACGGGTTAAACGAGA
-1700  CGGACCGGCAAGGTCGGCTCGTTTTAACACCCCTAATCAAAACAAAATCCAAATTAGTAG
-1640  TTGTTGTCCCTTAATTTTATGAATCTTTTTATAAATTAATTTTATCAATCCTTATTTACA
-1580  CGGAAATTAAACAACATAAAAAGTTGAGGAATAGATCATCTCATTAAAATTTTATACTGA
-1520  TTTGGTGGGGTATTTTTACAAGTATATTTAGTGGTTTTTCATCACAAATTTTATTAGAAT
-1460  CTCATCTCATCTATCTAATATATTTATAGAGCGATGGATACAAAACTTTCACATGATACC
-1400  ATTTGATGAAATCATGCATTTCCGTATGAATATACTATTTGTTTTATATTGGGATAGGAG
-1340  ATATTGTGGCCGTGTGCATAGAGACAAAAAGAAGACAAAGCAAAGTGTTAGTTTATTTGT
-1280  CATCTCCTCCAATAATTTGTAACTAAATCACTTCAAAAAGCTAACTATATAAAGTTTAAG
-1220  AATAATTCCCTTTACATTTCAACCCTATCATATACATATCTCTCTATTTTATTATCTTCA
-1160  CATAAAGTGCAATCACTTTAGTAGAAAAATGTGACATGTTAAAGAGAATCCTAACCTTAT
-1100  TGTGTGATCTTGACTGAGAGAAAGAAAGATTAATTAATTACAAGAAAATGAATATTAATT
-1040  GAAAGGAACGTATAACTAAAAAAAGGCTTTATTTACATACTATATTTTCTGTTTTTGGTC
 -980  ATTTCCCAGTACTGATCATAATCAAGTCTTGTGCAGATATAATTATTAATTAGACATTTT
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-920  GAGAGTACCTATTCTCTTATAGAGACTCAAGAAACTGTATAATTCAAAATCTTTGTCACA
-860  CATATATAGAAATTGTACCATACAAAACCATAATATATAGTCTTCTTTGCTCATCAGTAT
-800  CATCAATTCTACCACTGCATCAAAAGTACAGTTAGAAAAGTTATACTTCCTGATATAACT
-740  TTAGAATATGTGTACCAATATGCAGAACAAAATAAATTTAAAACCCTAATGAAAACAAGG
-680  TTCATATATATTGGGACCCATACCATACATATGAGATTCATACTAATGAAAAATACATTT
-620  TTACCCTCCACTTTTTTTGCTCTAGTACAACAATATCTTAGTAGCTATTGAAAAATGATA
-560  TATTCATGTAGGGGCCATGTCTGATTTCCACATCAAAAGAAGAACCTATTGAGAACTCCC
-500  AAATCACACTAGCATCATTCTATTTCATTCATTCATTCAGTAACATCTACTTTTTTTCAT
-440  ATCATTTCATACATAGATACATTACAGTACATTTACATACATACCCTTTATTTTCTAATT
-380  TAAAATTTAATCAATGTATGCAAATACATTATTTTTTACAATATACCTGGCATACAATGC
-320  CTTTTGATCCAAGTAAATTCCTGGTCACTTTCTAAGCTCTCTTTTGTCCCTTCCCTTTTT
-260  ATGAACATCTCTCTGTCAACTTCAGTCCTTCTCTCCCAGATATCATTTCTGATTTTCTCT
-200  CTTTATCACTCCATGTACCATGTGCATGCCCTATATCATCATATTCATATTATTTATATT
-140  TATATATATAACCTACCAATACAATACATTACATGCAGTAGTACTCTCTTTCACACTCAC
 -80  CTACCCTCTCTATTCTATCTTTTATTTTATTTTTTGTTTTCTTCTCTTTGTTTTGTTCTT
 -20  GTTTTCAGTCCAACAAAAAC ATG
                          +1
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MtWOX5

cDNA: sequence obtained from BF649819 and predicted from CU326389

```
   1  ATGGAAGAGAGCATGTCAGGCTTTTGCATGAAATCATCTGGAAGTAGTAATTTCCATGGT
  61  AAAAGTGGAAGCAGCAGCACTGGCACAAAGTGTGGTCGTTGGAATCCAACAACAGAACAA
 121  GTTAAACTTCTGACTGAACTTTTCAGAGCAGGGCTCAGAACTCCAAGCACTGATCAAATT
 181  CAGAAAATATCCAATCAACTCAGTTTTTATGGTAAGATAGAGAGCAAGAATGTGTTCTAT
 241  TGGTTTCAAAATCACAAAGCTAGAGAAAGACAAAAACGTCGTAAGGTTTCTTTTGATGAT
 301  GACAAGGATGTCATAGTTCATAGAGACAACTCTATGAATGCTTCAACACAAAG
      -Intron-
 354  GTTTGCT
 361  GAGATGTACTCAGAGCCTGATAGAGTGATTGAGACTCTTGAGCTTTTTCCATTGAACTCA
 421  TTTGGTGAATCAGAATCAGAAAAGTGGAGTATGCATGCAAATGAATGCAGGGAAAATACA
 481  ATGTTTGCATACACATGTACAATGGGAGAACAAATGGAACACCCACCATTGGACTTACGG
 541  TTGAGTTTTGTGTAA
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Protein: 184 amino acids. Underline indicates WOX homeobox region.

```
   1  MEESMSGFCMKSSGSSNFHGKSGSSSTGTKCGRWNPTTEQVKLLTELFRAGLRTPSTDQI
  61  QKISNQLSFYGKIESKNVFYWFQNHKARERQKRRKVSFDDDKDVIVHRDNSMNASTQRFA
 121  EMYSEPDRVIETLELFPLNSFGESESEKWSMHANECRENTMFAYTCTMGEQMEHPPLDLR
 181  LSFV
```

Promoter sequence: 5120 base pair upstream from coding site (Bold type)

```
-5120  CTAACTTGGGAGTTCTGTCAAACTAAAATGACCTATTTAAAACTTTCGAAACTTAAAGAC
-5060  TAACTTGAGAATTCTATCAAATTAAAATGACTAAAAGTGATATTTGACCTATAAATTACT
-5000  TATTTTTAGTTGTCCCTTTAGAGGTTTTTATACATACCAAGAAAATTAATAAATATTATT
-4940  ATTTTGATAGAATTATTATTTATTTTTTAACATGTAAACTCAACGGCTTCTGTCCAAAAG
-4880  ACATAGGAGTTAGAGCAGGCAAGACAAAACAAATCCTAGTGCCATGCGAAGTTTCAAGAG
-4820  ACAAAATAGCATTAGTAAATAGTTGAACATCTTTGGGAATTGTAGATAATGCACAAAAGA
-4760  ATGTCGTCTGTCTTTTTGTGATTATCAGATCTTGGGAGTAACTTAATTGTGGAAATTAGG
-4700  GTTGGGTAGTTTAATTGATTTTGAACTAATTAGTTAAGGATTTTGAGTTTCATGATTCAA
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-4640  GTTCTGGTAGAAGAAAAACTAACATAATCATTAATATACTAACATTTATTTTAGAGCAAA
-4580  TTAATATACTACTCCCTCCGTCCCAAATTGTATGACGTTTTGGGCATTTCACACATATTA
-4520  AGAAATGTAATTAATATTGTGTGGGAAAGAGATATTATGAGTTGTTTTACAAAATTGTCC
-4460  TCAATAAATGATATGGAAAAGATAAATGAATGAATTGAAAGAAGAGAGTAATAAATAGTT
-4400  AAGGATATAATAGGAAAAGTAACATTAATTTTTCATTGATATTGTAAAGCGACATACAAT
-4340  TTGGGACAAATATTTTTTCTAAAATGACATACAATTTGGGACGGAGGGAGTAACATTTAT
-4280  CATTAAAAAAATTGTGGAAATTAAAATCAACACGAAAAGCATGCATCTCAATCTATCAAG
-4220  AATGGATCATAAACTTGATAACTTATTATGATATATACGTCATGCATGTTGATCTTTCAA
-4160  ATATAATTAGAATAATTGATTGCTAATATTCTCTTAGCGCTAATCTTGAAATACAAAAAG
-4100  AGAGATACATGTGATTAGAGATGTAAATGGATATCCATGGATGCGGATAGTGTGATATTC
-4040  GTGTCCGCTCCGTTAGATAAATATGAAATCTGTATCCTATCTATATCCGTGCGAATATCC
-3980  ATTTAGCGGATAATCCATGGATTTTTAGGTATCCGCAGGTTTATACAAATATCCATGGAT
-3920  AACATTTATAAAAATAAAATAAAAAATTTGTTTTAATTTTTTTTAACTAAAATTTAGAAA
-3860  CAAAGGTTATTCACATGTATTTTCTTTTCTTTTTAGCAAAACATAATATTCATAAATTAC
-3800  AATAACAACAAAGATCATTGACTCAACAAAAACATAAATAAAGTCCCTTACATTTAATAA
-3740  AAATAAAGTTATTTGATTTAACAAAATCATAAGTAAAGCTCATTATATTCAACATAAATA
-3680  AAGCTTTTGCTTCAACAATCTCAACAACTTTCATTTCATTTTAAAGGAGCATAATAACTG
-3620  ATAACACTCACAATACCTAAAGTTGAATGATATTGAGGTTATTTATGTAATTTACTCGGT
-3560  TTAATAGCGGGTGTGGATACCACTAAATCCGCATCCGTATCCATTAATTAGCGGGTAGTT
-3500  AAAAAATCCGTTTATTTTATCCATGGATATCCATTAAGGTATCCGCTCCGTGTCCGTGGC
-3440  GGATTTTATCCGCGGATATCTACGGGTGCGGATTTTTTTGCCATCCCTACATGTGATATG
-3380  GATAGATCGAGAATGTTAGTTGAACTTGGGCAACGTTTCCTTGCAACCAACTCTGACCCT
-3320  CTCATTGTCATCCTTTATTCAAATCGAAGCCATTATACACAGAAAAATTGATAACTTCCC
-3260  TGAATTCTTCCTTTGATGAGAATTTACATCCTAATGTCCATCGTTTACAAACTTAATTGC
-3200  CACATCATCGTTTTTATAACGTTTGATTAACATAAACAGTAAATTAAAAATGTTTACAAA
-3140  CTTAAATGATTTATGTGTGACCAAAATAACTTAAAATACCTATATATATTACAAACCTCA
-3080  AAATTTTTTTTGAAGGATAATACAAACCTCAAACTAAAAGTTAAAGAATCGTTGGTCTAA
-3020  TTTAGCCAATTTTAAAATAAAACATCAGTTTCATCACTCATTTTTGAACCAACATCAATT
-2960  AATTAATTATATAATTAAAAATCTGATTAAAATTATTACTTAATTTCATTACTAATTAAG
-2900  TAAATTAAGGGCTACATCATAATCATATCATATGGTATATAATGTGAACATTGCCTGCCG
-2840  GTCAAAGTTTTGGGATAGATGAAGTATACTATTAAGTAAAGGCAATTTGCTTCGCATTGT
-2780  GGCAGTTACAACATCTCCTTCTTAAGGTTTTCATTTGACAAAGCCTGCTAATATATATAT
-2720  ATATATATATATATATATATATTAAAGTAGAGTTGTTTAATAAAGATCCTTTACCCTTCA
-2660  ATTTTCAAGAGGAAATGATCCTGTAATTTAGGAATCAAATTCAGATTTTCTCGAATAATT
-2600  TTGATAATAATTTTTCTCAGTAGGTATACTTTAAATAGTTTTGTGTAATCTACATTGTCT
-2540  TATCTTTATCATAGTTATCTTTAATTGAAAATTTTAAATATTAAATAAAAATATGCTTTT
-2480  AATTATGTATATTTATCAATTAGTTCATTCACAAAATTTATCAAACCTTCAAAATTTTGA
-2420  TTCATATATTAGTTTTGTATGATATCTTACTTCGCATGTTATCAGTTAGTTTATCCATTA
-2360  TCTGTTTTTTACGGACCCAATTCTAAATCTATTAATTTGGGGACTTAAATTCCTATCATC
-2300  CAATTTTTGGCGACCAGATTCTAAATCTCTCAAAAATAAATTGTTAGACCTAAATTATAC
-2240  TCCCACGCCTTAATTAAAAAGTATATTACCATGTAACATTAAGTCAACCTAATTCAATAT
-2180  CTCTGTCAGACTTTATTGAGATCACTGTAATTCAATATCTTTGTTATCACAGCCGTCCAA
-2120  CAAAGTTCATAAAGTATATTACTTTGACTTGCATGACTGCCATTGCCACTCCTCAAAATT
-2060  CAATACATGATTTGTTTTTTCATCTAATATAGTGCATATTACTTGATAAATAGTCCTAAT
-2000  TAATAATTAAGTCTTTTTTCTTTCATATTATCATAATATACAACAACAACCAAGCCTTAT
-1940  CATAGTATATTTACATATTAAATTACTAAGTTCTTCATACTTTTAATCAAAAATAGTTTA
-1880  ATGGATAATTACAACTACATTATAATAAAATTATCTTATTTTTCTTCACTTTAAAGAAGT
-1820  TTATATTACATTACAATATATAACAACTTTGAACCATTTGTATATGAACTTTAGCGGACC
-1760  AACCACTTAATTTTAAAAGCAAAATTTGTCTTAACTCACACGTCTTGATTGAATGGGATC
-1700  AATCTTATAAGTATGGTGTCGAAGCACTAAGTATAAAAGATAAGACAATGAAAATTACAC
-1640  CACATATGTAGAATAGTACAATCATGGAAGTAAGTGCACACTTTACTGAAAAACACATTG
-1580  TGTTGTGTCTTAGAATATGAACTCGAGGAAATAAAAAAAACATAACATAAATAAAGAATCA
-1520  CTAAATACTGTTTAATTTCAATTAGTGAAACTTATATGGTCACTGTTCCAAGACCAAAAC
-1460  GTTGAACAATGTCTCCTTTTACAAATACCAATATCTTTCTACTTGTTTATTAGCATCAGT
-1400  CTAAATGAAACCGATCAAATCCACATATTATAGTGCCTCACATTCCCTAAATTCAAAGCA
-1340  GACAGAACGTATATTATATGTTCACTACATGTTTGATTTTACTTCTTTTGTGTGCCAAAG
-1280  TTTAAGTATAAATTGATTTTGATATGTTTGACCAATAAGAATTAAGTTTAACTTTTTTTT
-1220  TTTAAAGAAGAATTAAGTTTAACTTGAAGAGATTAGTCTCATATAACGCGGTGAATTAAA
-1160  ATTGAACTCTAGTTAGAAGATATTTAATAAAAATGATTTCTTCGAGTAGAAATTTTTTAA
-1100  GGGAAAAATATGATTTTGACATATTTAAGGGTTGATGAATAAAAATTCTTTTGTATGAAT
-1040  TTTAACTTGATTTCAAGATTTTAACTTTTGCTTCCCAACATAATTTGTAACCTCATATTT
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-980  ATTGTTGAATTATCTTTTGAGTGAATTGCACCAAAATCAAATTTAGAGAATGAGTTAATT
-920  GATAGTCATTATGGTAACCATAAAACTAATCACAAGGATTCACCATTCTTGCACACGAGA
-860  CAATCATATTTGAGACCAAAAGGAAAAAAAAGGCATACGTTCAAAAAATCATCAATTTAG
-800  TTAGGAAAATGCTAATAAATATGTCCATGTAACACTTGTAACCCACTTGGATCGGTCTAG
-740  TGTCGTTGGATTGAAACTTTGAAGTGTGCTCCTCTCAAAATCTTTGATTTTATTTCATAT
-680  GGTGTCAATTTTGGTGGACTGTTTCCCTACAAAGCTTGTTCTTGTGGCCCTGCAAGAGCA
-620  CGAAGAGATTGCTCCAATCGGATAAGTCGTTCATTGAGCCGGATATGGAGTTTTCAAAAT
-560  AAATATATAGTAAAATTGTCTTAATACTTGTGTCGTTAATTTTTAAAGGGTCTTGTTAAC
-500  TGGTGCCCCCGGAGCACTGGTTAAAGAATCTATAAATAGAATTTTATGTCTTGAAAATAT
-440  AAACTGTCACTTTTATTTAGTAATAATTACATTACTTTTTAATAAAAACTTATTTTATTT
-380  GGTGCTTAACCAATACCCGAAGCATCGGTTAACATTCTCCATTTTTTAAAATGTAAAACA
-320  AACAAAAAAAAGCTCTTTTCATTTTTTTCCTGCCTTTAACAAATGTCAATTTACTTGTTTT
-260  CTTGTCTTTAACAAGTGTCCAAGAGACATTAGTTAACCCAACACATTAAGTAATCTAAAC
-200  TCACTCTCTCTAATTTAGTGGAATAGCCCTTAAACTATTTTTTTTTTTCTATTTTCAAAA
-140  CAAGCATGCTTCCATTGTGCAAACCCTTTTGATTGTTAGTGTATTAATCTATTATATATA
 -80  TGCACACGGATTGGGATATTGTTCAGATAAACTCAGAACTAAAGTATTGAGTTCAGAGTA
 -20  AAAACATCTAGAATTGAAAT ATG
                          +1
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MtWOX4

cDNA: sequence obtained from TC102100 and predicted from AC148486

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   1  ATGAAGGTGCATCAATTCACACGTGGATTTTGGGAGCATGAACCTTCCCTCACACTTGGT
  61  TGCAAGCGTTTACGTCCTCTTGCACCTAAAATTTCTTCTAATAACACCGATAATTCTACT
 121  ACTAACACCCCTTCTCTTTCTTTTGATCTCAAGAGTTTCATTAGACCCGAAACTGCTCCC
 181  AGAATTAGTCTTTCTTCCTCTGATGATAACAAGAAAGATCCACCTTCACCACAAAGCCAG
 241  G
      -intron-
 242  TTGAAACGCATATTCCAGGAGGGACAAGATGGAATCCAACACAAGAACAAATAGGAATA
 301  TTGGAGATGTTATACAGAGGTGGAATGAGAACTCCAAATGCACAACAAATAGAACAGATT
 361  ACAGTTCAACTTAGCAAGTACGGTAAAATTGAAGGGAAAAATGTGTTCTATTGGTTCCAA
 421  AACCACAAAGCACGCGAGAGACAAAAGCAGAAACGTAACAGTCTTGGCCTTCCTCATAGT
 481  CCTCGAACTCCCACCACCACACTTGTGTCTATGTCCCCCACCTTTAGTACTATTACAACT
 541  TTGGACACCCCAAAAAGGG
      -Intron-
 560  TGCAAGTAATGGAAAGAGATCAAGAAGATAGCCCATTGAAG
 601  AAGAGTAGGAGTTGGCCATTTGAGTATTTGGAAGAGAAAAATTGGTCAATGTGCAAAGTA
 661  GAGGAACATAAAACTCTAGAGCTTTTCCCATTACACCCAGAAGGCAGATGA
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Protein: 236 amino acids. Underline indicates WOX homeobox region.

```
   1  MKVHQFTRGFWEHEPSLTLGCKRLRPLAPKISSNNTDNSTTNTPSLSFDLKSFIRPETAP
  61  RISLSSSDDNKKDPPSPQSQVETHIPGGTRWNPTQEQIGILEMLYRGGMRTPNAQQIEQI
 121  TVQLSKYGKIEGKNVFYWFQNHKARERQKQKRNSLGLPHSPRTPTTTLVSMSPTFSTITT
 181  LDTPKRVQVMERDQEDSPLKKSRSWPFEYLEEKNWSMCKVEEHKTLELFPLHPEGR
```

Promoter sequence: 5000 base pair upstream from coding site (Bold type)

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-5000  GATTTTCAGCTATCAGGTAGCTTATAGTTTTTTTTACATAACACACCCTTCAACTAGTTT
-4940  ATCAGTCATCAGCTATCCGCTATAAGCTATCAGCTATTAGCTATAAGCTATCCTCTATTT
-4880  TTACCAAACAGAGCCTAGGATTTTTTTTTTAAAATAGATTTACTAAAAAGCTCACACTAC
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-4820  AAACCAAAAAAAAAAAAAAACTTTTTAAACCATTGAGGTGATAAGTGCCACTTTTACCAA
-4760  GTATTGGAGATATTTTGAAAGCATTGTGGACTAAGTTTAATAGAAAACATATGATTTTGC
-4700  CCCAATTCTTGAATATAGATATTTAGTTTTTTTTTTTTATTTAATCATGAGAAATAAACTT
-4640  TATGGAATATATTAATTTTTTAAAACAAGACAGAATGAGTTAAAATGAGACAGGGTCAGT
-4580  AAACTGTAATTTTATGAGACGACCGACATTATTAGCCGGAAGAGGAAAGTTTATGTTTGG
-4520  GCAGTGACATACCTGAGTTGGTTTGCAAAGTGGCAAGCAAAGTAGCACAACGGCATTCAC
-4460  AATCATAACCATAGTTTATCTATCCGTCAAAAAAATCATAAATATACTGTGAAGTGTAAG
-4400  TGGAATAATATTCACACTTCCATTGCAGGTGCATCATTGTGATGATTGGTGATCATGTAT
-4340  TTTGACTATAACACACGTGTGCCATGAGTCTCCCACTCGTGCGCCGATTTTGAATCCCAA
-4280  TTCACTTATAGCGGTTTTTTTTTTTAAGGTTTATAGCAGAAAAAAAAAAATTAAGCTTTT
-4220  TTTTGGGTGTCTAAATTAATGGGACTTGGGCACTATTTTTTAGCAATAAATTGGTTTTTG
-4160  ACGACGAAAAAGAAAGTACTACCTCCGATCATATTTATAAGCAAAAAATAGCTTTTTAAT
-4100  TATATATTGAATAATTAATGTATCCGATCATATTTATAGATCAAATGCATTAATTATTCA
-4040  ATGTACTTAAAAAGTTGTTTTTTGCTTATAAATGTGACTAGATGAAGTATGTTTTTAAAG
-3980  AATATTTTTATCGAAAAAAAAATAGCGGAACTTACTTGTATTATATTGTTGAGTGTGAGTT
-3920  AAAATCTCATTATATATAAAAGAGAAGATTTTGAAAAAGTTTTTTTTTGGCCTCGCATGC
-3860  TAGGCATATGATGTTTATGAGCGCATAGGCTTGTACGTTGTGCATGTTGTTAGTGATGCG
-3800  CTTGGCGCATGGCTCGGGTTTTGGACCTCTATTTAAACTTTCTTCTTGTTCTTTTTGTAT
-3740  AATCCTTGAAGAACCAAATGAATATACTCTTGGAAAACGACTTATGCCTTTTGGGAGAGA
-3680  TTCTAGGGGTGGGAAACACTTTTATAAGAGGGTAAATGAGAGAAATTTAAGAATGTTCTT
-3620  GAGAACATTGGGAGCTAGATTTCTTATGACTTTTGTAATTCTCTTGTTCATGAGAAGATA
-3560  GTGGAAAATGGTACGATTAGGTTTGGTCTTGGAGAGTTTGTGGAAAACTAATTTATTGTG
-3500  TTTGTTTAAAGTTAAATCTCTTTGTAATGACTCGTTGATATTAGATTGGAGATATGGCCA
-3440  CACCCTTGTTAGCATTTTTCCTCTATTTTTAGTGCATTAAGTACCGAAAAAATCAAAATT
-3380  ATGTGTAGGTCATTTATATATTTACATGTACACAAGGCTCTAACCATCCCAAATCCCGAT
-3320  GACATTTATCGATCAATTTCATAGTATATAAATGAATTCATATATTGAACTTAAAATTTT
-3260  CATCTTAACTAATTGATTTTGATCTGTAGAAAGAATATAAAAACACCTTGCCATGTTGAT
-3200  TCAATCATTTTTTATATGCAGTAAGTAAAAAAACTATATAACCTAACTAATGAGAACTGA
-3140  GAATAAAATAATAACTTAAACTCTTGATAGAATCTCTCCCTCTCCAATAAGCCGGCCCTA
-3080  GTTTTCTCTCTCTAAGTTTTCTCTTACAATTTCTTTATTGAGGGGTGACTTTGGGTCAAT
-3020  ACTTGACCTGAAATCACCCCTCTTCATCTTTTTCTCTTTATTTCAATTTAAATAAGTGAT
-2960  TTTCACATAGATCTAGGTTTTTTTTTCTTTGTAATTTCATCATCTAAGTGTGGTGATTTG
-2900  TTGTTCGTCGTCTTGTGCGGCGTTGTTTGATTTCCCGTCTTCGTGCGGTGTTCATTTGAT
-2840  TCATATTGAAATATCGATTGTTCAATCAAAGATTTGGGCATCAACGTTGCAGATCCGGAG
-2780  ACAATGGATATTCCGGTCACTTTAATTTCATCATATATTTGTGTAGGCATTATGCCGTTA
-2720  TATGCTATTAACTTGGATGTTGTGAGTTTGTTCGCAGATTCATCCTTTACGTTTTTAGCG
-2660  GTGTTGAATGATGTAATCTCTCAATTTGAATGTTTGTTATATCCACTAAGTTATAGTTTA
-2600  TAGTTTTTTTAAGGGAGTAGTTTATACTTATATCTTGTCAAATTTTATATATATAATGTA
-2540  TGTATAATTATATTGTTAATTCAAAGAACTACGACGGAAAAATTCCCAGCTACTCAAGAG
-2480  TACTCAATCATATGCTAGTAGGGAAGTGAGAATTATTGCCCGGCCACTCCTCATGTCGCT
-2420  TTGGGAAGTGAGAATATGTTTAACCAATACGAATGAAAATGCACATACACATTTCTATAT
-2360  ACATTTGAATCTTTGATTGGTTATTATATAGTAATATGCATAAGACATGGCTCCACTTGA
-2300  GGAGTCCATATAAACTATAATAATACACCAACAGAGTCAATTTGGATAAAAAGATTAATG
-2240  AAATAAAATTTTAAGGCTATATCACGAATGCATGGTCTATACGTTGAGTATTAAAGTAGA
-2180  ATTTGGGAAATTGGTGCTACAGTTCCTAAAGTAGAGGAGTGGATATTATGGGGTTCGATC
-2120  CGATAAGGAAACTATTTTTGAAAAATATTTATATGGTCATAAGATTATAGACACAAGATT
-2060  TAGCTTATATTCAAGGTTTTGTTTAATATATTTGTATATAGGATCTAATACATCTTTATA
-2000  AAACGGGTAACTCGTTTTTTAGATGAGATGTCACTTCTCACAAATTTATGTCACGCTTTA
-1940  TATTATTTTATGTGAGAATATTTTTTGCAATACACCGCACCCCTATTTAACTTGATGCAT
-1880  GTATTTTTTTTTTTAAGCGACTTGATGCATGGATCTAATTTATGGTGTTCCAAACCAATA
-1820  TACATATATGTTATGTAATTCGTCCTTACAAAATTGGTTTATAAACACGTTTTAAACTTT
-1760  AATTTTATTTTATAGTATACTTAGTTTTTCTCAATATGGTCGCCATAATAAGTCCACATC
-1700  TTTCCCTACTCTTGCATTTTTTCCTCATATAAAAATCTGTATCTTGAAAAAGCAAATAAA
-1640  AGGGGTTCCATCTTCAAGTTTTGGGATTGGAGGAATCTTTTTGCATTCATTCCTCACTTC
-1580  ACTAACTTTAATTGATGAAGGCCACCGAAAAGTGAGAACACCTTATGATGAAAGTAAGAA
-1520  AATTTTAAACGACCCGATTTGACGGGCCAACCTATAAACTCCTGTGTCCACACACGTTTA
-1460  CATCCTCTTCATTCATTCTGATTCAGATATGTGTACAACATTCCCTTTCTTGAGTGTCAA
-1400  AAATAATAGGGAAAGAGAAAGAAAAAAAAAAAGATAAATTAAAAGTAAAAAAATGTGTGA
-1340  TATTTGATTTGATGAATGAAAAGGTTAAAACAAGAAGAAATTTTAAAGTCATCGACATGA
-1280  GAAAAGAAAAGAAAGGTGTGATCACCGTAAACCTTTTTAAAGATGTTCTATTGCTTTTTA
-1220  TTATCAAATAAATTTAAACATATTTTGATCTTAATACATTCTATCATGTGCTAACAATAT
```

APPENDIX 3 ***Medicago truncatula*** **WOX genes –DNA and protein sequences**

```
-1160  ATAGTTTTTTTTTTTTAAGGAACAATATATAGCTTTATTGCCTCTCAAAAATTAATATAT
-1100  AGTTTTATTACGCATATATACAACTAATAATTAGACATAGCAACAAAGCCCATACATGGG
-1040  GTACTCGTTCGAACCAAATCTAAATTGATGAGTTTTACCTGCTTTGATTGAGTTTGGGTT
 -980  ATCCCCGATTTTAAAATACAGATACGGTACAGGTAATGCGGATATAAGTACTCACCCCGA
 -920  ACTCATAACCATACCTGAAGGTAAAAAATCAATTTATTATATCTTTTATATAATATAAAC
 -860  TCAAATCCTAAATCATTTCACTAACACGATCAAAGTCTCAGTATTCATTTCTCAAAGTTT
 -800  TCTCTCTTCTCCTTCCACATCTCACACCCTGAGTATCTTTCTTCTCCGTCACCATAGCAA
 -740  TCAATCACCATCATTTATTTGCAGGATAATGTATTACAACATTGAGTTTTGGGCTGAAAA
 -680  ATACATTTTTTTATTAAAAAAAATTATCCACTACGGGGATGGGGATGAGATACCCGAACC
 -620  TGTCAGGGACGGAAATGAAATTTGTTTTCTCATCCCCGTTGGATATAGGTAAGATAACGT
 -560  GTAAATATTTGAGATTAGGATATGAGGACAAGAAAGGTAAAAATTGTCTCCACCCCGCCC
 -500  ATTGCCATGCCTACTAATTAATAATCAAAATACACTATAATAATGTCATTTTGCTATATT
 -440  AGTTACTAAGTTTTTCTATATATAAAATAATGAAATATGAATGAATGTACCAGTGATAAA
 -380  GTTTTACAATAATTTTAATAGTGTATAAAAATCAAATCCAAAGGTTGTTACTCATCTGGC
 -320  AGCTGGAATGAACCCAGGCACAGTAGTATTCTCTGAAGGTACAAAATGTAAGTTACAGTA
 -260  TTTGATTTGATTTGAAGAATTTTGTCGTGTTTTACTTTTGGTTTAATTTAGAGAGGGAAA
 -200  ATCAAAAGTCAACTGCACCTCATCATTTGGTCTTGGTTCTTACACGCAACACCTCATTTC
 -140  CCACTTTGTATACTTTTCACTGTTGTTTTGTTTTTCTCATCATCATAATTTCCATTCATT
  -80  CATTCTCTCTATATAATCACATTCCCTGTCATTCATTTCTTCATCACACCGTGTGGCAAA
  -20  TTATATTAAACATAAACATT
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ATG

+1

APPENDIX 3 ***Medicago truncatula*** **WOX genes –DNA and protein sequences**

APPENDIX 4

Medicago truncatula CLAVATA-like genes - DNA and protein sequences

SUNN

cDNA: sequence from AY769943

```
   1  ATGAAAGGAGAGAAAGCCAAAGATGATGCACTCAAAGACTGGAAATTTTCAACTTCTGCT
  61  TCAGCTCACTGTTCATTTTCCGGTGTAAAATGCGACGAAGATCAACGTGTGATTGCTTTG
 121  AACGTGACGCAAGTTCCACTCTTCGGACACCTTTCCAAGGAGATCGGAGAGTTGAACATG
 181  CTCGAGAGCCTTACAATCACTATGGACAATCTCACCGGCGAGCTTCCAACTGAGCTATCC
 241  AAACTTACTTCTCTTAGAATCCTCAACATCTCTCACAACCTCTTCTCCGGTAACTTCCCC
 301  GGCAACATCACTTTTGGAATGAAGAAACTTGAGGCTCTAGATGCTTATGACAATAATTTC
 361  GAAGGTCCTCTTCCAGAGGAAATCGTTAGCCTGATGAAACTCAAGTACTTAAGTTTTGCT
 421  GGAAACTTTTTCTCCGGTACAATACCGGAGAGTTACTCGGAGTTTCAGAAGTTGGAGATT
 481  TTAAGGCTGAACTATAACAGTTTAACAGGGAAGATTCCTAAGAGTTTGTCGAAGTTAAAG
 541  ATGCTAAAGGAACTCCAATTAGGTTATGAGAATGCTTACTCCGGTGGAATTCCACCGGAG
 601  TTAG
      -Intron-
 605  AAATTCCACCGAGTCTTGGAAATTTAGAAAACCTCGACTCCTTGTTTTTGCAAATG
 661  AACAACCTCACCGGAACAATTCCACCCGAACTCTCTTCAATGCGGAGTCTCATGTCGTTG
 721  GATCTCTCCATCAACGGACTCTCAGGGGAGATTCCAGAAACCTTCTCAAAGCTGAAAAAT
 781  CTCACTCTCATCAATTTCTTCCAGAACAAGCTTCGCGGTTCAATTCCAGCGTTCATCGGC
 841  GATCTTCCTAACCTCGAAACGCTTCAGGTTTGGGAAAACAATTTCTCTTTTGTATTGCCG
 901  CAGAATCTCGGTTCAAACGGAAAGTTCATATACTTTGACGTTACGAAGAATCACCTCACC
 961  GGATTGATCCCACCGGAGTTATGCAAATCAAAGAAGTTGAAAACGTTTATCGTTACTGAC
1021  AACTTCTTCCGCGGTCCAATACCTAACGGAATTGGCCCGTGTAAGTCACTTGAAAAAATC
1081  AGAGTGGCTAATAACTACTTGGACGGCCCGGTCCCACCGGGGATTTTTCAGTTGCCTTCT
1141  GTACAGATAATAGAGCTTGGAAATAACCGTTTTAACGGCCAACTACCAACGGAGATTTCT
1201  GGCAATTCTCTCGGGAATCTCGCTCTTTCTAACAATTTATTTACCGGGAGGATTCCGGCG
1261  TCCATGAAGAATCTCCGATCACTGCAGACGCTGTTACTCGATGCCAATCAGTTTCTCGGA
1321  GAAATTCCGGCAGAGGTCTTTGCTTTACCGGTGTTGACTAGAATCAACATAAGTGGCAAT
1381  AATCTCACTGGTGGAATTCCAAAGACGGTTACTCAATGTAGTTCACTGACTGCAGTTGAC
1441  TTCAGCCGAAACATGCTTACCGGTGAGGTTCCTAAAGGGATGAAGAATCTGAAGGTTCTA
1501  AGCATTTTTAATGTTTCGCATAATAGCATATCTGGGAAAATCCCTGATGAGATTAGATTC
1561  ATGACGAGTCTAACGACGCTGGATTTATCTTACAACAATTTTACCGGAATTGTCCCCACA
1621  GGTGGTCAGTTTTTGGTCTTCAACGACCGGTCATTTGCCGGAAATCCTAGCCTATGTTTC
1681  CCCCACCAAACAACATGTTCTTCATTGCTCTATCGTTCGAGAAAAAGCCATGCAAAGGAG
1741  AAAGCTGTCGTCATAGCAATCGTCTTCGCCACAGCGGTGTTAATGGTAATTGTAACACTG
1801  CACATGATGAGGAAGAGGAAGCGTCACATGGCAAAAGCATGGAAGCTAACAGCGTTTCAG
1861  AAGTTGGAATTCAGAGCAGAGGAAGTAGTGGAGTGTCTGAAAGAAGAGAACATAATAGGA
1921  AAAGGAGGAGCTGGGATTGTCTACAGAGGGTCCATGGCAAACGGAACAGACGTTGCGATA
1981  AAGCGTTTAGTTGGACAAGGAAGTGGTAGAAATGATTATGGATTCAAAGCTGAGATAGAG
2041  ACATTGGGAAGGATTAGACACAGAAACATAATGAGGCTTTTGGGATATGTTTCAAACAAG
2101  GATACAAATTTGTTGTTGTATGAGTACATGCCTAATGGTAGTTTAGGTGAGTGGCTTCAT
2161  GGTGCAAAAGGTTGTCATTTGAGTTGGGAAATGAGGTACAAAATTGCTGTGGAAGCTGCT
2221  AAGGGACTTTGCTATTTGCACCATGATTGTTCACCTCTTATCATTCATAGGGATGTTAAG
2281  TCTAATAATATATTGCTTGATGCTGATTTTGAGGCTCATGTTGCTGATTTTGGACTTGCT
2341  AAGTTCTTGTATGATCCAGGTGCTTCTCAATCCATGTCCTCAATTGCTGGCTCCTACGGC
2401  TACATTGCTCCAG
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      -Intron-
 2414  AATATGCATACACTCTCAAAGTGGATGAAAAAAGTGATGTGTATAGT
 2461  TTCGGAGTGGTGCTATTGGAGCTGATAATAGGAAGGAAGCCAGTTGGTGAATTTGGAGAT
 2521  GGAGTAGACATCGTTGGATGGATCAATAAAACTGAATTAGAACTTTATCAGCCATCAGAT
 2581  AAAGCATTAGTGTCAGCAGTGGTGGACCCACGACTCAATGGATACCCTTTGACTAGTGTT
 2641  ATCTACATGTTCAACATAGCTATGATGTGTGTTAAAGAAATGGGACCTGCAAGGCCTACC
 2701  ATGAGGGAAGTTGTTCATATGCTCACTAATCCACCTCACTCTACAAGTCACAACTTGATT
 2761  AATCTCTAG
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Protein: 974 amino acids.

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    1  MKNITCYLLLLCMLFTTCYSLNNDLDALLKLKKSMKGEKAKDDALKDWKFSTSASAHCSF
   61  SGVKCDEDQRVIALNVTQVPLFGHLSKEIGELNMLESLTITMDNLTGELPTELSKLTSLR
  121  ILNISHNLFSGNFPGNITFGMKKLEALDAYDNNFEGPLPEEIVSLMKLKYLSFAGNFFSG
  181  TIPESYSEFQKLEILRLNYNSLTGKIPKSLSKLKMLKELQLGYENAYSGGIPPELGSIKS
  241  LRYLEISNANLTGEIPPSLGNLENLDSLFLQMNNLTGTIPPELSSMRSLMSLDLSINGLS
  301  GEIPETFSKLKNLTLINFFQNKLRGSIPAFIGDLPNLETLQVWENNFSFVLPQNLGSNGK
  361  FIYFDVTKNHLTGLIPPELCKSKKLKTFIVTDNFFRGPIPNGIGPCKSLEKIRVANNYLD
  421  GPVPPGIFQLPSVQIIELGNNRFNGQLPTEISGNSLGNLALSNNLFTGRIPASMKNLRSL
  481  QTLLLDANQFLGEIPAEVFALPVLTRINISGNNLTGGIPKTVTQCSSLTAVDFSRNMLTG
  541  EVPKGMKNLKVLSIFNVSHNSISGKIPDEIRFMTSLTTLDLSYNNFTGIVPTGGQFLVFN
  601  DRSFAGNPSLCFPHQTTCSSLLYRSRKSHAKEKAVVIAIVFATAVLMVIVTLHMMRKRKR
  661  HMAKAWKLTAFQKLEFRAEEVVECLKEENIIGKGGAGIVYRGSMANGTDVAIKRLVGQGS
  721  GRNDYGFKAEIETLGRIRHRNIMRLLGYVSNKDTNLLLYEYMPNGSLGEWLHGAKGCHLS
  781  WEMRYKIAVEAAKGLCYLHHDCSPLIIHRDVKSNNILLDADFEAHVADFGLAKFLYDPGA
  841  SQSMSSIAGSYGYIAPEYAYTLKVDEKSDVYSFGVVLLELIIGRKPVGEFGDGVDIVGWI
  901  NKTELELYQPSDKALVSAVVDPRLNGYPLTSVIYMFNIAMMCVKEMGPARPTMREVVHML
  961  TNPPHSTSHNLINL
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Promoter sequence: 4040 base pair upstream from coding site (Bold type)

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-4040  TTGATTGGAGGCGCCCCCTTTTTGTTTGGGAGAATGAGTTGCTAACGGAATTATTACAAG
-3980  ACTTGGATGGCTTTAGAGGGTCTCTAGAGGAGGATGCTTGATGGTGGAGATTGGAAGGGA
-3920  ATGGTGGATTTACGGTGAAATCTATGTACAAGAAGTTGGAGGTGTTGATGCTGGAGGAGA
-3860  GCATGGCTTCCATAGAGGAAAGGAGGATGTTTTCTCATATTTGGAAAAGTCCGGCTCCGT
-3800  CTAAAGTGGTAGAATTCTCTTGGAAATTGCTTCATGATTGGATACCGACGAAAGTCAATT
-3740  TATGGCACAAGCAAGTTCTTCCACCAGATACTTCGGTGAGCTGTGTTTTGTGTGAGGGGA
-3680  ATGCAGAGTCCGTGAATCATTTGTTCATTCATTGTAAGTTTGCTATGGAGGTGTGGAAAG
-3620  GCGTGTGGAGGTGGTTTGATGTTTTTCTTGGAGCTTCCGCCGAATTTGTTCCTGTTGTGG
-3560  GAGGGCTTGAATGAGCTTGTGGGTAATAATAGGATCCGGAAAGGTTTTCGTTTGATTTGG
-3500  CATGCCGTGGTGTGGAGTATATGGAGGGCGAGAAATGAGCGAATTTTTAACAATTCAAAT
-3440  TGTGGGGTGGAGGAGATTCTGGAGGCTATGAAAGTGCTGTCGTGGAGATGGACTTTAAGT
-3380  CGTTTGAAGATATGAGCGAATTTTTAACAATTCAAATTGTGGGGTGGAGGAGATTCTGGA
-3320  GGCTATGAAAGTGCTGTCGTGGAGATGGACTTTAAGTCGTTTGAAGATATCGGCTTGTTT
-3260  GTTTTATGAGTGGAGTTGGAATCCGAAAGGGTGTCTTATGCGACAAGGGAGCGGTTTTAC
-3200  CTGTGCTGCATCGACAAATTTTGAGGCTGCGGTATGCGAGTTTTTATGTTATCAAGTGCT
-3140  AATCGGTTTAGTGGCCTTTAGCTGTTCGTGCTATGGCTTAATTGTTTCCTGTTTCTTTGT
-3080  GCTGTTTTCTGCTTTTTGTGGGACTGAGGTGTTTGTGGGGTTTCTGCTATATCAGTGCCT
-3020  TTGTATTCCTGTTTTTCGCCGTGAGTTTTTTTGCTGTGCTTCTTGCACACACTCGTCGTG
-2960  AATAAATTTAGCCGTTTCTAAAAAAAATAAAAAAATTGAAAGCATTTAACAAATTTGTGA
-2900  ATACTTCCCGAAGCTCTAGAGGCAGCTACTTTGCTAATTGATCGAAAAGCTAGCAAGGGT
-2840  CTGCGCAAGTTTCTACGTGTTCATTATGAAAATGAAACGTTAGGTGTAGCCAAGTCAAAG
-2780  CTTGGGAATATCATTAAGGAGAAATAGGTATGCTACCTAAGTTACTCTTAACAAAATTTA
-2720  TGATTGTTTACATAAGTAGTAGTTGTTTCTCACCTTTATTGTTGTACTAAGTTTTCAACC
-2660  TGATCAATATATTGAAGTCACTTCAATTTTTCTCATGTTAATGTCCATATTCTCCTTTGC
-2600  CATATGCTTTGTTCTATTTAGTCCCCATCTCTGATCCACTTTCTCGCAAAAAATGACCGG
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-2540  ATAATATTTGAGTTTTAATCCCTTGTGCCACATGTATTACTTCACAAGTGTGGGCACCAT
-2480  AAACATTGGGTTTCCTTTTCCTCTTGAAAGGGGTAGATTCATTCAATGAAATGACACGGT
-2420  TCAAAGTGGTATTTTTGTGTAAGCACCACTTGTTTCTCGAGTGAGAGAGACTAATCACTC
-2360  AAGGCATTGAAACTTCCCTACAACTATTCCTCTACAGTCACATATCTGAGATTGAATCTA
-2300  TAACCACTAAAGAAACTCAATTTACTGAGATCGAAAATTTACTTCAACAAAATTGTGGGG
-2240  ACTCCGTTTAAAGATGGAGCAAAGTTCTTTATGAACTAACCCACAAAAATTGGCACACTA
-2180  TTAGACGAACTCAATTGAGCTTCTATGACTGCATGATTAAAAGTGACAGATAACTCATTA
-2120  CGACTTTATTTTCTGTTCGCTAGAGGATGCAACCAACGGTATTGCCAATTTGCTATTCAC
-2060  ATGATGAGTATAAGGACCTGAATTAGAGAGACTTGTGGATAAGAGTCTTGGTACCAAGTT
-2000  CTTCACCAAATGTGGCTTATAGTATAACTAGAAACTAAGTGGTTATTGGCTAAAGTAGGA
-1940  TATTTTTATCTGAACTGAATCAATCTAATTACAAAACAAAATGATTATGTTATGCTATTT
-1880  GTGGTTTATAGAGGTCAACTGCCGGAACAAACGTGATCCACACTTTAGAGGTGTTCCATC
-1820  ATGTCAACATAATGCACAAAAGAGGTCATAAGCATAGTAAAAACGAGTAATTTATGAGAG
-1760  ATTATCTGTTCAAATTTCATAGATAAAAAAATAACGATAAAGTAAGTGCTCAAAACATGG
-1700  ATACTTATTACAGAAATATTATTTCTGTAATTGCTATGTCTTTATACTTCTCCCCGACAC
-1640  AAGGAATAATTCCTCAATTGAACACACACCTTGCTCGATTATTAACAAGATCCTTTTAAA
-1580  GAAGTGTCAAAACCCCAGTTTTGCCTTTCGCTCTAACGAGCTGCGTTATAGTTACTGTCC
-1520  TTGCTCTTCCGAGAATTACACAATGATTTACGCTTCATCGACATCCAAGGACAGTAGTTA
-1460  CTCTTACTGTTACTGCTCTACACTGCATTACAAGAGAGAAAACATTGAGAGAATATGTGA
-1400  AAGAGAAATGTAAAATGGGTGGGGACAACATCCATTTTTGTATATGGGAACCAAAATGTT
-1340  GAAAAAGTATATAAAAGGAAAAATAATGTGTGGGGGAAATCACTCAATATTATGAGCAGA
-1280  TCCGCCATTGTAGCTAAGTACAGGAGCTTGCCATAGAGAATATAAACTGTCTGCCATTGT
-1220  AAAAGAGCAAGAAAAATTTAGAAAAATGATATTTGAACATCATTTTATGACAACTTTCTC
-1160  TCTCATACTCACATTCCTTTTTATTTTATCTCTCTATTGCTTTGGTTTTCGTGTAAATAC
-1100  CAACTTTTTCTTTGTAAATTATGGTTGTCCCAAAAGTTGTCAACCAAATGGTTATTCAAA
-1040  TAACACTTCTCAAAAATTTAAGGGCCAATAATGGAAGATAATTTTGGTCGCTTCACGTAC
 -980  ACTAAACTAGTTGTCACTACTCACTATCATTAGAGGGGTTGACATACCACCCATCCATTA
 -920  TTAGTGGATGTGGATCCCAATTCCAAAAAACTATGAAAGAAATGTTGAAGAATAACACGA
 -860  ATCCATTTTGGAAGCATTGCTATGTGGCAAATCTTGACCTACTGTTTCATGACAAAATTA
 -800  TTGATTGGGAAAATGAAATACTTTCCCTTAGCAATATTCACACTTGTTAGTAAAAATAAA
 -740  TAAATAAAAGCTACATATATTATCTTTTTGTATGTTCATGTTCTTGTAAATTATTTTTTC
 -680  TTTATGCTCTTCTAGATGAAACATGACACGTTTATACGGTATATTTTTAAAATAATAGTA
 -620  ATATTTTTCTTCCCTTATTATTAATTTTTGGCTTAATAAGATAAGATACTTGATCCCTTT
 -560  CACACACAAAATGATATCTGTTTCCTTATATGACGAGTTAAGTGTAGTCTCTATCACGAG
 -500  TTCGTAGGTCTTCCAAAATGGTGTTATGTAGGAATGGAAATCCGGGACAGACAATGCTGA
 -440  TTATGGATGGTAGCAGAGTGATGTTATGGGGGCATGATAACTGATAAGCAGTGGTGCATC
 -380  TAGCTTCCGATGGTGGTGCTATGAGTGTCACAAACTGATGTAACATTTAAAATTTTGAGA
 -320  ACTCAAACAAATTTTTTAATACACAAATAAACACTAGACAAAGTACTTTTGGACACCACA
 -260  GAAAACTAGGATGAGGTTTCAGTGAAGTCGTGTAGTCGGCCTATTTTCGCACATATTCTC
 -200  TAGAGAATGAAACGTTCTTAGAGAAACTTCATACTAAAAAGGTTTTCTTACAACTTGCTT
 -140  TGTACTCTCGTAGAAGCCTTATTTATAGGTCTAAGGACCCTACTACTATTCCGTCATAAT
  -80  ACTATTCACTATAGTTCTATTTACACAAATATTGTTCATTTCTTACATGATTTATAACAT
  -20  AAAGAATTACATTTCATTGT ATG
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MtRLK1

cDNA: predicted from AC141862

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    1  ATGCTTCCCTTTCTACCACTCCTCCTCTTCCTCCTCTCATTCAACCTTAACCAAGTACTT
   61  TCCACACCCCACATCTCTGAATACCACTCCCTCCTCTCCTTCAAATCCTCCATAACAAAC
  121  GACCCACAAAACATCCTCACCTCATGGAACCCAAAAACCCCTTACTGTTCCTGGTACGGC
  181  ATTAAATGTTCCCAACATCGCCACGTCATTTCTCTCAACCTCACCTCCCTCTCCCTCACC
  241  GGCACCCTTTCCCTCTCCAACCTCCCTTTCCTCACCAATCTCTCCCTCGCCGACAACAAA
  301  TTCTCCGGCCCAATACCCTCTTCTCTCTCCTCTCTCTCTTCTCTCCGCTTTCTCAACCTC
  361  TCCAACAACATCTTCAATGGCACCCTCCCTCAAGAACTCTCTAACCTCTTCAATCTTCAA
  421  GTTCTTGACCTCTACAACAACAACATGACCGGTTCACTTCCTGTTTCGGTCACCCATCTT
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 481  TCTTTTCTCCGCCATTTGCATCTTGGTGGTAACTTCTTCACCGGAAAAATACCACCGGAG
 541  TATGGCTCTTGGACTCATTTAGAATATCTTGCTGTTTCTGGTAATGAACTTTCCGGTCAC
 601  ATTCCACCGGAGATTGGTAACATTACTTCTCTTAAAGAGCTTTACATTGGTTACTACAAC
 661  ACCTACGACGGTGGTATTCCACCGGAGATAGGAAATTTGTCGGAGATGGTGAGATTTGAT
 721  GCTGCTTACTGTGGTTTAACCGGTGAAGTGCCACCGGAGTTGGGTAAGCTTCAGAAGTTG
 781  GATACTTTGTTTCTTCAAGTAAATGCACTTTCTGGGTCGCTTACATCGGAGTTAGGGAAC
 841  TTGAAAAGCTTGAAATCGATGGATTTGTCGAATAATGCTTTCACAGGTGAAGTTCCGGTG
 901  AGTTTTGCTGAGTTGAAGAATCTGACACTTTTGAATTTGTTTAGAAATAAGCTTCATGGT
 961  GCTATACCGGAGTTTATCGGGGAAATGCCGTCGCTTGAAGTGTTGCAGATTTGGGAGAAT
1021  AACTTCACTGGAAGTATTCCTCAGAGTTTGGGAAAAAATGGAAAACTCACTCTTGTTGAT
1081  GTTTCTTCAAACAAGTTAACCGGTTCTCTTCCACCTTTTATGTGTTTTGGTAACAAGCTT
1141  CAAACTTTGATAGCTTTAGGGAACTTTCTTTTTGGTCCTATTCCAGATTCACTTGGAAAG
1201  TGTAAATCTTTGAATAGAATAAGAATGGGTGAGAATTTTCTTAATGGGTCAATTCCAAAA
1261  GGACTTTTTGGTCTTCCTGAATTGACACAAGTTGAGCTTCAAGATAATCTTTTATCTGGT
1321  AACTTTCCTCAACCTGTTTCAATGTCTATAAATCTTGGTCAAGTTACTCTTTCCAACAAT
1381  AAGCTTTCTGGGCCATTACCACCTTCCATTGGGAATTTCACAAGTGTTCAAAAGCTTATA
1441  CTTGATGGTAACCAGTTTTCAGGTAAAATCCCTGCTGAGATTGGAAAGTTACATCAGCTT
1501  TCTAAGATAGATTTTAGTCATAACAAATTCTCTGGTCCTATTGCGCCCGAGATAAGTCAC
1561  TGTAAGCTTTTAACTTTCGTTGATCTTAGTCGTAACGAGCTTTCTGGTGAGATTCCTAAA
1621  GAAATCACTAAGATGAAGATATTGAATTACTTGAACCTGTCAAGAAACCATCTTGTTGGT
1681  ACCATTCCAGGTTCTATTGCTTCAATGCAAAGCTTAACTTCTGTTGATTTTTCATATAAT
1741  AATCTCACTGGTTTGGTTCCTGGTACTGGCCAATTTAGTTACTTTAACTATACTTCTTTC
1801  CTTGGTAACCCTGAACTTTGTGGACCTTATTTGGGTCCTTGCAAAGATGGCGTTGCTAAT
1861  GGACCTCGTCAACCTCATGTTAAAGGACCACTCTCTTCTACTGTGAAGCTTTTGCTTGTT
1921  GTTGGGTTGCTTGTTTGCTCTGCTATATTTGCTGTTGTAACGATATTTAAAGCTAGGTCT
1981  TTGAAAAAGGCGAGTGAAGCTCGTGCATGGAAATTAACCGCATTCCAACGATTGGACTTC
2041  ACGGTTGATGATGTTCTCGATTCCTTGAAAGAGGATAACATCATAGGAAAAGGTGGTGCT
2101  GGCATTGTTTACAAAGGTGCTATGCCTAATGGTGATCTTGTTGCTGTGAAAAGGTTACCG
2161  GCTATGAGTAGAGGCTCTTCACATGATCATGGTTTCAATGCTGAGATTCAAACTTTGGGG
2221  AGAATTCGACACAGACACATTGTTAGATTATTGGGTTTCTGCTCAAACCATGAGACAAAT
2281  CTTTTGGTTTATGAGTACATGCCTAATGGAAGTTTAGGCGAAGTTCTTCATGGTAAGAAA
2341  GGTGGTCATTTGCATTGGGATACAAGGTACAAAATTGCTGTGGAAGCTGCAAAGGGTCTT
2401  TGTTATCTACATCATGACTGTTCACCTCTAATTGTTCATCGTGATGTGAAATCAAACAAT
2481  ATCTTGCTTGATTCAGGCTTTGAAGCCCATGTTGCTGATTTTGGACTTGCTAAGTTCTTG
2521  CAAGATTCTGGAACTTCTGAATGCATGTCTGCTATTGCTGGTTCTTATGGATACATAGCT
2581  CCAG
      - Intron-
2585  AGTATGCATACACATTGAAAGTCGACGAAAAAAGCGACGTCTACAGCTTCGGTGTG
2641  GTACTTCTAGAGCTTGTAGCAGGAAGAAAACCAGTTGGAGAATTTGGTGATGGAGTAGAC
2701  ATTGTGCAATGGGTGAGAAAAATGACAGATTCAAACAAAGAAGGTGTTCTAAAAGTTCTT
2761  GATCCTAGACTTCCTTCAGTTCCACTTAATGAGGTGATGCATGTCTTCTATGTAGCCATG
2821  TTATGTGTCGAAGAACAAGCTGTAGAAAGACCAACTATGCGCGAAGTTGTTCAAATGCTC
2881  ACTGAGCTTCCAAAACCACCTAGCTCAAAACATGTTGAAGAAGACTTAACAACATTAACA
2941  ATCAATGAATCCTCTTTGTCTTCATCAAACAGTTTAGAGTCTCCATCTAAAGATCCTAAA
3001  GATCTTCTTAGCATATGA
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Protein: 1005 amino acids.

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   1  MLPFLPLLLFLLSFNLNQVLSTPHISEYHSLLSFKSSITNDPQNILTSWNPKTPYCSWYG
  61  IKCSQHRHVISLNLTSLSLTGTLSLSNLPFLTNLSLADNKFSGPIPSSLSSLSSLRFLNL
 121  SNNIFNGTLPQELSNLFNLQVLDLYNNNMTGSLPVSVTHLSFLRHLHLGGNFFTGKIPPE
 181  YGSWTHLEYLAVSGNELSGHIPPEIGNITSLKELYIGYYNTYDGGIPPEIGNLSEMVRFD
 241  AAYCGLTGEVPPELGKLQKLDTLFLQVNALSGSLTSELGNLKSLKSMDLSNNAFTGEVPV
 301  SFAELKNLTLLNLFRNKLHGAIPEFIGEMPSLEVLQIWENNFTGSIPQSLGKNGKLTLVD
 361  VSSNKLTGSLPPFMCFGNKLQTLIALGNFLFGPIPDSLGKCKSLNRIRMGENFLNGSIPK
 421  GLFGLPELTQVELQDNLLSGNFPQPVSMSINLGQVTLSNNKLSGPLPPSIGNFTSVQKLI
 481  LDGNQFSGKIPAEIGKLHQLSKIDFSHNKFSGPIAPEISHCKLLTFVDLSRNELSGEIPK
 541  EITKMKILNYLNLSRNHLVGTIPGSIASMQSLTSVDFSYNNLTGLVPGTGQFSYFNYTSF
 601  LGNPELCGPYLGPCKDGVANGPRQPHVKGPLSSTVKLLLVVGLLVCSAIFAVVTIFKARS
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661  LKKASEARAWKLTAFQRLDFTVDDVLDSLKEDNIIGKGGAGIVYKGAMPNGDLVAVKRLP
721  AMSRGSSHDHGFNAEIQTLGRIRHRHIVRLLGFCSNHETNLLVYEYMPNGSLGEVLHGKK
781  GGHLHWDTRYKIAVEAAKGLCYLHHDCSPLIVHRDVKSNNILLDSGFEAHVADFGLAKFL
841  QDSGTSECMSAIAGSYGYIAPEYAYTLKVDEKSDVYSFGVVLLELVAGRKPVGEFGDGVD
901  IVQWVRKMTDSNKEGVLKVLDPRLPSVPLNEVMHVFYVAMLCVEEQAVERPTMREVVQML
961  TELPKPPSSKHVEEDLTTLTINESSLSSSNSLESPSKDPKDLLSI
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Promoter sequence: 4040 base pair upstream from coding site (Bold type)

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-4040  CTCCTTTAATCTACTCGCTACCCCTAAAATTGCCTCCCTAGCTACTAGCTGGACTATATA
-3980  TACCCCACAAATATTGTTTGTTTTAAGTCATACGATCACGGATCACGTGAAGTGTGATTA
-3920  ATTAGCCGTTTCTAACATCAACAACAAGATTAATATGCAGACTCCGTATCATGATTAATT
-3860  TTGTCAAGTATAATATACCACCTAACGAGGCTTTGAGTGGTTAATGAAAAGGGATCATAA
-3800  GAACAAACTTAACCTCTTGTGATAAAGTAGTACTAGTAATTTATTGGTAGGAACCTGAAG
-3740  TGACCACTATTGATGCATATATTTTGGATTCATGAGTCTAGGCCACCACTCTTCTAATGA
-3680  CAAAATTTAACTTTAATGGCAACAAAATCAAAAAGCTTCCCCTTGATAGAAACTATTGCA
-3620  CATGACTTTGTGAATGATTATGAGGCTTTATGCTAAATCACTATTCCTAATTAATCAATG
-3560  TGGGGATCAACCTACATAGCTAAGTACCGTCCTCGTCAATTAAGATCCTTGTTTGATTCT
-3500  TGAGTTGGACAAGTTCATGGTGCGCACAAATTTGGTACCCTACTAGTTGAAATTGAAACC
-3440  AATTAATTAAAAGGTTTATCCTTTTATGTGTATATATATTTGCTTTCTATTTTCTGTGAT
-3380  GTTCAGAATGTTTTGATGTCACAAGAACATGTTGTTCCTGCATTATTGGCTTTTAACCTA
-3320  GTCATTAGAATCATAATCAAAGGGTGCATGTGTTTAATATTACTATTGTTAAGGATCGTG
-3260  AAGCAATAGTGTATGATCATCACGTTTTTTTAGGCGCATTGTATATGACAAGAGAGGGGG
-3200  AAGTTTATTACCTCATCATAAGTGGATTACAATACCAACGTTTTTTTCACCCAAAAAAAT
-3140  GATTGATATGTCTCTCGTCAACAAACTCACTTTGGAGAGCAAAGTAACCAGTAATATCAA
-3080  TCATCTATTCAAAAAAGTTAATTGTAATATTACATACGTGAGTATATTTTTTAATGACAA
-3020  AATTTTAACATACACTTCGAGGATTTGCAAATACTTCATGGGTATAAATCAAATAGAAAT
-2960  CCTAGCAATGTGGACATATGAACAAAAAAAACGGTCGGCACTCCACAATAATCTCGCAAG
-2900  TTTGATTTTAAGATGAAGTTGAAGAAATATCGACATGTAGAAGATAATAGTAACAAACGA
-2840  CAAGTAATTATTGACAACCAATCAACAAAGTTGACAAGCGAATGACAACTATTGGAACAT
-2780  TGGTATGATGTTAGGAAGAAAAATTTTAGAGAAATGATATTTGTACATCTACTTTGTGAC
-2720  AGTTTTTTGACAACTTTCTCTCTCATACTCACATTATGTTTTTATTCCCTCTCTTTCTTT
-2660  TTCTCTCTCCATTATTTTTGATCAATAAAAAGTGAGAAAAAAGAAGTTGTACCAAAAGTT
-2600  GGCTAAAATAGTTGTTCAAATATCACTACTCAAAATTTTAAGATTAAACACTAAATGATT
-2540  ATACTTTGATCTAATGTTGTATGAATTAAAAAAATAGTGTGGGGTACCAAATAAAATAGA
-2480  ACACACATATTATTAGTGTTTAAATTGCGGTATTTAATTTTAAATATTTGATATGGTAAA
-2420  AATAATAGTAGTAGTAATTATTATTTTTATTAGTGAATATCACTTCCCATACATTTAGCT
-2360  TATGGTGGTAGTGATAGAAATAAGAATAAGAATTCAATTGAATTTTTTATTTTTCTTTCA
-2300  TTCAAATACAATGAGTTAATAAGAGTGTTCTTATTCATTAGTCTCTGTTCTGTATAATGT
-2240  CCCTTCTAAACCAAGAAAAGTCTTAAAAACATAGTCTCTGTGATAATGTATGTTCATTTG
-2180  GAATAAAAGAGTACTTTTAAGATCATAGTTCCTTTGAGAACAATAATGTCTCATCGGAAT
-2120  TAAGAATGATTTTAACAGTATATAAATATTCTTTCAATGTTTTTCTATTTGAGAAGAAAT
-2060  TAAAAAGTCAAAATGGTGGTATCATCGTATTTGAATTATCATAGGGTCATATTGATATTT
-2000  TGGAGAACTTAAAGTTAGAATGGTGGTGGCACCATATTTGAGTGGTCCCAGCACCATAAA
-1940  CAGAGTGCTAACAATACCATATTAAAGTGATTTCAATATTAAAAAGTGGTCTTAGTACCA
-1880  TATTTAAGAATGTAAATCTTGAGAACGGTTTTCTACAGTACAGTAATTAACCACACAATT
-1820  GTAGTTGAACTTGTTTTATCCTAAAAGCGGTGTGATTGGATAATCTATCTTGCACAACTT
-1760  TAAGCAGTGCTACAAAACGTCTTAAAGAGAGTGACCTGGTCGTAACTCAAACTAATTCGA
-1700  TAGATATTTTTTTCAAAAAAAATAAAAATAAAAATCCAAATTCAACAGTTTAGAAATTCA
-1640  AATACAACAACGGTACAACGAGAGTAACATCGATGGGTGAAACCAACACCTGCTCGTCGG
-1580  CCTCTAATTGAAAAACCAATAATTTAAGTAGAAATAATAATGGTCACAAACGTAGTGCGC
-1520  GATTACCTAGTTTAATTTTCTATAAATATTAGTTCCTACGACGTACATAAGGTTCAGGGC
-1460  TCATTCAGACATTTAGTGCATGTGGGGCTAAAATCTGACTAAGTTGCTAACACTTACAAT
-1400  TGCTATCGACAAAAGCAAAACCAACTTTAAAAGCTTATAAATTATTGCAAATATGTTGCA
-1340  CTTATTTGTTTCCACAAGTTTTGTACAATGTTGTTGACTTGTTGGTCTCATTCTTAACAT
-1280  ATTGTGTTTTATTTATAAAGGGTAATAATTATTTTGGTCCCTGAATGTGTAAGGAGTAAC
-1220  CATAATAGTCCTTCAATGTATCAAAATTTTAAAATAGTCATTGATTGTGCATTTGCTAGT
-1160  CAAAATTGCCATTAACATTAAAATAATTTGTTAATATTATCATATTGATCTTTTAATATA
-1100  ATTACTTATTGTCACTTCAAGACTATTTTGACTAACAAAGTGTAAAATCAAACACTATTT
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-1040  TTAAAATTTTAATTCATTAAGACATTATCGTGACTACTCGTTACACATTTGGATATCAAA
 -980  ATAATTATTACACTATTTATAATAAAAAAAAAAAAAAAACAGGAAAGCTCAATGTGGAGT
 -920  TTTGGCCCCACATAGCCCCTTAATGAATTCACCCTAATAAGCTTTGATACGTATATTATG
 -860  AAAGCCTACTCCTAATGTGAAAAATTAATTAAAGCTATTAGGATCTAAGTATCTCTTCCA
 -800  ATATCTTTAAAAAATTTACAATTTTATGGAATTTACTATTTCCGATCAAAACTATGATAA
 -740  TTAATTTTTTTTATTATTACTTTCAAATAATTTTTAGTATGATCACTTTATATAGTGACA
 -680  CAAAACTAACGTGAATCTTATAATTTAAAAATTAAAGTATATTAAAAATATAGATTCCAA
 -620  CGTCAAGATAAAATTTATTAAATGATAAGAACATGATTATTTATCCCCATGAGCTTAGCT
 -560  CATTTGGTAAGGGATAATGCACAATATGTGCAGGGGCCGGGGGTTCGAACCCCGGACACC
 -500  CCACTTATTCATCTTTAAGGTGAATTTCTCTAGCCACTAGGCTGCTTGACCAAAAAAAAA
 -440  AAAAAAAGAACATGATTATTTATACTTTAGTTAGAGCGGCTCTTAAGCTAATCACTGTAT
 -380  TTTGTAATTTTATCATTCTTGCAATCAACAAGATTAATAACATCAACTAACTTTGCTTAT
 -320  GTAAAAAAAAAAAAAAAACTAAAGTATAGAAATTAGATATTTTTATTTAATGAGCTGGTA
 -260  CCAACCAAATCTGATATTATATAAAAAAAAAAAATTGAATAGATTTTATGTTTTTGTTTT
 -200  TTGAATAACTAAAAGCTAAATATGAAATAGACATTGGTACTCCTCCTTTGCAAGGATATA
 -140  CAAAATTGTTCATCATCATGTTGGCTGTATTCATGAGAGTTGAAAAGGGCCACCACCACC
  -80  ACCACAACCCTTGAACAATACTTACTTATTCCTTCACTCTCACTTTCACTCATTCATCTC
  -20  ACTTCCCTCTCTCCCTCAAA ATG
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MtRLK2

cDNA: predicted from CR955004.1

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    1  ATGCGTTTCCTTCTTCTCCTTTTCTTTCTCTTCCATTTCCACTACCACCATGTTCTCTCT
   61  GCATCTGCACCAATCTCAGAATACCGTGCACTTCTCTCTTTCCGTCAATCAATTACCGAT
  121  TCTACCCCTCCTTCACTCTCTTCATGGAACACTAACACCACACACTGCACGTGGTTCGGT
  181  GTTACATGCAACACGCGCCGCCATGTCACCGCCGTTAACCTCACCGGACTTGACCTCTCC
  241  GGCACACTCTCTGACGAGCTCTCTCATCTCCCATTTCTCACTAACCTCTCGTTAGCTGAC
  301  AACAAATTCTCCGGCCAAATCCCGCCGTCGCTCTCCGCCGTCACTAACCTCCGTCTCCTC
  361  AACCTCTCCAACAATGTCTTCAACGGAACTTTCCCTTCCGAGCTTTCTCTTCTCAAAAAC
  421  CTTGAAGTTCTTGATCTCTACAACAACAACATGACCGGAACTCTTCCTCTCGCCGTAACG
  481  GAGTTACCAAATCTCCGTCATCTTCATCTCGGAGGTAACTACTTAACCGGCCAGATCCCG
  541  CCGGAGTATGGTTCATGGCAGCATTTACAGTACTTAGCAGTTTCCGGTAACGAACTCGAC
  601  GGAACAATTCCACCGGAGATCGGAAACTTAACAAGTCTCCGGGAGCTTTACATCGGATAC
  661  TTCAACGAGTACACCGGCGGCATTCCGCCGCAAATCGGAAACTTAACAGAACTCATCCGT
  721  CTCGACGCGGCGTACTGTGGTTTATCCGGTGAGATACCACATGAAATCGGTAAACTTCAA
  781  AACCTAGATACACTGTTTCTTCAGGTGAATGCACTTTCAGGCTCTTTAACATGGGAACTT
  841  GGAAATTTGAAGAGCTTAAAATCAATGGATTTGTCTAATAACATGTTAACCGGTGAGATT
  901  CCGACGAGTTTCGGTGAACTGAAGAATTTAACACTTTTGAATTTGTTCAGAAACAAGCTT
  961  CATGGTGCTATACCGGAGTTTATCGGTGATATGCCGGCGTTGGAGGTTATTCAGTTATGG
 1021  GAGAATAACTTCACCGGTAATATTCCGATGAGTTTGGGAACAAATGGAAAATTGTCTCTT
 1081  TTGGACATTTCATCAAACAAGTTAACTGGAACACTTCCACCTTATTTGTGTTCTGGGAAT
 1141  ATGCTTCAAACTTTGATAACTCTTGGGAATTTTTTGTTCGGTCCAATTCCTGAATCTCTT
 1201  GGTGGTTGTGAATCATTGACTCGGATTCGAATGGGTGAAAATTTTTTCAATGGTTCAATT
 1261  CCTAAGGGTTTGTTTGGATTACCAAAATTGAGTCAGGTTGAGCTTCAAGATAATTATTTA
 1321  TCTGGAAATTTTCCTGAGACTCATTCTGTTTCTGTTAATCTTGGTCAGATTACTTTGTCG
 1381  AATAATCAACTCTCAGGTCCTTTGCCTCCTTCTATTGGGAATTTTTCGGGCGTGCAGAAG
 1441  CTTCTGCTTGATGGTAACATGTTTGAGGGTAAAATTCCATCTCAGATTGGAAGGTTGCAA
 1501  CAGCTTTCAAAGATTGATTTTAGTCATAACAGGTTTTCTGGTCCGATTGCACCGGAGATT
 1561  AGTAAATGCAAGCTGTTAACTTTTGTTGATCTTAGTCGAAATGAGTTATCTGGGATTATC
 1621  CCTAATGAGATAACTCATATGAAGATATTGAATTACTTCAATATTTCTAGAAATCATTTG
 1681  GTTGGTAGTATTCCGGGTTCTATAGCGTCGATGCAGAGTTTAACTTCTGTTGATTTTTCG
 1741  TATAATAATCTATCTGGTTTGGTTCCTGGCACTGGTCAATTTAGTTACTTTAACTATACG
 1801  TCTTTCTTGGGAAACCCTGATCTATGTGGACCTTATTTGGGTGCTTGTAAAGATGGTGTT
 1861  CTTGATGGTCCTAACCAACTTCATCATGTTAAGGGTCATCTTTCTTCTACTGTGAAGCTG
 1921  TTGCTTGTTATTGGGTTGCTTGCATGTTCGATTGTGTTTGCTATTGCAGCAATAATCAAG
```

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1981  GCTCGGTCTTTGAAGAAGGCTAGTGAGGCTCGTGCATGGAAATTGACTTCATTCCAACGT
2041  TTGGAATTCACTGCTGACGATGTTTTAGATTCCTTGAAGGAGGATAACATCATAGGAAAA
2101  GGAGGTGCTGGCATTGTCTACAAAGGGGCAATGCCGAATGGGGAACTTGTTGCTGTTAAA
2161  AGGCTTCCGGTTATGAGCAGAGGCTCTTCTCATGATCATGGATTCAATGCTGAGATTCAG
2221  ACACTGGGGAGAATTAGACATAGGCACATTGTTAGGTTGTTGGGTTTCTGTTCAAACCAT
2281  GAGACAAATCTTTTGGTTTATGAGTACATGCCTAATGGAAGTTTAGGCGAAGTTCTCCAT
2341  GGAAAGAAAGGCGGTCATTTGTATTGGGATACAAGGTATAAAATTGCTGTGGAGGCTGCA
2401  AAGGGACTTTGCTATCTACACCATGATTGTTCCCCGCTTATTGTTCATCGAGATGTGAAA
2481  TCAAACAACATCCTTCTTGATTCTAATTATGAAGCCCATGTTGCTGATTTTGGACTTGCC
2521  AAGTTCTTGCAAGATTCTGGAACATCTGAATGCATGTCTGCTATTGCTGGTTCATATGGA
2581  TACATAGCTC
      -Intron-
2591  CAGAGTATGCCTATACATTGAAAGTTGATGAGAAAAGCGATGTATACAGC
2641  TTCGGTGTTGTTCTTTTAGAGCTTGTAACTGGAAGGAAACCAGTTGGAGAATTTGGCGAC
2701  GGGGTGGACATTGTGCAATGGGTCAGAAAAATGACAGATTCCAACAAGGAAGGAGTTCTA
2761  AAAGTTCTTGATCCAAGACTTTCCTCAGTTCCTCTCCAGGAGGTCATGCATGTTTTCTAT
2821  GTAGCCATTCTGTGTGTTGAAGAACAAGCAGTAGAAAGGCCAACTATGCGCGAAGTTGTT
2881  CAAATTCTGACTGAGCTTCCGAAGTCAACCGAGTCTAAACTAGGAGACTCAACAATTACA
2941  GAATCGTCTTTGTCATCATCAAATGCTTTAGAATCTCCAACTGCAGCCTCTAAGGATCAT
3001  CAACATCCTCCTCAATCACCACCGCCGGATCTCCTAAGCATTTGA
```

Protein: 1014 amino acids.

```
   1  MRFLLLLFFLFHFHYHHVLSASAPISEYRALLSFRQSITDSTPPSLSSWNTNTTHCTWFG
  61  VTCNTRRHVTAVNLTGLDLSGTLSDELSHLPFLTNLSLADNKFSGQIPPSLSAVTNLRLL
 121  NLSNNVFNGTFPSELSLLKNLEVLDLYNNNMTGTLPLAVTELPNLRHLHLGGNYLTGQIP
 181  PEYGSWQHLQYLAVSGNELDGTIPPEIGNLTSLRELYIGYFNEYTGGIPPQIGNLTELIR
 241  LDAAYCGLSGEIPHEIGKLQNLDTLFLQVNALSGSLTWELGNLKSLKSMDLSNNMLTGEI
 301  PTSFGELKNLTLLNLFRNKLHGAIPEFIGDMPALEVIQLWENNFTGNIPMSLGTNGKLSL
 361  LDISSNKLTGTLPPYLCSGNMLQTLITLGNFLFGPIPESLGGCESLTRIRMGENFFNGSI
 421  PKGLFGLPKLSQVELQDNYLSGNFPETHSVSVNLGQITLSNNQLSGPLPPSIGNFSGVQK
 481  LLLDGNMFEGKIPSQIGRLQQLSKIDFSHNRFSGPIAPEISKCKLLTFVDLSRNELSGII
 541  PNEITHMKILNYFNISRNHLVGSIPGSIASMQSLTSVDFSYNNLSGLVPGTGQFSYFNYT
 601  SFLGNPDLCGPYLGACKDGVLDGPNQLHHVKGHLSSTVKLLLVIGLLACSIVFAIAAIIK
 661  ARSLKKASEARAWKLTSFQRLEFTADDVLDSLKEDNIIGKGGAGIVYKGAMPNGELVAVK
 721  RLPVMSRGSSHDHGFNAEIQTLGRIRHRHIVRLLGFCSNHETNLLVYEYMPNGSLGEVLH
 781  GKKGGHLYWDTRYKIAVEAAKGLCYLHHDCSPLIVHRDVKSNNILLDSNYEAHVADFGLA
 841  KFLQDSGTSECMSAIAGSYGYIAPEYAYTLKVDEKSDVYSFGVVLLELVTGRKPVGEFGD
 901  GVDIVQWVRKMTDSNKEGVLKVLDPRLSSVPLQEVMHVFYVAILCVEEQAVERPTMREVV
 961  QILTELPKSTESKLGDSTITESSLSSSNALESPTAASKDHQHPPQSPPPDLLSI
```

MtCLV2-1

cDNA: predicted from AC124218

```
   1  ATGAGGCTTAAGCATCTCACCGAGCTTGTTTTGAATGAGAATCCACCTTTGGGAGGTTTA
  61  TTGCCTTTTTGGATAGGTAACTTCTCTGCAAATCTTGAAAGAGTTCAGCTTGGTTATTGT
 121  TCATTCAGTGGAAGTATACCTGAGAGTTTGCTTTATTTGAAGTCTCTTAAGTATTTGGAC
 181  CTTGGAAGCAATTTATTGTCTGGTAATCTTGTTGATTTTCAACAGTCTTTCGTTTTTCTC
 241  AACCTTGGTTCCAATCAGTTTACAGGTACTTTGCCTTGTTTTGCAGCTTCAGTTCAGTCC
 301  TTAACTGTGTTGAATTTGTCTAACAATTCTATTGTGGGGGGTTTACCTGCTTGTATTGCT
 361  AATTTTCAAGCTTTGACTCATTTGAACTTATCAAGGAACCATTTGAAGTATAGAATATAT
 421  TCAAGGCTTGTGTTCTCAGAGAAACTTGTTGTTTTGGATTTGAGTAATAATGAATTGTCT
 481  GGTCCTATTCCTAGTAAAATTGCAGAGACCACCGAAAAACTTGGTCTTGTTTTTCTTGAC
 541  CTTTCTCACAATCAATTCTCTGGTGAAATTCCATTGAAAATTACTGAGTTAAAAAGCTTG
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 601  CAGGCTTTGTTCCTTTCTCACAATCTTCTTTCTGGAGAAATTCCTGCTAGAATTGGAAAT
 661  TTGACTTATCTTCAAGTCATTGATATTTCACACAACTCTTTGTCTGGTACCATTCCATTC
 721  AGTATTGTTGGATGCTTTCAGTTGTATGCTCTAATACTTAATAATAACAATCTTTCTGGT
 781  GTGATTCAACCGGAGTTTGACGCGTTGGATATCTTGAGGATACTAGATATAAGCAACAAT
 841  AGGTTTTCCGGGGCTATTCCACTCACTTTGGCTGGTTGTAAATCTTTGGAGATTGTAGAT
 901  TTTAGTTCCAATGATCTTTCTGGATCCTTGAACGATGCAATAACCAAATGGACAAACCTC
 961  AGGTATCTTTCTCTTGCGTGGAACAAATTCAATGGAAACTTGCCTAGTTGGTTGTTCGCA
1021  TTTCAAGCTATTGAAACAATGGATTTGTCACACAACAAATTTTCTGGCTTTATACCTGAT
1081  ATTAATTTGAAGGGTAGCTTATTATTTAACACACGGAATGTTACTGTTAAAGAGCCTTTT
1141  GTTGAAGCTACAAAGGTGTTTGAACCAAGAGTTTCAGTAGTTGTTTCTGATAGCAATCAA
1201  CTCAGTTTCACATATGATCATTCATCGATGTTCGGAATCGATCTCTCCGATAACTTGTTG
1261  CATGGTGAGATTCCAAGGGGCTTATTTGGCCTATCTGGCTTAGAATATCTGAATTTGTCA
1321  AACAATTTTCTCAACGGACAGCTTCCTGGTTTGCAGAAAATGCAGAGTTTGAAAGCTATA
1381  GATTTGTCGCATAATTCCCTATCAGGACATATCCCGGGAAACATTTCCAGCCTTCAAGAT
1441  CTTACCATCTTGAATCTGTCTTACAACTGTTTCTCTGGATATGTTCCTCAGAAGCAAGGT
1501  TACGGAAGATTTCCTGGTGCATTTGCTGGAAATCCAGATTTATGCTTGGAATCTCCGAGT
1561  GGAGTGTGTGAGGATGGGAGAATTCCATCAAATCAAGGAAGTTATTTTAAGGAAGATAAG
1621  ATGGATGGACCAATATCTGTAGGGATTTTCTTTATCAGTGCCTTTGTTAGTTTTGATTTT
1681  GGTGTTGTTGTTCTATTCTGTTCTGCTCGAACAAGAAAGTACATTCTCCAAACCAAAACT
1741  TGA
```

Protein: 580 amino acids.

```
   1  MRLKHLTELVLNENPPLGGLLPFWIGNFSANLERVQLGYCSFSGSIPESLLYLKSLKYLD
  61  LGSNLLSGNLVDFQQSFVFLNLGSNQFTGTLPCFAASVQSLTVLNLSNNSIVGGLPACIA
 121  NFQALTHLNLSRNHLKYRIYSRLVFSEKLVVLDLSNNELSGPIPSKIAETTEKLGLVFLD
 181  LSHNQFSGEIPLKITELKSLQALFLSHNLLSGEIPARIGNLTYLQVIDISHNSLSGTIPF
 241  SIVGCFQLYALILNNNNLSGVIQPEFDALDILRILDISNNRFSGAIPLTLAGCKSLEIVD
 301  FSSNDLSGSLNDAITKWTNLRYLSLAWNKFNGNLPSWLFAFQAIETMDLSHNKFSGFIPD
 361  INLKGSLLFNTRNVTVKEPFVEATKVFEPRVSVVVSDSNQLSFTYDHSSMFGIDLSDNLL
 421  HGEIPRGLFGLSGLEYLNLSNNFLNGQLPGLQKMQSLKAIDLSHNSLSGHIPGNISSLQD
 481  LTILNLSYNCFSGYVPQKQGYGRFPGAFAGNPDLCLESPSGVCEDGRIPSNQGSYFKEDK
 541  MDGPISVGIFFISAFVSFDFGVVVLFCSARTRKYILQTKT
```

MtCLV3

cDNA: predicted from AC151522

```
   1  ATGGCTTCTAAGTTCATCTTTTCTTCTGTCTTTTTACTTGTTCTTTTCTGCTTGCTTTTT
  61  ATCAAGGAGACTTCTGCAG
      -Intron-
  80  GTTGCAATACTGCATATGCATGCTCTTATGCTAATGGCGAA
 121  AGTCTCAAAATGTTCCAAAACAGGAAG
      -Intron-
 148  ATGCTGTCTGGTTTGAAGGTTAGTTTGGAAGGA
 181  TCTTCAACCAAGATCAAGTATGGTGAAAAGACAGTGGTTGGAGAGTTGAGAAAGGTTCCT
 241  ACAGGTCCAGATCCACTGCACCATCATAACATTGGCAACCCTATTAAGCCTGAAAACCCT
 301  TGA
```

Protein: 100 amino acids. Underline indicated CLE family sequence.

```
   1  MASKFIFSSVFLLVLFCLLFIKETSAGCNTAYACSYANGESLKMFQNRKMLSGLKVSLEG
  61  SSTKIKYGEKTVVGELRKVPTGPDPLHHHNIGNPIKPENP
```

Promoter sequence: 4100 base pair upstream from coding site (Bold type)

```
-4100  AAACAAACTGAATCATCACCGTCGGTCATCATCATCACCACCAGAATCTTCAATCTCATC
-4040  ATCAACACCGTAATTCATCATCATCACCGTCATTCACCATCATCATCACCGTACCGAAAT
-3980  CATCATCATCATCACCATCGTCATTCATCATCATCACCGTCAATCAAAATTGTATATGTT
-3920  AGTTGGAAATGATTTCAGAGTGTTGATTTGGTTCAATGACAGTCACAAATGTCCTTATTA
-3860  TAGTATGTTTTGTATAAAATTATGCCAAAATCATTTTGCTGTGGAAATGGTTTTTGTGAG
-3800  TTGTACTCCAAAACCATTAGATGTATTTAATGGTTTCCAGCAGGTGAAAAAAACTAAGGA
-3740  CCTAAAGCCATTGCCATCATTCAATTTTGGATTGACACAATTGGAGGAGGAGGATATAAA
-3680  TGGTTTATGTGACTTGTACTCCAAAACCATTTTGCTGTGGAAATGATTTATGTGAGTTGT
-3620  ACTTCAAAACCATTAGATGTACTTAATGGTTTTCTTTGATCCAGCAGGGTGCATATTACA
-3560  ATCAAAAATAAAAAACATATGGAATTTTCTAACCATAACAGAAAATTTTCAAAACCATTC
-3500  AGTCACCAACTTTGAATTCTTAGCAACAAAATCACAATTATCAGAAAAATTTCCAATTTG
-3440  ATTTCGACATTTTCTCAAACCAGATCCATCATCAATTTTCCCAATCAAATTCAAATCAAT
-3380  CCCTAATCCCTCCAACTCATTACTCCAAATTCCCCTCCTCTTCAACGCTGTCCTTTCACA
-3320  ATCAACTTCAATTGAACTTCTCTTCCGTCGCCGACGCGTGTAAACAAACAAAAGCGGCGG
-3260  TTTTGGAAAATCCTCCACAAAAAACAACGTCATCTTAGACATGGTTTTTTCAATTTCAAT
-3200  CGTTGAATCATCGATTTCACCATTACTGAACTGAATCTCGTTCTCCAGGATTATTACTGA
-3140  ACTGAATCATCAATTTCACCATTACAAACAAAAGTTGGTGCTTGAATTCAAAGTTGATAG
-3080  ACTTAGGATTATTACACGTGTAAAACAAATCTGATGGTTGTGAAGTGTTTATGTAGGGTA
-3020  CATAAGAAAATGACTTATGCATAATAACTTTTGCCAATATTTATGTAGGGGAATCTTAAT
-2960  GCATATCCATTCAAAAATTAAGAGATGTTCATTAACAATGTGCACTTTTATATTTTGAAA
-2900  AGAAAAAAATTACATCTGTATAATTATTTTGTGATAACTTTCTTTCTACAAATGCTTGTT
-2840  CAAATAACACACTTTAACGTAAAAGGTGTACATTGCTAATCAACGTCTTTTATATTTGTT
-2780  GCTCCATGTTGTAATAGGTTTAGAGTACTTCTTGTTCTCTTGTTGTCTATTTTATATAAT
-2720  CTTCTATTTTTTTTTTTTTTAAAAAAGTTGTTTGTCCTCATAATCTCAATTGTTAGAAAA
-2660  GATCTCCAAAACATTTGTTGAAATATAACAAAAAAATTTCACATACACAACTTTTGACAT
-2600  CAGACATCGTATAAATATGTGTTGGATTTTGTTACTATTATTTCTCTCACTTTGGTTGGA
-2540  AAACAAACTAGCTCGAGATGTTTTTTATATACGGTGTCGGTTTTGTGTGTTAAAATAACA
-2480  CTCCTCGAAATATAATGGTCATGCAACATTCTCAAATAATGTGGTCGGTTTTAGAATGTG
-2420  TTTATTATGTGTGTGTGTGATTGAGGTTGGTAATTTCCTTCACAATAGTGCTATATGTCC
-2360  CGAGTTAAAACACAACGAATTTATCTAAAAGAGTTTCTCACGTTAGATTAGTCCAAGGCT
-2300  GAAAAAACATATGGTGGGATGCAAAAGAGGGACCTACTACAATAAACAGTGTATGGGAAT
-2240  CTGTTCGGGATACATTCGCGTCAATTGTGCTCGCGATAAGAAGCATTTTCCTTTCCTTCA
-2180  AGGAAAAAATCTCCAATTGCTGGTCAAGGTTAAACATTCATGAAGGTATGTCATTTGGTT
-2120  TGCTTCATGCTATGCAATAGAGATGAGAGCTAGACATGGGTATTGACAACGTCACTTTTG
-2060  AACTCAATTCAAAATAATTAGTGGTGAGATTCAATAGTTAGGATCAATGCAATCCATCCA
-2000  AACATTCTAAATCCTTATGCTTTATACCCTAGAAATACATTTTTGTTAAAGACATCTAGG
-1940  CCTCCAAACAAATTAACTGATACTATTGAATTGACACAATTTCTTCCACGTCTTTTATCT
-1880  TATTTATTTGTAACACTTTAATCGTCATTTATCTCTTTTGACACTTTCAAATCTTTTATT
-1820  TCTATGGTTTCCCTTTATCTTTCTCTTTTGACACGTTCAAATTCTTTATCTCTAAAAATG
-1760  CGTACAAGTTAGTCATTATTTTTTGTATTTTATTAAAACAACGCTGAGGTGGCTTGCTAA
-1700  GAATTATTATTAGAAGTAATACTTTTATTTTTATATAACTTTTTTACTAAAAAAACCTGA
-1640  AAAAAAATATCTTCATCTTTTTCGTATATTTATCGAGTTCAACATCATCTTCAAACCCAT
-1580  AAAACTTTATTATCAAATCAACATCCTAAATCTTCAAATTTGTAGTGGCTCAAGATCAAA
-1520  ATCCATGATCTATCATAAAAAAGAAACTCAACTAAGAAAGAAGAAGTTTACAACACATCT
-1460  TAATCATCCAATTGTTGATGATTCGAAGTTTGCAACACATATCAAAATCTCAATTTAATT
-1400  TCATCAATTTCCGAGTTTAAGATGATGATTAAAAGAGAAGAAAATTTGTAGGTTTGGTGT
-1340  TTTTTGGGTTCTAATTCGAGCTACTAAGATTTAGGGTTTTGATTTAACTTTATGGGTTTG
-1280  AAGAAGAAGATAGCTATAGAAGATATTTAAGATGATAAACATATAAAAATAAAGATGAAG
-1220  ATTGCTTGATTTCAATTTTTTTATTGAAAAAGAATATTTAAAAATCAAATTATTATTAAT
-1160  AATCCTACATGGCAAACCGCCTCAACGTTTTAAGATAAATAAGATAAAGAACAATTTTTT
-1100  TGCTAAAAAAAAAGATAAAGAACCATTTCATTTGAATAAGTTTGCCTACTTAATACTAAT
-1040  AAGTTGATATTTTGCATAATTTCCATGTAGCTTAGTTGGTTAGTTGATTGAAGTGTGTGT
 -980  TTGGTTTTCCATTTGCAACATACTAAATCACATTTTCCAATACGTTTTGTCGTGACTTTT
 -920  AGAAATTCCAAATATTAACTTCTACGATAACATAATTTTTTGTTGTGATTTTCATCAAAC
 -860  TCCGTTTTGCAAACGTTGTATCAAATAGACACAATTTTTGTTTTTTTTTTTTTTTACTC
 -800  TTGGGTGGGAGACAAAGTTATGCATAAAACAAGCTGATTGTTTGGTTTAACCCGTCCTAT
```

APPENDIX 4 ***Medicago truncatula*** **CLAVATA-like genes – DNA and protein sequences**

```
-740  GTAAGTAACTTCGATAATCTGTTGTTAAGCCAAGAGGCTAGCATTAGCCAATAATTATTC
-680  AAATTTAAATGAGTACTCCAATAAGAAAGATTTCATGAAATGTGCATGTTATTATTATAA
-620  TAGTAAAGATTCATAGAAAAATCGGATGAAGACCTTCAATGATACTCTTTAATTTTGAAC
-560  TTGAGTAAAAATTTAGTAAATAATTCTTACAAATTGGAATATACAAAAGTGAAATCATGT
-500  AAGCAGATTATGCGACAGAGAGAATCAGATATGGCCACATCCCCAAAAAAGTTGTATAGA
-440  CTCAGAAAGGAAACATGACGTATATATTGATGCTCCTCCTATCTTTATTTGACATTCCAA
-380  ATCAAATCATAATAATAATATAAAATCATATCATATCCTTTCTACTTTTTATAGTTCTTT
-320  ATTAACTTTTGATATCATATTTTTTAAGATAGATTTTGCATATGGCAGCACCTGCAACCA
-260  GGTAATTTTCGACAACCAAATATATAAGACGGCTCTGTCACATAGATACACAGAGGTAGC
-200  AGTGAAAACGTACAGATAATGCTTCCTCATTAGTGTCTTTAATCATTAATTTCTTCCCAA
-140  ATGGCTGCAACACCCATCATTCTAATTTCTAATTTTTAATTATTAAATCCTTGAATTATC
 -80  CCACCATTATAAATATATGTGTCTTCTTCTTCACAACATGCATATTAAATTAACCTAGCT
 -20  ACGTATCTCCAATATTTATA
```

ATG

+1

APPENDIX 5

Solution preparation

MOPS buffer (10X):

MOPS	0.2 M
Sodium Acetate	50 mM
EDTA	1 mM

Adjust pH to 7.0 using NaOH.

TAE (Tris-Acetate-EDTA) (50X for 1 L):

Tris base	242 g
Acetic acid	57.1 mL
0.5M EDTA	100 mL

Add ddH_2O to 1 L and adjust pH to 8.5 using KOH.

LB medium (1 L):

Bacto Trypton	10 g
Yeast extract	5 g
NaCl	5 g

Add ddH_2O to 1 L and adjust pH to 7.2 using NaOH.

YEP medium (1 L):

Bacto Trypton	10 g
Yeast extract	10 g

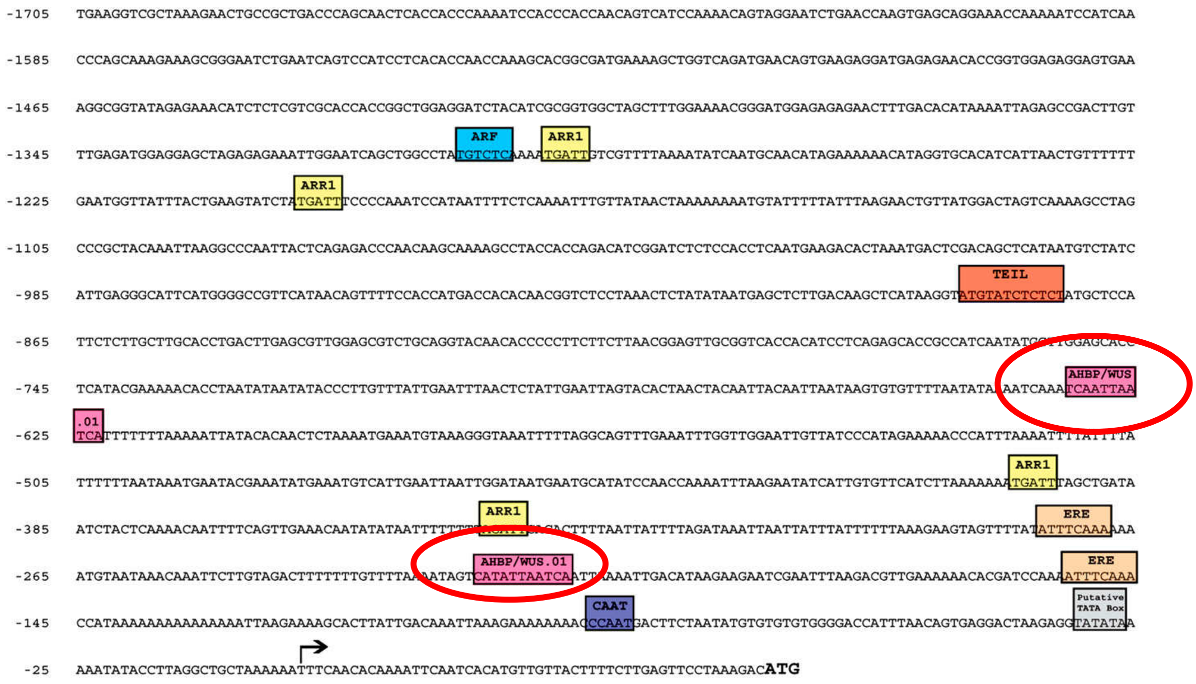
Add ddH_2O to 1 L and adjust pH to 7.0 using KOH.

APPENDIX 5 Solution preparation

APPENDIX 6

WUSCHEL binding site in promoter region

The WUSCHEL binding site in the promoter region was first identified in the *AGAMOUS* (*AG*) promoter region of Arabidopsis (Lohmann et al., 2001) and *WUS* regulates *AG* expression in the floral system by this binding site. This binding site also influences the expression patterns in other genes (Takada and Jürgens, 2007). *MtSERF1* which express in somatic embryogenesis has this WUS binding site in its promoter region (Fig. A6.1, Mantiri et al., 2008a). The possible linkage between these two genes required for SE was discussed in a joint study (Mantiri et al., 2008b) with other researchers in the lab and will be investigated.



AHBP/WUS.01: Arabidopsis Homeobox protein/WUSCHEL
ARF : Auxin Response Factor binding site
ARR1 : Arabidopsis Response Regulator 1 binding element
CAAT : CAAT box
ERE : Ethylene Response Element
TEIL : Tobacco EIN3-like binding site

Figure A6.1 Core and responsive element motifs in a 1758-bp region upstream from the transcription start site of *MtSERF1*. Red circle indicates the WUSCHEL binding site. Diagram is modified from Mantiri et al., 2008a.

APPENDIX 6 WUSCHEL binding site in promoter region

APPENDIX 7

Nodulation of 2HA in response to ethylene

The nodulation of 2HA in response to ethylene was carried out by Ulrike Mathesius at the Australian National University. The ethylene biosynthesis inhibitors AVG (aminoethoxyvinylglycine) and stimulators ACC (1-aminocyclopropane-1-carboxylic acid) were added to the medium for *M. truncatula* A17 and 2HA root nodulation experiments (Fig.A7.1). The nodule numbers increased in response to the ethylene inhibitor AVG and decreased with the ethylene stimulator ACC, indicating that ethylene inhibits the nodulation. In 2HA, the number of nodules is higher than A17 and the ethylene stimulator ACC did not decrease the nodule numbers as in A17.

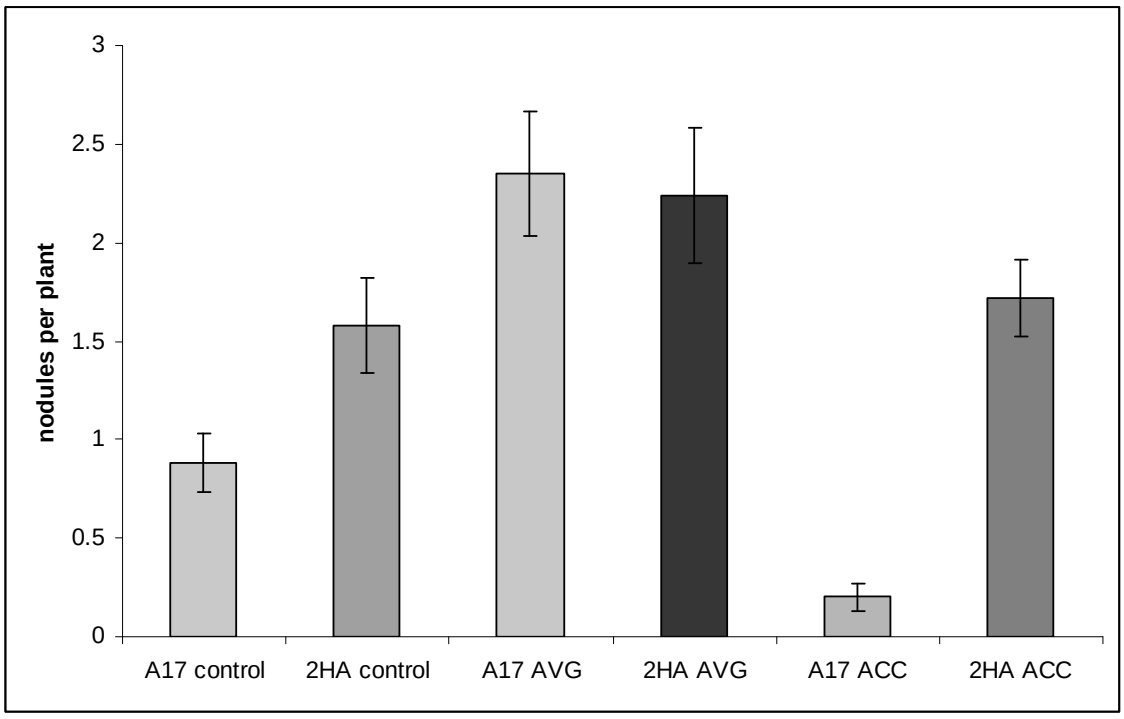


Figure A7.1 Nodules numbers of *M. truncatula* A17 and 2HA in response to an ethylene inhibitor of biosynthesis (AVG) and an ethylene biosynthesis stimulator (ACC). The data are supplied by Ulrike Mathesius in a collaborative study.

APPENDIX 7 Nodulation of 2HA in response to ethylene

APPENDIX 8

Cell proliferation in 1 week cultured leaf explants

The cell proliferation in early stage of tissue culture was investigated through tissue clearing and staining. The *M. truncatula* 2HA leaf explants were cultured in auxin plus cytokinin medium for 1 week, and then the tissue was cleared and stained by fuchsin and investigated using light microscopy. It shows the cells have started to proliferate not only at the edges of the explant but also near the vascular tissues, probably from procambium cells (Fig. A8.1). This result suggests cell division will occur throughout the explant in the early stages of tissue culture, and explains why the pr*MtWUS*::GUS results in 3 d cultures show staining across the surface of the explant (Fig. 5.8A). These data were supplied by Dr. Xin-Ding Wang in the RR Laboratory.

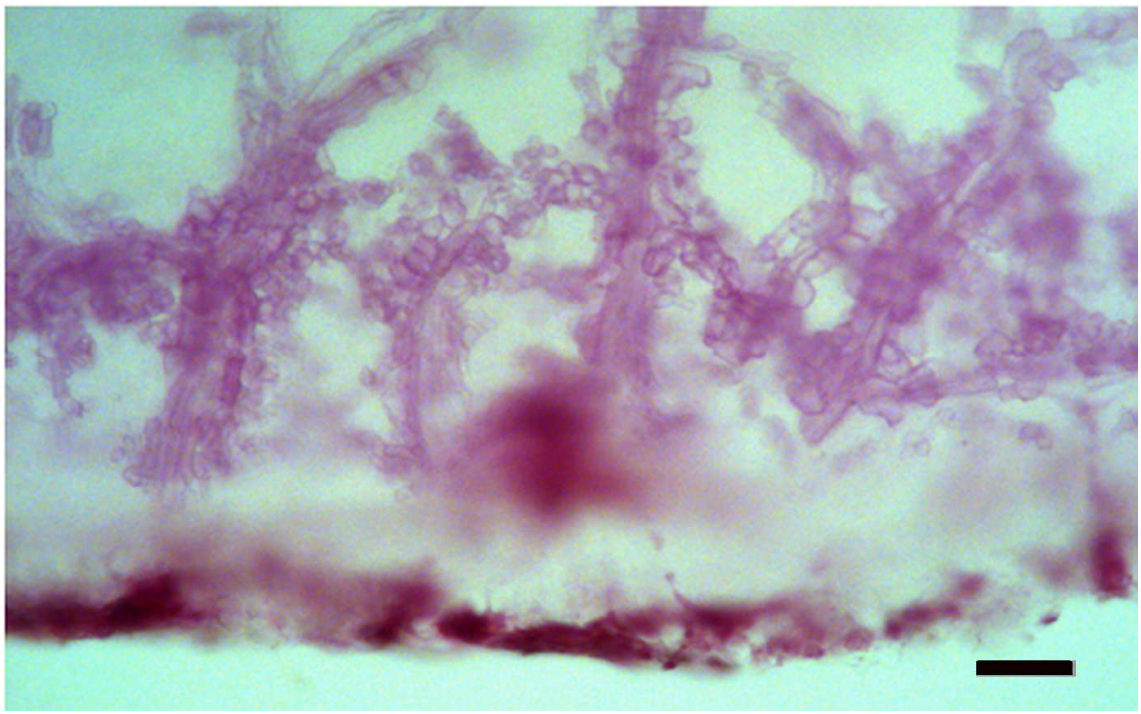


Figure A8.1 Photograph of 2HA leaf explant cultured in auxin plus cytokinin medium for 1 week. The tissue was cleared and stained by fuchsin. Bar = 80 μm.