Sterol gene expression in Arabidopsis

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STEROL GENE EXPRESSION IN ARABIDOPSIS

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2009

Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisor, Professor Keith Lindsey, who gave me the chance to work and study in his laboratory and helped me with his patience and knowledge throughout the study and writing of this thesis. This would be much more difficult without his arrangement for me to finish this off in Nottingham University and use facilities there after I moved to Loughborough in 2007 with my family. I feel deeply indebted for his kindness in making this happen. He is a great supervisor. I attribute the level of my Masters degree to his encouragement and efforts. Without him this thesis would not have been completed or written.

My special thanks go to Ellie Short. It would not be the same without her in this whole process. Her kindness, patience and knowledge kept encouraging me to continue to this very end in every possible way and helped me in all the time of research and writing of this thesis. I really appreciate her permission to use her data in my thesis.

I am also highly thankful to Jen Topping for her invaluable support, encouragement, supervision and very useful suggestions throughout this research work. Many thanks also go to Stuart Casson who was willing to help me answer all my questions without hesitation.

I would like to express my gratitude to all those in Nottingham University who helped me to finish my laboratory work. My sincere thanks go to Professor Malcolm Bennett for giving me the permission to continue my research work in his laboratory, Morag Whitworth for helping me with various things, Ranjan Swarup for his assistance in microscope work and valuable hints, and Li Zhang for looking after my plants when I were not there.

In my daily work I have been blessed with a friendly and cheerful group of fellow students and colleagues. Roya Mannis, a former technician, who is always calm and helpful and always there for me, Saher Mendi who is so kind to offer me all kinds of help and became a good friend of mine, Margaret Pullen who is a hard working model for me and a good accompany during lunch time. I also thank these people from

Hussey's lab with whom I had a good time.

My families have been very supportive throughout. Thank my mother and sister for their encouragement over the years, my mother and father in-law for taking care of my son Michael during my study. I also see this thesis as a memory to my father. He would certainly be pound of me if he was alive. Thanks for my lovely son Michael. I have grown up as a mother and you are growing up with me. Final thanks go to my husband Dapeng whose patient love enabled me to complete this work.

Declaration

I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others.

Dongbin Liu

Statement

Dongbin Liu

Abstract

Sterols are recognized as playing vital roles in plant development, as has been demonstrated from mutational studies. The study of the *hydra* mutants of Arabidopsis provides new tools to dissect sterol function in development at the molecular genetic level.

Both *HYD1* and *HYD2* genes encode sterol biosynthetic enzymes and their mutants exhibit pleiotropic phenotypes including defective embryogenesis, defective cell shape, polarity and patterning, multiple cotyledons and short roots and hypocotyls. The fact that neither *hyd1* nor *hyd2* are rescued by the exogenous application of BRs suggests other sterol-dependent signaling defects in these mutants.

Interestingly, *HYD* genes are not expressed to detectable levels in all the tissues that show developmental defects. The aim of the work described in this thesis was to determine whether there is a non-cell autonomous sterol-dependent signal required for the correct growth and development of the root. The approach was a) to silence *HYD* gene expression in specific cell types of the root using RNAi; and b) to activate *HYD* gene expression in specific root cell types in a null *hyd* mutant background.

It was found that attempts to silence the *HYD1* and *HYD2* genes in the root meristem by RNAi-mediated gene silencing had a small effect on root meristem function and patterning in individual transgenic lines for *HYD1* gene repression. However, by expressing the *HYD1* gene in cells of the root meristem of the *hyd1* mutant, there was a dramatic effect on the development of the root. These data are consistent with the view that sterols function in a non-cell autonomous way to regulate root development in plants.

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CHAPTER 1: INTRODUCTION

1.1 Plant development

Plant development is the process by which cells, tissues and organs originate and mature as a plant grows. The process of development in plants has some fundamental differences from that seen inhttp://en.wikipedia.org/wiki/Vertebrate animals. When an animal embryo begins to develop, it will very early produce all of the body parts that it will ever have in its life. By contrast, the plant embryo is a relatively simple structure, and new tissues and organs are produced throughout the life of the plant from meristems located at the tips of organs (the shoot and root apical meristems), or between mature tissues (e.g. the vascular cambium). A vascular plant begins from a single celled zygote, formed by fertilisation of an egg cell by a sperm cell. From that point, it begins to divide to form a plant embryo through the process called embryogenesis. By the end of embryogenesis, the young plant will have all the parts just necessary to begin in its life. Once the embryo germinates from its seed or parent plant, it begins to produce additional organs (leaves, stems, and roots) through the process of organogenesis.

1.1.1 The study of plant development

Developmental biology is the study of the processes by which organisms grow and develop from a fertilized egg to a mature adult. Modern developmental biology studies refer to the genetic control of cell growth, differentiation and morphogenesis using expertise and tools from genetics, molecular biology and cell biology.

Plants development and growth is characterized by the execution of cell division, expansion and differentiation along the apical-basal axis and the radial axis. These are regulated by the coordinated expression of genes and groups of genes in space and time. There are certain heuristic concepts derived from the study of wide range of organisms that geneticists rely on to explain the development process in molecular basis. The variable gene activity theory (Britten & Davidson 1969) assumes that each cell contains an entire genome and that differential transcription of selected genes control the development and differentiation of that cell. The transcription of certain

genes in the cell at certain developmental stages is influenced by its internal environment and signals sent from the external environment, and which in turn affects the next stage of transcription.

The adaptation of single species as "model systems" has been used for the exploration of specific gene function to address biological questions. These model systems include *Arabidopsis thaliana* (e.g. Valvekens *et al.* 1988; Mayer *et al.* 1991; Lindsey & Topping 1993; Goldberg *et al.* 1994), *Zea mays* (Frame *et al.* 2002), *Antirrhinum majus* (Coen *et al.* 1986) and *Petunia hybrida* (Harbord *et al.* 2000).

A number of important technological breakthroughs have been made in recent years and widely used to push the study of the plant development to new levels. The *in vitro* DNA amplification via the Polymerase Chain Reaction (PCR) (Mullis *et al.* 1987), efficient *Agrobacterium tumefaciens*-mediated transformation methods (Valvekens *et al.* 1988, Frame *et al.* 2002) and the use of the green fluorescent protein (GFP) from jelly fish (Haseloff *et al.* 2006) have revolutionized plant biology.

This burst of new techniques has promoted the study of the early development process. The identification of essential genes and transcription factors has allowed new understanding of embryo development (e.g. Jürgens *et al.* 1991, Mayer *et al.* 1991, Laux *et al.* 2004, Kaplan & Cooke 1997), many of which are unique to the plant (Schena M *et al.* 1992, Martin C *et al.*1997).

One of the successes is the identification of the key regulatory genes that control plant axis formation. To do this, phenotypes predicted from defects along the apical-basal or the radial axis were obtained upon mutagenesis and screened for seedling-lethals (Mayer *et al.* 1991). Corresponding genes that are involved in cytokinesis and auxin transport have been identified (Shevell *et al.* 1994; Lukowitz *et al.* 1996; Hardtke, 1998; Assaad *et al.* 2001). Mathematical modelling and computer simulation of plant development patterning (Prusinkiewicz *et al.* 2006) are under construction using massive amounts of experimental data.

1.1.2 The use of mutants to study plant development

In the process of understanding the molecular basis of plant development, mutational approaches have been widely used in recent years to identify genes required for the signal transduction pathways. This can be done by creating a mutant allele of a gene in which the influence of the gene on the phenotype can be assessed (a 'forwards genetics' approach). Mutants can be generated by irradiation, chemical means or insertional mutagenesis. On the basis of an available complete genome sequence, a gene of interest can also be studied using the phenotypes generated by over-expressing or blocking the gene using gene manipulation approaches (a 'reverse genetics' approach). By crossing, double, triple or even higher multiple mutants can be produced to study gene interactions. It is very useful if genes are members of functionally related pathways and act redundantly. Chemicals can also be used to phenocopy known mutants, in a pharmacological approach to understand its function. For example, the primary function of the *PIN1* gene in auxin polar transport in the inflorescence axis is found by *pin1* mutants showing the same phenotypes in wild-type plants cultured in the presence of chemical compounds known as auxin polar transport inhibitors 9-hydroxyfluorene-9-carboxylic acid or *N*-(1-naphthyl)-phthalamic acid (Okada *et al.* 1991). Together with the generation of the lethal *auxin binding protein1* mutant in *Arabidopsis* (Chen *et al.* 2001) using T-DNA tagged methods, these mutant lines can be studied for the function of these important genes to investigate the role of auxin in development.

1.2 Mechanisms of *Arabidopsis* Embryogenesis

Unlike the situation in animals, in which the embryo represents a miniature version of the adult form, embryonic development in plants results only in the formation of the essential body plan, while the growth and development of the major organs is primarily a postembryonic process (Mayer *et al.* 1991). Mature plant embryo therefore has a very simple body plan, in which the apical-basal and radial axes are specified. By the end of

embryogenesis, the root and shoot apical meristems are established and become active upon germination, and generate the primary root and shoot, respectively. Across the radial axis, patterning of progenitor tissue layers occurs during early embryogenesis, ultimately leading to concentric rings of epidermal, grouns and vascular tissue. To establish this organization, the cells of the embryo need to become specified and must differentiate into cell types in an integrated manner. Furthermore, the correct apical-basal polarity need to be established on which post embryonic processes continue the elaboration of this pattern in response to the external environment. One of the key questions has been the identification of regulators that control the switches from globular to heart stage or from heart to torpedo stage.

1.2.1 Stages of *Arabidopsis* embryo development

Embryogenesis in *Arabidopsis* is a highly predictable, regulated and organized process. The fixed pattern of cell divisions in early stages makes it possible to trace the origin of seedling structures back to regions of the early embryo (Fig. 1; Mansfield & Briarty, 1991; Jürgens & Mayer, 1994)

First, the zygote (fertilized egg) divides asymmetrically to form the apical and basal daughter cells with different sizes and cytoplasmic densities (Mansfield & Briarty, 1991). The establishment of the apical-basal axis is the earliest patterning events in plant embryogenesis. Through perpendicular shifts during three successive rounds of cell division, the apical daughter cell gives rise to an eight-cell embryo proper (the octant stage) comprising an apical and central embryo domain. The apical domain, composed of the four uppermost apical cells of the embryo proper, will remain largely isodiametric prior to the initiation of the shoot meristem and most of the cotyledons. The central domain, consisting of the four lower cells of the embryo proper, will divide strictly oriented either parallel or perpendicular to the apical-basal axis and form the hypocotyl and root, and contribute to part of cotyledons and the root meristem. By contrast, the basal cell of the zygote divides only horizontally to form a filamentous structure: the suspensor and the hypophysis. The embryonic suspensor pushes the embryo into the lumen of the ovule and provides a connection to the mother tissue which will facilitate the embryo-proper's nutrition.

1.2.2 Pattern formation

The main elements to be positioned along the apical basal axis in *Arabidopsis* embryo are the shoot apical meristem (SAM), cotyledons, hypocotyl, radical and the root apical meristem (RAM). The establishment of the apical-basal axis in the early stage of embryogenesis is crucial for subsequent plant growth. The mechanisms regulating this process are discussed in the following sections.

1.2.2.1 The First Division of the Zygote: Embryo-Proper versus Suspensor

One of the earliest patterning events in plant embryogenesis is the establishment of the apical–basal axis which can be traced back to the first asymmetric division of the zygote. Two daughter cells, an apical cell and a basal cell, adopt different developmental fates in later development. The apical cell of the embryo-proper will divide vigorously and form most of the embryo while the uppermost cell of the

suspensor, the hypophysis, undergoes a sequence of reproducible divisions giving rise to part of the primary root meristem, comprising the quiescent centre and the central (columella) root cap initials (Scheres *et al.* 1994). The most basal suspensor cell enlarges dramatically and has abundant contact with surrounding maternal tissues, likely facilitating the supply of nutrients to the embryo. The suspensor appears to have two different functions: it physically projects the embryo into the endosperm, and provides both a conduit and a source of hormones and nutrients for the developing embryo. Perhaps the clearest difference between the embryo-proper and suspensor is the programmed cell death of the suspensor which is not present in mature seeds while the embryo reaches the torpedo-stage of development (Yeung & Meinke, 1993).

There is increasing evidence that the embryo-proper and suspensor express distinct gene expression programmes. Direct evidence for different gene expression profiles in embryo and suspensor comes from promoter trap analysis in Arabidopsis (Topping *et al.* 1994), which has led to the identification of the difference of gene expression between the apical and basal cell following the first zygotic division. For example, mutants in the *HYDRA1* (*HYD1*) gene of *Arabidopsis thaliana* show embryo-specific

defects (Topping *et al.* 1997). Cells of the embryo proper of the mutant exhibit abnormalities in size and shape and fail to develop an embryonic root. The normal development of the suspensor suggests that the *HYD1* gene is expressed in the embryo but not in the suspensor (Souter *et al.* 2002).

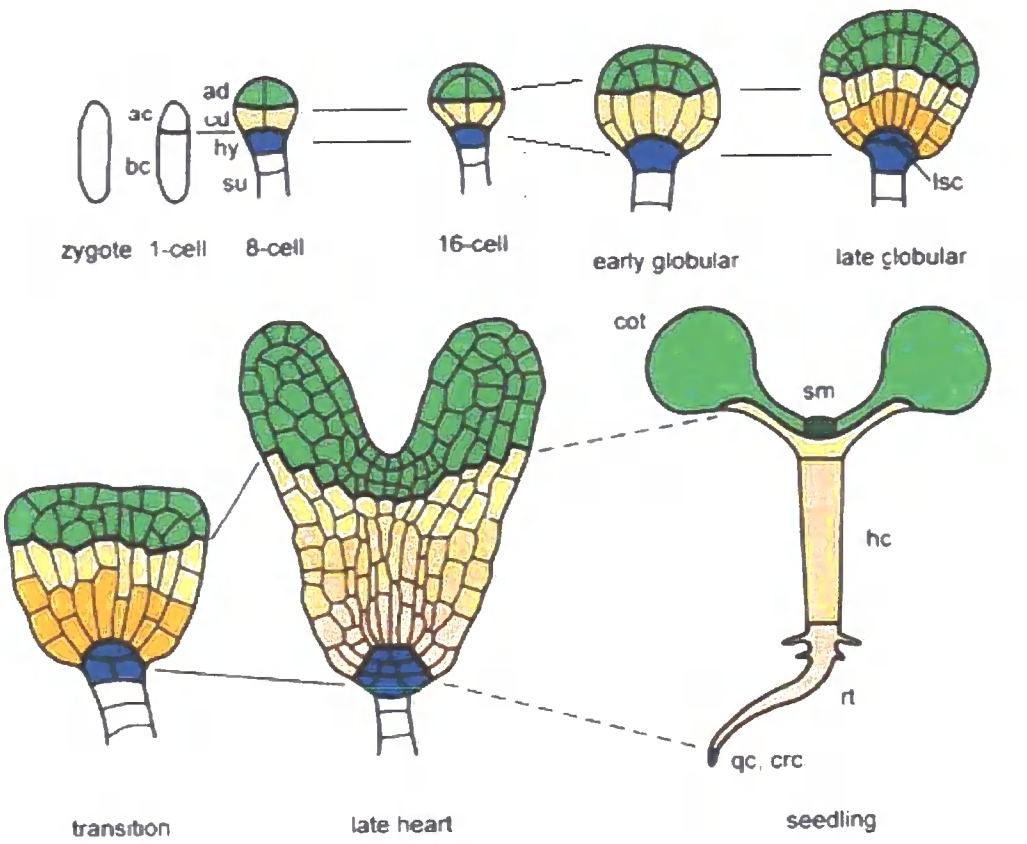


Figure 1.1: Embryonic origin of seedling structures. Colors identify corresponding regions in embryo and seedling. ac, apical daughter cell; ad, apical embryo domain; bc, basal daughter cell; cd, central embryo domain; cot, cotyledons; crc, central root cap; hc, hypocotyl; hy, hypophysis; lsc, lens-shaped cell; qc, quiescent center; rt, root; sm, shoot meristem; su, suspensor. (Figure taken from Laux *et al.* 2004)

Several observations indicate that it is the embryo itself that represses embryonic development in the suspensor and normal development of the embryo-proper limits growth and differentiation of the suspensor. The formation of a secondary embryo from the suspensor cells has been induced through the experimental abortion of the embryo (Gerlach-Cruse, 1969). Moreover, mutations in several genes result in the same effect either after the primary embryo arrests development or even if it continues to develop, resulting in polyembryony.

Two or more embryos capable of developing into mature plants upon germination were contained in the *twin* mutant of Arabidopsis (Vernon & Meinke, 1994; Zhang & Somerville, 1997). Secondary embryos produced by this mutant were shown derived from the suspensor of the primary embryo. The *TWIN* gene is likely required for normal development of embryo proper as well as the suppression of embryonic potential of the suspensor. Similarly, a similar spatial and temporal pattern of several embryo cell-specific mRNA accumulation occurs within the enlarged suspensor region of *raspberryl, 2, 3* mutant embryos (Yadegari *et al.* 1994, Apuya *et al.* 2002), suggesting that a defect in embryo-proper morphogenesis can cause the suspensor to take on an embryo-proper-like state and differentiate a radial axis.

Gene expression studies indicate differences of expression in the apical and basal cells. Two *WUS-RELATED HOMEOBOX* (*WOX*) genes, *WOX2* and *WOX8*, are found expressed in the zygote cell (Haecker *et al.* 2004). The asymmetric division of the zygote separates these mRNAs, in which *WOX2* is expressed only in the apical and *WOX8* exclusively in the basal daughter cell thereby establishing two cells of different identities and setting up the apical–basal axis of the embryo. Mutations in *WOX2* cause an aberrant apical cell division pattern which has a similar phenotype to the mutant *pin7,* and *PIN7* is also expressed asymmetrically in the daughter cells of the zygote. *PINFORMED7* (*PIN7*) encodes a member of the *PIN* family, which are part of the auxin efflux transport machinery (Galweiler *et al.* 1998; Friml *et al.* 2003). *PIN7* is restricted in expression to the basal daughter cell of the zygote, where it is localized at the apical cell wall and mediates the efflux of auxin into the apical cell. Thus, asymmetric *PIN7* and *WOX2* expression is important to establish apical cell identity at this stage considering the importance of the timing and orientation of cell divisions to keep the aspect of cell identity during early *Arabidopsis* embryogenesis.

During A*rabidopsis* embryogenesis, the zygote divides asymmetrically in the future apical-basal axis; however, a radial axis is initiated only within the eight-celled embryo. Mutations in the *GNOM*, *KNOLLE*, and *KEULE* genes affect these processes (Mayer *et al.* 1991, 1993): *gnom* zygotes tend to divide symmetrically *knolle* embryos lack oriented cell divisions that initiate protoderm formation; and in *keule* embryos, an outer cell layer is present that consists of abnormally enlarged cells from early development. Pattern formation along the two axes is reflected by the position-specific expression of the *ARABIDOPSIS LIPID TRANSFER PROTEIN*

(*AtLTP1*) gene, a homeobox gene. In wild-type embryos, the *AtLTP1* gene is expressed in the protoderm and initially in all protodermal cells; later, *AtLTP1* expression is confined to the cotyledons and the upper end of the hypocotyl. Analysis of *AtLTP1* expression in *gnom*, *knolle*, and *keule* embryos showed that *gnom* embryos also can have no or reversed apical-basal polarity, whereas radial polarity is unaffected. *knolle* embryos initially lack but eventually form a radial pattern, and *keule* embryos are affected in protoderm cell morphology rather than in the establishment of the radial pattern (Vroemen *et al.* 1996).

1.2.2.2 Development of the embryo proper

The O' boundary separates the upper tier and lower tier at the octant stage of the embryo-proper, which later give rise to the apical and much of the basal portion of the seedling respectively, and follow their own division programme.

As mentioned above, the two related homeobox genes, *ATML1* and *PDF2* are expressed early on in development (Lu *et al.* 1996, Abe *et al.* 2003). In the 'octant' embryo, both of these genes are expressed in all of the cells. By the 8- to 16-cell transition, *ATML1* and *PDF2* expression is found in the outer layer but not the inner layer, and comprises a radially-organized differential gene expression within the cell mass. This means that on the basis of gene expression, the radial organization of the embryo proper is the first 'pattern' to be established.

Subsequently the protoderm follows a series of predominantly anticlinal divisions and remains essentially separated from inner cells throughout development, while all inner cells adopt a common orientation of cell division, in which newly formed cell walls are aligned along the apical basal axis. As the result of this, the inner cells remain organized in two tiers, upper and lower, but amplify the number of the cells within the each tier. There is no difference in the cells behavior along the apical-basal axis (upper tier versus lower tier).

1.2.2.3 Shoot Apical Meristem

The daughter cells of the upper tier form the SAM and most of the cotyledons. The SAM is in the centre of postembryonic organ formation in the shoot. It consists of a

central zone which contains stem cells in the outermost three cell layers, and the signaling niche cells (Mayer *et al.* 1998).

The formation of the SAM requires the expression of novel homeodomain proteins (Mayer *et al.* 1998). For example, one of them, *WUSHCEL* (*WUS*) is active at this stage is to maintain the stem cells in an undifferentiated state (Lenhard & Laux, 1999). Meristem development requires the expression of the *WUS* in the four inner apical cells at the 16-cell stage *Arabidopsis* embryo (Mayer *et al.* 1998). In the late heart stage, the expression is restricted to the signaling niche cells which locate under the three layer stem cells. The expression of the *WUS* is then suppressed by *CLAVATA3* (*CLV3*), the putative ligand for the *CLV1* receptor kinase signalling pathway (Fletcher *et al.* 1999), expressed in the stem cells to limit the size of the signaling niche cells.

The *SHOOT MERISTEMLESS* (*STM*) gene, encoding a homeodomain transcription factor from the Knotted class, is required for the SAM formation in embryogenesis (Long *et al.* 1996). Seedlings homozygous for the strong *stm* alleles do not develop a SAM. The cotyledons and other embryonic structures can be formed in these mutants but a population of self-renewing stem cells can not be established (Long *et al.* 1996, Barton & Poethig, 1993). The expression of *STM* is initiated at the late globular stage in the central region of the embryo apex and then expands into a band across the apex at the transition to heart stage which is independent of *WUS* action (Mayer *et al.* 1998). It turns off as cells are recruited towards the formation of lateral organ primordial. The persistence of the expression of *STM* in the SAM of seedlings and adult plants suggests the importance of STM in the initiation and maintenance of the SAM development.

In the heart stage embryo, shortly after the appearance of UFO transcripts (Long & Barton 1998), *CLAVATA1* (*CLV1*) transcription appears in the stem cell population in the shoot apex. Both the *UFO* and *CLV1* subdomains fall within the domain of *STM* expression (Clark *et al.* 1993; Lee *et al.* 1997), and act independently of *STM* (Long & Barton, 1998). The *UFO* gene is required for the correct placement of floral organs and the correct specification of floral organ fate (Levin & Meyerowitz, 1995; Wilkinson & Haughn, 1995) while *CLV* genes promote the progression of meristem cells towards organ initiation and are required to limit the size of the SAM (Leyser & Furner, 1992; Clark *et al.* 1993).

A feedback loop is formed by three genes: *STM*, *WUS* and *CLV* (*CLV1* and *CLV3*) to regulate the size of the stem cell population and maintain meristem homeostasis. *WUS* function is required for the expression of *CLV3* and *STM* in mature embryos (Laux *et al.* 1996; Mayer *et al.* 1998; Brand *et al.* 2002) and correct spatial *WUS* expression in turn requires *CLV3* activity from the heart stage on. *CLV3* binding to *CLV1* represses the expression of *WUS* at the transcript level to limit the size of the signaling cell niche. This regulatory feedback loop between stem cells and the signaling niche cells provides a mechanistic framework to explain how the plant could dynamically assess and adjust the size of the stem cell pool (Schoof *et al.* 2000).

1.2.2.4 The central and basal regions of the embryo

The inner cells in the central domain, developed from the lower tier in the octant stage embryo, first divide horizontally to give apical descendants and basal descendants alone with the daughter cells from protoderm. The apical descendants will contribute to the base of the cotyledons and the basal descendants will form hypocotyls, embryonic root and proximal stem cells of the root meristem (Scheres *et al.* 1994). During embryonic root meristem formation, the proximal stem cells are derived from the most basal cells of the central embryo domain, whereas the QC and the stem cells of the central root cap are derived from the basal domain, the hypophysis (Mansfield & Briarty, 1991; Jürgens & Mayer, 1994).

The transport of auxin from the embryonic shoot to root is essential for the specification of the hypophysis to form the root meristem. The function of the auxin transporters *PIN1* and *PIN7* is to redirect and regulate the efflux of auxin, probably by a *GNOM*-dependent pathway (Friml *et al.* 2003, Mayer *et al.* 1993, Geldner *et al.* 2003). Failing to do so will result in the compromise both of the specification of the apical cell lineage and the specification of the hypophysis as the root meristem precursor.

One more piece of evidence for auxin in the role of regulating the basal cell lineage for the root development is the study of *AUXIN RESISTANT6 (AXR6)* (Hobbie *et al.* 2000). The first defect in *axr6* embryo is the aberrantly orientated cell divisions in the derivatives of the basal daughter cell of the zygote; subsequently, no root meristem is formed. *AXR6* encodes a subunit of the ubiquitin protein ligase

SKP1/CULLIN/F-BOX protein which stimulates degradation of the *Aux/IAA* auxin response inhibitor proteins through the action of the ubiquitin protein ligase SCF^{TIR1}.

Both *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*) genes play roles in the formation of the central and basal part of the embryo and the establishment of the axial pattern (Przemeck *et al.* 1996, Hardtke & Berleth 1998, Hamann *et al.* 1999, 2002). The *MP* gene encodes a putative transcription factor related to *AUXIN RESPONSE FACTOR1 (ARF1)* which binds to the promoters of auxin-inducible genes and regulates their transcription (Ulmasov *et al.* 1997). *BDL* encodes *AUX/IAA PROTEIN12 (IAA12)*, a putative inhibitor of *MP*. The apical daughter cell in the zygote in the recessive *mp* mutant and gain of function *bdl* mutant divides horizontally instead of vertically, forming four tiers instead of two tiers of cells in the octant stage of the embryo. Therefore the placement of division planes in the globular embryo is highly irregular. Both mutants are defective in the development of the hypophysis and fail to generate the embryonic root. This suggests that *MP* and *BDL* are required for correct cell axialisation within the hypocotyl and correct alignment for the vascular tissue. The fact that the auxin response reporter DR5::GFP is not expressed in *mp* or *bdl* embryos, including their hypophysis, indicates the requirement of the *MP/BDL*–dependent auxin response for specification of the hypophysis and initiation of the embryonic root (Friml *et al.* 2003).

1.3 Root structure of Arabidopsis

The growth of the Arabidopsis root post-embryonally starts from the division of the cells within the root meristem region. The newly divided cells elongate and differentiate to form mature roots which provide for the collection and distribution of water and nutrients to the rest of the plant.

Indeed, the root can be divided into three distinct developmental regions along the root axis, and that corresponds to the processes of cell division, cell expansion and cell differentiation (Esau, 1977). Fig. 1.2 highlights these three regions.

1.3.1 Cell division zone

The apical root tip contains the meristem or zone of cell division. Cell division is initiated by stem cells and there can be considered to be four types of stem cells located at the tip of the root: a): the epidermal/lateral root cap initials giving rise to the epidermals and the lateral root cap, b): the columella initials, c): the cortex/endodermis initials and d): the quiescent center (QC) (Fig. 1.3; Dolan *et al.* 1993).

The origin of the quiescent centre and the columela root cap can be traced back to the hypophysis which was derived from the basal cell of the zygote. The remaining cells of the root will form from the apical cells of the zygote (Scheres *et al.* 1994), as described above (Section 1.2.1).

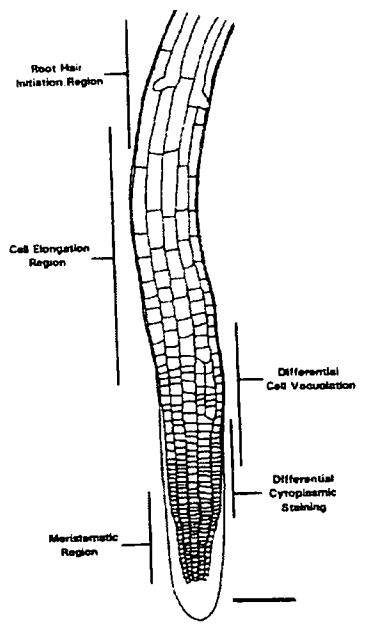


Figure 1.2: The Developmental Regions of the Root. Schematic diagram showing longitudinal files of epidermal cells originating at the meristem initials. The developing regions of the root and the location of differences between differentiating root hair and hairless epidermal cells are indicated. The single curved line at the root apex indicates the outline of the root cap. Bar = 100 μm (Fig. taken from Masucci & Schiefelbein, 1996).

Containing the non-dividing cells, the quiescent centre has traditionally been thought to serve as the backup of the initials when it is dead or loses the function of dividing

(Barlow, 1974) and inhibits the differentiation of the initials. This view latter has been confirmed experimentally, whereby laser ablation of the QC leads to differentiation of the surrounding stem cells (Van den Berg *et al.* 1997). Several genes, including *CYCAT* (Doerner *et al.* 1996) and *CDC2a* (Hemerly *et al.* 2000) have been found to be required to regulate the plant cell cycle, but it is beyond the scope of this thesis to consider these mechanisms in detail.

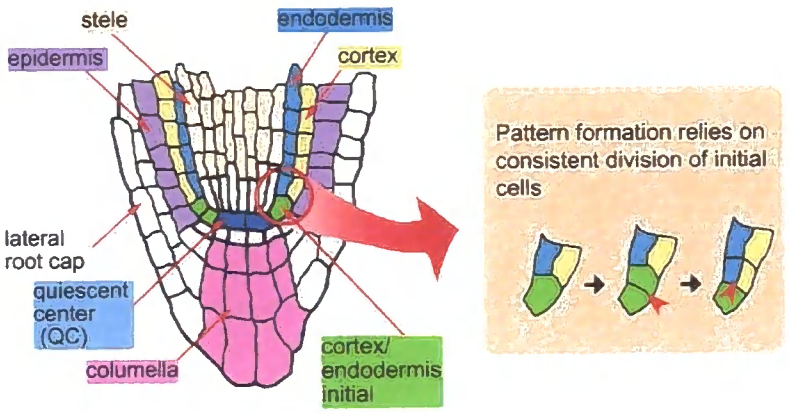


Figure 1.3: Four types of the Arabidopsis root stem cells and the structure of the root tip. (Figure taken from Scheres 2002).

1.3.2 Cell expansion zone

Cell expansion begins when the cell separation is completed. It is tightly regulated via the control of cell wall plasticity, and involves the cytoskeleton-mediated localization of cellulose synthesis components (Dolan *et al.* 2004). Diverse hormones and other (eg sterol-dependent) signals are involved in coordinating cell expansion processes, including auxin, ethylene and gibberellins. Gibberellins (*GAs*) are key regulators of cell expansion which determine plant growth and development. They promote growth by targeting the degradation of *DELLA* repressor proteins (Dolan *et al.* 2004). Recently, *gai*, a non-degradable, mutant *DELLA* protein, has been expressed in selected root tissues to map the site of *GA* action in regulating root growth. Root growth was retarded specifically when *gai* was expressed in endodermal cells. It is suggested that the endodermis represents the primary GA-responsive tissue regulating organ growth and that endodermal cell expansion is rate-limiting for elongation of other tissues and therefore of the root as a whole (Ubeda-Tomás *et al.* 2008).

1.4 The role of sterols in plant development

Recent evidence from mutational studies in Arabidopsis implicates sterols as playing vital roles in plant development. Sterols are isoprenoid-derived lipids synthesized via the mevalonate (sterol biosynthesis) pathway. Plant tissues contain an average quantity of 1-3 mg of sterols per gram dry weight and they are essential components of fungal, plant and animal membranes, whereby they regulate fluidity and interact with lipids and proteins. They also function as precursors for steroid hormones, the brassinosteroids (BRs).

The sterol biosynthetic pathway (Fig. 1.4) in plants can be viewed as comprising three domains (Lindsey *et al.* 2003): 1) a precursor-forming domain that leads to 2) a branch that produces the bulk membrane sterols, and to 3) a second branch that gives rise to the BRs (Clouse, 2000; Diener *et al.* 2000; Schaeffer *et al.* 2001).

The discovery of the *hydra1* and *hydra2* mutants (Topping *et al.* 1997; Souter *et al.* 2002; Souter *et al.* 2004) provides an opportunity to investigate the requirement for sterols in the control of embryogenesis and early seedling development. They were identified in a screen of insertion mutant populations as recessive seedling-lethal mutants, and exhibit pleiotropic phenotypes, including defective cell shape, polarity and patterning, multiple cotyledons and short roots and hypocotyls. Both *HYD1* and *HYD2* encode sterol biosynthetic enzymes. *HYD1* encodes Δ8-Δ7 sterol isomerase, the plant orthologue of yeast *erg2* and mammalian emopamil-binding protein (Souter *et al.* 2002). *HYD2* is allelic to *FACKEL (FK)* (Souter *et al.* 2002), and encodes sterol C14 reductase (Jang *et al.* 2000; Schrick *et al.* 2000), which is adjacent to *HYD1* in the sterol biosynthetic pathway (Lindsey *et al.* 2003; Fig. 1.4). The *sterol methyltransferase1* (*smt1*) mutant of Arabidopsis is also defective in various facets of cell polarity (Willemsen *et al.* 2003). Furthermore, mutation of the sterol methyltransferase gene *SMT2* is accompanied by reduced growth and low fertility, which is not modified by BRs (Schaeffer *et al.* 2001; Carland *et al.* 2002). Intriguingly, neither *hyd1*, *fk* nor *smt1* are rescued by the exogenous application of BRs, though mutants defective in enzymes in the distal BR biosynthetic branch of the sterol pathway are. This suggests specific signaling defects in these mutants, which also are unusual in showing defective embryogenesis that does not happen in BR

mutants.

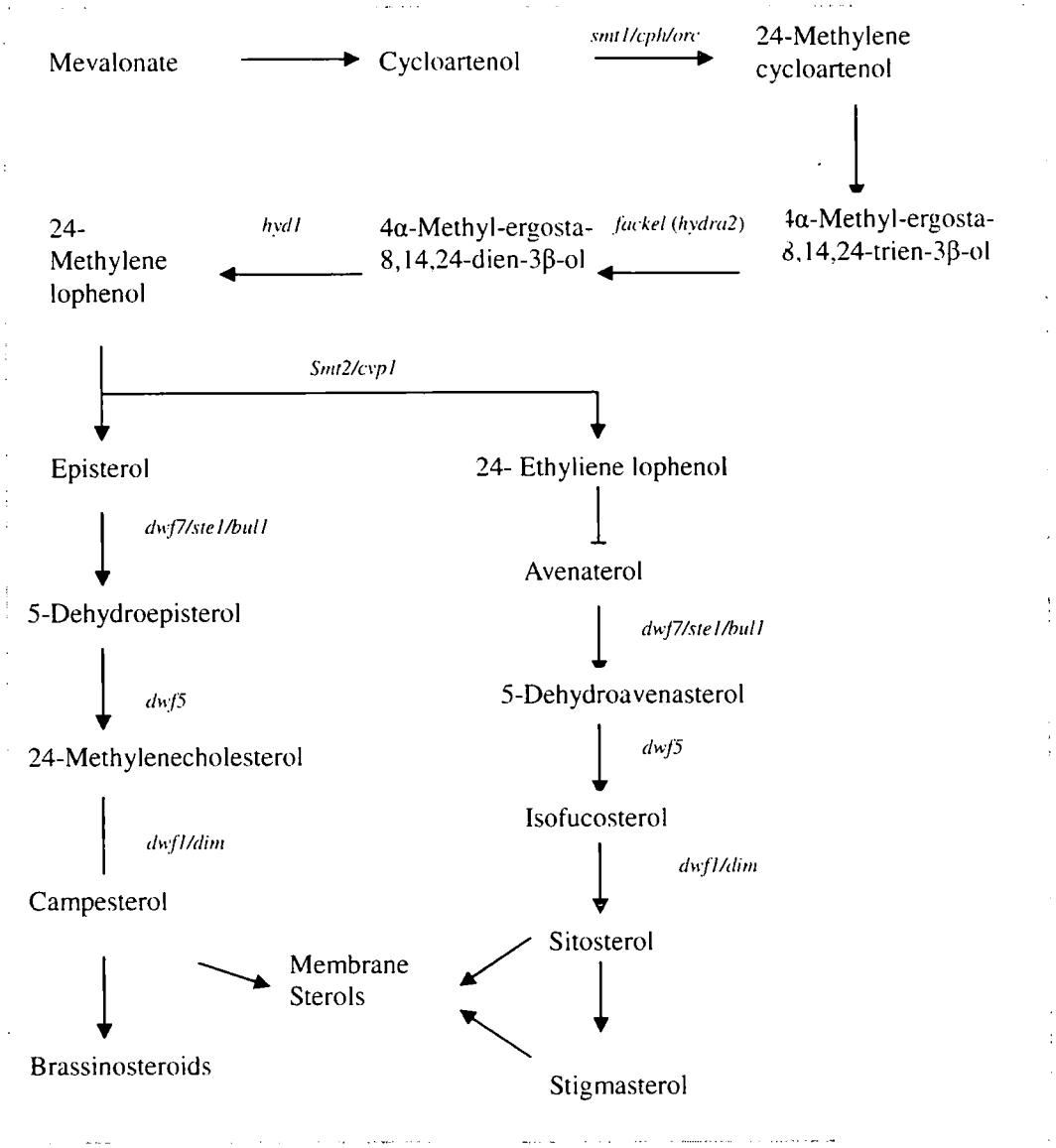


Figure 1.4: Plant sterol biosynthesis pathway. Scheme taken from Lindsey *et al.* (2003) Annotations refer to known biosynthetic mutants from *Arabidopsis thaliana*.

Several other mutants have been found defective in the sterol synthesis pathway in Arabidopsis and these defective genes are downstream of 24-methylene-lophenol (the joining point between the branch producing sitosterol and the one producing campesterol). Such mutants, including *dwf5*, *dwf7/ste1/bul1* and *dwf1/dim*, are typically dwarfed and each has phenotypes similar to BR-deficient mutants downstream of campesterol. The defective phenotypes of these mutants can be rescued by the application of the brassinolide, the most biologically active BR in many plants, and biosynthetic intermediates in the BR-specific pathway. *DWF5*, *DWF7/STE1* and *DWF1* catalyse the conversion of avenasterol to sitosterol and the

episterol-to-campesterol conversion as well. Thus, both sitosterol and campesterol, which are components of membrane, are deficient in these three mutants. As a result, the mutant may be affected in membrane structure and function as well as the defects caused by the deficiency of BRs. However, the rescue of these mutants by the application of BR suggests that it is the hormone deficiency rather than the altered membrane structure that plays an important role in the development of these mutants.

A key question is how defects in sterol biosynthesis are linked to the pleiotropic developmental defects seen in the *hyd1* and *fk/hyd2* mutants. Of significant interests is the observation that the *HYD1* and *FK/HYD2* genes are not expressed to detectable levels in all tissues that show developmental defects. For example, the *FK* gene is expressed in embryos and in the shoot and root apical meristems post-embryonically, but not in the hypocotyls or root epidermis, which each show abnormal cellular patterning in the *fk/hyd2* mutant (Jang *et al.* 2000; Schrick *et al.* 2000; Souter *et al.* 2002). The *hyd1* mutant shows similar developmental abnormalities, including aberrant leaf and hypocotyls morphogenesis. The *HYD1* gene is expressed in early embryos, the root apical meristem and the stipules, though not in the shoot apex (Souter *et al.* 2002, 2004).

1.5 The mechanism of the RNAi

One technique that can be used to investigate gene function, and has been used in this thesis to investigate the tissue-specific requirement for sterol gene expression, is gene silencing via RNAi, whereby dsRNA (double stranded RNA) for specific genes can be expressed in transgenic organisms to suppress gene expression.

The first evidence that dsRNA could lead to gene silencing came from work in the nematode *Caenorhabditis elegans*. On the basis of knowing that the injection of both sense and antisense RNA into the *C. elegans* shut down the expression of one particular gene, Fire and Mello injected dsRNA — a mixture of both sense and antisense strands — into *C. elegans* (Fire *et al.* 1998). This injection resulted in much more efficient silencing than injection of either the sense or the anti-sense strands alone.

So how does injection of dsRNA lead to gene silencing? Now it has been found that it is the small interfering RNA strands (siRNA) which have complementary nucleotide

sequences to the targeted RNA strand guided the specific RNAi pathway proteins to target mRNA (Hannon 2002). The mRNA was cleaved and broken down into smaller fragments, and as result of this and can no longer be translated into protein.

Exogenous dsRNA initiates RNAi by activating the ribonuclease protein dicer (Bernstein *et al.* 2001)http://en.wikipedia.org/wiki/RNA_interference - cite_note-Bernstein-6#cite_note-Bernstein-6 which binds and cleaves dsRNAs to produce double-stranded fragments of 20–25 base pairs. These small interfering RNAs (siRNAs) are then separated into single strands. One of the strands, called guide RNAs, was integrated into an active RNA-induced silencing complex (RISC) while another strand degrades themselves. After integration into the RISC, siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template (Hannon 2002, Ahlquist 2002).

hpRNA- induced RNAi has been shown to be much more efficient in plants than anti-sense-mediated gene silencing (Chuang *et al.* 2000), in which, gene silencing is achieved by transformation of plants with constructs that express in self-complementary (termed hairpin) RNA containing sequences homologous to the target genes (waterhouse *et al.* 2003). In hpRNA-producing vector, a strong 35S *CaMV* promoter drive the inverted repeat of target gene separated by intron was inserted.

The control of tissue-specific of gene suppression in plants is another application of the RNAi. By using variable promoter which is expressed in various tissues, the function of gene in this particular tissue and the effect for other tissue can be examined. However, RNAi is known to spread systemically (Waterhouse *et al.* 2001). When suppressing the target gene in one particular tissue, it is possible that the gene in other tissues is also down-regulated.

1.6 Aims and Objectives

It has been proposed that, in view of the restricted expression of the *FK* gene, sterols may act as mobile signal molecules in a non-cell autonomous manner, to regulate development at a distance from the site of biosynthesis, such as signaling between hypocotyls and apical meristems (Schrick *et al.* 2000). This would be analogous to the

effects of brassinosteroids, which interact with membrane-bound receptors (Li & Chory, 1997).

An alternative possibility is that the long-distance signal is not a sterol molecule itself, but a component of a signaling pathway regulated by, or otherwise dependent upon, sterols. Of relevance here is the evidence that both *hyd1* and *fk/hyd2* mutants are defective in auxin and ethylene signaling, and the mutant phenotypes can be rescued partially by the experimental inhibition of auxin and /or ethylene signaling, such as in double mutants with *axr1*, *axr3*, *etr1* or *ein2* (Souter *et al.* 2002, 2004). Recovery of auxin-mediated gene expression in double mutants with either *etr1* or *ein2*, which are ethylene insensitive, suggests that the loss of correct ethylene signaling in the *hyd* mutants leads to defective auxin-mediated gene expression (Souter *et al.* 2004).

It is possible therefore that *HYD/FK* gene function is required for correct plant cell signaling in a non-cell autonomous way, via effects on ethylene and auxin signaling. However, in possible contradiction to this idea, it has been found that the antisense expression of the *HYD1* gene under transcriptional control of a tapetum-specific promoter leads to male sterility but other development appears normal (Topping & Lindsey, unpublished). This suggests the effects of sterols can be cell-autonomous, at least in some developmental contexts. If sterol-dependent signaling is cell-autonomous, we would not expect to see phenotypic rescue of for example the mutant epidermis if the *HYD/FK* genes are transgenically expressed in restricted cell types such as the root meristem or stele, and this is a testable hypothesis. Therefore, a major aim of the work in this thesis is to investigate this question of the possible cell autonomy of sterol function in plant cell signaling control.

The model system used to study sterol-dependent signaling is the Arabidopsis root. This was used because one readily detectable aberrant phenotype in the *hyd1* and *fk* mutants is defective meristem function, root hair patterning and morphogenesis. Interestingly, despite the epidermal defect, available evidence indicates that neither the *HYD1* gene is expressed in the meristem, nor that the *FK* gene is expressed in the root epidermis. This evidence derives from promoter-GUS fusion analysis (Jang *et al.* 2000, Schrick *et al.* 2000, Souter *et al.* 2004), and also from a transcriptome analysis of specific root cell types, carried out by the Benfey laboratory (Birnbaum *et al.* 2003). Analysis of the transcriptome data has revealed that both the *HYD1* and *FK* transcripts

are expressed poorly, if at all, in the differentiating epidermal cells (Souter *et al.* 2004).

A number of possibilities are considered to explain the apparent discrepancy between the spatial expression of the *HYD1* and *FK* genes and the cellular defects in the epidermis:

- A very low level of transcription of the *HYD1* and *FK* genes occurs in the differentiating epidermis in wild-type roots, but loss of expression in the *hyd1* and *fk/hyd2* mutants is sufficient to generate the mutant phenotype in the epidermis.
- The *HYD1* and *FK* genes are not expressed in the epidermis, but a non-cell autonomous sterol-dependent signal is required for the correct patterning and polarity of epidermal cells.
- The non-cell autonomous signal could be either a mobile sterol or a different mobile sterol-dependent signaling molecule, such as auxin or ethylene (or a precursor of these).

Key to the experimental strategy will be: a) the suppression of *HYD1/FK* expression in a tissue-specific way in wild-type seedlings, using RNAi; b) the activation of *HYD1/FK* gene expression in a tissue-specific way in *hyd1* and *fk* null genetic backgrounds. By determining whether these modifications affect the phenotype of the seedlings, it is the objective to establish whether sterol biosynthesis regulates cellular development in a cell-autonomous or non-autonomous way.

CHAPTER 2: MATERIALS AND METHODS

2.1 Material

2.1.1 Chemicals

All chemical reagents used were analytical reagent grade and were obtained from Fisher Scientific (Loughborough, UK), Sigma (Poole, UK), and BDH (Lutterworth, UK), unless otherwise stated.

2.1.2 Plant Lines

A class of recessive seedling-lethal mutants defined by two loci was isolated in a screen of T-DNA and EMS mutants of *Arabidopsis* and designated *hydra1* and *hydra2* (Topping *et al.* 1997). Among these, several alleles of *hyd1* are available and the only one we used for the experiments was *hyd1-2* in the *Arabidopsis thaliana* ecotype Wassilewskia (Ws) background which will be referred as *hyd1* unless otherwise stated. One allele is available for *hyd2* in the Ws background. *Arabidopsis thaliana* ecotypes Wassilewskia seeds were obtained from Nottingham Arabidopsis Stock Centre (NASC). The DR5::GUS transgenic line (Ulmasov *et al.* 1997) and the *hyd1* and *hyd2* plant lines were supplied by Professor Keith Lindsey (Durham University).

2.2 Plant Growth Conditions

2.2.1 Plants Germinated on Soil

Arabidopsis plants used for bulking seeds, dipping, segregating mutant lines were grown in soil. Soil was made up of a 5:1 mixture of Gem multi-purpose compost

(LBS Horticulture LTD) and horticultural silver sand (LBS Horticulture LTD) to enhance drainage.

All compost was treated with Intercept (Levington Horticulture LTD, UK) at the concentration of 64 mg/tray (24 small pots per tray) by watering after being dissolved in 5ml plastic tube first, to reduce insect damage. The mite *Amblyseius cucumeris* (Syngenta, UK) was used for bio-control of thrips by being hung on the aerial parts of plants.

Seeds were first sown in propagators in a controlled environment room (22°C, 16 hours light and 8 hours dark) for 7 days before being transferred to a greenhouse (22°C, 16 hours light and 8 hours dark). Plants were then separated and put into appropriate pots. For these for seeding collection, plastic bags were used to cover the plants. The Aracon system (Lehle Seeds, USA) was used to collect the seeds and seeds were air dried for at least 2-3 days before being used.

2.2.2 Plants Germinated on Culture Media under Sterile Conditions

All seedlings for analysis and DNA (RNA) extraction were germinated under sterilized condition on $^{1}/2$MS10 agar medium. The medium was made of 2.2g/l half-strength Murashige and Skoog basal mix (Sigma, Poole, UK), 10g/l sucrose and 8g/l Bacto-agar (Difco, Detroit, USA), adjusted to pH 5.8 with 1 M KOH (Murashige and Skoog 1962). After being sterilized by autoclaving at 121°C for 20 minutes, $^{1}/2$MS10 medium was poured into Petri dishes under sterile conditions in an air-flow cabinet, or stored in cool dry place and melted by microwaving before using.

Seeds were sterilized in an Eppendorf tube by gently shaking in 70% ethanol for 2 minutes. After removing ethanol, 10% commercial bleach with a drop of Tween 20 was added before being shaken for 20 minutes. Seeds were washed with autoclaved distilled water three times. They were then transferred to 100 mm^2 sterilized Petri dishes (Fred Baker, UK) at 22°±2°C and at a photon flux density of approximate 150 µmol/m2/second. For seedlings which need to grow vertically in root length analysis experiment, square Petri dishes were used. Plates were labeled and sealed with

Micropore medical tape (Industricare Ltd, Leicestershire, UK). To ensure uniform germination, plates were wrapped using aluminum foil to keep the seeds away from light before being placed into Fridge (4°C) for 2 days (*hyd* mutants for 7 days) to vernalize and transferred to growth chamber at 22°C, 16 hours light and 8 hours dark.

2.3 Methods for making gene construction

2.3.1 Polymerase Chain Reaction (PCR)

2.3.1.1 Standard PCR

The Oligdeoxynucleotide primers (in Appendix 3) were obtained from MWG-Biotech and then re-suspended to make 50 pmol/µl working concentration using sterile distilled water. *Taq* DNA polymerase (Bioline, UK) was used for standard PCR.

The standard PCR reaction using *Taq* DNA polymerase

A typical reaction contained the following components:

Mg^{++} free 10x reaction buffer	5 µl
DNA template	10-100 ng
primer 1 [forward]	1 µl (2 pM final concentration)
primer 2 [reverse]	1 µl (2 pM final concentration)
50 mM $MgCl_2$	1 µl (1.5 mM final concentration)
10 mM dNTPs mix	1 µl
Taq DNA polymerase	1 ul

The volume was then made up to 50 µl with nuclease-free water provided and assembled on ice in a sterile 0.5 ml micro-centrifuge tube. A tube of reaction components with no DNA template was also made as negative control.

Tubes containing reactions were mixed gently and centrifuged briefly before being placed in a DNA Thermal Cycler (Perkin Elmer; CA, USA) with a drop of mineral oil. The cycle parameters were and initial 5 minutes denaturation at 94°C, followed by 30 cycles comprising 1 minute denaturation at 94°C, 1 minute in annealing temperature

and extension at 72°C for 1 minute per kilo-base of expected product. A final extension step of 10 minutes at 72°C was performed after amplification.

The standard PCR reaction using *KOD* Hot Start DNA polymerase

A typical reaction contained the following components:

10x reaction buffer	5 µl
DNA template	10-100 ng
primer 1 [forward]	1 µl (0.3 mM final concentration)
primer 2 [reverse]	1 µl (0.3 mM final concentration)
25mM MgSO4	1 µl (1.0 mM final concentration)
10mM dNTPs mix	5 µl (0.2 mM final concentration)
KOD Hot Start DNA Polymerase	1 ul

The volume was then made up to 50 µl with Nuclease-free water, and assembled on ice in a sterile 0.5 ml micro-centrifuge tube. The cycling condition was carried out in thermal cycler as 2 minutes at 94°C, followed by 30 cycles comprising 15 seconds at 94°C, 1 minute at annealing temperature, 1 minute at 72°C. A final step of 10 minutes at 72°C was performed before the tubes were chilled in 4°C.

2.3.1.2 Colony PCR

Colony PCR was performed to select the colony with desired insert using Bioline *Taq* DNA polymerase.

10x Mg^{++} free 10x reaction buffer	2 µl
10 mM dNTPs mix	0.5 µl
Primer 1 [forward]	0.5 µl
Primer 2 [reverse]	0.5 µl
50 mM $MgCl_2$	0.5 µl
Bio *Taq* DNA polymerase	0.1 µl

Sterilize distilled water was used to make the total volume up to 20 µl. Master Mix was made before an aliquot of 20 µl was used in sterile 0.5 ml tube. The cycling condition was carried out as the one described in standard PCR using Bioline *Taq* DNA polymerase.

2.3.2 DNA Agarose gel electrophoresis (after Sambrook *et al.* 1989)

Agarose gel electrophoresis was used to analyse DNA (RNA) concentration, size, and to separate different fragments from each other. Gels were typically made of 1% agarose in 1x TAE buffer (40 mM Tris-acetate 1 mM EDTA, pH 8.0) although the concentration of the agarose can vary from 0.7% w/v to 2% w/v according to the size of interested DNA fragments. Gel was melted in a microwave and allowed to cool down to below 50°C before adding a drop of ethidium bromide. The gel was then poured into a gel tray with appropriate comb and allowed to solidify at room temperature for about half an hour before being used.

10-20 µl of DNA sample was loaded in gel with loading dye for separation of DNA fragments. For checking the concentration, 1 µl of DNA sample, loading dye and dH2O in a total of 10 µl was loaded with DNA marker (Hyper ladder I or Hyper ladder IV depending on the size of fragment) on both sides. Electrophoresis was performed at 80 V/cm and a Gel Doc 1000UV trans-illuminator system (Bio-Rad) was used to visualize DNA.

2.3.3 cDNA synthesis

- **RNA extraction**

Qiagen RNeasy plant Mini kit was used to extract RNA from 2 weeks old seedlings according to manufacturer's instructions.

- **Removal of DNA from RNA samples using RQ1 RNase-Free DNase**

For 10 µl of reaction, the solution was set up as follow:

RNA sample	1-8 µl
RQ1 RNase-Free DNase 10x buffer	1 µl
RQ1 RNase-Free DNase	1 µl/µg RNA

The mixture was shaken gently before being incubated at 37°C for 30 minutes. 1 µl (every 10 µl of total volume) of RQ1 RNase-Free DNase was added and incubated at

65°C for 10 minutes to terminate the reaction.

- **cDNA synthesis**

1-2.5 μg of RNA sample was mixed with 1 μl of Oligo dT in a sterile Eppendorf tube and made up to 10 μl with sterile distilled water. The sample was heated at 70°C for 10 minutes before being cooled on ice for 5 minutes. The following components were then added in this order: 5 μl of AMV RT 5x buffer (supplied with AMV reverse transcriptase, Promega Ltd.), 2.5 μl 19 mM dNTPs mix, 1 μl RNasin (Promega Ltd) and 3 μl (20 units) AMV reverse transcriptase, giving a total volume of 20 μl. After mixing and brief centrifugation, the sample was incubated at 42°C for 45 minutes and 50°C for 25 minutes. The reverse transcriptase was then heat inactivated by incubation at 95°C for 5 minutes. The synthesized cDNA was being checked using standard PCR using KOD Hot Start DNA Polymerase.

2.3.4 Digestion and ligation

The reaction was carried out according to manufacturer's instructions. The typical digest reaction was as follow:

10x buffer	2 μl
BSA	0.5 μl
Enzyme A	1 μl
Enzyme B	1 μl
Plasmid DNA	(20 μl in total)

The mixture was incubated overnight at 37°C before being heated to 65°C for 20 minutes to inactivate restriction enzymes. The result was then checked using gel electrophoresis. In the ligation reaction, 3 different ratios of vector and insert were used to optimize the reaction: 1:1, 1:3, 3:1. The amount of samples being ligated was calculated using the following formula:

ng vector × kb size of insert/ kb size of vector× ratio=ng insert.

1 μl 10x ligase buffer and 1 μl T4 ligase was added into the tube and mixed gently with vector and insert using sterilize distilled water to make up to 10 μl. The tube was

then incubated at room temperature for 15 minutes before being heated at 65°C for 10 minutes to terminate the reaction.

2.3.5 Extraction and purification of plasmid DNA

- **High copy number plasmid DNA extraction**

Promega WizardPlus SV mini-prep kit was used for the extraction of high copy number plasmid DNA from 1-5 ml culture volumes. The resulting DNA was suitable for sequencing, PCR and all cloning purposes. All centrifugation were carried out at 13,000 rpm using a bench-top micro-centrifuge. Bacteria containing the plasmid of interest were grown overnight at 37°C with constant shaking in selective LB liquid medium. A 1.5 ml Eppendorf tube was used to pellet the culture in several batches by centrifugation for 2 minute and the supernatant was removed each time. 1 ml of each culture was left to store at 4°C to make glycerol stock (300 µl 1:1 LB : glycerol and 700 µl bacterial culture containing plasmid DNA, stored at -80°C) for use later on if required. The cells were re-suspended in 250 µl of Re-suspension solution by vortex mixing. 250 µl of Lysis solution was then added and mixed by inversion for a few times before the tube being incubated at room temperature for no more than 5 minutes until all suspension is clear. 350 µl of Neutralization solution was added, immediately mixed by inversion before the tube was centrifuged for 10 minutes to pellet the cell debris. The cleared supernatant (containing the plasmid DNA) was then applied to a Mini Spin Column in a collection tube, and centrifuged for 1 minute. 750 µl of Wash solution was added to the column and centrifuged for 1 minute. The flowthrough was discarded and washing procedure was repeated using 250 µl of Wash solution before the column was finally centrifuged for 5 minutes to dry the membrane. To elute the plasmid DNA, the column was transferred to a new collection tube. 50 µl of sterile water was applied to the membrane and the column was left to stand for 2 minutes before centrifugation for 1 minute. The eluted DNA was stored at -20°C.

- **Low copy number plasmid DNA extraction**

A Qiagen Midi Prep kit was used for the preparation of low copy number plasmids such as pΔ-CIRCE from large culture volumes (100-200 ml). 5 ml bacteria in selective LB liquid media containing the plasmid of interest was grown overnight at 30°C with constant shaking before being transferred to 100 ml selective LB media at 30°C for another 2 days. The culture was then transferred to steriled 50 ml Falcon centrifuge tubes before being pellet by centrifugation at 6,000xg for 15 minutes at 4°C. The supernatant was discarded and the cells were re-suspended in 4 ml of Buffer P1. 4 ml of Buffer P2 was then added and mixed thoroughly by inversion before incubation at room temperature for 5 minutes. 4 ml of chilled Buffer P3 was added and mixed by inversion before incubation on ice for 15 minutes. The resulting mixture was then centrifuged at 20,000xg for 30 minutes at 4°C and the supernatant was removed to a new tube before centrifugation for a further 15 minutes at 20,000xg at 4°C.

During these centrifugation steps, a QIAGEN-tip 100 column was equilibrated by adding 4 ml of Buffer QBT to the column and allowing it to drain by gravity flow. The cleared supernatant containing the plasmid DNA was applied to the column and allowed to drain again. The QIAGEN-tip 100 was then washed with 2x 10 ml of Buffer QC, before the DNA being eluted into a sterile 15 ml Falcon tube with 5 ml of Buffer QF. The DNA was precipitated by adding 3.5 ml (i.e. 0.7 volume) of room temperature isopropanol. The suspension was transferred to Eppendorf tubes before centrifugation at 15,000xg for 30 minutes at 4°C. The resulting pellet was washed with room temperature 70% (v/v) ethanol and centrifuged at 15,000xg for 15 minutes at 4°C. The supernatant was removed and the Eppendorfs containing the pellet were air-dried until all visible droplets of liquid had disappeared. The DNA was re-suspended in 100 µl of sterile distilled water and stored at -20°C.

2.3.6 Bacterial transformation

- **Transformation of TOP10 competent cells**

TOP-10 competent cells were supplied with the TOPO-TA® cloning kit from Invitrogen and stored at -80°C. An aliquot of cells was defrosted on ice and at the same time the tube containing plasmid DNA of interest was centrifuged before being placed on ice. 1-5 µl of the plasmid DNA was added directly into the cells and mixed by gently tapping before being incubated on ice for 30 minutes. Heat-shock at 42°C

was performed for 30 seconds to induce uptake of the DNA, and then the tube was returned to ice for recovery for a further 2 minutes. 250 µl of pre-warmed LB media was added before the tube being incubated for 1 hour at 37°C with gentle shaking. 50-100 µl of cells was spread onto selective LB agar plates grown overnight at 37°C and the resulting colonies were checked by colony PCR.

- **Transformation of chemically competent *E.coli* with plasmid DNA**

The method we use for making competent *E. coli* cells is described by Ausubel *et al.* (1994).

Solutions

- TfbI buffer: 30 mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride, 15% v/v glycerol, pH 5.8 with dilute acetic acid, filter sterilized
- TfbII buffer: 10 mM MOPS, 75 mM calcium chloride, 10 mM rubidium chloride, 15% v/v glycerol, pH 6.5 with 1 M NaOH, filter sterilized

E.coli strain DR5α was used for the preparation of chemically competent cells. A single colony from a fresh LB plate of DR5α was used to inoculate 100 ml of sterile LB media, and grown overnight at 37°C with vigorous shaking to A550 = 0.48. The culture was chilled on ice for 15 minutes in 50 ml Falcon centrifuge tubes and the cell was pelleted by centrifugation at 4000xg for 5 minutes at 4°C. The supernatant was discarded, and the cells were gently resuspended in 40 ml (0.4 of the original culture volume) of buffer TfbI. The cells were stored on ice for 15 minutes before the centrifugation step was repeated during which the supernatant was removed. The cells was re-suspended in 4 ml (0.04 volumes of the original culture volume) of ice cold buffer TfbII and stored on ice for 15 minutes. 80 µl aliquots were pipetted into ice cold Eppendorfs and flash frozen in liquid nitrogen before being stored at -80°C until required.

For transformation, an aliquot of cells was defrosted on ice. Around 1 µl of ligation mixture was added and mixed gently before incubation on ice for 20 minutes. The cells were then heat shocked at 42°C in water bath tank for 30 seconds without shaking before being placed on ice for 2 minutes. 1ml of liquid LB was added and the cells were shaken at 37°C for 1 hour. Aliquots of 50 µl, 100 µl and the rest of the cells

(centrifuge and re-suspended using LB media again) were spread onto selective LB agar plates and grown overnight at 37°C.

2.3.7 Mobilization of p-CIRCE into *Agrobacterium*

2.3.7.1 by Electroporation

Solution

1 mM HEPES, pH 7.0 with 1 M KOH, filter sterilized
20% Glycerol, filter sterilized
LB medium

Preparation of competent Agrabacteria cells

5 ml of LB medium with defrosted *Agrobacterium* (C58c3) was grown with 20 μg/ml nalidixic acid and 100μg/ul streptomycin at 30°C with constant shaking for 24 hours. 200 ml of LB medium with the same concentration of antibiotics was then added with constant shaking for 2 days. The cells were pelleted in centrifuge at 3000xg for 20 minutes at 4°C and then re-suspended in 1/3 initial volume of ice cold 1mM HEPES solution after discarding the supernatant. The bacteria were pelleted again in a 4°C rotor. After repeated re-suspension and pelleting, the cells were re-suspended in about $1/75^{th}$ initial volume of 20% glycerol. 100 μl aliquots were pipetted into ice cold Eppendorfs and flash frozen in liquid nitrogen before being stored at -80°C until required.

Electroporation

For transformation, an aliquot of cells was defrosted on ice before adding 1 μl of DNA. The mixture was pulsed using eletroporator in 2.5 kV, 125 μF and 400 Ω. 1 ml LB medium was then added before being transferred to an Eppendorf tube. The Eppendorf was placed at 30°C for 6 hours with constant shaking. Aliquots of 100 μl and 250 μl of the cells were spreaded onto LB agar plates containing 20 μg/ml nalidixic acid, 100 μg/ml streptomycin and 50 μg/ml kanamycin and grown for 2 days

at 30°C. The plasmid DNA pelleted from colonies were being checked by restriction digestion and sequencing.

2.3.7.2 Tri-parental mating

Some copies of the p-CIRCE vector carrying the desired sequences were mobilized into *Agrobacterium* using tri-parental mating (Bevan 1984; Hooykaas 1988).

Two days prior to Tri-parental mating, 10 ml of LB medium containing antibiotic appropriate for the selection of recombinants was inoculated with *Agrobacterium* C58C3 and left at 30°C with constant shaking. The day before, two 10 ml LB overnight cultures containing 100 mg/l kanamycin sulphate was inoculated, one with *E.coli* strain HB101 carrying the pRK2013 plasmid, and the other with *E.coli* strain containing the construct to be transformed. Both cultures were grown at 37°C with constant shaking.

For mating, 100 µl aliquots from each of the three cultures were mixed together in a sterile 1.5 ml Eppendorf tube, and the cells were pelleted by centrifugation at 12,000 rpm for 5 minutes. After discarding the supernatant, the pellet was then re-suspended in 10 µl of 10 mM $MgSO_4$. This droplet was spread onto a non-selective LB agar plate and allowed to incubate overnight at 30°C. During this time, mobilization of the plasmid occurs.

Triple-selective LB agar plates were prepared containing 100mg/l kanamycin sulphate, 100 mg/l streptomycin and 25 mg/l nalidixic acid. A sterilized microbiological loop was used to streak out a patch of the bacteria from the overnight LB-agar plate onto a selective plate. Controls of each of the parental strains were also streaked, each onto a separate plate, and all plates were left overnight at 30°C. Only the *Agrobacterium* strain carrying the pΔ-GUS-Circe plasmid should grow under these selection conditions. The isolated *Agrobacterium* strain was re-streaked for single colonies, and the extracted plasmid checked by restriction analysis.

2.3.8 *Arabidopsis* transformation using the dipping method

This protocol was described by Clough & Bent (1998) and modified by Jennifer Topping (Durham University).

Preparation of the plants

5cm square pots containing 9 seedlings in compost were used. Plants were grown for 3-4 weeks until they were approximately 10-15 cm tall and displaying a number of immature, unopened flower buds. Open flowers and any young siliques were removed.

Preparation of the *Agrobacterium* culture

A culture of *Agrobacterium* strain C58C3 carrying each construct in the p-CIRCE based vector was prepared. The *Agrobacterium* was grown in culture flasks for 48 hours at 30°C in 200 ml of sterile liquid LB supplemented with 100 mg/L kanamycin sulphate, 100 mg/L streptomycin and 25 mg/L naladixic acid. The cultures were transferred to sterile 50ml Falcon centrifuge tubes and pelleted by centrifugation for 10 minutes at 3000xg at 4°C. The cells were gently resuspended in 1 litre of a freshly made solution of 5% w/v sucrose with 0.05% v/v Silwett L-77™ detergent (Lehle seeds, Texas, USA).

Method

Trimmed plants were dipped fully into the *Agrobacterium* solution and gently agitated for 10 seconds before removal. Trays containing Dipped plants were covered using a large transparent plastic bag to maintain humidity and placed in the greenhouse in a shaded position overnight. The next day, transparent bags with open tops were used to cover each pot and plants were returned to their normal greenhouse growth conditions. Plants were allowed to set seed and dry out in the greenhouse. Seeds were then collected and labeled.

Dried seeds were surface sterilized (as described in section 2.2.2) and germinated on 1/2MS10 plates made with Bacto-agar supplemented with 35 µg/ml kanamycin

sulphate (to select for transformants) and 200 μg/ml augmentin (to remove *Agrobacterium*). Seedlings that developed bleached cotyledons were considered genetically wild type. Primary transformants were transferred to soil and seeds from these plants were tested for segregation on selective plates.

2.3.9 DNA Sequencing

All DNA samples needed for sequencing were sent to DNA Sequencing Laboratory at the University of Durham using an ABI 373 DNA sequencer and dye terminator labelling reactions (Perkin Elmer Applied Biosystems). M13F and M13R primer sites which are included in the vectors on either side of the relevant multiple cloning sites were used for sequencing unless the special primers were stated in which the concentration of 3.2 pmoles/μl should be supplied. Samples were supplied at the concentration of 100 ng/μl.

2.4 Plant Analysis

2.4.1 Root length measurement

Root length measurement in the mutants was required for the quantification of root growth in order to assess the impact of different mutations and transgenes on seedling development. The primary root was used for all the root growth experiments. The same plants were used for all measurements with each developmental stage and marks were made on plates every three days until six days, to monitor growth. The plates were then photographed and the pictures were used to measure root length (from the root-hypocotyl junction to the mark) using software Image J. The mean and standard deviation of the root measurements taken for each day were determined. The standard deviation was used to calculate the standard error of the mean.

2.4.2 Lugol Staining

In order to view the root cap columella and then determine the activity of root meristem, Lugol solution was used to stain the starch granules within columella cells. The following method used was as modified by Ranjan Swarup (University of

Nottingham). Seedlings cleared using Malamy and Benfey's method (Section 2.4.3) were stained directly in Lugol solution for a few seconds and immediately put on a slide and viewed under a light microscope with DIC optics. Images were captured quickly using a digital camera so as to avoid photo-bleaching.

2.4.3 Histology and histochemistry

This method has been adapted from Malamy and Benfey (1997). Seedlings were incubated at 57°C in a 0.24 M HCl 20% methanol solution for 15 minutes, and then transferred to a 7% NaOH 60% ethanol solution for 15 minutes at room temperature. Seedlings were re-hydrated through an ethanol series of 40%, 20% and 10% ethanol (5 minutes incubations in each). Seedlings were then mounted in 50% glycerol and viewed using DIC optics. Lugol stained tissue was viewed in this way.

2.4.4 DNA extraction from small amount of materials

The method was used to genotype mutants and transgenics. A small leaf was placed into an Eppendorf with a small amount of sand before adding 400µl extraction buffer (200mM Tris-HCL pH 7.5, 250mM NaCl, 25mM EDTA, 0.5% (w/v) SDS). The suspension was grounded with an Eppendorf pestle for about 15 seconds and then vortex mixed. The tube was centrifuged at 4000xg for 5 minutes to pellet the cell debris and sand. The supernatant was transferred to a new Eppendorf, and then an equal volume of isopropanol was added. The mixture was then centrifuged for 5 minutes, and the supernatant was removed. The pellet was washed with 70% ethanol and centrifuged for 1 minute. The ethanol was removed and the Eppendorf containing the pellet was left to air dry. The pellet was re-suspended using 50 µl of sterilize distilled water and stored at -20 °C.

Chapter 3: Construction of *HYDRA* RNAi and sense gene fusions for the tissue-specific regulation of sterol biosynthesis

3.1 Introduction

The aim of the project was to use transgenic techniques to either repress *HYD* gene expression in specific cells of the root, or to activate *HYD* expression in specific cells of the *hyd* mutant roots, and to determine effects of sterol gene expression on root development. Repression of *HYD* gene expression (by post-transcriptional gene silencing) in specific cell types of the root was approached experimentally by expressing RNAi gene constructions. In a second approach, HYD gene expression was activated in specific root cell types in *hydra* mutants using sense cDNAs under tissue-specific transcriptional control.

This Chapter describes the construction of RNAi and sense expression fusions, using a meristem-active gene promoter. The promoter used was the *DR5* synthetic promoter (Sabatini *et al.* 1999), which is auxin-responsive and known to exhibit highly specific expression confined to the root meristem and columella, where the maximum auxin response is found.

3.2 The construction of RNAi constructs

The experimental strategy for the construction of the RNAi gene fusions is illustrated in Fig. 3.1. (The strategy for *HYD1* and *HYD2* RNAi constructs was essentially the same). Primers were designed to two regions of the relevant gene coding region, for cloning either side of an intron sequence, to generate a double stranded hairpin structure (illustrated in Appendix 4). The sequences of primers used in this work are shown in Appendix 3. Oligodeoxynucleotide primers used in PCR reactions were obtained from MWG-Biotech (Ebersberg, Germany).

PCR using each of the primer pairs was carried out using the *Taq* DNA polymerase (see Section 2.4.1). The template for PCR was cDNA from 2 weeks old *Arabidopsis*

ecotype Ws seedlings. After being purified using QIAquick PCR purification kit, these PCR products were cloned into the TOPO TA vector using Top 10 competent cells (see Section 2.4.6). The universal primers M13F and M13R were used to check the existence of the right fragment by colony PCR or by sequencing. Each plasmid was then digested using the appropriate restriction endonucleases (Promega Ltd, see Section 2.4.4) to release the insert from the PCR TOPO vector. The bands corresponding to the fragments were then cut out from the gel and purified using the Qiagen QIAquick® Gel extraction kit (Qiagen, UK). At the same time, the p-CIRCE vector was digested using the same pair of the enzymes and purified using the High Pure PCR Product Purification kit (Roche Applied Science). Each of these two fragments was then ligated together using T4 ligase (Promega, see Section 2.4.4) and introduced into chemically competent *E.coli*. The colonies were then checked by colony PCR using the primers originally used to amplify the fragments. A colony containing the insert of the correct size was then used to prepare plasmid DNA using the Sigma GenElute™ Plasmid Mini-prep kit before being sent for sequencing. The next fragment was added into the p-Circe vector which already contained the previous fragment. The complete construct with the promoter and sense fragment and the anti-sense fragment and intron was added in the p-CIRCE vctor (see Appendix 4 for the diagram of the RNAi structure). The construct which contains all the fragments needed were confirmed finally by sequencing with the universal primers M13F and M13R.

The plasmid DNA was introduced into electro-competent *Agrobacterium tumefaciens* strain C58C3 (see Section 2.4.7). A few colonies were selected to make cultures and the plasmid DNA was prepared using the Qiagen Midi Prep kit before being sent for sequencing (see 2.4.5). Validated plasmid was used for introduction of the construct to Arabidopsis Ecotype Ws by the dipping method (see 2.4.8). Seeds from the dipped plants were sown on ½MS10 medium supplemented with 35 mg/L kanamycin sulphate and 200 mg/L augmentin. Transformed plants were identified and seeds were collected as individual lines.

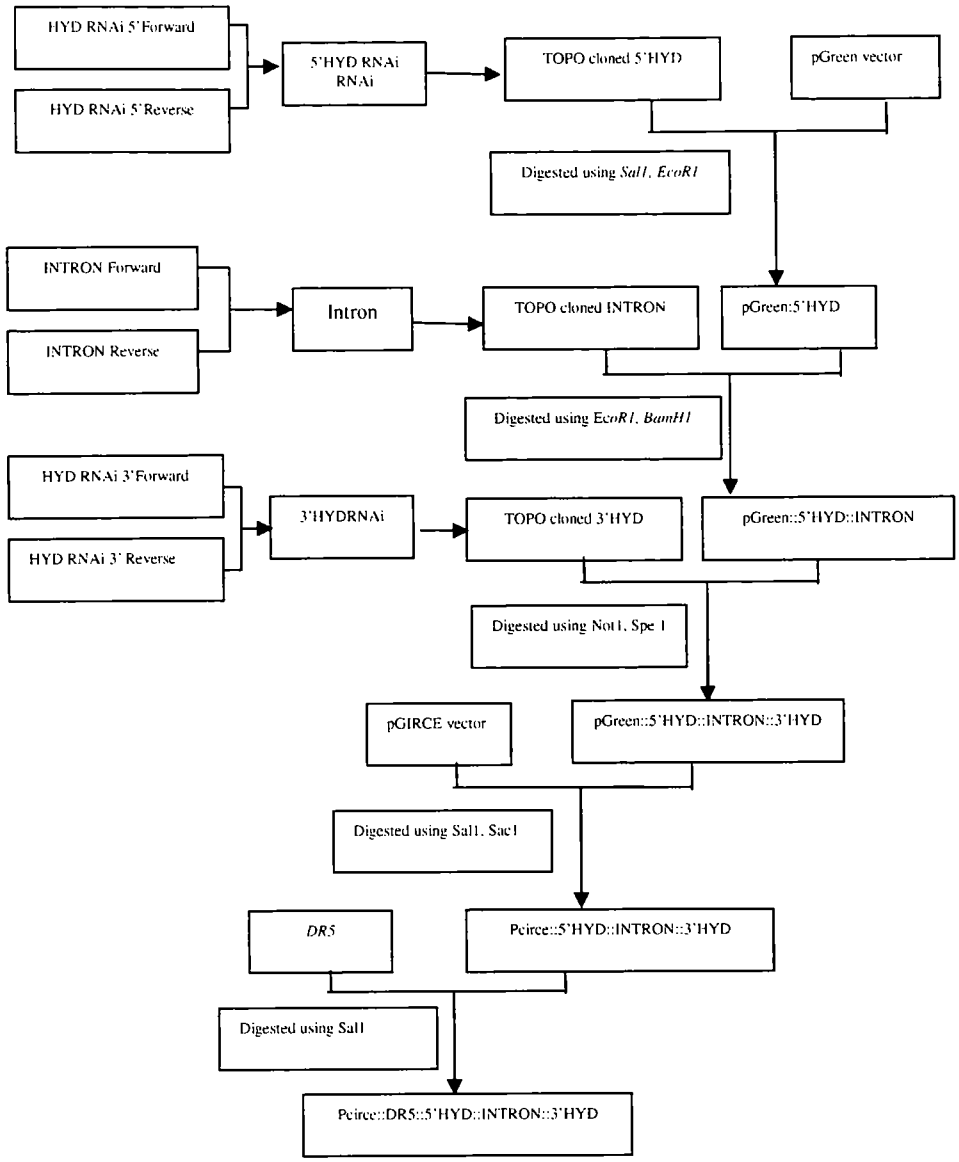


Figure 3.1: Cloning strategy for *HYD* RNAi construction.

3.3 The construction of the sense gene construct

The experimental strategy for the constriction of the sense gene fusions is illustrated in Fig. 3.2. Instead of the sense plus antisense fragments and intron being inserted, the appropriate *HYD* cDNA (*HYD1* or *HYD2*) was used in the constructs. Primers were designed to allow amplification of the whole of the coding region of the gene (illustrated in Appendix 3). Instead of using the standard *Taq* DNA polymerase, the KOD hot start DNA polymerase enzyme (from Novagen) was used to amplify these

two genes from cDNA of the Ws wild type Arabidopsis in order to increase the specificity (see 2.4.1). The constructs were cloned into the Topo vector to sequence before transferring into p-CIRCE with the DR5 promoter.

When the *HYD2* constructs were sequenced, a single base-pair difference with Col-0 was noted. This was found to be due to an ecotype-specific difference and was deemed to be acceptable as it did not give rise to a change in the amino acid sequence.

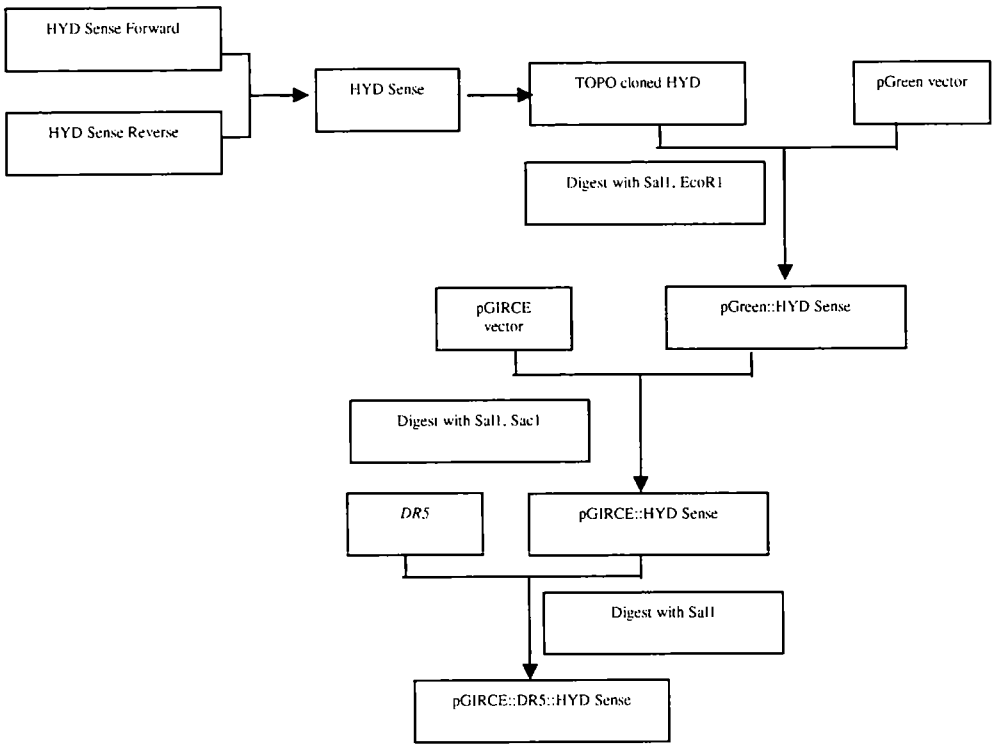


Figure 3.2: Construction of sense *HYD* gene fusions.

3.4 Binary vector construction

pGreen0179 initially was used as a vector to make the constructs (See the structure of pGreen 0197 in Appendix 1). Different methods were used to transform the pGreen vector into the *Agrobacterium*, including electroporation and tri-parental mating. However, none or a few small colonies were obtained from the transformation. None of these small colonies were detected as positive when PCR were performed (data not shown). Therefore, pCIRCE binary vectors were used to ligate the constructs cut from the pGreen. Appendix 2 shows the structure of the pCIRCE vector. The constructs were then transformed into Ws wild type, and subsequently crossed with *hyd* mutants

for further analysis. The crosses were performed by Dr. Ellie Short (Durham University)

3.5 Summary

This Chapter is described the construction of RNAi and sense expression gene constructs and transformation of *Arabidopsis* (as described in Section 2.3.8) for analysis of the effects of repression or activation of *HYD* gene expression in transgenic plants. The next Chapter describes the analysis of the transgenic plants.

CHAPTER 4: RNAi-mediated repression of *HYDRA* gene expression in root cells

4.1 Introduction

The pleiotropic phenotypes of *hydra1* and *hydra2/fackel* mutants include defective root meristem function, root hair patterning and morphogenesis which could not be rescued by the exogenous application of brassinosteroids. However, despite the epidermal defect, evidence derived from promoter-GUS analysis (Jang *et al.* 2000, Schrick *et al.* 2000, Souter *et al.* 2004), and also from a transcriptome analysis (Birnbaum *et al.* 2003) indicates that neither the *HYD1* nor the *FK* genes are strongly expressed in the root epidermis. The aim of this chapter is to present the results of a number of experiments designed to investigate whether there is a non-cell autonomous sterol-dependent signal required for the correct patterning and polarity of epidermal cells.

4.2 DR5-mediated RNAi of *HYD* gene expression

To test whether the repression of *HYD1* and *HYD2* gene expression in the development of root meristem has any effect on root growth and development, RNAi versions of the *HYD1* and *HYD2* genes, under the transcriptional control of the DR5 promoter, were transferred into Arabidopsis Ws ecotype by *Agrobacterium*-mediated transformation. The objective was to silence expression of each gene respectively in restricted cells of the root, and then to determine the effect on cells developing at a distance from the site of expression. The DR5 promoter is known to have highly specific expression confined to the root meristem and columella, where the maximum auxin response is found. The DR5 promoter consists of 7 tandem repeats of an auxin-responsive element (Ulmasov *et al.* 1997), and is activated rapidly by auxins within the 10^{-8} - 10^{-4} M range. Expression of the reporter shows a "maximum" in the root meristem region, in the columella initials of wild-type seedlings (Sabatini *et al.* 1999; Fig. 4.1).

Figure 4.1: DR5::GUS expression in the wild type root at 3 days after germination showing activity restricted to the root meristem and columella. Magnification ×100. Picture taken from Souter *et al.* 2002

pC::DR5::HYD1RNAi and pC::DR5::HYD2RNAi constructions were made and transformed into Ws wild type Arabidopsis (Section 2.3 and 2.4 in M&M chapter). Four homozygous lines of each transformed line derived from from T2 seeds were selected to study. DNA was extracted from T2 homozygous lines and RT-PCR was carried out using DR5 and intron primers to confirm the presence of the transgene (Fig. 4.2). The presence of the expected DR5 sequence amplification product (244bp) can be seen in the transformants, but not in the Ws wild type control.

4.2.1 Development of the root meristem in the transgenic lines

To investigate the development of the root meristem in the transgenic plants, the Lugol staining method was used, which reveals the organization and specification of the columella, derived from the meristematic columella initials.

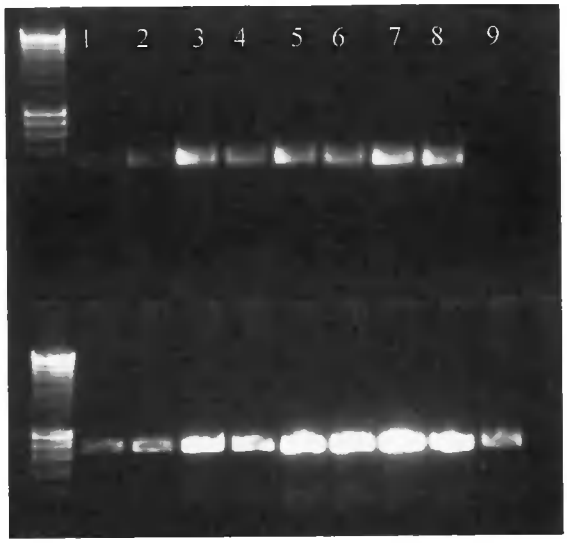


Figure 4.2: Genotyping of T2 DR5 RNAi lines. The top panel shows RT-PCR products generated with DR5 primers and the lower panel shows RT-PCR products generated with wild-type intron primers as a loading control, to show sufficient template is present in the wild-type (Ws) sample. Lane1: DR5::HYD1 RNAi 2.1, Lane2: DR5::HYD1 RNAi 3.2.7, Lane3: DR5::HYD1 RNAi 4.1.1, Lane4: DR5::HYD1 RNAi 4.2.3, Lane5: DR5::HYD2 RNAi 2.2, Lane6: DR5::HYD2 RNAi 4.3, Lane7: DR5::HYD2 RNAi 11.1, Lane8: DR5::HYD2 RNAi 13.3, Lane9: Ws. Size markers are shown on the far left of each gel.

Seedlings were germinated and grown on vertical agar plates for up to 15 days, and then stained with Lugol as described in Section 2.5. In *hyd* mutants, microscopic examination showed a loss of correct columella patterning and specification, with a loss of Lugol staining of the columella, indicating a loss of that tissue (Fig. 4.3 B, C, D). Lugol staining of the four independent transgenic lines suggested there was no adverse effect of RNAi of the *HYD2* genes on columella specification or organization (Fig. 4.3 G). However, there was some evidence of disruption to the columella patterning following *HYD1* RNAi expression in seedlings at 15 days post germination, with unclear or poorly defined tiers of columella cells (Fig. 4.3 E, F). However the phenotype was much less severe than in the *hyd2* mutants (Fig. 4.3 D).

4.2.2 Root length determination

One of the severe phenotypic defects found in *hyd1* and *hyd2* mutants is the very reduced growth of the root. The *hyd2* root grows to only about 0.21 cm in 7 days and

ceases growth at about 10-14 days (Souter *et al.* 2002, 2004) while *hyd1* root continues to grow slowly until the seedling dies at about 30-40 days after germination; although the root meristem it ceases cell division within 2 weeks. The *hyd1* root length typically reaches about 0.31 cm in 14 days (Ellie Short unpublished). This corresponds to the loss of correct cell patterning of the root meristem (Souter *et al.* 2002). To investigate whether the suppression of *HYD* gene expression in the root meristem has any effect on root growth, primary root length was measured in transgenic lines, compared to wild-type. Seedlings were germinated on vertical agar plates and root lengths were measured over 6 days.

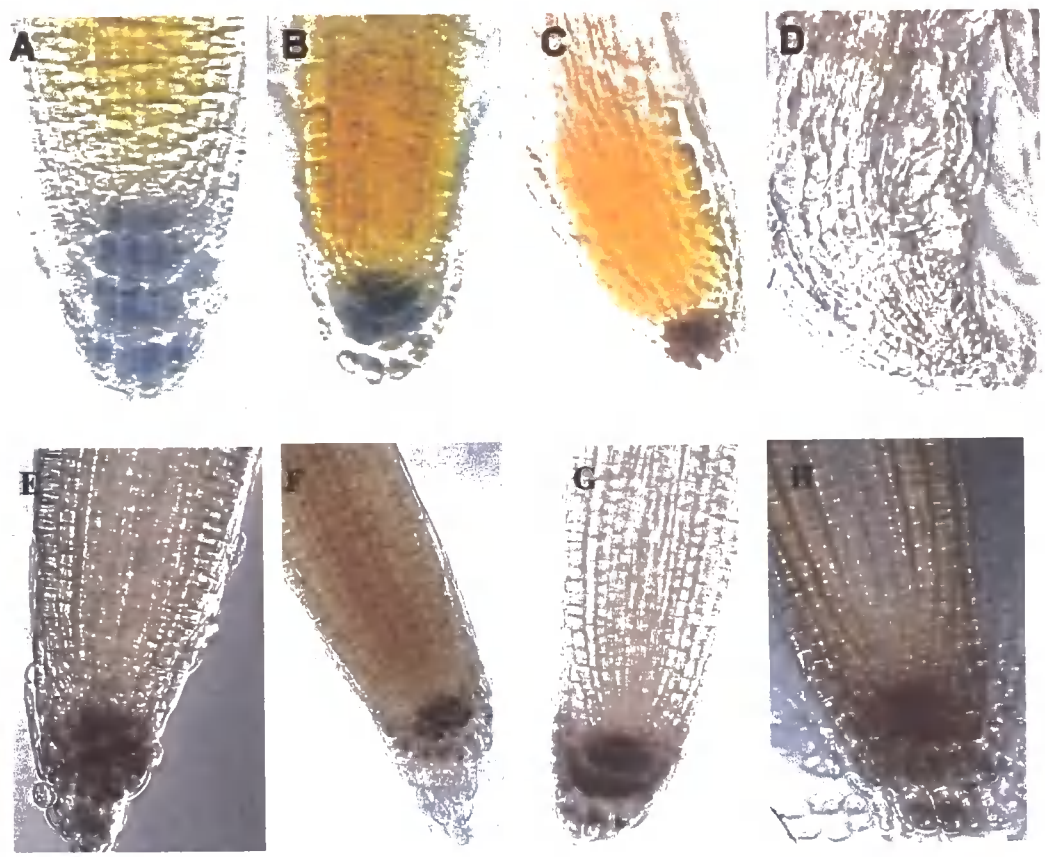
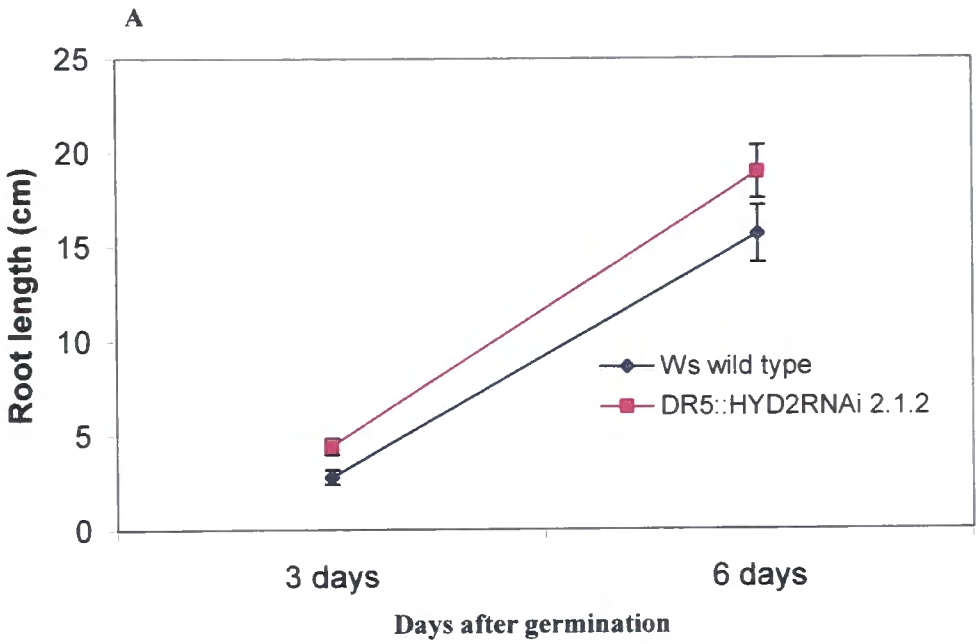
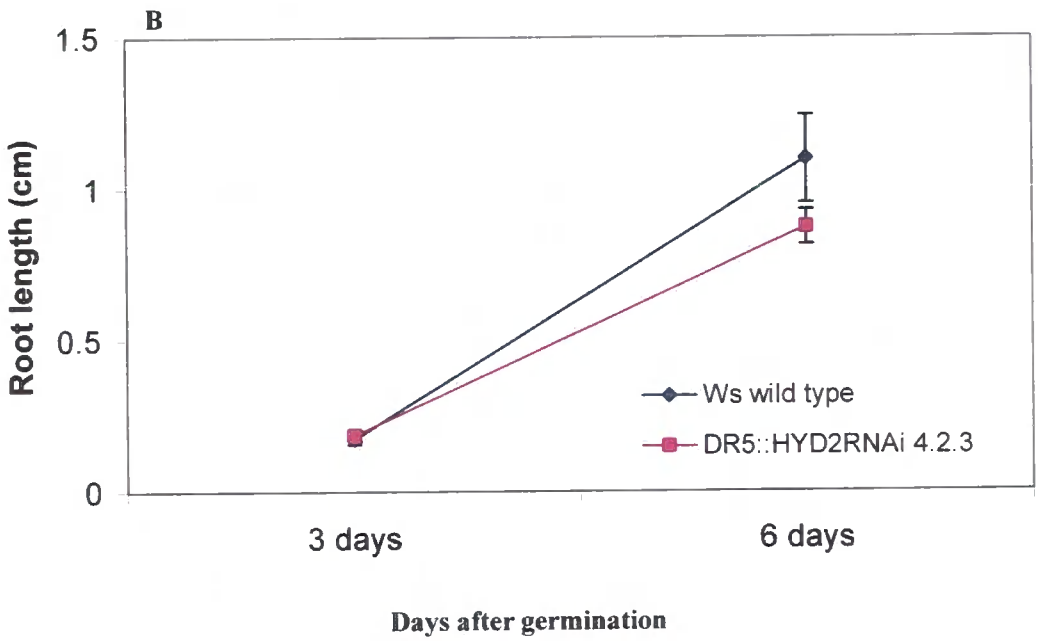


Figure 4.3: Lugol-stained roots of wild-type ([A]; 14 days after germination, [H]; 15 days after germination), *hyd1* ([B]; 14 days after germination), *hyd2* ([C]; 10 days after germination), and *hyd2* ([D]; 14 days after germination), DR5::HYD1 RNAi homozygous line ([E], [F]; 15 days after germination), DR5::HYD2 RNAi homozygous line [G]; 15 days after germination), to reveal the columella organization. Magnification x200 (Picture A-D taken from Souter *et al.* 2002).

The results, shown in Fig. 4.4, reveal no consistent significant root growth differences between Ws wild type and either the *HYD1* or *HYD2* RNAi transgenic lines seedlings. However, individual lines did show evidence of reduced growth at 6 days post-germination (eg DR5::HYD2 RNAi line 4.2.3; DR5::HYD1RNAi 13.3).





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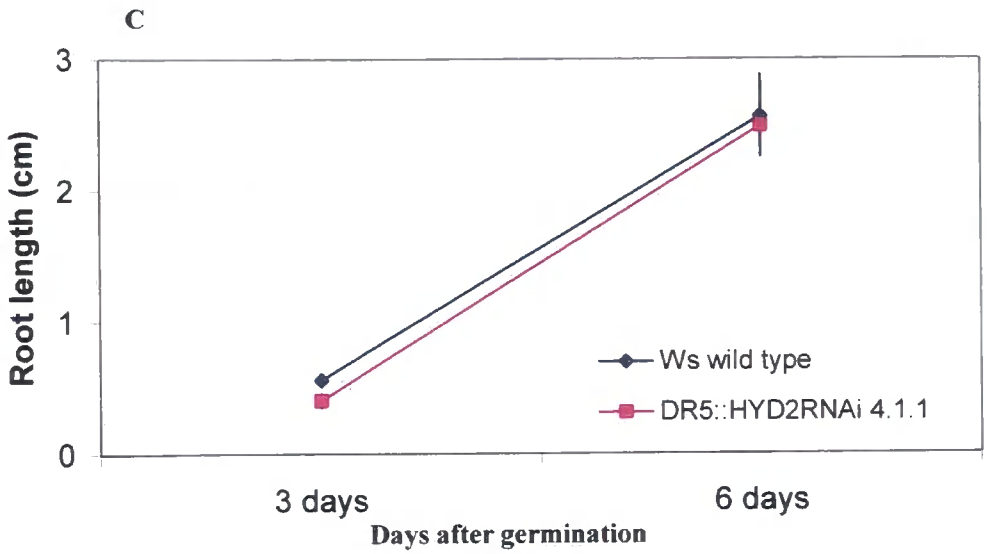

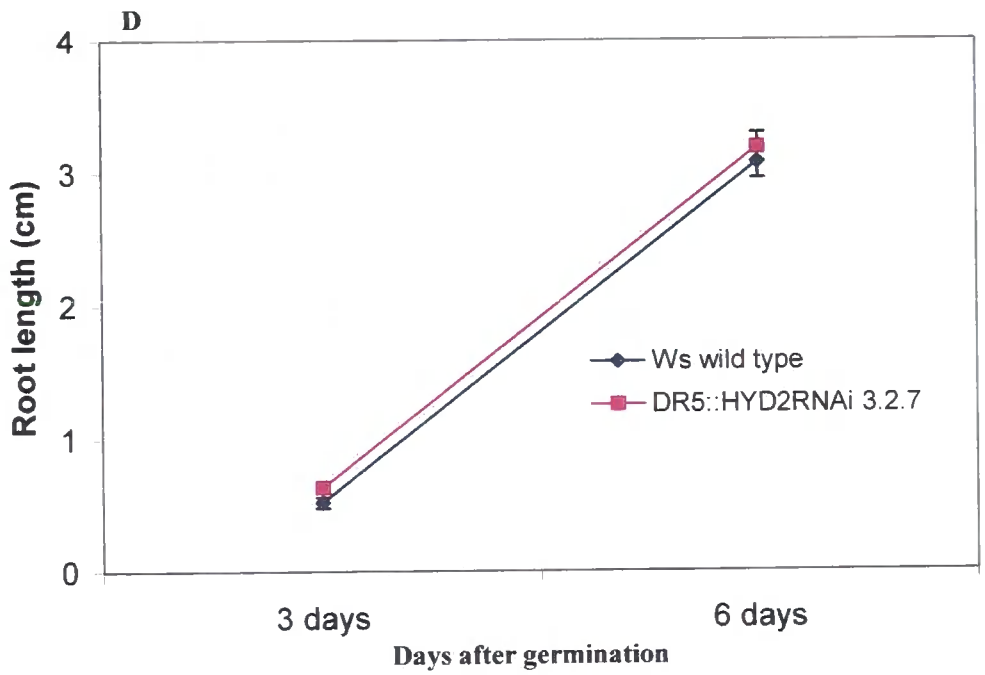

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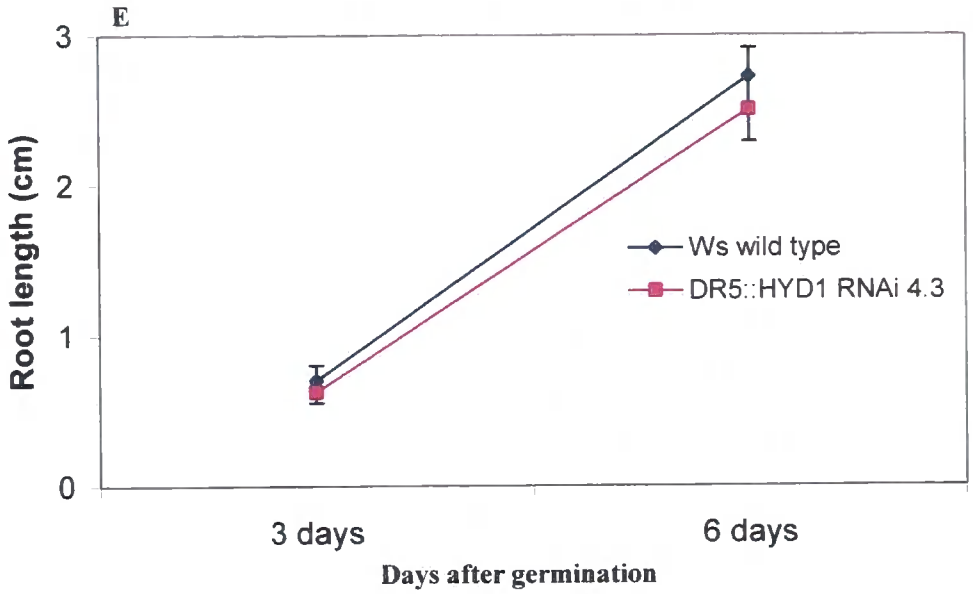

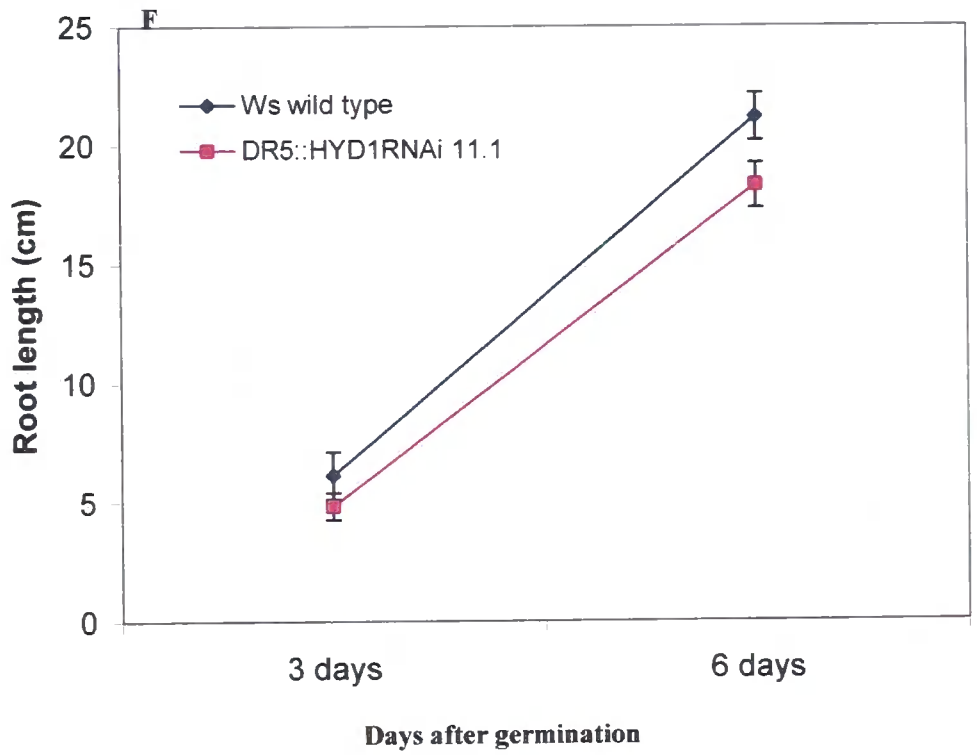

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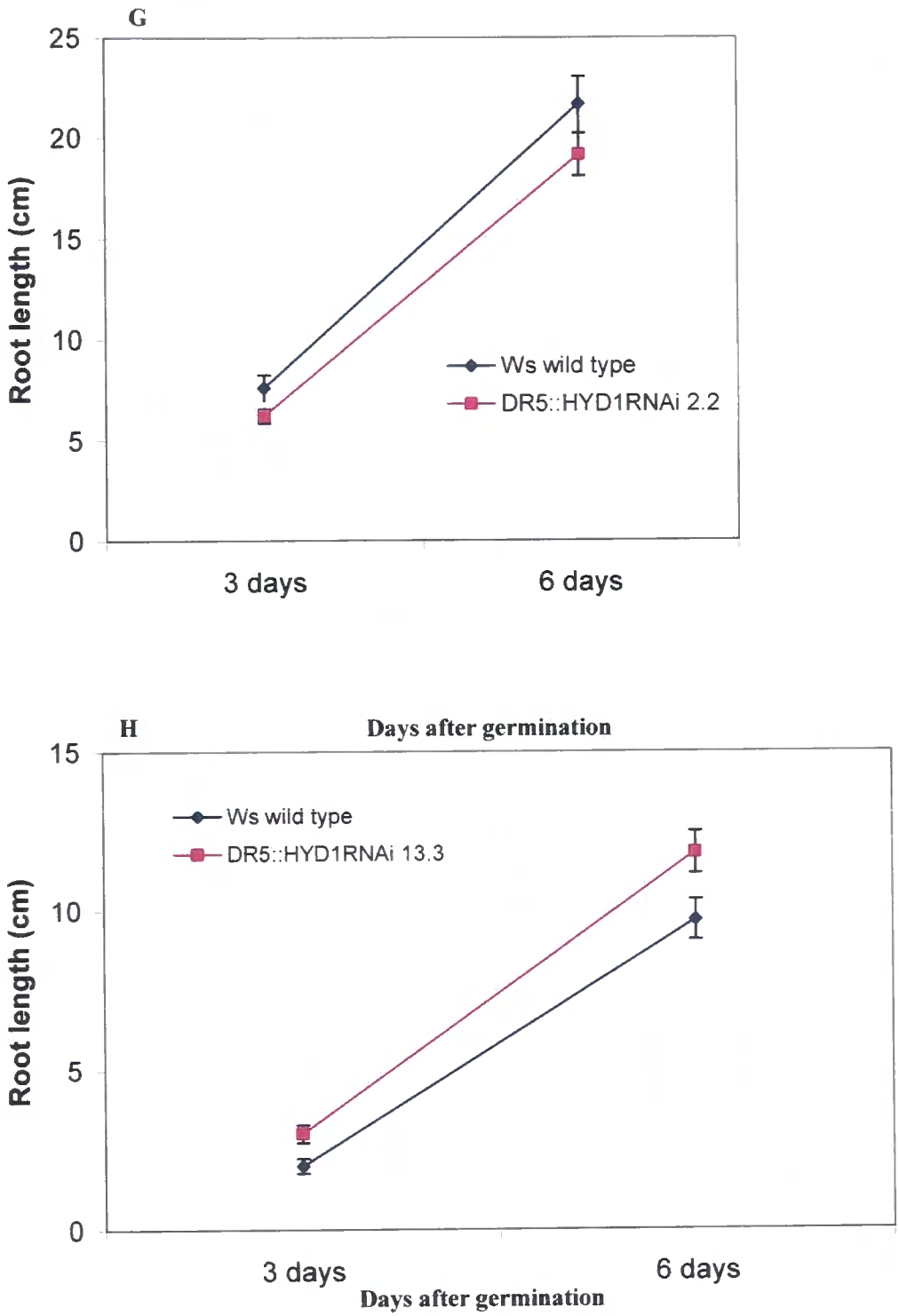

Figure 4.4: Comparison of the root length change between transgenics and the Ws wild type. A-D: four transgenic lines of DR5::HYD2RNAi, E-H: four transgenic lines of DR5::HYD1RNAi.

4.2.3 Branching root hairs found in all trangenics and Ws-wt control as well

To investigate whether the expression of RNAi-HYD genes in the root meristem has any effect on the development of the root epidermis, the root hair phenotype was examined. In *hyd* mutants, a defect in the mechanisms controlling *GL2* gene expression has been suggested (Souter *et al.* 2002). The alternating expression of the cell file marker *GL2*::green fluorescent protein (*GFP*), expressed in atrichoblast (non-hair-forming) files in the wild type (Masucci *et al.* 1996) (Fig. 4.5A), was seen in all root epidermal cell files in a *hyd2 fk* background (Fig. 4.5B). This suggests that the *GL2*-regulatory system is defective. All cell files produced root hairs in the mutants, and up to five root hairs can form from individual trichoblasts (Fig. 4.5C). Given that *GL2* is a negative regulator of hair formation (Masucci *et al.* 1996), this result also suggests the *GL2* protein is non-functional in *hyd* mutants.

In the homozygous RNAi-HYD1 or -HYD2 lines, seedlings with branching root hairs were found, but were surprisingly also found in Ws wild type. In some batches of plates, seedlings with branching root hairs could reach 100% in some of transgenic lines and in Ws wild type as well. Branched root hairs were variably found in the upper part of the root or spread widely in the whole root in both RNAi populations and in Ws wild type (Fig. 4.5). Some seedlings, including trangenics and the wild type, had up to 10 branched root hairs in one primary root although it was more common to find 2 or 3 branching hairs in one root. No branching root hairs were found near root tip, which is commonly observed in the *hyd* null mutant roots (Topping *et al.* 1997; Souter *et al.* 2002). In some seedlings (both transgenic and wild-type), branching root hair were often also short (Fig. 4.6). Since wild-type as well as trangenic RNAi lines showed defective hair development, this suggests that culture conditions rather than RNAi effects were the cause of hair growth defects.

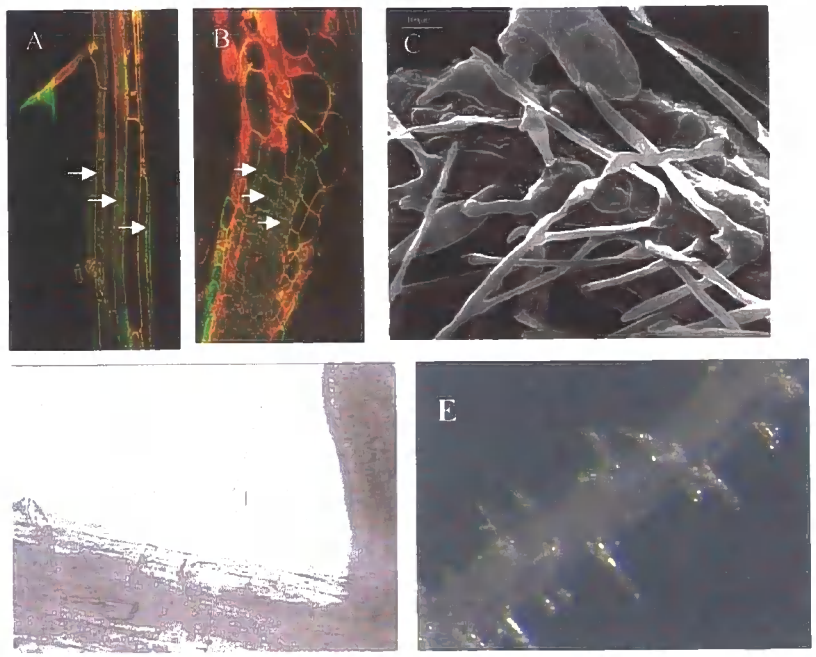

Figure 4.5: Confocal images of wild-type (A) and *hyd2* (B) roots expressing GL2::GFP fusion in epidermal cell files (arrows). Note in *hyd2* the expression in all cell files and the abnormal cell shape. Magnification x200. (C) Scanning electron micrograph of a *hyd2* root showing ectopic root hairs (arrows) and abnormal root hair shape. Bar: 100 μm. (D) DR5::HYD2RNAi Homozygous lines with branching root hairs, (E) Ws wild type with branching root hairs.

Figure 4.6: Ws Wild-type seedling with branching root hairs (arrows).

4.3 Summary

Four homozygous lines each containing DR5::HYD1RNAi and DR5::HYD2RNAi transgenes were generated and analysed for root patterning, growth and root hair morphology. There was some evidence that individual lines exhibited defective columella pattern (DR5::HYD1RNAi) and a statistically significant reduction in root growth compared to wild-type (one line each of DR5::HYD1RNAi and DR5::HYD2RNAi). Seedlings with branching root hairs can be found in the transgenic populations. However this phenotype was also found on Ws wild type, and it is concluded that this is not a significant effect of RNAi.

CHAPTER 5: Activation of *HYDRA* gene expression in specific cell types of the *hydra* mutant root

5.1 Introduction

In the previous chapter, it was found that attempts to silence the *HYD1* and *HYD2* genes in the root meristem by RNAi-mediated gene silencing had some effect on root meristem function or patterning in some transgenic lines. A second approach, and the aim of the work described in this chapter, is to investigate the role of sterol gene expression in specific root cell types by activating sterol gene expression in specific cell types of sterol null mutants, and then determine the effects on root structure and function. Therefore the objectives of the work were to activate the expression of the *HYD* genes in the root tips of Arabidopsis *hyd* mutants under the transcriptional control of the DR5 gene promoter, which is active in the root meristem, as described previously (Sections 4.2).

5.2 Confirmation of transformation

To confirm DR5::HYD transgenic lines contained the transgene prior to further analysis, DNA was isolated and PCR was used to genotype the plants. 3 homozygous lines from each of DR5:HYD1 and DR5:HYD2 were obtained, and representative data for the genotyping of one line (DR5::HYD1 homozygous line 0.5.1) is shown in Fig. 5.1. Because of time limitations, only DR5::HYD1 transgenics were studied, and the results are presented here.

5.3 DR5::HYD1 expression and root phenotype

Seedlings transgenic for the DR5::HYD1 gene fusion were germinated on vertical agar plates and grown for 14 days under standard growth conditions (Section 2.2). Fig.

5.2 shows the activity of the DR5 promoter, as a GUS reporter fusion (DR5::GUS) in the *hyd* mutant background, revealing expression in the remaining meristematic cells exhibiting auxin-mediated gene expression.

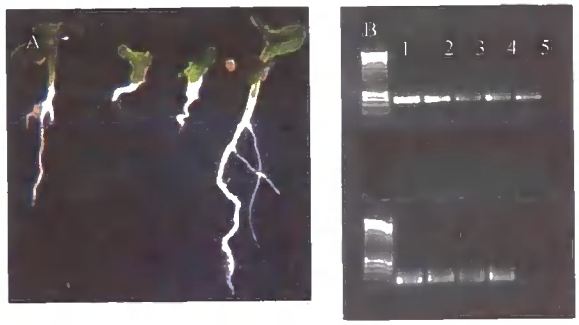

Figure 5.1: DR5::HYD1sense 0.5.1 x hyd1 A: 7dag, used for DNA extraction, B: PCR on DNA extracted from seedlings showed on picture A and Ws is in lane 5. Top row using intron primers as a control, bottom row DR5 primers to show presence of construct.

To determine the effects of DR5::HYD1 expression on the *hyd1* root organization, seedling roots from line 0.5.1, 0.1.3 and 0.1.5 were analyzed by Lugol staining, to reveal starch-accumulating columella cells, and were compared with wild-type and *hyd1* mutant root tips. Lugol staining after 7 days post germination of the DR5::HYD1 transgenic seedling roots shows a patterning of the root tip that is similar to wild-type (Fig. 5.3).

To determine the effects of DR5::HYD1 expression on the *hyd1* root growth, primary root length of the transgenics was compared to that of wild-type and *hyd1* mutants. It was found that the expression of sense gene in *hyd1* mutant in line 0.5.1 has a significant effect on the root growth (Fig. 5.3).

Compared to the *hyd1* mutant, the DR5::HYD1×hyd1 line 0.5.1 showed better root growth by 7 days post germination. After 14 days of germination, the root length of the transgenic line was 10 times greater than the of the *hyd1* mutant, showing that *HYD1* gene expression in the meristematic region of the root is sufficient to allow approximately wild-type root organization and growth.

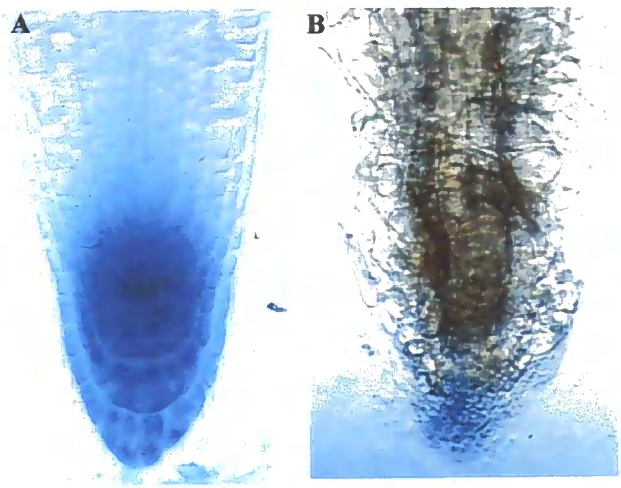

Figure 5.2: DR5::GUS expression in (A) wild-type and (B) *hyd* roots at 15 days post-germination.

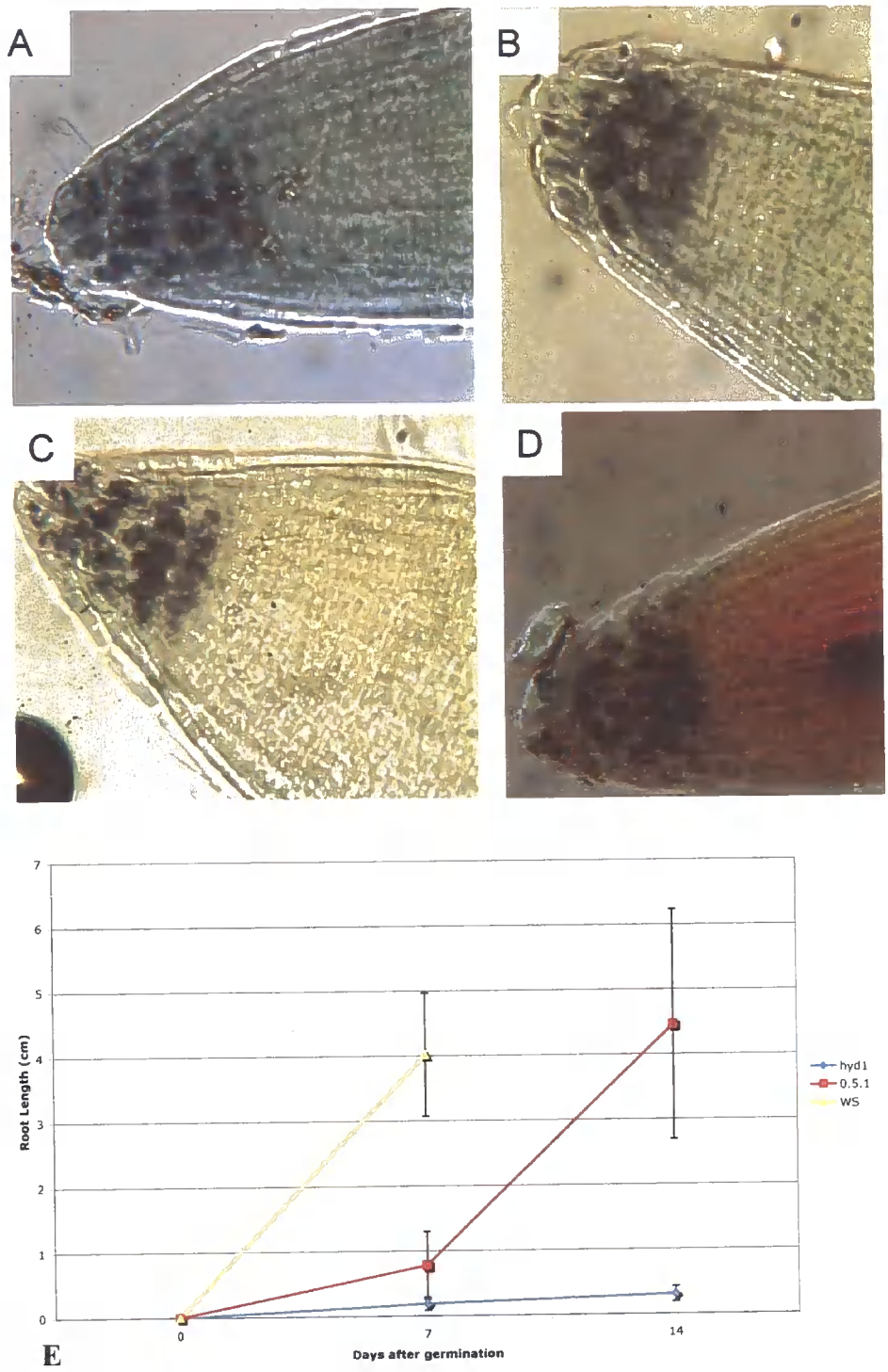

Figure 5.3: DR5::HYD1 expression in the *hyd1* mutant background leads to partial rescue of root columella patterning and growth. Lugol stained root tip of (A): wild-type; (B, C): DR5::HYD1 line 0.1.3; (D): DR5::HYD1 line 0.1.5. (E), root growth of DR5::HYD1 line 0.5.1. All seedlings are at 7 days post-germination.

5.4 Summary

Three homozygous lines each containing respectively the DR5::HYD1 and DR5::HYD2 sense expression gene constructs were obtained. Only the DR5::HYD1 transgenics were analysed. Analysis of the root growth showed that activation of *HYD1* expression led to partial rescue of root columella patterning and primary root growth. Further analyses are being carried out in the laboratory on effects of the expression of *HYD1* and *HYD2* genes in the respective *hyd* mutant background.

CHAPTER 6: Discussion

The aim of the work described in this thesis is to understand better the role of sterols in plant development. Previous studies indicate that genes encoding sterol biosynthetic enzymes are not expressed in all cell types (Schrick *et al.* 2000, Souter *et al.* 2002, 2004), and so the question is raised as to how cell function is regulated in cells that do not express these genes. Mutants defective in these show abnormalities in cells not expressing the genes, suggesting that they function in non-cell autonomous ways. To address this, the approach was to a) inhibit the expression of the *HYD1* and *HYD2* sterol genes, both essential for normal development, in specific cell types in the root, using RNAi, and determine the developmental consequences; and b) to activate *HYD1* expression in specific cell types of the root in the null *hyd1* mutant background, and determine whether root development is rescued. These experiments were aimed at determining whether there is a sterol-dependent signal that is able to act at a distance from the site of expression of the gene.

6.1 RNAi to inhibit *HYD1* expression

The *HYD1* gene is expressed in the root tip, though not in all cell types of the root. Recent work in this laboratory has shown that the *HYD1* promoter is not active in the root meristem, but is active in the columella, lateral root cap and epidermal cells of the elongation and early differentiation zones of the Arabidopsis root (Fig. 6.1). *FK/HYD2* is reported to be expressed in the root meristem (Schrick *et al.* 2000), but detailed expression studies describing the specific cell types in which the gene is expressed have not been carried out to date.

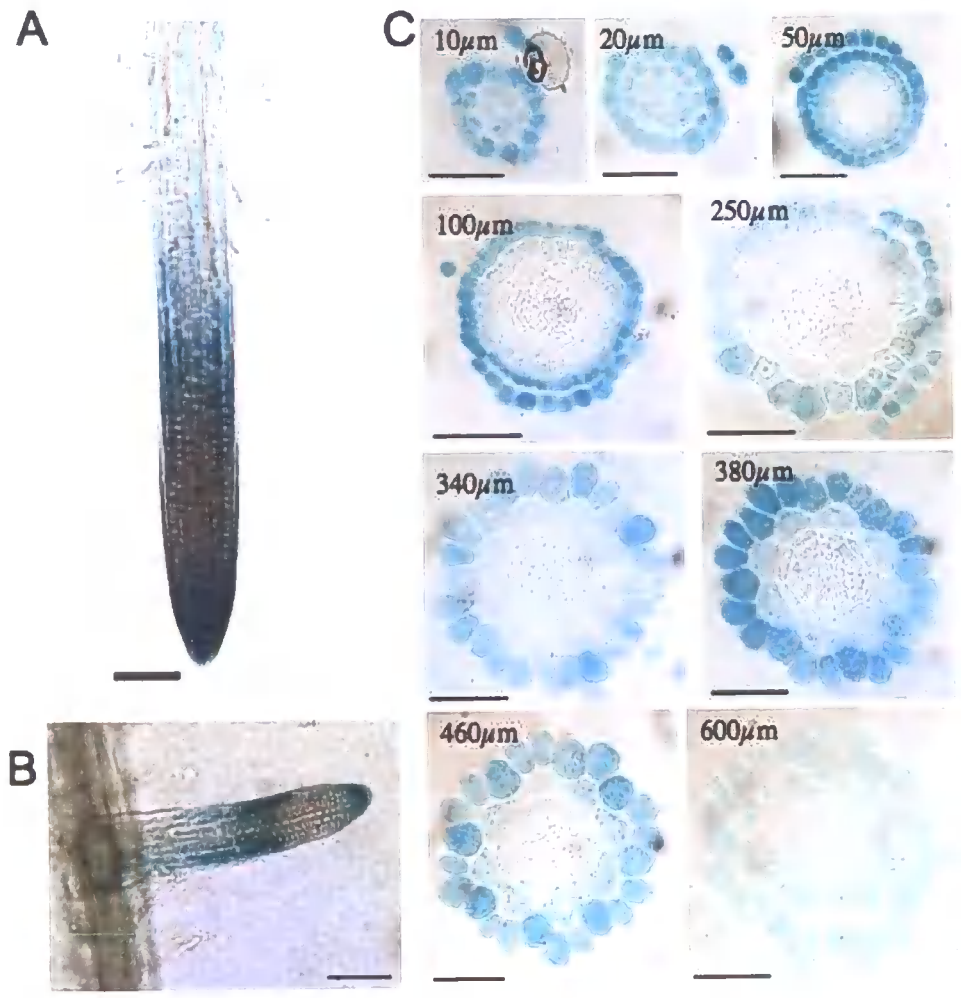

Figure 6.1: proHYD1::GUS expression, A: primary root, B: emerging lateral root, C: transverse sections of primary root-distance from tip indicated (um). Taken from M Pullen and K Lindsey, unpublished.

The *hyd1* and *fk*/*hyd2* mutants show cellular disorganization at the root tip, associated a reduced frequency of cyclinB-GUS-posistive cells between days 10 and 14 after germination, indicating a loss of mitotic activity of the mutant root meristems during development (Souter *et al.* 2002, 2004). DR5 expression is also abnormal, indicating a defect in auxin localization or response in the mutant roots, which is likely to account for a failure of meristem function and cell specification and patterning in the root tip. In some homozygous RNAi lines for the down-regulation of the *HYD1* and *HYD2* genes, development of the root meristem was apparently normal, as determined by Lugol staining. The cells are of normal shape and growth of the root was as in wild-type. However, in specific DR5::HYD1RNAi lines, there was evidence of defective columella patterning, seen as a 'messy' patterning of the tiers, similar to the *hyd1* mutant at 14 days post-germination. Furthermore, one line each of the

DR5::HYD1RNAi and DR5::HYD2RNAi transgenics showed a small but significant reduction in growth rate of the primary root. Together these data suggest that HYDRNAi gene expression in DR5-positive cells can cause structural and functional defects in the root tip.

There are a number of reasons why RNAi of the *HYD1* and *HYD2* genes gave only relatively weak *hydra*-like phenotype, and these are now discussed.

6.1.1 RNAi expression

The RNAi transgenics were demonstrated to contain the transgene, by PCR (Fig. 4.2). However, due to time constraints, it was not possible to determine whether the level of *HYD1* or *HYD2* expression was reduced significantly below wild-type levels in the transgenic lines; or if it was, the extent of repression of the genes. This could be carried out by quantitative or semi-quantitative PCR to determine the relative abundance of the *HYD1* and *HYD2* transcripts. Northern blot analysis could also be used to detect the presence of small RNAs, expected to be produced by cleavage of double-stranded *HYD* gene RNAs.

6.1.2 *HYDRA* genes may not be normally expressed in DR5-positive cells

The DR5 promoter is active in cells showing relatively high auxin responses, considered to be due to sites of enhanced auxin accumulation rather than enhanced sensitivity (Sabatini *et al.* 1999). Typically DR5 activity is found in the quiescent centre, columella initials and columella of the root, with some expression also seen in the lateral root cap (Fig. 4.1). As seen in Fig. 6.1, the *HYD1* promoter is not strongly expressed in these cells. It is also not clear whether the *HYD2* promoter is expressed in these cells either. Therefore, it is possible that, if these gene promoters, and so the genes themselves, are not normally or strongly active in DR5-positive cells of the root, then inhibition of their expression in these cells would not lead to a dramatic defective phenotype. More information on the cell type-specific expression of the *HYD2* gene is currently in progress in this laboratory.

6.1.3 Instability of RNAi expression

The phenotype suppression by hpRNAi may be inherited unstably (Mitsuhara I *et al.* 2002). It was found that the effectiveness of the RNAi was lost after a few generations. The phenotypes the RNAi transgenics were examined in the homozygous lines, ie T2 plants, and it is possible that by the time the homozygous plants were analysed, the phenotype may have been already lost or reduced. It is also possible that more independent transgenic lines need to be examined in order to detect an RNAi effect. Only a relatively small number of lines were examined due to time constraints, and it is likely that, due to position effects, the level of RNAi expression would vary between lines. Screening more lines might be expected to lead to the identification of lines showing stronger levels of RNAi expression, and so gene inhibition and *hydra*-like phenotype.

6.2 Sense expression of the *HYD1* gene

A second approach used to investigate the cell type-specific requirement for sterols in plant development was to activate *HYD1* gene expression in specific cells of the root tip in null (loss-of-function) *hyd1* mutants. By expressing the wild-type cDNA of *HYD1* under the control of the DR5 promoter in the mutant, the expectation was that the sterol biosynthetic pathway downstream of the HYD1 sterol isomerase would be activated in those cells (ie in the most distal cells of the *hyd1* root tip; Fig. 5.1). It was hypothesized that, if sterols function in a cell autonomous way, then expression of the *HYD1* gene in these cells would not lead to significant rescue of plant development. On the other hand, if root growth and development were rescued in the transgenic mutants, this would suggest a non-cell autonomous function for sterols.

It was found that the DR5::HYD1 x *hyd1* transgenic mutants showed a significant rescue of root growth and development. This was seen as an improved patterning of cells in the root tip, revealed by Lugol staining of the columella cells; and improved growth rates. Root hair development is also more similar to wild-type. More detailed analysis can be carried out in the future, for example to study the expression of genes normally expressed in the root tip, such as the *PLETHORA* genes (Aida *et al.* 2004, Galinha *et al.* 2007) and *SCARECROW* and *SHORTROOT* (Di Laurenzio *et al.* 1996;

Helariutta *et al.* 2000) that regulate meristem cell specification in response to auxin. One or more of these would be expected to be defective in *hyd* mutants, which fail to maintain meristem function.

6.3 Sterols may function in a non-cell autonomous way

The data generated are therefore consistent with the hypothesis that a mobile sterol-dependent signal is transported from the root tip (specifically, DR5-positive cells) to exert a long-distance effect on the rest of the root, including the root epidermis. The effect is to allow significant phenotypic rescue of the *hyd1* mutant root, such as increased root length, improved cell pattern and epidermal cell morphogenesis.

A key question is, what is the mobile signal? It has been suggested that steroids (including sterols) would not be likely mobile signals for thermodynamic reasons – they are highly hydrophobic and very poorly soluble in aqueous solution, and so would not be able to diffuse more than a very short distance in the plant (Christiansen *et al.* 2003). Brassinosteroids are known to signal to cells at a distance from the site of synthesis (Reinhardt *et al.* 2007), but it is not likely that the brassinosteroid molecules move beyond the immediate intercellular space, if secreted. Salvadi-Goldstein *et al.* (2007) have shown that brassinosteroid signalling in the shoot epidermis is able to regulate shoot development, by allowing internal layers to undergo cell expansion correctly. Similarly, Reinhardt *et al.* 2007 have shown that activation of brassinolide synthesis at the leaf margin in Arabidopsis rescues the development of correct leaf shape in the *dwf4* mutant, which has abnormal leaf morphogenesis.

Currently we can only speculate about the nature of mobile signals that are dependent on sterol biosynthesis. A possible clue comes from the observation that the *hyd* mutants are defective in auxin responses (Souter *et al.* 2002, 2004). This is revealed by aberrant DR5::GUS localization (Fig. 5.1) and sensitivity to auxin, revealed as ectopic DR5::GUS expression in response to 2,4-D. It is also known that the localization of PIN proteins, which are involved in the auxin efflux process, is defective in sterol mutants such as *hyd* (Souter et al. 2002) and *orc/smt2* (Willemsen

et al. 2003). Other defects in sterol mutants such as abnormal vascular tissue development, which is dependent on correct auxin distribution and signalling, are defective in several sterol biosynthesis mutants (Carland *et al.* 2002; Souter *et al.* 2002). *PIN* proteins require vesicle trafficking for correct localization to the plasmamembrane; and this is a sterol-dependent process (Men *et al.* 2008).

Therefore we can suggest a model in which sterols, synthesized in the root tip under the control of the DR5::HYD1 gene fusion, provide a means for correct *PIN* localization, auxin transport and then root development, such as via the activation by auxin of the *PLETHORA* genes necessary for meristem function. In this model, auxin is the mobile signal that allows long-distance signalling between tissues in the root to regulate growth. Further work is required to test this model.

6.4 Future work

New experiments are suggested by the work carried out for this thesis. It would be useful to generate and characterize more RNAi lines, to determine whether silencing of the *HYD1* or *HYD2* genes under the transcriptional control of a meristem-specific promoter can inhibit root development. It would also be important to characterize in better detail the cell type-specificity of the *HYD2* promoter, which currently is not well characterized. If these promoters are not in fact active in the meristem, this would explain the results observed in this study. It would also be interesting to both silence, and activate, the *HYD1* and *HYD2* genes in other root cell types, such as epidermis, cortex, endodermis and vascular tissue, to determine whether sterol-dependent signalling occurs from these cells to regulate root growth. This work is ongoing in the laboratory.

To investigate further the hypothesis than auxin is the mobile signal dependent on sterol biosynthesis, it would be useful to analyse *AUX1* and *PIN* protein localization in mutants rescued by *HYD1* or *HYD2* transgene expression. One possible explanation for the rescue observed is that these auxin transport proteins become correctly localized in the rescued plants, allowing improved auxin transport and so root growth and development. It would also be possible to monitor auxin distribution in the rescued plants, for example by measuring auxin levels directly using mass specrtromtery (eg Ljung *et al.* 2005), or by monitoring DR5::GUS expression.

Since the *HYD1* gene is activated in the mutants, it would be valuable to know whether correct sterol profiles are found in the rescued plants. It is known that the sterol profiles in the *hyd1* and *hyd2* mutants are severely abnormal (Souter *et al.* 2002), and the profiles in the rescued plants could be determined. The expectation is that they would be closer to wild-type.

In summary, the work described in this thesis provides new clues to the roles of sterols in plants, but opens up new ideas for experimentation.

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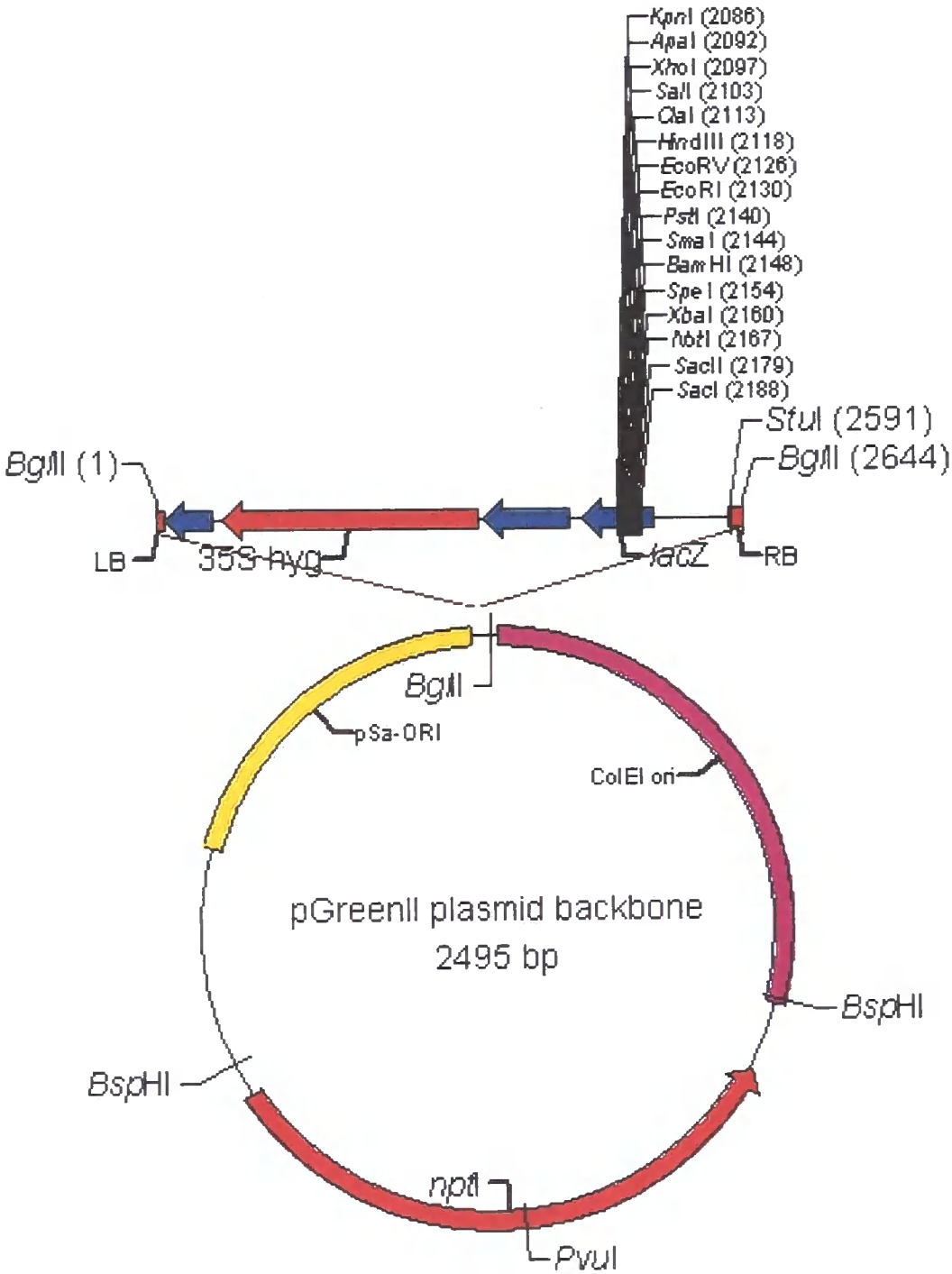
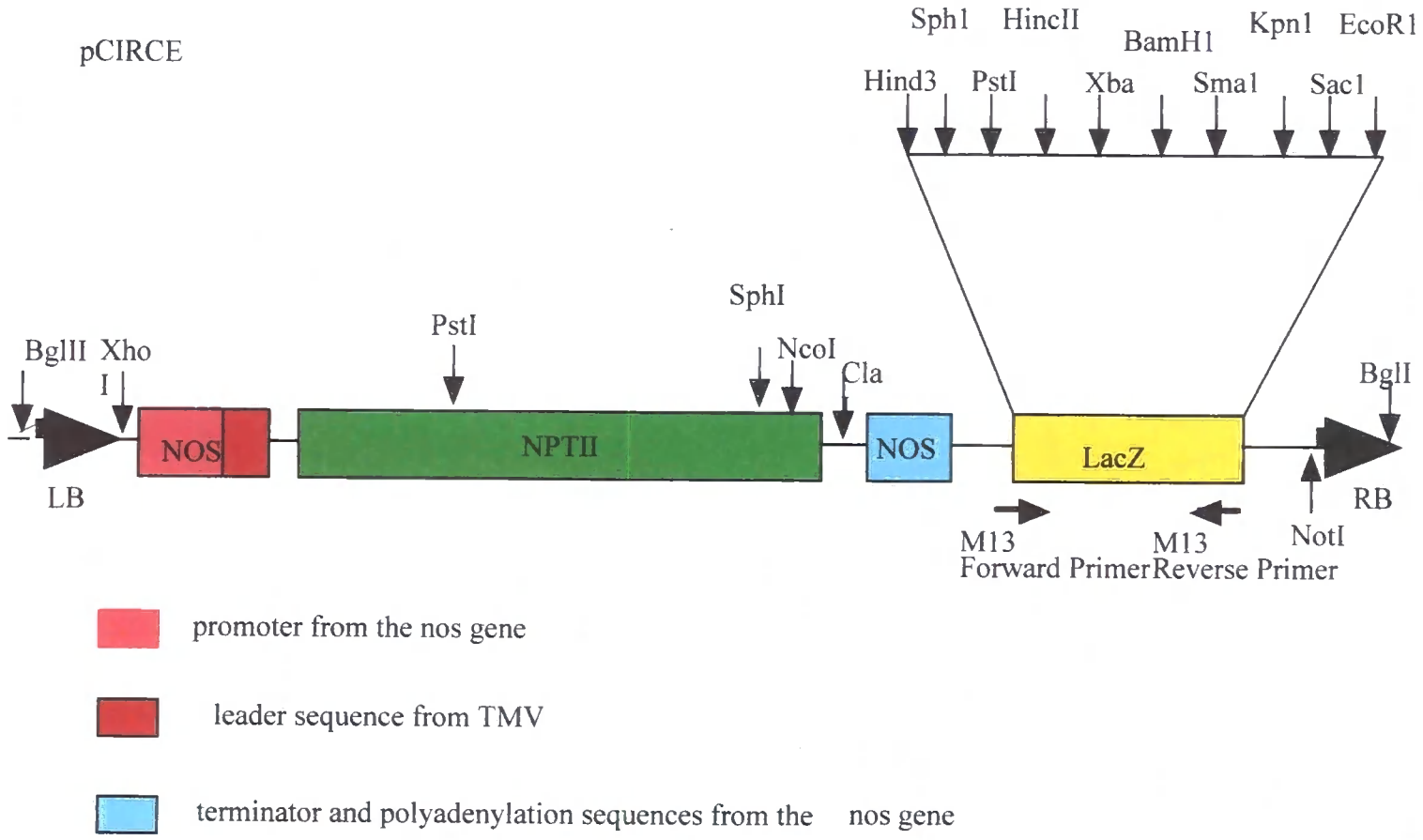
Appendix 1: The structure of pGreen vector

PGreen0179:



Appendix 2: The structure of pCIRCE vector



Appendix 3: Sequences of primers and cDNAs

Primer sequences used to clone differential lengths of the *HYDRA gene* from *Arabidopsis thaliana* ecotype Wassileskia, are shown below.

HYD1 Sense:
Forward: GTCGACAACATGGAGGAGTTG (Sal I)

Reverse: CGGAATTCTCAACGGGTTTTCTTC (Eco I)

HYD2 Sense:
Forward: GTCGACATGCTGCTAGATATGGAT (Sal I)

Reverse: CGGAATTCGGCACATCTAATAAAC (Eco I)

HYD1 RNAi:
Forward5': GGTCGACGAAAGCTAAACTTGATA (Sal I)

Forward3': ATGCGGCCGCAAAGCTAAACTTGATA (Not I)

Reverse5': CGGAATTCGTGGCAAAGTTGTCT (Eco I)

Reverse3': GACTAGGTGGCAAAGTTGTCTCC (Spe I)

HYD2 RNAi:
Forward5': GTCGACTGCTAGATATGGATCTC (Sal I)

Forward3': ATGCGGCCGCTGCTAGATATGGATC (Not I)

Reverse5': CGGAATTCCAATGCTCATAAACTGAG (Eco I)

Reverse3': GACTAGTCAATGCTCATAAACTGAGG (Spe I)

DR5 :
Forward: GGATCCCTTTTGTCTCCCTTTTG (Only)

GUS Reverse: GGGCCCGCTAAGCTTACCATT

Intron:
Forward: CGGAATTCCGCTCGTCAGGTAT (EcoR1)

Reverse: CGGGATCCGCCGCATTCTACAA (BamH1)

The coding sequence of HYD1:

ATGGAGGAGT TGGCGCATCC GTACGTTCCG AGAGATCTGA ACCTACCCGG
ATACGTACCA ATCTCAATGT CAATGTCCTC CATCGTCTCT ATCTACCTCG
GTTCTTCCCT CCTTGTTGTC TCCCTCGTCT GGCTTCTCTT CGGGAGGAAG
AAAGCTAAAC TTGATAAGTT GCTTATGTGT TGGTGGACAT TCACTGGTCT
CACTCATGTT ATTCTCGAGG GCTATTTCGT TTTCTCCCCT GAGTTTTTCA
AGGACAACAC TTCTGCTTAT CTTGCTGAAG TTTGGAAAGA ATATAGCAAA
GGTGATTCGA GATACGTAGG TAGAGATTCT GCAGTTGTAT CTGTTGAAGG
GATCACTGCT GTTATCGTTG GCCCAGCTTC TCTCCTAGCT ATTTATGCCA
TTGCTAAGGA GAAGTCGTAT AGCTACGTGC TTCAGCTTGC GATTTCGGTT
TGCCAGCTCT ACGGATGTTT GGTTTATTTC ATTACTGCTA TCTTGGAAGG
AGACAACTTT GCCACAAACT CTTTCTACTA TTACTCCTAC TACATCGGGG
CTAACTGTTG GTGGGTCTTG ATACCTTCAC TCATTTCTTT CCGTTGCTGG
AAAAACATTT GTGCAGCTGC TGCCATTGCC AACAACAACT
TCGAGACAAA GACGAAGAAG AAAACCCGTT GA

The coding sequence of HYD2:

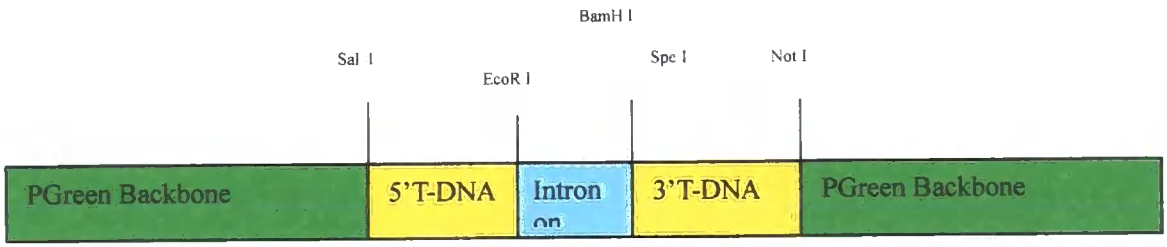
ATGCTGCTAG ATATGGATCT CGGTGTTCTT CTTCCATCAT TGCAATCTGT
TTATGTGCTG GTGTTTTACT TCGTTTACTT GGCCGTTGCC GGAGAAATTC
TCCCCGGGAA AGTTATTCGC GGCGTCCTTT TATCAGATGG CTCTCAACTT
CGTTACCGAT GCAATGGTCT ATTGGCACTA ATATTGTTGG TAGCTATTTT
GGGAATCTGT GCAAAACTTG GCATTGTATC ACCTCTTGTG GTTGCGGATA
GAGGACTTGA GTTACTCTCA GCTACTTTTA TTTTCTGTGT TTTGGTGACA
TTAGCATTGT ATGTTACTGG GCGAAGTTCC TCGAATAAGG GTTCTTCCCT
AAAGCCTCAT GTCTAAGGAA ATCTTGTACA TGACTGGTGG TTTGGAATAC
AGCTGAATCC TCAGTTTATG AGCATTGATC TCAAGTTTTT CTTTGTCAGC
GCCGGGATGA TGGGATGGCT GCTTATCAAT CTCTCTATTC TGGCAAAAAG
TGTGCAGGAT GGTTCCTTGA GTCAGTCGAT GATTCTTTAC CAGATCTTCT
GTGCGTTATA TATATTGGGAC TACCTTGTTC ATGAAGAATA CATGACCTCT
ACGTGGGACA TAATTGCAGA GAGACTAGGC TTCATGCTAG TGTTTGGAGA
TCTCCTGTGG ATTCCTTTCA CTTTTAGCAT TCAGGGCTGG TGGCTTTTGC
ACAACAAAGT AGAACTAACA GTTCCTGCGA TTGTAGTCAA
TTGCCTTGTC TTCTTGATAGG GTACATGGTT TTTCGAGGA GCTAACAAAC
AAAAACATAT CTTTAAGAAG AACCCAAAAA CACCAATATG
GGGCAAGCCT CAGTGGTAG TTGGTGGAAA CTTACTGGTT TCAGGCTATT
GGGGAATTGC AAGGCACTGT AATTACCTTG GCGACTTGAT GCTTGCTCTG
TCCTTCAGTT TGCCATGTGG AATAAGTCTC CCGGTTCCAT ATTTCTACCC
GAGATACCCT CTGATACTAT TGATATGGAG AGAACGAAGA GACGAGGTTC
GATGTGCAGA GAAGTACAAG GAGATATGGG CAGAGTATCT
TAGACTTGTC CCCTGGAGAA TACTTCCTTA TGTTTATTAG

The sequence of DR5:

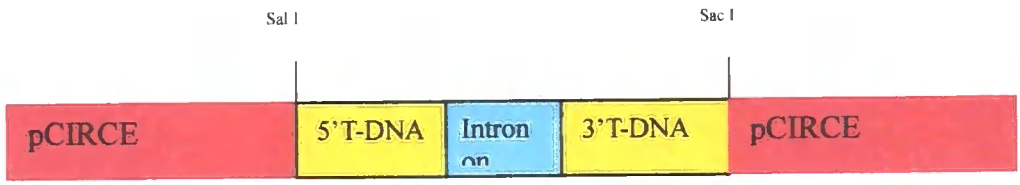
GCAGCCCGGG CAGGCCTTT GTCTCCCTTT TGTCTCCCTT TTGTCTCCCT
TTGTCTCCC TTTTGTCTCC CTTTTGTCTC CCTTTTGTCT CCCTGGGCAG
GCCTCGATAA GCTTGATATC GAATTAATTC CTGCAGCCCC GCAAGACCCT
TCCTCTATAT AAGGAAGTTC ATTTCATTTG GAGAGGTATT TTTTACAACAA
TTACCAACAA CAACAAACAA CAAACAACAT TACAATTACT ATTTACAATT
ACAATTACAT GGTAAGATTA GCGGGCCC

Appendix 4: The sequence of making RNAi constructs

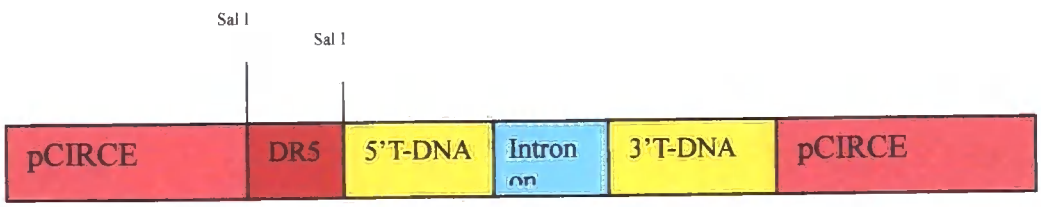
1. The T-DNA and Intron was put into PGreen0179 vector separately using the enzymes shown blow.



2. Intron together with 5', 3' T-DNA was cut out using the enzymes showed below.



3. DR5 promoter was put in using only Sal I enzyme



Appendix 5: Root Growth Data

DR5::HYD2RNAi homozygous line 4.2.3

Ws/cm	3 days	6 days
1	0.13	0.61
2	0.184	0.806
5	0.206	1.105
6	0.128	1.237
7	0.235	1.736
8	0.155	1.094
mean	0.173	1.098
standard deviation	0.039268	0.353515
standard error	0.016031	0.144322
DR5::HYD2 RNAi 4.2.3		
3	0.136	0.873
4	0.155	0.873
5	0.25	0.805
6	0.275	1.259
7	0.269	1.003
8	0.172	1.016
9	0.107	0.78
10	0.163	0.841
11	0.197	0.69
12	0.132	0.58
mean	0.1856	0.872
standard deviation	0.056893	0.178877
standard error	0.017991	0.056566

DR5::HYD2 RNAi homozygous line 2.1.2

Ws /cm	3 days	6 days
1	2.165	8.993
2	2.001	16.796
3	3.034	15.96
4	2.1	14.865
5	4.464	20.335
6	2.949	16.902
mean	2.7855	15.64183
standard Deviation	0.935544	3.73785
standard error	0.381934	1.525971
DR5::HYD2 RNAi 2.1.2		
1	1.639	3.662
2	6.314	21.88
4	5.386	25.046
6	5.818	23.901
8	5.203	22.297
9	5.993	23.356
10	2.225	16.999
11	1.554	18.063
13	4.127	18.368
14	4.812	20.088
15	5.223	17.831
16	4.847	15.882
mean	4.428417	18.94775
standard Deviation	1.689377	5.652742
standard error	0.422344	1.413185

DR5::HYD2 RNAi homozygous line 4.1.1

Ws/cm	3 days	6 days
1	0.74	2.78
2	0.573	1.018
3	0.473	2.686
4	0.541	2.993
5	0.52	3.073
6	0.495	2.84
mean	0.557	2.565
standard deviation	0.096198	0.770837
standard error	0.039273	0.314693
DR5::HYD2RNAi 4.1.1		
1	0.412	2.265
2	0.513	2.913
3	0.582	3.242
4	0.548	3.15
5	0.55	2.813
6	0.666	2.141
7	0.071	2.192
8	0.241	2.128
9	0.32	2.168
10	0.441	2.769
11	0.372	2.308
12	0.141	1.792
mean	0.40475	2.490083
standard deviation	0.1835	0.465001
standard error	0.052972	0.134234

DR5::HYD2 RNAi homozygous line 3.2.7

Ws/cm	3 days	6 days
1	0.633	3.329
2	0.537	3.238
3	0.511	2.491
4	0.531	2.671
5	0.667	3.16
6	0.553	3.091
7	0.314	3.151
8	0.41	3.5
mean	0.5195	3.078875
standard deviation	0.113699	0.335755
standard error	0.040199	0.118707
DR5::HYD2RNAi 3.2.7		
1	0.828	3.407
2	0.872	3.716
3	0.518	2.936
4	0.217	2.334
5	0.575	3.325
6	0.709	3.667
7	0.616	3.556
8	0.649	3.236
9	0.736	3.283
10	0.812	3.605
11	0.583	2.753
12	0.46	3.095
13	0.555	3.094
14	0.721	2.672
mean	0.632214	3.191357
standard deviation	0.170972	0.407735
standard error	0.045694	0.108972

DR5::HYD1 RNAi homozygous line 4.3

Ws /cm	3 days	6 days
1	0.516	2.549
2	1.097	3.386
3	0.792	2.521
4	1.004	3.237
5	0.827	3.012
6	0.298	2.07
7	0.348	1.901
8	0.722	3.106
mean	0.7005	2.72275
standard deviation	0.291416	0.548654
standard error	0.103031	0.193978
DR5::HYD1 RNAi 4.3		
1	0.499	2.377
2	0.229	1.332
3	0.708	2.309
4	0.239	1.39
5	0.543	2.272
6	0.884	4.225
7	0.474	2.259
8	0.83	3.056
9	0.943	3.066
10	0.492	2.536
11	0.513	1.954
12	0.988	2.993
13	0.776	2.734
mean	0.624462	2.500231
standard deviation	0.250521	0.761595
standard error	0.069482	0.211229

DR5::HYD1 RNAi homozygous line 11.1

Ws /cm	3 days	6 days
1	6.893	19.357
2	4.876	19.357
3	7.031	22.251
4	5.509	23.33
5	7.934	22.149
6	7.338	24.653
7	6.089	22.712
8	7.003	21.79
9	2.423	15.114
mean	6.121778	21.19033
standard deviation	1.67969	2.848934
standard error	0.559897	0.949645
DR5::HYD1 RNAi 11.1		
1	4.983	18.528
2	4.999	16.278
3	6.659	22.895
4	0.936	11.277
5	6.054	20.035
6	5.815	21.031
7	5.774	20.184
8	5.212	19.454
9	3.154	14.589
10	6.273	22.934
11	3.987	17.138
12	6.536	21.83
13	3.748	14.699
14	2.652	13.015
15	5.747	20.568
mean	4.835267	18.297
standard deviation	1.632988	3.635397
standard error	0.421636	0.938655

DR5::HYD1 RNAi homozygous line 2.2

Ws wild type	3 days	6 days
1	5.762	18.062
2	10.356	24.485
3	8.524	23.27
4	8.138	24.618
5	4.789	15.312
6	7.09	21.107
7	6.897	19.79
8	9.146	26.877
mean	7.58775	21.69013
standard deviation	1.819024	3.846663
standard error	0.643122	1.360001
DR5::HYD1 RNAi 2.2		
1	9.474	24.948
2	5.648	17.234
3	7.3	22.394
4	6.867	21.711
5	8.12	22.675
6	5.576	17.775
7	2.824	5.453
8	5.791	20.299
9	7.048	19.946
10	5.397	15.465
11	6.204	23.135
12	6.423	21.342
13	4.138	12.451
14	6.163	22.135
15	8.204	20.474
16	6.456	22.661
17	3.983	13.091
18	5.902	20.181
19	6.133	20.799
mean	6.192158	19.16679
standard deviation	1.546946	4.719154
standard error	0.354894	1.082648

DR5::HYD1 RNAi homozygous line 13.3

Ws wild type	3 days	6 days
1	2.01	10.01
2	1.42	8.15
3	3.01	12.06
4	1.84	9.16
5	1.72	9.15
mean	2	9.706
standard deviation	0.604276	1.471574
standard error	0.270241	0.658108
DR5::HYD1 RNAi 13.3		
1	3.2	13.26
2	2.91	11.38
3	2.46	12.89
4	2.49	12.91
5	3.65	12.6
6	2.3	9.31
7	2.83	8.9
8	4.22	13.38
mean	3.0075	11.82875
standard deviation	0.657827	1.791571
standard error	0.232577	0.633416

DR5::HYD1sense homozygous line 0.5.1

0.5.1 7day	0.5.1 14day	hyd1 14 day				
1.452	7.326	0.295				
2.115	6.748	0.154				
1.956	6.884	0.205				
1.134	7.287	0.385				
0.516	4.175	0.34				
1.771	6.398	0.426		0	7	14
1.163	5.545	0.068	**hyd1**	0	0.19	0.31
0.636	6.718	0.415	**0.5.1**	0	0.779	4.46
0.16	0.387	0.231	**WS**	0	4.02	
0.398	3.243	0.254				
0.329	2.786	0.486				
0.034	6.077	0.324				
0.48	4.47	0.264				
0.74	5.165	0.486		0	0.1	0.12
0.744	4.646			0	0.52	1.77
0.809	2.174			0	0.95	
0.585	4.161					
0.5	5.038					
0.953	5.11					
0.581	3.8					
1.183	4.181					
0.829	4.735					
0.847	4.245					

0.338	1.514		
0.517	4.804		
0.869	3.588		
0.104	1.876		
0.337	3.297		
0.691	3.06		
0.785206897	4.46337931		0.3095
0.522129867	1.769812024		0.123660049
0.095327435			
***hyd1* 7dag**	**DR5:HYD1sense*hyd1* 7dag**	**Ws 7dag**	
0.19	0.79	4.02	
0.1	0.52	0.95	
hyd1	**0.5.1**	**Ws**	
0.19	0.79	4.02	7dag
0.31	4.46		14 dag
0.1	0.52	0.95	
0.12	1.77		