The effect of hydra mutations on HD-START domain genes in Arabidopsis thaliana

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The Effect of *hydra* Mutations on HD-START Domain Genes in *Arabidopsis thaliana*

by

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Summary

The study of sterol defective mutants of *Arabidopsis thaliana* has provided an invaluable opportunity to investigate the importance of sterols in plant development. The *hydra* mutants in particular - seedling lethal mutations - exhibit great phenotypic variation, leading to the supposition that sterols have a far more complex role in plant development than previously anticipated.

It has been postulated that some sterol molecules are directly involved in the regulation of gene transcription during plant development. The binding of some sterols to START domains (sterol binding domains) found in some mammalian proteins has been noted, and it is possible that plant transcription factors containing START domains could be localised to the nucleus by the binding of specific sterols. This hypothesis may be tested by creating HD-START::GFP fusion proteins to act as a reporter, permitting the subcellular localisation of the produced protein to be viewed using confocal scanning laser microscopy.

In order to determine if the expression of START domain transcription factors is itself regulated by sterols, independent of possible roles for sterols in their localization to the nucleus, the comparative steady state transcription levels of five HD-START domain transcription factors were studied by real-time RT-PCR. A comparison of *hydra* mutant mRNA levels to their wild type counterparts was carried out to determine whether there is a requirement for sterols in the regulation of the tested genes. Although the results do appear to suggest this to be the case, the data and the conclusions require further validation.

Contents

List of Figures

List of Tables

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Part 1: Introduction

1. Introduction

1.1. Background Information

The regulation of genes and their activity is a very important component in the development of all living creatures. Genes regulate all functions in development and indeed throughout life; many genes also regulate the activity of other genes, perhaps the most classic example being those coding for transcription factors required for the expression of other genes.

1.1.1. Sterols and the Sterol Biosynthetic Pathway

1.1.1.1. Sterols

Sterols are essential molecules in all eukaryotes, playing a crucial role in many aspects of cellular activity. They play a key structural role, with their presence in membranes allowing for increased and regulated fluidity which is essential for cells to function, allowing the modulation of protein function and preventing membrane freezing at low temperatures. They also play an important role in both extracellular and intracellular signalling, often acting as secondary messengers, and are precursors of a number of other molecules. Ergosterol, typically found in fungal cell membranes, can go on to form Vitamin D2 with the aid of ultraviolet light (Rajakumar, 2007). In plants sterols take on an extra significance as the precursors of the hormones known collectively as brassinosteroids (BR) (Hartmann, 1998). The BRs are involved in any plant processes, including the promotion of vascular differentiation (Cano-Delgado, 2004), pollen tube formation (Hewitt *et al.*, 1985) and, alongside auxin, the promotion of cell elongation and expansion (Clouse and Sasse, 1998; Nemhauser *et al.*, 2004) Therefore it is difficult to overestimate the importance of sterols in plants, and much work has been carried out in order to elucidate the mechanisms by which they are formed, transported, and function (Bishop and Yokota, 2001).

1.1.1.2. The Sterol Biosynthetic Pathway

Since the functions of sterols are clearly so many and varied, it is important to understand how the molecules themselves are made. In plants the pathway by which sterols are produced is complex, involving many reaction steps and a multitude of enzymes. Such complexity can be beneficial to the plant, since it allows for a great diversity of products and also allows for tight regulation by the many enzymes and other molecules involved at each step.

Figure 1 shows a simplified representation of the sterol biosynthetic pathway in *Arabidopsis thaliana*. It is evident that much of the pathway is arranged as steps which follow directly on from one another. It is also evident from the diagram that the *Arabidopsis* sterol biosynthetic pathway forks into two major and distinct branches, each of which leads to the production of very different products. One branch primarily synthesises those sterols which have roles in membrane fluidity (such as cholesterol) and intracellular signalling (i.e. the bulk sterols). The second branch, whilst forming other sterols early on, ultimately goes on to form the brassinosteroid hormones, (Hartmann,1998; Clouse, 2000).

1.1.1.3. Mutations in the Sterol Biosynthesis Pathway

Much of the sterol biosynthesis pathway and many of the enzymes involved with it have been identified and characterised based on comparison with the sterol biosynthetic pathway in yeast, which has been well studied (Gachotte et al., 1996). Studies in plants themselves have yielded a wealth of information, and genetic studies have proved valuable in identifying enzymes involved in the production of sterols and further understanding signal transduction (Clouse, 2000; Diener et al., 2000; Schaeffer et al., 2001). In such a highly complex pathway there are a large number of enzymes and other molecules required in order for the pathway to function. Any one of the steps, if affected by a non-redundant mutation, would prevent the formation of sterols essential to the development of a healthy plant.

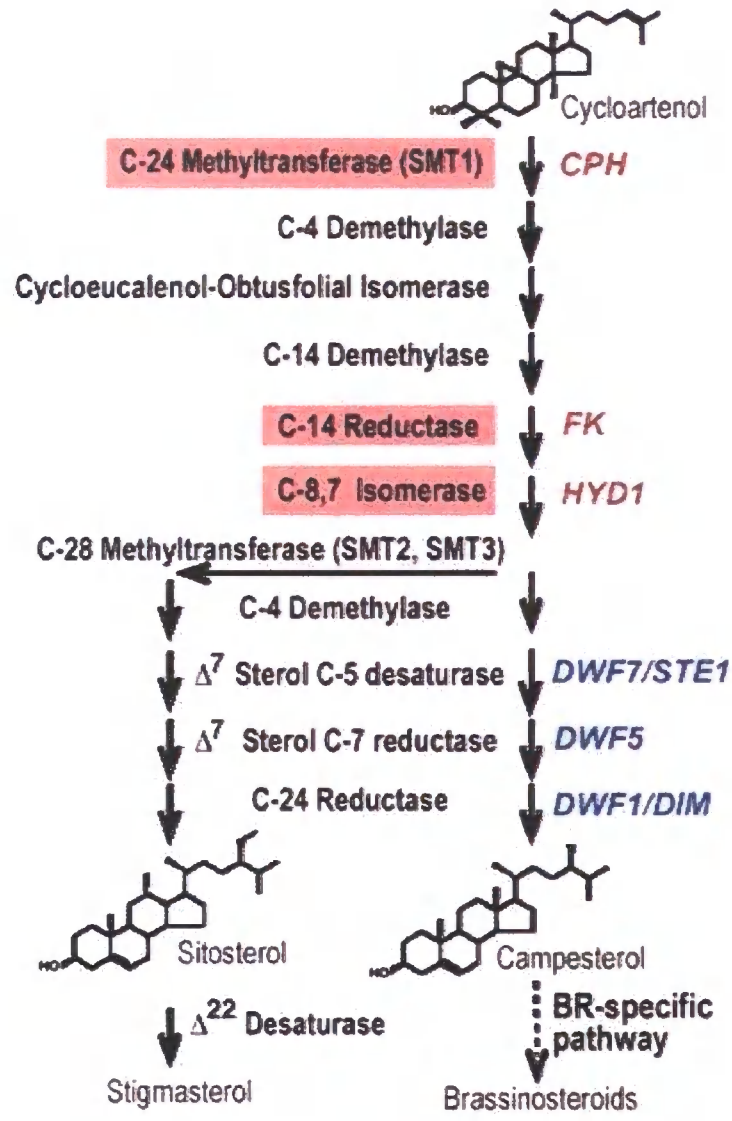


Figure 1: Diagrammatic representation of the sterol biosynthetic pathway in *Arabidopsis* (from Schrick, 2002) showing the relative positions of the *hydra 1-2* and *hydra 2* (also known as *fackel*, and marked above as *FK*) mutants. It should be noted that the *hydra* mutations are located upstream of the division of the pathway into two main branches, and consequently the *hydra* mutations would affect the bulk sterols and the brassinosteriods. (http://www.uni-tuebingen.de/ZMBP/research/dgschrick.html)

Numerous mutations which affect the *Arabidopsis* sterol biosynthetic pathway have already been identified, and the study of mutants such as these has proved a valuable tool in further elucidating the mechanisms by which plant development is governed. Some defects in embryogenesis and hormone signalling appear to be the result of abnormal sterol levels, and in some cases the application of a sterol, otherwise downregulated by a particular mutation, results in at least partial phenotype rescue. Typically this phenotype rescue is possible when an enzyme involved in the BR branch of the sterol biosynthesis pathway is faulty, where the exogenous application of BRs allows the plant to develop far more normally than would otherwise have been possible (Szekeres *et al.*, 1996; Choe *et al.*, 1999), as is the case with the tomato *dwarf* mutants, with a mutation directly affecting the mutation of the BR brassinolide (Bishop *et al.*, 1999). However, many other mutations are unable to be overcome by the addition of BRs. One such mutant is the *Arabidopsis* mutant *sterol methyltransferase 1* (*smt1*), in which sitosterol is not formed (Diener *et al.*, 2000).

Another sterol biosynthetic mutant which was unable to be rescued by exogenous BR application, despite being BR deficient, was *fackel* (*fk*). Encoding a sterol reductase the *fk* mutants are typically embryonic lethal, although they are able to be grown for a time in MS media (Jang et al., 2000; Schrick et al., 2000). Further studies have since shown that FK is allelic to the *hydra2* (*hyd2*) mutant, first described along with *hyd1* in 1997 (Topping, 1997). It is these hydra mutants which will be studied in further detail in this dissertation.

1.1.2. The *hydra* Mutants

The sequential organisation of the *Arabidopsis* sterol biosynthesis pathway is such that a mutation further upstream in the sequence would affect many sterols located further downstream, likely resulting in pleiotropic phenotypes. Such is the case with the *Arabidopsis* mutants *hydra1* and *hydra2*, where the mutations affect steps of the biosynthetic pathway upstream of the fork which splits the pathway into the bulk steroid and BR branches (Diener *et al.* 2000; Jang *et al.* 2000; Schrick *et al.* 2000; 2002; Souter *et al.* 2002). These mutants therefore suggest a critical role for sterols in signalling pathways involved in early plant development and patterning.

1.1.2.1. The *hydra* Mutant Phenotype

The *hydra* mutants were first identified by a mutational screen in 1997, and were found to contain significantly reduced concentrations of major sterols than the wild type. The resulting pleiotropic phenotypes were highly variable. Generally seedling lethal, these mutants were dwarfed, characterised by misshaped cells, multiple, fused cotyledons, a truncated hypocotyl, and a greatly reduced root system. If grown in greenhouse conditions these mutants are embryonic lethal, because although embryo development is completely normal up to the octant stage, the embryos rapidly fall behind what is expected from the wild type in terms of cellular arrangement, with very poorly defined vascular tissue and embryonic root when present at all (Topping *et al.*, 1997). Figure 2 shows a wild type seedling as compared with a *hydra* mutant (pictured at 3 weeks old).

1.1.2.2. The *hydra* Mutations

The mutations which cause the *hyd1* and *hyd2* phenotypes have been identified, allowing a greater understanding of the roles of the *HYD1* and *HYD2* genes in sterol biosynthesis. *HYDRA1* is a gene which encodes a Δ7- Δ8 sterol isomerase enzyme first characterised in yeast (Grebenok, 1998). There are no similar mutants in other organisms, although the protein itself has been found to have much homology with EBP, a mammalian sterol receptor located in the nuclear envelope and endoplasmic reticulum of cells (Jblio *et al.*, 1997; Dussossoy *et al.*, 1999). The *HYDRA2* gene has been identified as coding for a C14 sterol isomerase located 1 step upstream from *HYDRA1* in the sterol biosynthesis pathway.

1.1.2.3. What the *hydra* Mutants Can Teach Us

The study of the pleiotropic effects of two identifiable mutations which occur in such close proximity within the sterol biosynthetic pathway, provides further evidence which may be used to determine possible mechanisms by which sterols are involved in plant development.

A)

B)

Figure 2: A typical *Arabidopsis* plant (A) as compared with a *hydra* mutant (B) both aged three weeks. In the images above the *hydra* mutant has been magnified x4, whereas the wild type plant has been magnified x2. Here the truncated shape exhibited by *hydra* mutants is visible, as well as atypical cotyledons, however the exact phenotype of *hydra* mutants is highly variable.

As mentioned previously, the *hydra* mutants, while low in BRlevels, cannot be rescued by the exogenous application of BRs. Since other mutants can be rescued, whether fully or partially, by this method, the unresponsiveness of the *hyd* mutants is suggestive of difficulties either in perceiving or utilising the BRs applied or of a role for sterols upstream of the BR pathway. This in turn indicates that the problems encountered by the *hyd* mutants during development are not purely the result of a lack of BRs, and therefore it is likely that some bulk sterols, or indeed minor sterols, may have important roles to play in plant development.

Whilst it is possible that a failure to respond to applied BRs is the result of decreased membrane permeability or similar problems, another possible mechanism is being investigated which could shed further light on the varied and complex sterol interactions required for development.

1.1.3 START Domain Genes

1.1.3.1. What START Domain Proteins Are

START domains are sterol binding regions found in some proteins and are approximately 200 amino acids in length. They are highly conserved in plants and animals and appear to have a regulatory role (Ponting and Aravind 1999; Schrick *et al.* 2004).

Proteins with this sterol binding region were first identified in mammals, which make use of a protein which binds and transports cholesterol. This cholesterol binding protein is known as the steroidogenic acute regulatory protein (StAR) and problems with the expression or function of this protein are known to result in a variety of disorders in humans, including autoimmune diseases and some cancers (reviewed in Alpy and Tomasetto 2005).

The StAR-related lipid transfer (START) domains were again first identified in mammals, where the structure of several START domains has been determined. The

evidence shows they form a hydrophobic tunnel into which a ligand, typically a sterol but may also be a lipid, is bound (Schrick, 2006). START domain proteins are far more numerous in plants than in mammals, with the human genome containing 15 as opposed to the 36 found in Arabidopsis. This dissertation will be focusing upon the START domain proteins found in plants, where they are thought to play a role in signalling and transport

Several theories for START-mediated lipid signalling have been proposed, and many different families of START domain proteins have been identified and investigated. Amongst these families are those START domain proteins which also contain a pleckstrin homology (PH) domain, whose function appears to be linked to phospholipid signalling and at least one of which is implicated in disease resistance (encountered in studies of faciogenital dysplasia, or Aarskag-Scott Syndrome: Orrico, 2000) . There are also two families of START domain proteins specific to plants which contain a C-terminal domain designated DUF1336, the function of which has not yet been determined. (Schrick, 2006). The family of START domain proteins to be considered and studied in more depth in this dissertation are the plant-specific family which contain both a START domain and a homeodomain (HD) DNA binding motif.

1.1.3.2. The Role of Sterols in HD-START Domain Transcription Factors

In Arabidopsis the majority of proteins containing a START domain also contain this HD motif and act as transcription factors. The HD-START transcription factor family were found through genetic analyses to be involved in plant development. This evidence, added to the knowledge that mutant HD-START phenotypes exhibit phenotypic similarities with a select group of sterol biosynthesis mutants, supports the hypothesis that the START domains in transcription factors have some role to play in regulating the expression of genes and therefore plant development as well.

More specifically, a subset of the HD-START domain proteins has been characterized which should contain a leucine zipper, a structural motif in proteins which allows the

arrangement of parallel alpha helices. This HD-ZIP subset of START domain transcription factors has been divided into 2 families, each of which contains genes which will be studied further in this investigation. Of the 21 HD-START domain transcription factors in *Arabidopsis*, 16 contain what is known as the class IV HD Leu-zipper (HD-ZIP) and therefore form the class IV HD-ZIP family (Schrick, 2006).

A number of these HD-START proteins containing the IV HD-ZIP region are known to be involved in development, allowing for cell differentiation within specific layers of tissue (reviewed in Schrick *et al.* 2004). Two members of this family have been chosen for further study: *Arabidopsis thaliana MERISTEM LAYER 1* (*AtML1*), expressed in the epidermis of developing embryos, and *GLABRA 2* (*GL2*), affecting epidermal cell fate, including trichomes, root hairs, and seed coat (Nakamura *et al.* 2006).

The remaining 5 HD-START domain transcription factors form the class III HD-ZIP family, in which the products contain a classic basic-region leucine zipper (Mukherjee *et al.* 2006). Of these five genes, three will be investigated further during this dissertation: *REVOLUTA* (*REV*), involved in meristem formation at lateral positions such as lateral roots and flower buds, and *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), both of which have been implicated in regulating abaxial/adaxial axis fate in developing leaves. All of these genes in the class III HD-ZIP family have roles in embryonic patterning, and their roles have been found partially to overlap (Prigge *et al.*, 2005)

Therefore five different genes have been chosen for study: *AtML1*, *GL2*, *PHB*, *PHV*, and *REV*. These genes were all chosen because they all have roles in patterning and cell fate during development. It is hypothesised that sterols interact with them and may therefore help to regulate or aid their function, in which case sterols would have a key regulatory role in plant development (McConnell *et al.*, 2001).

1.2. The Project: Aims and Objectives

The overall aim of this project is to elucidate further the critical role played by sterols in plant development. It has been hypothesized that the extreme pleiotropic phenotype exhibited by *hydra* mutants is a direct result of the role of sterols in gene regulation. The mutations involved may result in a lack of specific sterols required to allow some plant transcription factors to function, either acting as co-factors or as ligands. There are 36 proteins in *Arabidopsis* which are thought to contain a sterol binding site (a START domain), and the majority are either known or predicted to be transcription factors. The working hypothesis for this project is that the binding of sterol molecules to START domains of the transcription factors acts to regulate gene expression by targeting the protein-sterol complex to the nucleus, where the transcription factor may carry out its function. Were this hypothesis to be correct, the *hydra* mutants could be the result of a deficiency of specific sterols resulting in improper subcellular localization or activity of START domain transcription factors, leaving them unable to carry out their function and therefore the developmental genes which they regulate would not be expressed. Given the phenotypic defects in the *hydra* mutants, it is also possible that transcription factors, including START-domain transcription factors, are not correctly expressed in the mutants, a possibility that is distinct from the possible mis-localization of these proteins.

The role of this dissertation is therefore to attempt to further the bodies of evidence enable the understanding of two important processes:

1.2.1. The Regulation of START Domain Transcription Factors

In order to determine if the expression of START domain transcription factors are in some way misregulated in sterol mutants, the comparative transcription levels of the five HD-START domain transcription factors described earlier - *AtML1*, *GL2*, *PHB*, *PHV* and *REV* – are here determined by real-time RT-PCR. This was accomplished by isolating mRNA from developing seedlings at 2 day intervals, thus their steady state transcription levels could be analysed in both *hydra* mutant backgrounds over the a 14 day developmental period as compared with wild type. Any significant differences

between steady state transcription levels of the genes in question in a typical sterol environment versus an atypical sterol environment would likely indicate a requirement for sterols.

1.2.2. The Subcellular Localisation of START Domain Transcription Factors

A major hypothesis of this dissertation is that sterols, by binding to START domain transcription factors, target them to the nucleus where they are then able to regulate gene expression, it follows that transcription factors expressed in an atypical sterol background might be improperly localised such that they would be present in the cell cytoplasm, and this can be tested. It is possible to determine this localisaton by creating HD-START::GFP fusion proteins to act as an indicator enabling the subcellular localization of a specific transcription factor to be observed when samples are viewed using confocal scanning laser microscopy. In this case the GFP to be used was the pMP N-linked GFP fusion construct,described in section 1.3.1.1., within the alcohol inducible binary vector described in section 1.3.1.2.

1.3. Methods to be Used

1.3.1 GFP and the alc System

1.3.1.1. GFP as an Experimental Tool

Green Fluorescent Protein, or GFP, is a protein of 238 amino acids which fluoresces green when exposed to blue light (Tsien, 1998). It was first identified in the jellyfish *Aequorea victoria* in 1962 (Shimomura *et al.*, 1962) and its discovery and scientific applications have proven so important that the scientists involved in its identification have been awarded a Nobel Prize for their achievements. However, the true significance of their discovery was not immediately evident, and it was not until some years later that the potential of GFP as a reporter for use in molecular biology studies began to be realised. This was largely made possible by the publication of a nucleotide sequence for wild type GFP (Prasher *et al.*, 1992) along with the fact that GFP requires no additional chemicals to fluoresce, and is able to fluoresce at room

temperature. GFP has been adapted to a wide range of uses in molecular biology, though in each case it acts as a reporter, providing information on the location of the protein(s) to which it is bound. This reporting can be large scale, allowing detection of specific cell types *in vivo* (Chudakov *et al.* 2005) or, on a smaller scale, it has been used to facilitate further understanding of processes such as protein folding and transport. GFP has proven to be invaluable in laboratory studies, revolutionising fluorescence microscopy and enabling previously very challenging experiments to be carried out more easily and effectively than previously (Yuste, 2005). Its influence is so widespread that GFP has even been the subject of art: the German artist Julian Voss-Andreae has created several sculptures based upon the structure of GFP (Voss-Andreae, 2005).

In this project GFP was used in an attempt to determine the subcellular localisation of HD-START domain proteins in *hydra* mutant backgrounds as compared to wild type *Arabidopsis* plants. The GFP used through the course of this dissertation was pMP N-link GFP; a new experimental GFP construct created and kindly supplied by Dr Margaret Pullen. A map of the pMP-GFP construct may be viewed in appendix B. Since this GFP is experimental its use within this dissertation takes on a second purpose: to determine if the pMP N-linkGFP functions correctly.

1.3.1.2. The *alc* System

In order to study gene activity in plants, several expression systems have been developed in which the external application of chemicals is used to induce transcription of the target gene (reviewed in Gatz, 1997; Jepson *et al.*, 1998, Zuo and Chua, 2000). This enables the studied genes to be 'switched on' transcriptionally at specific times during development. This method therefore permits the study of specific genes by effectively introducing them into an already functional and healthy system.

Modified versions of these systems have already proven to be useful tools in the study of several other plant mutants. The external application of dexamethasone to a dexamethasone-inducible system has enabled the targets of both the *APETALA3* and *CONSTANS* transcription factors to be identified, greatly furthering the understanding

of how they function (Sablowski and Meyerowitz, 1998; Samach *et al.*, 2000)

The *alc* expression system has been developed based upon a group of co-regulated genes which have been identified in *Aspergillus nidulans* and which use ethanol to control gene exression (Pateman *et al.*, 1983; Sealy-Lewis and Lackington, 1984; Felenbok *et al.*, 1988). In this naturally occurring system a transcriptional regulator named *alcR* will only induce expression of the genes in the *alc* regulon if there is ethanol present (Caddick *et al.*, 1998; Salter *et al.*, 1998)

For this dissertation a modified version of the *alc* system from *A. nidulans* was used in an attempt to determine the subcellular localisation of the different HD-START domain genes in both wild type *Arabidopsis* and *hydra* mutant backgrounds. A graphic depiction of the binary vector *alc* system and how HD-START domain genes were cloned into it is displayed as Figure 3.

The system used here is a binary vector system. For this experiment the two vectors to be used are pACN (the smaller initial vector; 4024bp) and pSRNACNbin (the larger final vector; 16515bp). Maps of both of these vectors can be seen in appendix B.

The desired outcome of the cloning procedures for the fluorescence microscopy is that each full length HD-START domain gene should be linked directly to pMP N-link GFP within the pSRNACNbin vector, and thence be able to be added to *Arabidopsis* plants both with and without a *hydra* background to act as a reporter for the genes' subcellular localisation. This outcome can be achieved in two different ways. The first, as intended, is the process which was used for *Revoluta*: pMP N-link GFP would be inserted into the pACN vector, *Revoluta* would then be inserted into pACN such that it became linked to the GFP. Then both the *Revoluta* and the GFP would be excised as a single fragment using Hind III, before being inserted into the Pst site of pSRNACNbin (as depicted in Figure3). However, both *GL2* and *PHB* contain internal Hind III sites and therefore would be incapable of being excised from pACN as a single fragment. Consequently the process for *GL2* and *PHB* cloning would be different: pMP N-link GFP would be inserted directly into pSRNACNbin at the SnaB1 site, and the HD-START domain genes would have to be linked to it directly by addition straight into the larger vector.

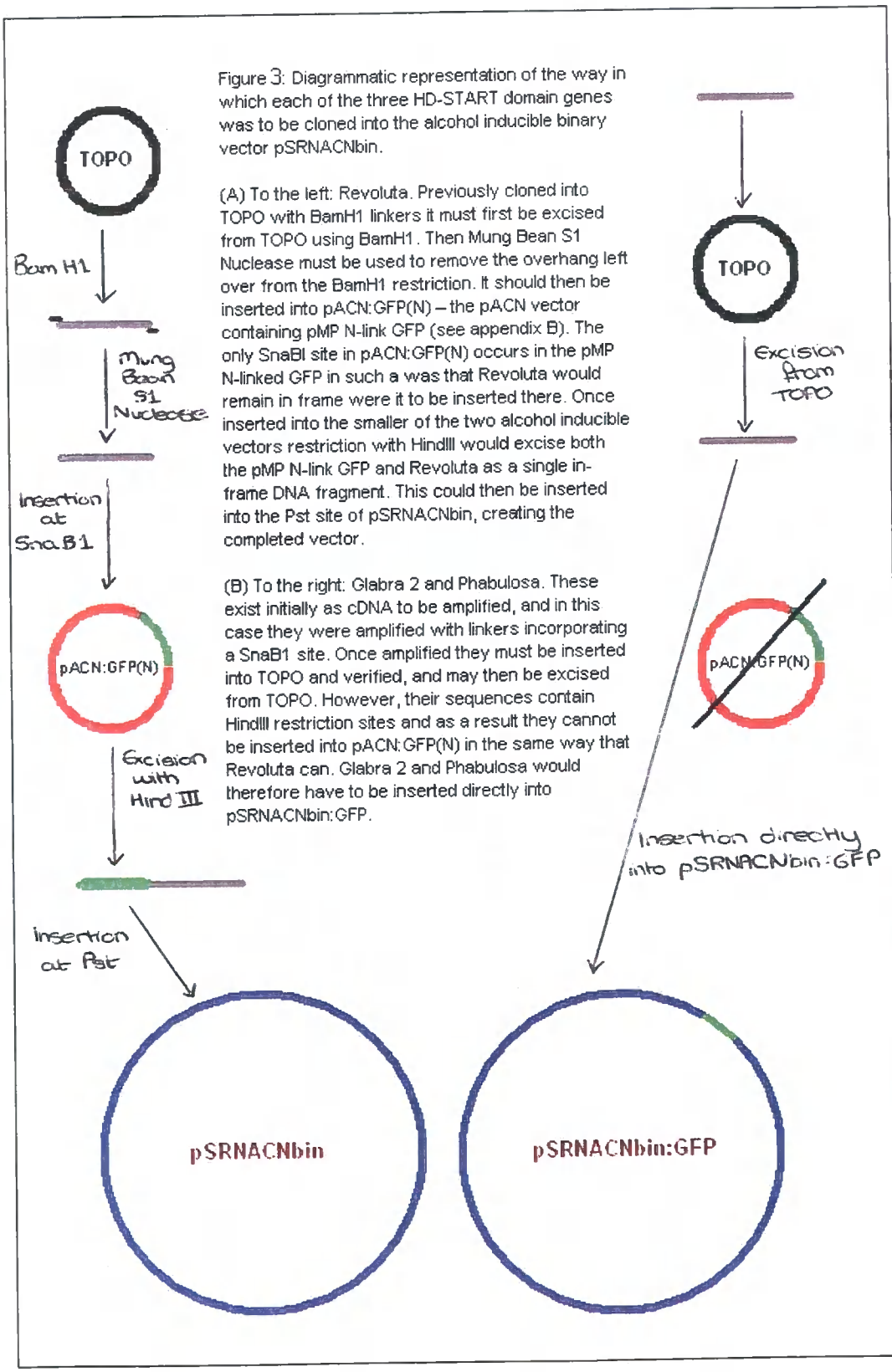


Figure 3: Diagrammatic representation of the way in which each of the three HD-START domain genes was to be cloned into the alcohol inducible binary vector pSRNACNbin.

(A) To the left: Revoluta. Previously cloned into TOPO with BamH1 linkers it must first be excised from TOPO using BamH1. Then Mung Bean S1 Nuclease must be used to remove the overhang left over from the BamH1 restriction. It should then be inserted into pACN:GFP(N) – the pACN vector containing pMP N-link GFP (see appendix B). The only SnaBI site in pACN:GFP(N) occurs in the pMP N-linked GFP in such a was that Revoluta would remain in frame were it to be inserted there. Once inserted into the smaller of the two alcohol inducible vectors restriction with HindIII would excise both the pMP N-link GFP and Revoluta as a single in-frame DNA fragment. This could then be inserted into the Pst site of pSRNACNbin, creating the completed vector.

(B) To the right: Glabra 2 and Phabulosa. These exist initially as cDNA to be amplified, and in this case they were amplified with linkers incorporating a SnaB1 site. Once amplified they must be inserted into TOPO and verified, and may then be excised from TOPO. However, their sequences contain HindIII restriction sites and as a result they cannot be inserted into pACN:GFP(N) in the same way that Revoluta can. Glabra 2 and Phabulosa would therefore have to be inserted directly into pSRNACNbin:GFP.

1.3.2. Real-time RT PCR

When investigating the mRNA levels of different genes, or of a single gene against different mutant backgrounds, there are several different experimental methods available. As well as northern blotting and cDNA arrays (Butcher, 1999; Parker and Barnes, 1999) one can use the quantitative real-time Reverse Transcription Polymerase Chain Reaction (real-time RT-PCR). This method of PCR is one of the most sensitive methods for the detection and quantitation of mRNA, and would therefore be suitable for use with plant tissue samples of limited mass (Bustin, 2000) which is a problem the *hydra* mutants pose, being both diminutive in size and having a low germination frequency.

Although real-time RT-PCR studies were used to quantify the HD-START domain mRNA levels by analysing the mRNA samples, mRNA itself cannot be used as a template for PCR reactions. Consequently all mRNA present must first be used to create cDNA replicas which may in turn act as a template for the PCR runs. The method by which this was carried out is detailed in section 2.2.1.4.

For the real-time RT-PCR studies in this dissertation the data collection is flouorescence-based, utilising the fluorescent dye SYBR Green: a cyanine dye which absorbs blue light at λ_{max} 488nm and emits green light at λ_{max} 522 nm (Zipper *et al.* 2004). When not bound to DNA, SYBR Green exhibits negligible fluorescence, however it binds indiscriminately to double-stranded DNA and, in doing so, fluoresces more strongly. Therefore the greater the quantity of double-stranded DNA which forms in the real-time RT-PCR reaction tubes, the greater the level of fluorescence will become (Morrison *et al.*, 1998). This fluorescence may be detected and this way the level of fluorescence exhibited in any PCR reaction tube will act as a representation of the amount of amplified product. For the mRNA studies in this dissertation the BioGene real-time RT-PCR system was used. For each PCR run the level of fluorescence in every PCR tube was automatically measured by the BioGene machine during the 'extension' step of each of the cycles - the step in which the DNA was double-stranded (details of the PCR conditions used may be seen in section 2.2.2.2). Transparant thin-walled reaction tubes enable both the addition of blue light and the recording of emitted fluorescence.

Due to the high sensitivity of real-time RT-PCR assays the conditions under which the PCR reactions themselves were carried out must be carefully chosen and strictly regulated. One such factor is the primer pair used to amplify the desired product. It is preferable to use primer pairs which amplify short sequences of the target gene (Bustin, 2002) however this is not the only aspect of the primers which requires attention. Primer dimmer formation is a subject of concern as it can prevent the completion of successful real-time RT-PCR assays. A number of computer programs are currently available to aid the selection of possible primer sites (Rychlik et al. 1990). This experiment made use of the Oligo program (Molecular Biology Insights Inc.) to identify which sections of the HD-START domain genes being tested would be likely to contain functional and distinct primer sites, although the exact sequences of each primer used was determined manually.

By using real-time RT-PCR and the other experiments described it is hoped that further evidence may be gathered to either strengthen or refute the hypotheses described in this introduction

Part 2: Materials and Methods

2. Materials and Methods

2.1. Frequently Used solutions/methods

2.1.1. LB (Lauria-Bertani) Medium

The bacteria used in this project were grown in Lauria-Bertani medium, used both as a liquid (for plasmid preps) and, with the addition of agar, as a thick gel used when growing colonies. When making LB stock, each litre was made with distilled water to include: 10g of peptone (Duchefa Biochemie), 5g yeast extract (Duchefa Biochemie), and 5g NaCl (Sigma). When dissolved, this liquid LB and is typically decanted into 5ml and 20ml aliquots. For LB agar, 15g of agar (typically bacto-agar or select agar) is added per litre of mix and decanted into 400ml portions prior to autoclaving. Some LB medium used during this project were selective, with antibiotics (such as kanamycin, used at 50μg ml^{-1}, and carbenicillin, used at 100 μg ml^{-1}) and X-gal (used at 40μg ml^{-1}) being added when appropriate.

2.1.2. Plant Growth Medium

All plants studied in this project were initially grown in ½ MS_{10} media. Plants are typically typically grown on a mix of salts first developed by Murashige and Skoog in 1962, which are still known as MS salts (composition details are given in: Murashige and Skoog, 1962). Ordinarily growth media would contain 4.4g l^{-1} of MS salts, however for ½ MS_{10} only half the concentration of salts are used (2.2g l^{-1}) along with 10g of sucrose. For all media used throughout this dissertation the pH was adjusted to 5.6/5.7 before the bactoagar was added at a concentration of 8g l^{-1}. Out of preference phytoagar would have been used, however this can degrade Kanamycin, which was used during this experiment.

2.1.3. TAE gel electrophoresis buffer

This solution is used both to buffer the electrophoresis gel during the process, and to make the gel itself. The buffer (10x) consists of the following components mixed in 5L of distilled water: 242g Tris (Analar), 57.1ml glacial acetic acid (Fisher Chemicals), and 37.2g $Na_2EDTA.2H_2O$ (Sigma).

The gel on which DNA samples are run during electrophoresis is a 1% agarose gel, such that a 50ml gel would be made with 0.5g of agarose added to 50ml of 1x TAE. After being heated in a microwave until it was fully clear, and allowed to cool until it

was approximately 60°C, 2µl of Ethidium Bromide was added.

2.1.4. Agarose Gel Electrophoresis

This is a DNA examination technique which enables fragments to be separated according to size. Once a gel – made as described above – sets, it is submerged into a tank of 1xTAE Electrophoresis buffer. The DNA samples to be separated are placed in wells at one end of the gel so that they sit within the gel itself. An electrical current is then passed through the gel with the negative electrode located next to the side of the gel with the DNA wells in and in parallel to them, and the positive electrode at the opposite end of the tank. Therefore the DNA, a negatively charged molecule, would move forwards through the gel itself; with smaller molecules moving more rapidly through the gel than larger molecules. The DNA is made visible under UV light by the incorporation into the gel of ethidium bromide, which binds to the DNA by slipping between adjacent base pairs (Southern, 1979; Sambrook and Russel, 2001).

The first, and sometimes also the last, lanes were loaded with hyperladder I (Figure 4; Bioline) to allow the determination of both the size of the DNA molecules being examined and an approximation of the mass of DNA contained in each band. The actual samples were loaded with the following mix; 2 µl of the DNA sample, 6 µl distilled water (to add volume in order to facilitate pipetting) and 2 µl of loading dye (where 10 ml contains 3ml glycerol and 25mg Bromophenol blue, made up to 10ml with distilled water).These samples were loaded into the wells of the submerged gel, and then run until about half way down the gel at between 68V and 130V, depending upon the size of the gel.

2.2. RNA extraction and analysis

2.2.1. Preparation

2.2.1.1. Growth Conditions for Plants

All *Arabidopsis* seeds, except those cultivated during the dissertation itself, were kindly provided by Dr Topping. The seeds used were the standard Columbia (Col-0) ecotype (to be referred to as wild type), from plants heterozygous for the *hydra 1-2* mutation, and from plants heterozygous for the *hydra 2* mutation. All seeds were

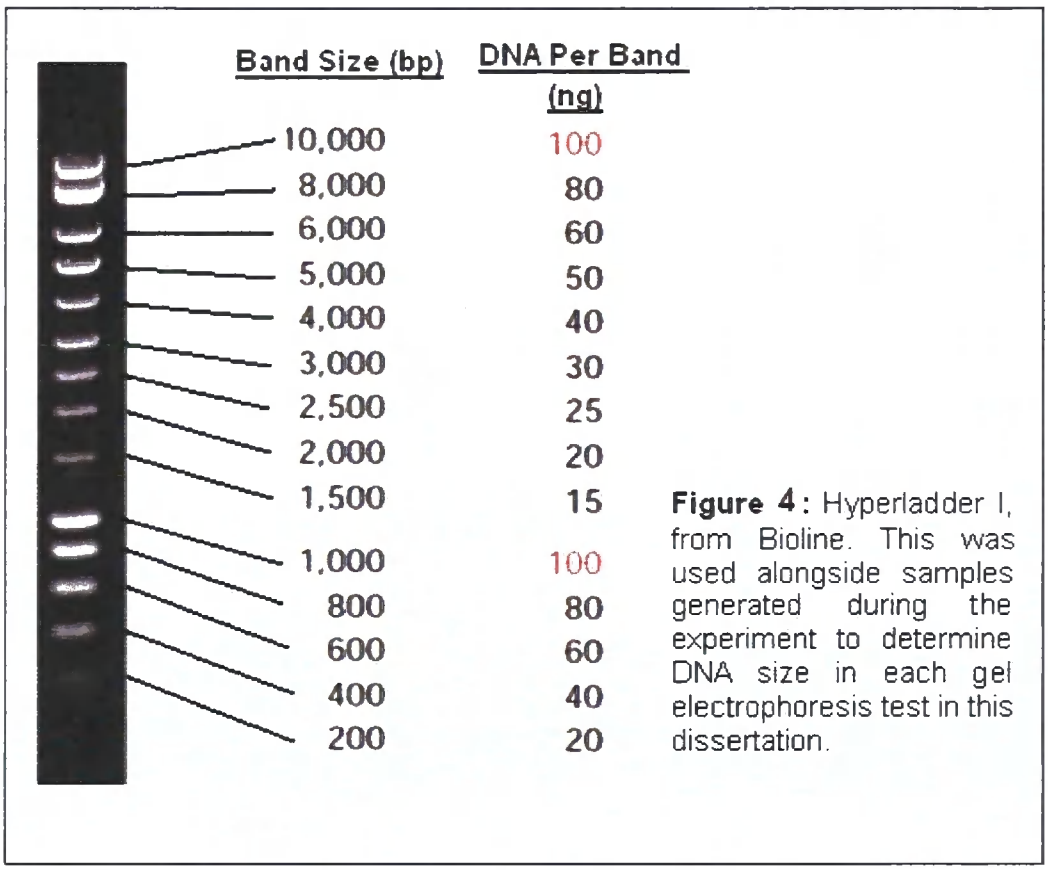


Figure 4: Hyperladder I, from Bioline. This was used alongside samples generated during the experiment to determine DNA size in each gel electrophoresis test in this dissertation.

sterilised and grown in the plant medium described in section 2.1.2 and, as it aids the germination of those seedlings homozygous for either *hydra* mutation, all were kept away from light and maintained at 4°C for two weeks prior to their removal to the plant tissue culture facility. In this facility plants were kept under light for 24 hours a day. Growing seedlings remained in the plant tissue culture facility for five or six days before being transferred to greenhouses, although all seedlings to be used specifically for RNA analysis remained in the facility indefinitely.

Although theoretically the heterozygotic seeds should result in one in four progeny being homozygous for *hydra* and therefore showing *hydra* phenotype, this was not the reality. The germination frequency for *hydra* mutants is much larger than would be expected by simple probability, especially for the *hydra 1-2* mutants. Therefore experiments requiring the use of homozygous *hydra* mutants had three times as much plant tissue with which to work for *hydra 2* studies than *hydra 1-2* studies.

2.2.1.2. RNA Extraction

Seedlings were sampled at days 9, 11, and 13 of development. It had been hoped a longer time frame could be tested, however before day 9 the *hydra* mutants were too small to sample, and after day 13 many began to die. Therefore for each sample point 60mg to 90mg of plant material was weighed out in sterile eppendorphs, frozen immediately in liquid nitrogen and stored at -80°C until needed (after approximately two weeks). Samples were kept frozen in liquid nitrogen as they were ground into a powder using a mini pestle in an eppendorph, with approximately 10µl of sand being used to aid tissue grinding (this was necessary due to how tough the tissue of *hydra* seedlings is). RLT buffer (containing 10µl ml^{-1} Beta-mercaptoethanol; Beta-ME) was added to prevent degradation of the mRNA as the tissue thawed, and the sample was spun down in a microcentrifuge for 30 seconds in order for the sand to separate from the sample and form a pellet. The supernatant was then removed carefully with a pipette and added to a spin column. Thereafter the rest of the RNA extraction followed the method set out in the Qiagen RNeasy RNA minipreps kit (the details of which are listed fully in appendix C) from step 3 onwards. Also, in order to remove

any possible contaminating DNA all samples were given a DNase treatment. For this treatment the RNeasy plant mini protocol was used (see appendix C).

2.2.1.3. cDNA Synthesis

In order to obtain a cDNA 'copy' of the extracted RNA samples the actual quantity of RNA in the purified sample was determined using a spectrophotometer. Each individual sample had its A_{260} and A_{280} measured to enable the RNA concentration to be determined by the following formula:

$$[RNA] = 40 \times A_{260} \times 20$$

Where 20 is the dilution factor (all tested samples were diluted in sterilised distilled water so that minimum RNA was lost for this particular calculation), and 40 is used because – for RNA – A_{260} is equal to 1 when the sample is at a concentration of 40 μg ml^{-1}. Samples may be considered pure if their A_{260} value divided by their A_{280} value falls between 1.8 and 2, therefore any samples which fell outside this range were discarded.

Having determined the samples' concentration, cDNA synthesis could proceed. For each sample 10μl total RNA (at 100ng μl^{-1}) was mixed with 2μl oligo(dT) primers, spun briefly in a microcentrifuge, and heated for 3 minutes at 70-85°C. They were then removed to ice, spun briefly, and replaced on ice before the following components were added: 2μl RT buffer (RetroScript), 4μl dNTP mix, 1μl RNase inhibitor (RetroScript) and 1μl MMLV-RT (Promega) as the reverse transcriptase. Samples were mixed gently, spun briefly in a microcentrifuge, then incubated at 50°C for 1 hour. They were then incubated at 92 °C for 10 minutes to inactivate the reverse transcriptase, and stored at -20 °C until required.

2.2.2. Analysis

2.2.2.1. Primers

Quantitative real time RT-PCR was used to investigate the expression of START domain transcription factors, and oligonucleotide primers were designed for each of these genes. Each of the primers was assigned a 'name' according to the following rules; rtRT-PCR (to indicate the experiment the primers were created for), the name of the gene (e.g. *REV* for *REVOLUTA*), then whether the direction of the primer is forward or reverse. These primers are detailed in Table 1.

Primer Name	Sequence (3' to 5')	Size of Product Created from cDNA/gDNA
rtRT-PCR_AtML1(for)	CCCTCAGCTCTCTTCTTCACACCA	438bp / 530bp
rtRT-PCR_AtML1(rev)	TGATACAATCCCGCAGAACACACT	
rtRT-PCR_GL2(for)	GAAGTCCCGTATTGCCGAGATTT	461bp / 645bp
rtRT-PCR_GL2(rev)	TCCACTATTGCCCATTTCTCAGG	
rtRT-PCR_PHB(for)	GCAGCAGCTCGTGACTTTTGGAC	473bp / 803bp
rtRT-PCR_PHB(rev)	CTCTGCACCATCGCTACCCATTG	
rtRT-PCR_PHV(for)	CGTCCTTGTGATGGTGGTGGTTC	421bp / 643bp
rtRT-PCR_PHV(rev)	ATAGAAGCTTTGGCACAGAGGAC	
rtRT-PCR_REV(for)	CAATGCTTCTCCAAAATGTTCCT	425bp / 723bp
rtRT-PCR_REV(for)	TGTACATCTCCCGTTTTAGCATC	

Table 1: Primers used for real-time RT-PCR studies. The table shows the sequences of the forward and reverse primers for each of the five START domain genes. The size of the products each primer pair would create is listed both for cDNA and for gDNA. This allows for the distinction of the desired cDNA from any contaminating genomic DNA (which contains introns and would therefore result in a larger product.) thereby enabling the purity of cDNA samples to be assessed.

2.2.2.2. Real-Time RT-PCR

Some of the factors which must be taken into consideration when designing real-time RT-PCR primers were mentioned in section 1.3.2. However, as well as the makeup of the primers themselves the concentration at which they are used in the reaction mix requires consideration. Indeed, due to the sensitivity of the real-time RT-PCR assays the optimal concentration of each component in the PCR master mix should be

determined to ensure accuracy of results. Prior to any reactions being carried out on the BioGene real-time RT-PCR machine optimisation runs were performed on standard PCR machines. For each of the five genes to be tested and the positive control gene *ACT2* (strongly expressed in all cells) sets of PCR reactions were carried out to determine the optimal concentration for every one of the components included in the PCR mix (by studying the gel electrophoresis results for each optimisation run). The final chosen volumes of PCR reactants for the real-time RT-PCR studies are listed in Table 2. Where possible it was decided that genes which were shown to provide clear results (bright and well defined bands on the electrophoresis gels produced during optimisation) for the same or very similar concentrations of reactants, would use the same PCR conditions.

PCR Reaction Mix		
Component	Volume	
	ACT2/AtML1/ GL2/PHB/PHV	REV
x10 Buffer (without $MgCl_2$)	2.0µl	2.0µl
dNTPs (10mM)	0.6µl	0.6µl
Forward primer (20pM)	0.2µl	0.2µl
Reverse primer (20pM)	0.2µl	0.2µl
cDNA (10ng µl-1)	0.4µl	0.4µl
$MgCl_2$ (50mM)	0.6µl	0.4µl
Sterile Water	15.5µl	15.7µl
SYBR Green	0.3µl	0.3µl
Taq Polymerase	0.2µl	0.2µl

Table 2: Table showing the volumes of PCR reactants used in the real-time RT-PCR studies.

As well as the Taq polymerase, the SYBR Green fluorescent dye was added and mixed moments before the reaction tubes were placed into the BioGene real-time RT-PCR machine. Both Taq and SYBR Green require storage at -20°C, therefore being exposed to room temperatures for any length of time before the PCR run started could only weaken their performance. SYBR Green must also be stored in complete darkness to prevent the fluorescence from becoming inactive, therefore it was the final PCR component added (to ensure the reaction tubes containing SYBR Green spent as little time as possible in the light).

The PCR machine was run according to the following requirements, determined by initial experimentation using standard PCR machines: After initially heating the sample at 94°C for 10 minutes a cycle was followed which consisted of denaturation for 30 seconds at 94 °C, annealing for 40 seconds at 58 °C, and extension for 60 seconds at 72 °C. This cycle was repeated 60 times before the final extension phase took place, in which the sample was maintained at 72 °C for 10 minutes.

2.3. Creation of START::GFP Fusion Vectors

2.3.1. Cloning

2.3.1.1. Amplifying the genes

The *REV, PHB* and *GL2* genes weresupplied as cDNAs by Dr Topping (at 10ng μl^{-1}). The full length *Revoluta* (*REV*) gene had previously been cloned into the TOPO vector in *E. coli* cells (strain JM109). The *Revoluta*–containing TOPO plasmid was sent for sequencing with the internal primers marked in the appendices. The sequence returned showed an open reading frame throughout, and displayed almost complete homology with the published sequence being used, and where any difference occurred it was consistent with a second published sequence (the 'table' for how the sequence lines up with both these published sequences may be seen in appendix C). The *Revoluta* cloning description from this point on shall be covered in section 2.3.2.

For each of the remaining genes, *Phabulosa* and *Glabra 2*, PCR was used to amplify the original template for use during the project. The primers used have been designed to amplify the genes with linkers to facilitate cloning into the GFP fusion vector, and restriction sites were incorporated into each primer at the 5' end to allow the amplified gene to be cloned into the vector.

Primers used for the reaction

Each of the primers was assigned a 'name' according to the following rules; FL (for amplifying the full-length gene as opposed to just the start domain), then the name of

the gene (e.g. *GL2* for *GLABRA 2*), pMPN-linkGFP (since the primer is designed to facilitate cloning into the pACN:GFP(N) construct) then whether the direction of the primer is forward or reverse, before finally stating which restriction enzyme has been added (in each primer here Sma I was used). These primers are listed in Table 3.

Primer Name	Sequence (3' to 5')
FL-GL2_pMPN-linkGFP(for)	TGCCCGGGATGTCAATGGCCGTCGACATGTCTTCCA
FL-GL2_pMPN-linkGFP(rev)	CCCCGGGCGCATGCAGCAATCTTCGATTTGTAGACTTCTC
FL-PHB_pMPN-linkGFP(for)	GCCCGGGATGATGATGGTCCATTCGATGAGCAGAG
FL-PHB_pMPN-linkGFP(rev)	GCCCGGGTCAAAGCAACGACCAATTCACGAACA

Table 3: Primers used for amplifying the *PHB* and *GL2* genes for full length cloning into the alcohol inducible vector. The table shows the sequences of the forward and reverse primers for each gene, with the Sma I restriction sites highlighted in blue.

PCR Protocol

The PCR protocol for amplifying the full length genes, including the restriction sites by which they are flanked, utilised the Expand High Fidelity PCR System (Roche see appendix C). The proofreading 3' to 5' exonuclease activity of the Expand enzyme ensures a higher degree of accuracy then many other enzymes (such as Taq polymerase), and therefore generates fewer errors during the reaction. The PCR solution used in each of the reactions can be seen in Table 4.

PCR Reaction Mix	
Component	**Volume**
x10 Buffer (without $MgCl_2$)	5µl
dNTPs (10mM)	1.5µl
Forward primer (20pM)	1.5µl
Reverse primer (20pM)	1.5µl
cDNA (10ng µl-1)	1µl
$MgCl_2$ (25mM)	5µl
Sterile Water	34µl
Expand Enzyme	0.5µl

Table 4: The PCR solutions used when amplifying the full length *Phabulose* and *Glabra 2* genes.

From Table 4 it is evident that the PCR solution would therefore have a final volume of 50µl, with the addition of 0.5µl of the Expand enzyme only after the rest of the PCR mix had been heated to 94°C in the PCR machine. This 'hot start' is required because the polymerase activity of Expand only occurs at this elevated temperature, whereas the proofreading function may occur across a much wider range, therefore adding Expand

before the solution is heated would lead to denaturation of the DNA.

The PCR machine was run according to the following protocol: After initially heating the sample at 94°C for 10 minutes a cycle was followed which consisted of denaturation for 1 minute at 94 °C, annealing for 2 minutes at 55 °C, and extension for 5 minutes at 72 °C. This cycle was repeated 30 times before the final extension phase took place, in which the sample was maintained at 72 °C for 10 minutes. 5µl of the enzyme Taq polymerase was added to the solution for this final extension phase in order to add an A-overhang onto the DNA to facilitate cloning into the TOPO vector.

2.3.1.2. Cloning into TOPO vector

The TOPO vector (pCR®II-TOPO®, Invitrogen ; shown in appendix B) is provided in a linearised form, with the enzyme DNA Topoisomerase I covalently bound, facilitating the ligation of complementary overhanging sequences. The linearised vector has a T-overhang which directly complements the A-overhang of the PCR products.

For each of GL2 and PHB the following mix was made: 0.5µl of PCR product was added to 3.5 µl sterile water 1µl MgCl2, and 1 µl TOPO cloning vector. This solution was left to incubate at room temperature for 30 minutes, before being returned to ice. Transformation of chemically competent *E.coli* cells (strain JM109, in tubes of approximately 300µl) began as 2µl of the cloning mix was added to a phial of cells, and the contents mixed gently. The cells were then incubated on ice for 30 minutes before being heat shocked at 42°C for 30 seconds and then returned to ice, at which point 250µl of liquid LB was added. The cells were then left to incubate for an hour at 37°C on a horizontal shaker before being spread onto plates containing kanamycin (to ensure only bacteria containing the TOPO plasmid would survive) and X-gal (to allow positive identification of cells which contained the START domain gene insert: colonies which would be white in colour as opposed to blue). Plates were then left overnight at 37°C to allow colonies to form.

2.3.1.3. Colony PCR

The following morning, after plates of transformed cells were left to incubate, colonies were tested to determine if they had taken up the gene of interest. Colony PCR was carried out on a sample of 20 colonies which appeared white or pale blue,

with a dark blue colony used as a negative control. The primers used for these colony PCR tests were those created for the real-time RT-PCR tests and are listed in Table 1. This and subsequent PCR tests were carried out using Taq polymerase (Promega) since a proofreading enzyme was not required.

Once these cycles had finished, 2µl of loading dye was added to each tube and the samples were loaded onto an agarose gel to undergo electrophoresis. Once this process was completed, five of the colonies (for each gene) identified as containing the correct insert were chosen to be grown in an overnight culture. Each was added to 5ml of LB medium containing 5µl of x1000 kanamycin stock. A flame-sterilised loop was used to lift bacteria from the colony into the culture medium, and the bacteria were then incubated overnight at 37°C on a shaker.

2.3.1.4. Plasmid Preps of Transformed Cells

In order to isolate the plasmids from the transformed bacteria, the Wizard® Plus DNA Purification System was used (Promega – see appendix C).

For each colony tested 1ml of overnight culture was placed in each of 4 eppendorphs, such that 4ml of culture was used per colony. The tubes were centrifuged at full power for 5 minutes so that a pellet formed. The supernatant was emptied and disposed of, and the pellet was tapped dry as much as possible.

To the first of the four eppendorph tubes (for a given colony's culture) was added 250µl of resuspension solution, it was then mixed thoroughly so that the pellet was completely resuspended. The mix from the first eppendorph was then added to the second, third, and final eppendorphs, with this resuspension being repeated such that the fourth tube contained 250µl resuspension solution along with the contents of the pellet from each eppendorph.

250µl lysis solution was then added to the mixture to break the cells open, the tube was inverted four times to mix. Then 10µl alkaline protease solution was added to break down cellular proteins, the mix was inverted four times before being left to incubate for 5 minutes. At the end of this incubation, 350µl of neutralisation solution

was added and the tube was inverted four times.

The solution was then centrifuged on full power for 10 minutes. The supernatant was then removed, without disturbing the pellet, and transferred to a spin column within a catch tube. This tube was then placed in a centrifuge on full power for 2 minutes, after which the flow-through was discarded. Into the spin column was then added 750µl column wash solution, before being centrifuged on full power for 1 minute. The flow-through was again discarded, and the wash repeated with 250µl column wash solution. Having emptied the through-flow, the tube was centrifuged whilst dry for 2 minutes.

For the final step, 50µl nuclease-free water was added to the spin column which was then centrifuged on full power for 1 minute. The through-flow from this step is the plasmid itself, so it was stored at –20°C.

2.3.2. Cloning of HD-START domain genes into the binary vector

The *REVOLUTA* gene, as previously stated, had been amplified using the Expand High Fidelity PCR System – and found to have no amplification errors at an earlier date. It was, however, amplified with BamH1 linkers. Before cloning could proceed further *REVOLUTA* needed to be excised from the TOPO plasmid and have the Bam H1 linkers removed. To remove *REVOLUTA* the TOPO plasmid was digested with Bam H1 as follows: 1µl of 10x buffer (for use with Bam H1) was mixed with 2 µl sterile water, 6t µl TOPO plasmid, and 1 µl Bam H1. This mixture was maintained at 37°C in a water bath for 2 hours.

Mung Bean S1 Nuclease (Promega) was then used to remove the overhanging DNA ends. The procedure used was essentially the same as for the Bam H1 digest but tailored for Mung Bean S1 Nuclease. All digests used through this experiment were performed in the same way, with buffer and incubation temperature being altered to the enzyme used. Shrimp Dephosphorylase (Promega) was then used to treat the blunt ended DNA in order to facilitate ligation into the next vector, pACN. Since pMP N-link GFP had been inserted into pACN to form pACN:GFP(N) the next step was to link *REVOLUTA* with pMP N-link GFP by inserting it into pACN at the SnaB1 site.

This is a blunt-ended ligation, and the intention was then to excise the REV::pMP N-link GFP fusion with Hind III in order to transfer it to pSRNACNbin. However, as shall be discussed in section 3.2., this objective proved unobtainable.

As shown previously (in Figure 3) both *Glabra 2* and *Phabulosa* contain internal Hind III sites which prevent them passing through the first stage of the binary vector system. It is for that reason that both genes need to be cloned directly into pSRNACNbin containing pMP N-tail GFP (pAIbinNGFP). Both of these genes were amplified with Sma I linkers, and both were added to pAIbinNGF at the Sna B1 site. This, as with *REVOLUTA*, is also a blunt-ended ligation.

For both of these blunt-ended ligations the enzyme T4DNA polymerase was used. This enzyme works at room temperature but works slowly. Therefore when cloning attempts were made the eppendorphs were left at room temperature on the bench overnight. As shall be described in section 3.2., these cloning reactions proved problematic, and were repeated often. In an attempt to increase vector take-up the vector mass:insert mass ratio was altered many times, ranging from 1:1 to 1:10.

When moving the DNA between vectors during this dissertation genes and constructs have, at times, needed to be excised from vectors and then isolated. In these situations the Roche High Pure® Purification kit for the purification of DNA from an electrophoresis gel was used.

2.3.3. DNA sequencing

All sequencing in this dissertation was carried out in the University of Durham DNA Sequencing Facility, using the ABI Prism 377 DNA sequencer.

2.4. Plant Preparation and Microscopy

2.4.1. Transformation and Growth of Agrobacterium

The method used in this project to transfer the desired vectors into *Agrobacterium tumefaciens* (strain C58C3) has been altered from that first described by Bechtold *et a.l* (Bechtold *et al.*,1993). Cultures of *Agrobacterium* were grown in 200ml LB, in which their initial stage of growth occurred, and the medium contained nalidixic acid (at a concentration of 25mg l^{-1}), streptomycin (at a concentration of 100mg ml^{-1}), and kanamycin (at a concentration of 50mg l^{-1}). Cultures were maintained at 30°C for between 40 and 48 hours whilst being shaken.

After two days of incubation at 30°C each culture tube was spun down in a cooled centrifuge until it formed a pellet. The supernatant was decanted and the pellet was resuspended in a 1 litre solution of sterile distilled water with a concentration of 5% sucrose, to which Silwett L-77 (Lehle Seeds) was added to a final concentration of 0.05%. This solution, once made, is ready for dipping instantly and cannot be kept.

2.2.2.1. Transformation of Plants

Young siliques and open flowers were trimmed from three week old *Arabidopsis* plants two days before dipping was due to take place (at roughly the same time as the initial *Agrobacterium* cultures were left at 30°C. These plants had been kept in a controlled *Arabidopsis* growth room (with warmer and more humid conditions than conventional greenhouses) during their development to ensure that no thick cuticles had formed and therefore dipping would be more likely to be successful. Each plant was dipped down into the *Agrobacterium* solution for approximately 20 seconds before being removed and placed away from the light in transparent platic bags (in order to create a humid environment for the *Agrobacterium*) overnight. The following morning plants were moved back into standard greenhouse conditions before the dipping process was repeated 7 days later. Thereafter the plants were kept permanently in standard greenhouse conditions and set seed. To facilitate seed collection the plants were placed in large transparent bags, also preventing cross contamination between other plants. Once the *Arabidopsis* plants had dried and turned

white in colour the seeds were harvested and left to dry for three weeks at 25°C.

2.2.2.1. Selection

When dry the seeds were sterilized and sown onto selective ½ MS_{10} media. The media contained kanamycin at a concentration of 35mg l^{-1}, to allow for selection for transformed plants, and vanomycin at 850mg l^{-1} to stop the growth of *Agrobacterium* on the plant growth medium, which could quickly grow and overwhelm the seedlings. Those plants which survived the kanamycin selection and remained looking healthy were later planted up into growth pots in the greenhouse, and as they became mature and formed seed the siliques were opened and studied under a microscope. Those plants found to have homozygous *hydra* mutants amongst their progeny were noted, and those which appeared only to have wild type progeny were discarded as they were of no further use in this study.

2.2.2.1. Induction

Ethanol induction of chosen plants was carried out in three ways to see which obtained the better results. All plants were sealed in 3l containers. Some of the plants were induced by root drenching with 0.3% ethanol, however these showed no signs of fluorescence when examined by confocal laser scanning microscopy. Those plants which were treated by ethanol vapour, where a beaker containing 100ml of 70% ethanol was placed in the 3l container with them, all wilted within hours most died. Therefore induction by ethanol vapour did not prove to be effective in this case.

The method which was consequently used for all plants tested in this experiment was the induction of GFP expression by ethanol spray. A 0.5% ethanol spray was used until the solution began to run off the leaves, and tissue was then sampled at 3 hours, 6 hours, and 18 hours after induction. Samples were immediately taken to the microscopy room for confocal laser scanning microscopy studies and images were taken.

Part 3: Results

3. Results

3.1. Quantitation of RNA Levels by Real Time RT-PCR

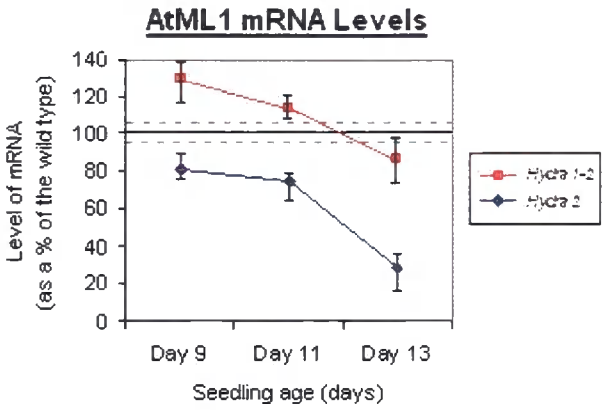
The aim of the work described in this section was to determine whether the mRNA levels of START domain transcription factors, which variously regulate vascular tissue and leaf dorsiventral pattern, which are defective in the *hydra* mutants, is altered in these mutants. As described in sections 1.1.5 and 2.2, the quantitation of mRNA levels in *Arabidopsis* seedlings was tested across several different factors. Trends across time, individual START domain genes, and type of seedling (wild type, *hydra 2*, and *hydra 1-2*) would be too complex to discuss at once, so results obtained for each gene shall be examined before more general trends are addressed. There is, however, one important point of information which is required to understand more fully the forthcoming results: real time reverse-transcriptase PCR is a tool being used here to measure cDNA created from mRNA, so the levels detected and described here directly represent those of DNA transcribed into mRNA. A number of factors could affect what proportion of the mRNA was translated into protein, and the activity of any protein produced. It is for this reason that the mRNA levels (steady state transcription levels) described below represent only one facet of gene expression.

ATML1

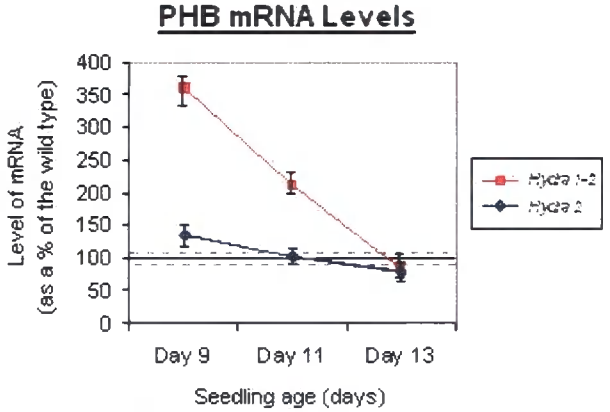
When both the *hydra 2* and the *hydra 1-2* samples were tested with the primers for isolating AtML1 (as described for all genes in section 2.2), the steady state transcription level of *AtML1* was shown to decrease with time with respect to the wild type steady state transcription level (see Figure 5A). The steady state transcription levels in the *hydra 1-2* mutants declined from an initial high of 30% above wild type steady state transcription (at day 9 of development) to 13% below wild type at day 13. Therefore, through a developmental period spanning five days, the *AtML1* gene dropped from being clearly upregulated to downregulated as compared with wild type seedlings.

The most significant result for *AtML1* was however seen in the *hydra 2* mutants. In these the steady state transcription levels were found to be consistently below those shown in the wild type. At 9 days this apparent downregulation was at 19% below

A)



B)



C)

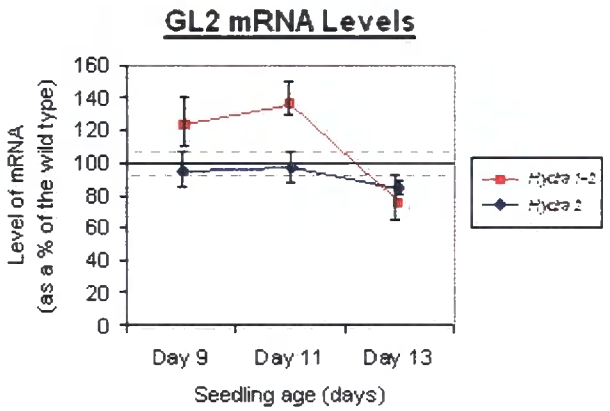
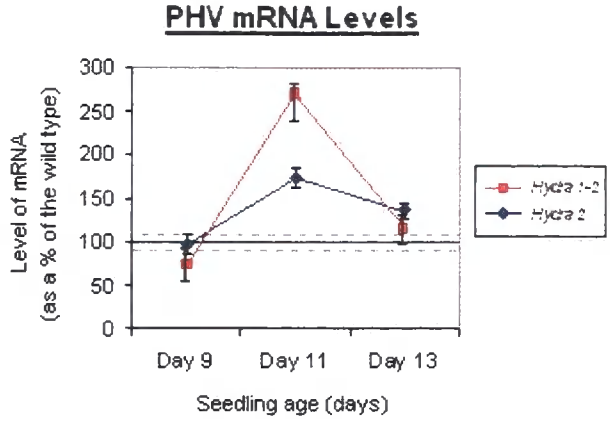
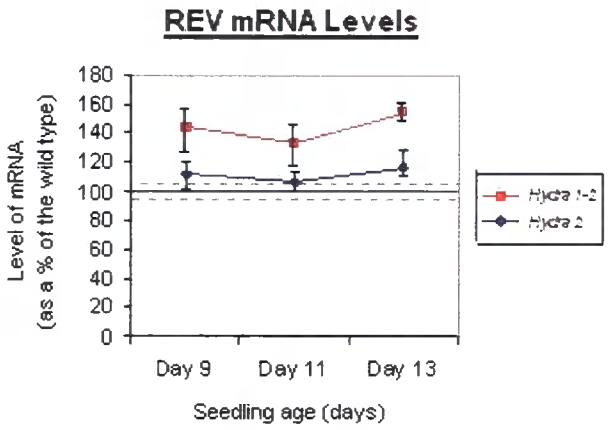


Figure 5: Level of mRNA present in *hydra* mutants compared to wild type plants. Developing plants were sampled aged 9, 11, and 13 days. Tissue was taken for each in the form of whole seedlings, and the level of mRNA was tested for specifically by real-time RT-PCR. Each data point for *hydra 2* represents the mean of 12 samples, for *hydra 1-2* it represents 4 samples. This figure shows data for the mRNA of; **A)** AtML1, **B)** PHB, **C)** GL2, **D)** PHV, **E)** REV, and **F)** ACT2.

D)



E)



F)

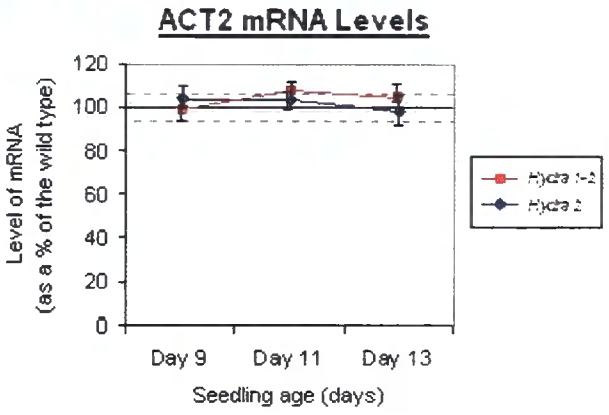


Figure 5: Level of mRNA present in *hydra* mutants compared to wild type plants. Developing plants were sampled aged 9, 11, and 13 days. Tissue was taken for each in the form of whole seedlings, and the level of mRNA was tested for specifically by real-time RT-PCR. Each data point for *hydra 2* represents the mean of 12 samples, for *hydra 1-2* it represents 4 samples. This figure shows data for the mRNA of; **A)** AtML1, **B)** PHB, **C)** GL2, **D)** PHV, **E)** REV, and **F)** ACT2.

wild type, however by day 13 this had fallen considerably to 72% below the wild type, meaning that steady state transcription levels were only a quarter of the value they would have been in a wild type *Arabidopsis* seedling. This downregulation clearly falls outside the standard deviation observed during the real time RT-PCR runs carried out for this experiment.

PHB

The pattern described above of the steady state transcription level in *hydra* mutants as compared with wild type over time, which was observed when studying *AtML1*, was very similar to that which emerged when samples were tested with the *PHB* primers: the steady state transcription levels all decrease with respect to wild type over time.

The *hydra 2* mutants show an initial upregulation of almost 40% compared to the wild type seedlings at day 9, but fall steadily (although more data points at intermediate times would be needed to prove this conclusively) until the final sample - at day 13 - was found to have 22% less *PHB* mRNA than the wild type. This matches quite closely with the actual numerical results of the *AtML1* steady state transcription in *hydra 1-2* mutants.

The most dramatic result of all those obtained for the START domain genes tested in this experiment was that of the level of steady state transcription of *PHB* in *hydra 1-2* mutants. At day 9 the level of mRNA in the *hydra 1-2* mutant seedlings was 359% that of the wild type plants, a 259% upregulation. This is clear evidence that *PHB* is upredulated during *hydra 1-2* development. Figure 5B also shows that this upregulation does not occur indefinitely: as with *hydra 2* by day 13 of development *PHB* mRNA levels may actually be downregulated compared to the wild type, however only by 14%.

GL2

Transcriptional analysis of the *GL2* gene produced a different pattern of results. In the *hydra 2* mutants the steady state transcription levels for *GL2* on days 9 and 11 were essentially the same as in wild type seedlings – with only 6% and 2% lower RNA levels measured with respect to the wild type, and falling well within the standard

variation exhibited in wild type seedlings (see Figure 5C). The only result which seemed indicative of abnormal gene mRNA levels was obtained for day 13, at which time steady state transcription was measured to be 14% lower than in wild type seedlings, though this value in itself was not significantly outside the standard range of error.

This pattern of almost constant steady state transcription levels with respect to the wild type through days 9 and 11, followed by a much larger drop in comparative steady state transcription was also evident in the *hydra 1-2* mutants. For these plants the pattern was exaggerated: the earlier stages (days 9 and 11) were upregulated, unlike in *hydra 2,* with 24% and 35% respectively greater steady state transcription than the wild type. However, by day 13 the steady state transcription level had fallen significantly with respect to the wild type, from 135% of the wild type mRNA levels to just 75%.

PHV

Perhaps the most intriguing result was obtained for the *PHV* gene. Instead of a general decrease of steady state transcription levels with respect to the wild type over time (as exhibited with each of the genes described so far) both the *hydra 2* and the *hydra 1-2* mutants showed a significant peak at day 11.

The steady state transcription level of *PHV* in *hydra 2* mutants at day 9 of development was essentially indistinguishable from that of wild type seedlings, whereas *hydra 1-2* mutants had 26% less PHV mRNA present than in the wild type. Two days later the mRNA levels of the *hydra 2* and *hydra 1-2* mutants with respect to wild type seedlings had drastically increased to 74% and 170% above the wild type value respectively. These are some of the most convincing results obtained in this experiment, falling clearly outside the standard deviation exhibited by wild type seedlings (see Figure 5D). However, as with *AtML1*, *PHB*, and *GL2* the results for day 13 of development indicate that the steady state transcription level with respect to the wild type seedlings again starts to fall, although it does not fall back to wild type levels in either of the *hydra* mutants.

REV

Another unusual result came from testing for levels of *REVOLUTA* mRNA over time, providing the only result for which the steady state transcription level appeared to increase towards day 13 of development with respect to wild type over time (see Figure 5E).

The *hydra 2* and *hydra 1-2* mutants remained consistently at or above the level of *revoluta* mRNA found in wild type seedlings. That is not, however, to say that their value with respect to the wild type remained constant. Both mutants exhibited a fall in comparative steady state transcription levels between days 9 and 11 of development, although this fall was more significant in *hydra 1-2* (from a 44% upregulation to a 32% upregulation) than in *hydra 2* (which fell from 12% upregulation to 6%; a small difference made even less notable by the error bars for these results, which show a large variability in the data concerned). Again the apparent increase in mRNA levels comparative to the wild type seedlings – which occurred between days 11 and 13 - was greater for the for the *hydra 1*-2 mutants (increasing from 32% to 54% upregulation) than the *hydra 2* mutants, where the rise was comparatively small.

ACT2

As mentioned previously *ACT2*, a housekeeping gene, was used in this experiment as a positive control. The *ACT2* mRNA levels were measured in exactly the same manner as those HD-START domain genes discussed above in order to provide a 'standard' to which they may be compared.

The steady state transcription levels of *ACT2* were observed to differ far less from the levels found in wild type seedlings than any of the HD-START domain genes were. Five of the six data points shown in Figure 5F fell within the standard deviation exhibited by wild type seedlings (indicated by the dotted grey lines). The levels of mRNA in the *hydra 1-2* seedlings were again generally slightly greater with respect to the wild type seedlings than the *hydra 2* seedlings, but with all six data points falling between 3% below and 9% above wild type levels.

OVERALL TRENDS

Upon examining the data collected through real time reverse-transcriptase PCR (as described in section 2.2) some overall trends become apparent with regard to the *hydra* mutants themselves

The most noticeable and consistent result to emerge is that the pattern of upregulation and downregulation of START domain genes – as compared with levels measured in wild type seedlings – is the same across both *hydra 2* and *hydra 1-2* mutants for each of the genes tested. This is largely to be expected considering the relative positions of the *hydra 2* and *hydra 1-2* mutations in the sterol biosynthetic pathway (as described in section 1.1.1.3). However, what was unexpected was the difference observed between the results for the *hydra 2* and *hydra 1-2* mutants with each gene tested. Table 5 is a table showing, for ease of reference, the greatest and lowest level of mRNA as a percentage of the wild type for both of the *hydra* mutants with each of the genes tested. From this table it is evident that whereas both *hydra* mutants follow the same pattern of upregulation and downregulation through time for each gene tested, the *hydra 1-2* mutant plants are displaying a more exaggerated version of the patterns observed in the *hydra 2* mutants. The only gene for which this was not the case was *AtML1*.

Sample		mRNA Level (as a % above or below the Wild Type)	
Gene	**Mutant**	**Highest Level**	**Lowest Level**
AtML1	*Hydra 1-2*	+29	-13
	Hydra 2	-11	-72
GL2	*Hydra 1-2*	**+35**	**-25**
	Hydra 2	-2	-14
PHB	*Hydra 1-2*	**+259**	**-14**
	Hydra 2	+38	-22
PHV	*Hydra 1-2*	**+170**	**-26**
	Hydra 2	+174	2
REV	*Hydra 1-2*	**+54**	**+32**
	Hydra 2	+17	+6

Table 5: Table showing the greatest and least level of mRNA (as a percentage above or below the wild type) for each of the five START domain genes tested by real-time RT-PCR. Data for the *hydra 1-2* and *hydra 2* mutants has been separated in order to highlight their differences. For each gene the values for the *hydra* mutant which displayed the greatest range of results have been highlighted in bold.

3.2. Creation of START::GFP Constructs

3.2.1. Cloning of START::GFP Fusion into alc System Vectors

The aim of this part of the project was to construct translational fusions between START domain proteins and GFP, for future localization studies in wild-type and sterol mutant plants. The cloning of *REV*, *PHB*, and *GL2* was carried out exactly as detailed in section 2.3. *Revoluta* was successfully cloned into TOPO with the linkers ready for insertion into the pACN vector containing the pMP N-link GFP. *Phabulosa* and *Glabra 2* were successfully cloned into TOPO with the linkers which would be required for their insertion into the pAIbin GFP binary vector (since, as stated in section 1.2.1.3, their internal Hind III sites precluded them from first being cloned into the pACN vector).

However, cloning was unable to progress successfully beyond this stage. Repeated attempts were made throughout the year; however in each case those vectors which had taken up the insert had done so in the incorrect orientation. It was also noted that the colonies were very slow-growing – requiring at least two full days at 37°C to reach the size ordinarily expected from overnight incubation at the same temperature. – and initially few colonies formed on each plate (typically about six were present). In order to increase the number of colonies per plate in subsequent cloning attempts 700µl of culture were spun down, the majority of the supernatant was removed, and the cells were resuspended in 150µl of LB medium. This enabled more colonies to be tested per plate but was to no avail: *GL2* and *PHB* cloning failed (due to being in the incorrect orientation) 243 and 251 times respectively, and *REV* was cloned backwards a total of 312 times. During the course of the year the experimental procedure was varied to ensure that it had no impact upon the cloning outcome, this included changing the *E. coli* cells used; using, amongst others, DH5α, TOP10, TOP1-F' (with IPTG), and XL Blue cells.

3.3. Microscopy

3.3.1. START::GFP Fusions and Fluorescence Microscopy

Since the START::GFP fusions could not be created, *Arabidopsis* plants containing these genes evidently could not be tested. Therefore the subcellular localisation of START domain proteins could not be examined using confocal scanning fluorescence microscopy. However, since the GFP due to be used in this experiment (pMP N-link GFP, described in section 1.2.1) is a new, experimental form, this opportunity was used to test the functionality of the GFP construct itself: essentially running the experiment purely to observe the positive control. Having transformed *Arabidopsis* pants using the pSRNACbin vector containing only the pMP GFP construction (i.e. pAIbin GFP), the remainder of the experiment was carried out as planned.

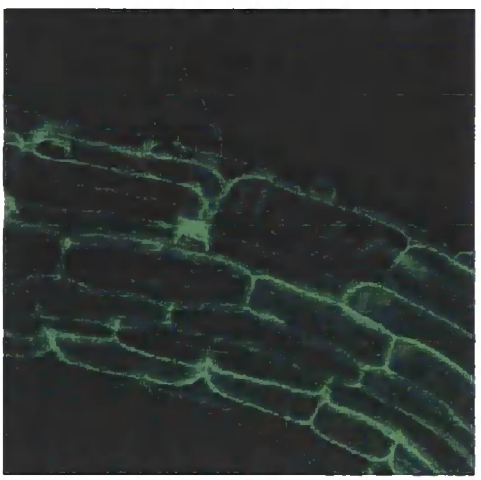
The time taken for the presence of fluorescing GFP to be evident under the fluorescence microscope varies greatly depending upon the specific GFP used in the experiment as well as the genes in question. Therefore since this is an experimental GFP which had not yet been tested, determining the most suitable time frame for fluorescence to be detected would still be worthwhile. After testing for fluorescence at 3 hours, 6 hours, and 18 hours it became evident that result could be best obtained 18 hours after induction (with samples taken at earlier times exhibiting no determinable differences to the negative control).

Figure 6 shows confocal scanning fluorescence microscopy images of root tissue in both a negative control plant (A) and an mpGFP-containing plant sampled at five days old. Images B to D show the roots of plants containing pMP N-linked GFP before induction, 3 hours after induction, and 18 hours after induction. These images show that GFP is fluorescing strongly at the edges of cells, indicating its presence in the cytoplasm around the large vacuoles found in root cells; as would be expected for GFP constitutively expressed under the CaMV35S promoter. This evidence is further supported by the strong result of the negative control, indicating that the GFP visible in the images taken could not simply be the result of autofluorescence. It is therefore reasonable to conclude that the pMP N-linked GFP functions correctly.

(A) Negative Control

(B) pMP N-link GFP before induction

(C) pMP N-link GFP 3 hours after induction



(D) pMP N-link GFP 18 hours after induction

Figure 6 : Confocal scanning laser microscopy images used to capture GFP. Unfortunately only the positive control was able to be tested, however the data provided has still proven to be useful. Images show *Arabidopsis* root tissue in a negative control plant which contains no GFP (A) which may be compared with the image of a GFP positive plant before ethanol induction (B) indicating that the expression of the alcohol inducible vector is not 'leaky'. GFP positive tissue 3 hours (C) and 18 hours (D) after ethanol induction together indicate show the time delay between induction and GFP detectable expression. (D) Also shows, by clear GFP expression in the cytoplasm surrounding the root cell vacuoles, that pMP N-link GFP functions correctly.

Part 4: Discussion

4. Discussion

It was hypothesised that the binding of sterols to the START domain of various genes enabled those genes to be targeted to the nucleus of a cell, to enable the transcription of genes essential for normal plant development. It was further hypothesised that the essential START domain protein genes themselves may be mis-regulated in sterol-deficient mutants, to produce the defective development seen. Such hypotheses are supported by the highly complex phenotypes of sterol mutants: for processes across such a wide range to be affected (multiple cotyledons, a lack of truly discrete vascular tissue, etc.) it is unlikely that the sole cause is abnormal membrane fluidity caused by deficient sterols, though this is still a likely important component of sterol function.

Studies have shown that START domains are important in transport and synthesis of sterols and those structures which have been elucidated are also suggestive of this (Tsujishita and Hurley, 2000). This is however, not the only possible mechanism by which nuclear localisation could occur; many other different proteins could be involved in this process. The hypothesis of how the START domain functions would provide an elegant mechanism by which this control could be mediated. Though it has not been expressly shown that sterols do bind to plant START domain transcription factors in this way, a body of evidence suggests this to be the case in mammalian systems (Christensen and Straus, 2001), and the archetypal protein STaR binds plant sterols (Kallen *et al.* 1998).

4.1. Creation of START::GFP Fusion Proteins

The aim of the GFP studies in this dissertation was to attach a reporter (GFP) to several START domain proteins, and then induce expression of those proteins in a living plant. In this way the subcellular localisation of START domain proteins could be viewed, catalogued, and compared between wild type plants and those with a *hydra* mutant background. Unfortunately the formation of START::GFP fusion proteins could not be completed, although the concept still has enormous potential.

It would be beneficial if GFP fusions could be made with the full length START domain proteins, thus it could be determined whether the presence of sterols

correlated with the nuclear localisation of the transcription factors studied, and if defective sterol backgrounds correlated with a lack of nuclear localisation. This experimental model could also be improved by incorporating the native promoters for each gene into the constructs, preventing the constitutive expression shown by the CaMV35S promoter. Genes with mutations in their START domain could also be introduced and linked with GFP in the same way. Confocal microscopy could be used to determine what effect, if any, this had on the subcellular localisation of the genes in question. Another approach to this study would be to identify any ligands for plant START domains, as they have thus far not been absolutely identified (Schrick *et al.* 2004).

Typically the inability to insert a gene in the correct orientation could be presumed to be a direct consequence of the inserted gene, when expressed, having such a deleterious effect upon the bacterium that it causes death and therefore no colony is formed from which a sample may be taken. Such toxicity of the inserted DNA would result in the data presented: low plasmid take-up, with those plasmids which have been multiplied containing the gene in an inexpressible form (i.e. 'backwards'). Although the use of the *alc* system was to ensure that the presence of alcohol was an essential component in initiating expression in plants, some leaky expression in bacteria may have been sufficient to induce toxic effects. Even if this were the case then due to the number of colonies tested the expression would have to be unstable enough to be fatal to every single bacterium which took it up. However, this hypothesis could be disproven by examining the results shown in section 3.3.1 (specifically those depicted in Figure 6, A and D). The positive control – GFP under the alcohol inducible promoter as these constructs would have been – was examined before induction and showed no visible signs of expression, appearing essentially indistinguishable from the negative control. Since 'leaky' expression would have been made manifest in the form of fluorescence it may be assumed that the alcohol inducibility of the promoter is functioning effectively in bacteria.

In a situation where a correctly functional and previously proven system, which should be able to contain a construct that is not expressed unless a foreign chemical (alcohol) is present, proves incapable of being cloned, suspicion is then drawn to the earlier stages of the experiment. Firstly, it could not be possible for the primers

attached to the genes to be influencing the orientation of the inserted DNA because all ligations used at this failed stage are blunt-ended (for full details see section 2.3.2). This considered, could the inserted DNA have been from the TOPO plasmid (or some other DNA) instead of the desired gene? This is impossible as every stage of the cloning acts as a checkpoint to determine the 'identity' of the insert as it is brought through the experimental process. The cDNA originally used was amplified with gene-specific primers and shown by gel electrophoresis to produce a DNA product of the correct size. When colony PCR testing was carried out a completely unrelated set of gene-specific primers was used to determine whether or not each specific HD-START domain gene was there (the real-time RT-PCR primers were used). Diagnostic digests indicated which specific START domain genes were present, and the actual sequencing data (used to check the orientation of the insert) clearly shows the genes to be present but in 'reverse'.

Therefore there is apparently no obvious explanation as to why the START domain genes would only be able to be inserted into the alcohol inducible vectors in the incorrect orientation, although the probability of the obtained results being down to chance is so low as to be negligible. Therefore there are evidently unidentified factors influencing the ability to clone these START::GFP fusions into the alc system, however it would likely be inefficient to investigate them further at this time when other lines of investigation are still available through which to glean more valuable information about the roles of sterols and START domain genes in plant development.

4.2. Real-time RT-PCR Assays

Although the real-time RT-PCR studies proved more successful than the HD-START domain gene cloning, the results are difficult to interpret. The aim of the real-time RT-PCR studies was to obtain more evidence to help determine if the expression of the START domain transcription factors is mis-regulated in sterol-defective mutants. The real-time RT-PCR assays provided valuable data on the difference in steady state transcription levels of five HD-START domain genes in both the *hydra 2* and the

hydra 1-2 mutants as compared with the wild type *Arabidopsis* plants.

The results provide evidence that the mRNA levels of the studied genes can vary greatly from those exhibited in the wild type, the greatest example being that of the observed steady state transcription level of *PHABULOSA* on day 9 of development in *hydra 1-2*, which showed *PHB* RNA levels 359% that of the wild type. For other genes, the differences were less dramatic, but were still significant, particularly when contrasted with the results for the positive control (housekeeping gene *ACT2*).

Therefore it is evident that the study of these steady state transcription levels did provide some evidence that defective sterol profiles of *hydra* mutants have an effect on the levels of mRNA present for the START domain proteins. The experiment also posed some questions: if the *hydra* mutants are similar enough consistently to exhibit the same pattern of upregulation and downregulation with time then why does *hydra 1-2*, the mutation further downstream in the sterol biosynthesis pathway, exhibit a more exaggerated pattern of differences in START domain gene steady state transcription levels than *hydra 2*? Moreover, a key question is, what is the biological basis, if any, for the observed mis-regulation?

One possibility is that the *hydra 1-2* mutation itself has a greater effect than the *hydra 2* mutation either directly upon START domain gene transcription or indirectly influences transcription by affecting other molecules involved in transcriptional regulation. This is somewhat unexpected, since the *hydra 1-2* mutation is located downstream of the *hydra 2* mutation in the sterol biosynthetic pathway (see Figure 1), which would might lead us to the assumption that it would have a lesser effect on the seedling. In addition, the phenotypes of the *hydra* mutants are very similar; this again could be anticipated due to the proximity in which the individual mutations occur. This in turn makes it seem unlikely that two such closely related mutations could cause the transcription of genes - themselves implicated in the control of transcription - to differ as shown. Further to this it must still be remembered that mRNA levels are not synonymous with gene expression, and that any manner of other factors could be affecting the actual expression of these START domain genes both before and after transcription. However, it is possible that the observed different sterol profiles in the *hydra1-2* and *hydra2* mutants (Souter *et al.* 2002) could be sufficient to affect

differentially the transcription of key transcription factors, such as those shown. The biological basis for this remains unclear, but given the defective hormone signalling, tissue organization and differentiation processes seen in these mutants, the disruption of gene transcription is not unexpected. For example, the observed down-regulation of *AtML1*, a gene expressed in the epidermal layers of the seedling, in *hyd2* may be the consequence of the observed defective differentiation of the epidermis in at least some *hyd2* plants (Topping *et al.* 1997; Souter *et al.* 2002). There is currently no information on the transcriptional control of the START domain protein genes by hormones.

In order to be convinced of the results obtained thus far it would be greatly beneficial if these real-time RT-PCR studies were to be repeated with additional samples. This would ensure that the observed trends discussed here are truly representative of the mRNA levels typically present in the *hydra* mutant seedlings. In addition, the data as collected in the course of this experiment cannot be used to identify the cause of these changes in steady state transcription levels. To tackle this and further elucidate the mechanisms and processes involved, further avenues of research must be followed (some are discussed in section 4.3)

Quality and Reliability of Results

When analysing the data obtained through real time RT-PCR the quality of the results themselves cannot be ignored. Indeed, the tentative conclusions drawn above could be rendered unusable if the quality of the data gathered is in dispute.

What is most noticeable about the results as they have been presented so far are the unexpectedly large variations in the individual results obtained, depicted graphically as the large error bars visible on Figure 5. This degree of variability amongst the results could suggest that the experiment was less precise than it should have been (and therefore, by extension, less reliable) however several key points when considered together are indicative of a different and altogether more positive explanation.

Firstly, were the large variations in the obtained results due to poor experimental procedure, low quality samples, or poorly optimised PCR conditions, the effect would

surely be that all data obtained shared large error bars. Whilst it is true that some of the data points are flanked by these large error bars this is not always the case. Not only are there many data points for HD-START domain genes which display comparatively very little variation (such as; *GL2*, *hydra 2*, day 13 and *REV*, *hydra 1-2*, day 13 – both in Figure 5) but the data obtained for the positive control, housekeeping gene *ACT2*, was consistently flanked by comparatively very small errors. These small standard deviations appear to indicate that the experiment itself is robust and the results simply reflect the differing levels of variation between samples. Furthermore, such variation is in itself cause for additional examination.

The most noticeable pattern to emerge when analysing the standard deviations is that they varied in an almost characteristic pattern according to whether the variation was for wild type, *hydra 2*, or *hydra 1-2* samples. The standard deviation amongst the results obtained for wild type seedlings averaged at 7% above or below the total mRNA content determined by real-time RT-PCR. This is demonstrated in Figure 5, A-F as the broken lines above and below the 100% mark as shown on the y-axes, and was almost invariably lower than the standard deviations for the *hydra* mutant results for any given test. In the majority of the real time RT-PCR runs the samples with the next greatest standard deviation in their results were the *hydra 2* mutants (with an average of 10% above and below) and the samples exhibiting the greatest standard deviation were the *hydra 1-2* mutants. It could be hypothesised that, since the *hydra* mutants by their highly variable phenotypes (Topping *et al.* 1997; Schrick *et al.* 2000, Souter *et al.* 2002) appear to indicate unpredictable changes in the levels and timing of gene transcription to a degree, greater variability in the actual levels of mRNA between samples would be anticipated. Although such variability would be minimised (and therefore produce smaller error bars) were all the samples of *hydra 2* or of *hydra 1-2* seedling RNA to be mixed before being dispensed into aliquots for each PCR run, this would simply mask the variability of the data, and knowledge of this variability is essential when analysing both the nuances and reliability of the results.

In the case of the *hydra 1-2* mutants the increased proportion of error inherent in the results may be a direct consequence of the smaller sample size drawn upon for real time RT-PCR studies (it was mentioned in section 2.2.1 that the *hydra 1-2* seeds had a lower germination frequency than the *hydra 2* seeds and therefore only a third as

many samples were able to be tested). Strength is added to this argument by the knowledge that the variation for *hydra 1-2* seedlings was slightly greater than for the *hydra 2* seedlings even for *ACT2*. Therefore, although several patterns have emerged from which it is possible to draw conclusions, the often large standard errors must still be considered when analysing the data.

In summary, data have been obtained that suggest that the expression of key transcription factors of the START domain family are mis-regulated in sterol-defective mutants. Given the phenotypes of the *hydra* mutants, this may be the consequence of the observed defects in hormone signalling, tissue specification and tissue patterning, which might be expected to affect transcriptional profiles, in a way that would be expected to be independent of a direct role for sterols in the control of gene regulation The data for ACT2 has demonstrated that this differential profile of mRNA levels in the *hydra* mutants is not common to all genes, thereby adding to the evidence suggesting a specific role for genes which utilise sterols. Another possible explanation for these patterns of differences is that, as stated previously, different sterol profiles within each of the mutants may in turn affect some sterol-dependant processes differently. It was not possible to determine more direct possible roles for sterols in transcription factor localization, and this should be the subject of future studies.

4.3. Directions for Future Research

An obvious direction for future work would be the completion of GFP::START domain protein construction, although evidently the experimental protocol suggested here will need to be re-examined. Once created these fusion proteins could be tested *in vivo* not only in the *hydra* mutants but in other sterol defective mutants. In this way more evidence regarding the subcellular localisation of START domain proteins could be obtained, allowing more definite conclusions regarding the role of sterols in START domain protein localisation.

This aim could be further investigated by the creation of START::GFP fusion proteins

in which the START domain itself has been altered by site-directed mutagenesis. Confocal scanning laser microscopy could then be used to examine the effect this would have upon subcellular localisation of the protein. In this way the necessity of the putative sterol-binding domain in subcellular localisation may be both determined and observed across both the wild-type and sterol deficient plants.

In order to obtain more definitive results it would be beneficial both to calculate a more accurate average by continuing experiments in this way, and to determine if the observed variation is indeed a result of variations in gene transcription levels and timing between individual *hydra* mutants. The former would provide a more accurate average by increasing the sample size, and could only be improved by also testing with a more sensitive PCR method. The latter would, however, prove more challenging as ideally RNA extractions should be carried out on individual mutants, and would also obviously not permit comparisons across time for individual plants, since RNA extraction is a destructive process. Further to this approach the data obtained through the real-time RT-PCR assays could be validated by visualising gene expression in *hydra* mutants. This may be done either by using *in situ* mRNA localisation, or by carrying out promoter::GUS fusion analyses of the HD-START domain protein genes.

An invaluable line of research would be, as suggested previously, the identification of those ligands which bind to the sterol binding domains in the genes tested. Moving away from those avenues of research dealt with directly during this project a range of other approaches could be used, including determining the extent to which the transport mechanisms of the mutant seedlings are affected (as is evidenced by the lack of response to the exogenous application of BRs, shown by Topping *et al.*, 1997) .

A combination of these and other studies in the future may prove invaluable in fully characterising the effect of sterol mutations upon plant development. In furthering the bodies of evidence regarding both the transcriptional regulation and the subcellular localisation of START domain proteins across wild type and sterol deficient plants this aim may begin to be achieved.

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Appendices

6. Appendices

6.1. Appendix A – Sequences

- AtML1 cDNA Sequence
- GL2 cDNA Sequence
- PHB cDNA Sequence
- PHV cDNA Sequence
- REV cDNA Sequence
- REV Amplified Sequence Aligned with cDNA Sequence and TAIR Sequence

AtML1 cDNA Sequence

ATGTATCATCCAAACATGTTCGAATCTCATCATCATATGTTCGATATGACG
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GL2 cDNA Sequence

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PHB cDNA Sequence

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PHV cDNA Sequence

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REVOLUTA cDNA clone BX830562 from genoscope

pink = START domain
orange = REV(for2)
purple = REV(for3) and REV(rev3)
red = REV(rev2)
green = FL-REV/NTAILGFP(FOR)BAM
light green = FL-REV/NTAILGFP(REV)BAM

```
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2001  TGTTGCAATG GCGATCTCAC CGTCTGGGAT AAGCCCGAGT CTGGGCTCCA
2051  AATTGTCCCC AGGATCTCCT GAAGCTGTTA CTCTTGCTCA GTGGATCTCT
2101  CAAAGTCACA GTCATCACTT AGGCTCGGAG TTGCTGACGA TTGATTCACT
2151  TGGAAGCGAC GACTCGGTAC TAAAACTTCT ATGGGATCAC CAAGATGCCA
2201  TCCTGTGTTG CTCATTAAAG CCACAGCCAG TGTTCATGTT TGCGAACCAA
2251  GCTGGTCTAG ACATGCTAGA GACAACACTT GTAGCCTTAC AAGATATAAC
2301  ACTCGAAAAG ATATTCGATG AATCGGGTCG TAAGGCTATC TGTTCGGACT
2351  TCGCCAAGCT AATGCAACAG GGATTCGCTC GCTTGCCTTC AGGAATCTGT
2401  GTGTCAACGA TGGGAAGACA TGTGAGTTAT GAACAAGCTG TTGCTTGGAA
2451  AGTGTTTGCT GCATCTGAAG AAAACAACAA CAATCTGCAT TGTCTTGCCT
2501  TCTCCTTTGT AAACTGGTCT TTTGTGTGA
```
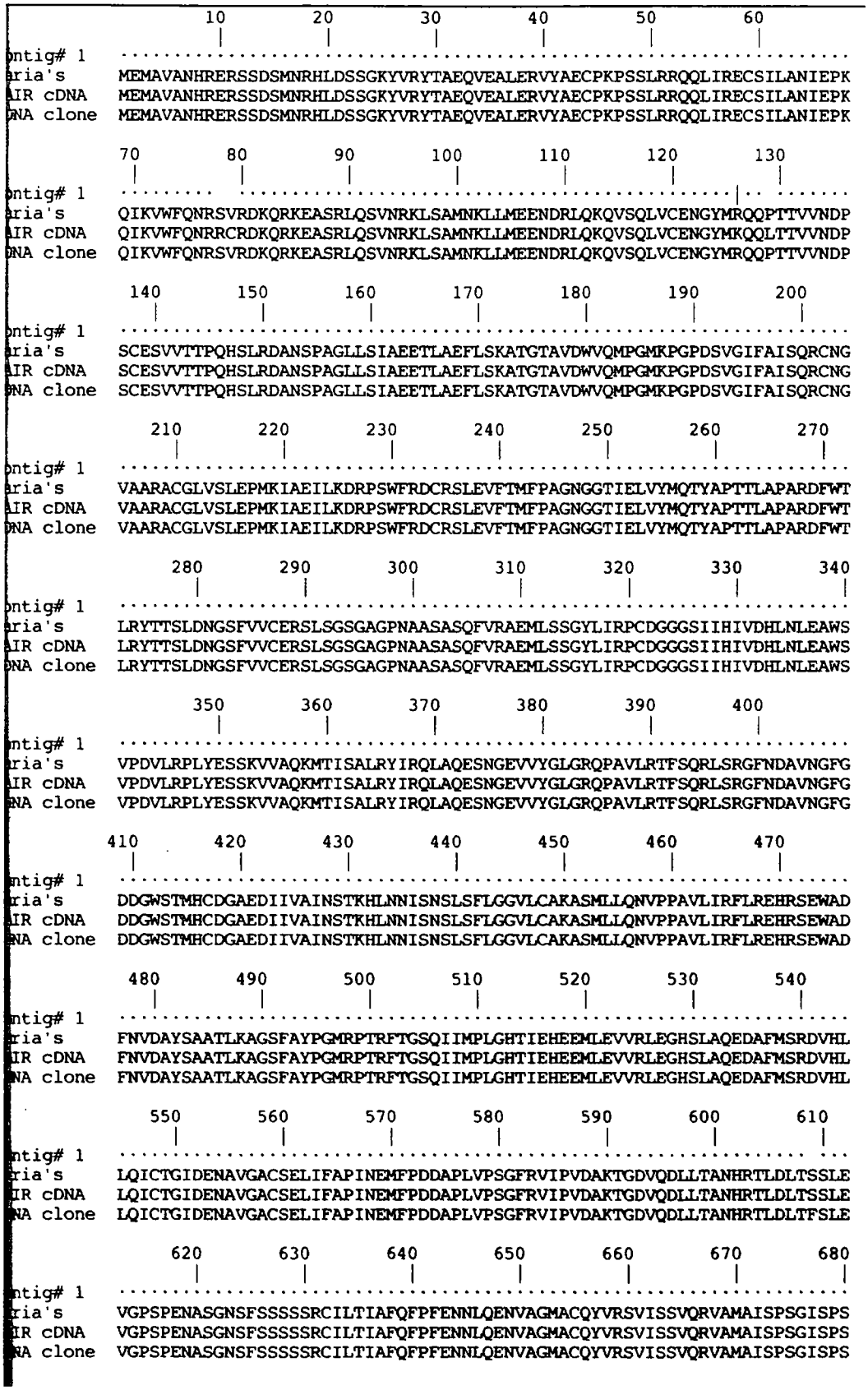
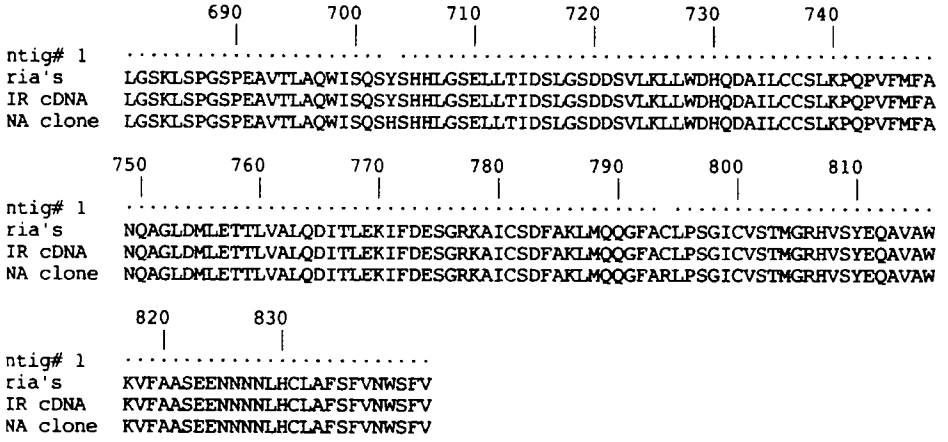

REV cDNA compared with two published sequences




690 700 710 720 730 740
ntig# 1 .....
ria's LGSKLSPGSPEAVTLAQWISQSYSHHLGSELLTIDSLGSDDSVLKLLWDHQDAILCCSLKPQPVFMFA
IR cDNA LGSKLSPGSPEAVTLAQWISQSYSHHLGSELLTIDSLGSDDSVLKLLWDHQDAILCCSLKPQPVFMFA
NA clone LGSKLSPGSPEAVTLAQWISQSHSHHLGSELLTIDSLGSDDSVLKLLWDHQDAILCCSLKPQPVFMFA
750 760 770 780 790 800 810
ntig# 1 .....
ria's NQAGLDMLETTLVALQDITLEKIFDESGRKAICSDFAKLMQQGFACLPSGICVSTMGRHVSYEQAVAW
IR cDNA NQAGLDMLETTLVALQDITLEKIFDESGRKAICSDFAKLMQQGFACLPSGICVSTMGRHVSYEQAVAW
NA clone NQAGLDMLETTLVALQDITLEKIFDESGRKAICSDFAKLMQQGFARLPSGICVSTMGRHVSYEQAVAW
820 830
ntig# 1 .....
ria's KVFAASEENNNNLHCLAFSFVNWSFV
IR cDNA KVFAASEENNNNLHCLAFSFVNWSFV
NA clone KVFAASEENNNNLHCLAFSFVNWSFV

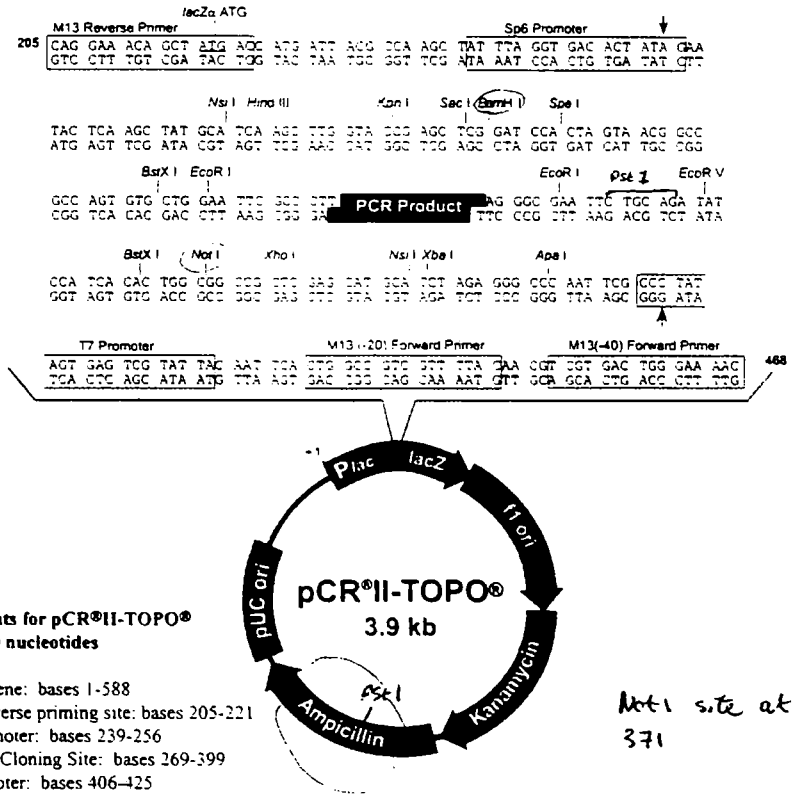
6.2. *Appendix B – Constructs and Vectors*

- pCR®II-TOPO® Plasmid
- pMP N-link GFP
- pACN (circular)
- pACN:GFP(N)
- pSRNACNbin (circular)
- pSRNSCNbin (linearised)

Map of pCR®II-TOPO®

pCR®II-TOPO® Map

The map below shows the features of pCR®II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for Sp6 and T7 polymerases. For the full sequence of the vector, you may download it from our web site or call Technical Service (page 19).



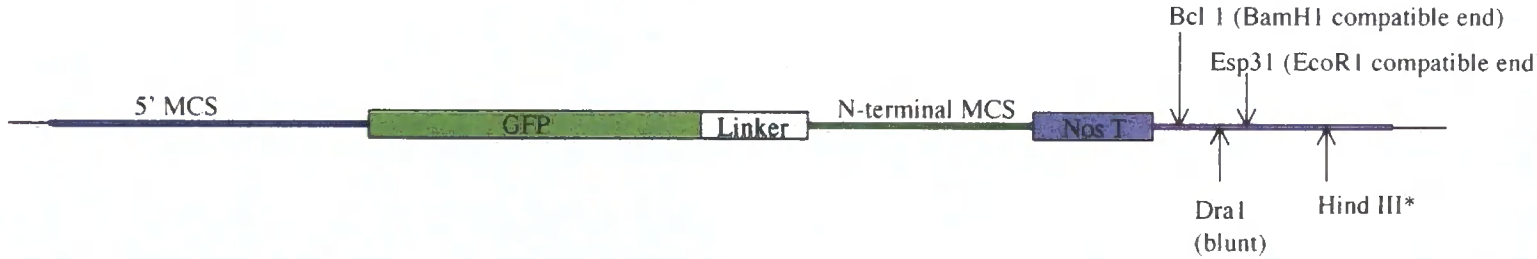
Comments for pCR®II-TOPO®
3950 nucleotides

LacZα gene: bases 1-588
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-399
T7 promoter: bases 406–425
M13 (-20) Forward priming site: bases 433–448
M13 (-40) Forward priming site: bases 453–468
f1 origin: bases 590-1004
Kanamycin resistance ORF: bases 1338-2132
Ampicillin resistance ORF: bases 2150-3010
pUC origin: bases 3155-3828

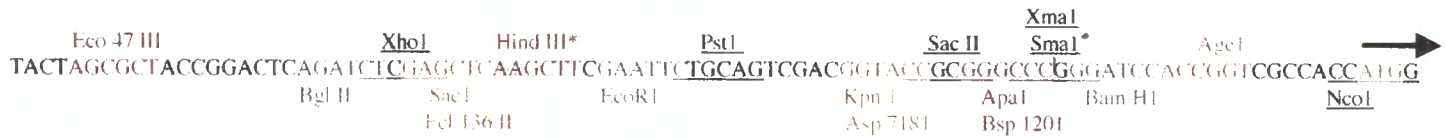
Not I site at 371

pMP-Nlink GFP

Modified GFP sequence with N-terminal linker and two unique multiple cloning sites; one for upstream promoter insertion and one for downstream N-terminal protein fusion. The Nos Terminator also has a choice of downstream restriction sites for transfer of the whole cassette into pCIRCE. The backbone is a high copy number vector, modified from the Clontech pEGFP-1 plasmid (Kan R). The completed protein fusion may be transferred into pCIRCE using 5' Bgl II (ends compatible with BamH1), BamH1, Sma1, Kpn1, Sac1, Hind III or EcoR1, coupled with sites from downstream Nos. NB ; HindIII site is not unique, being found in the 5' MCS and downstream of Nos.

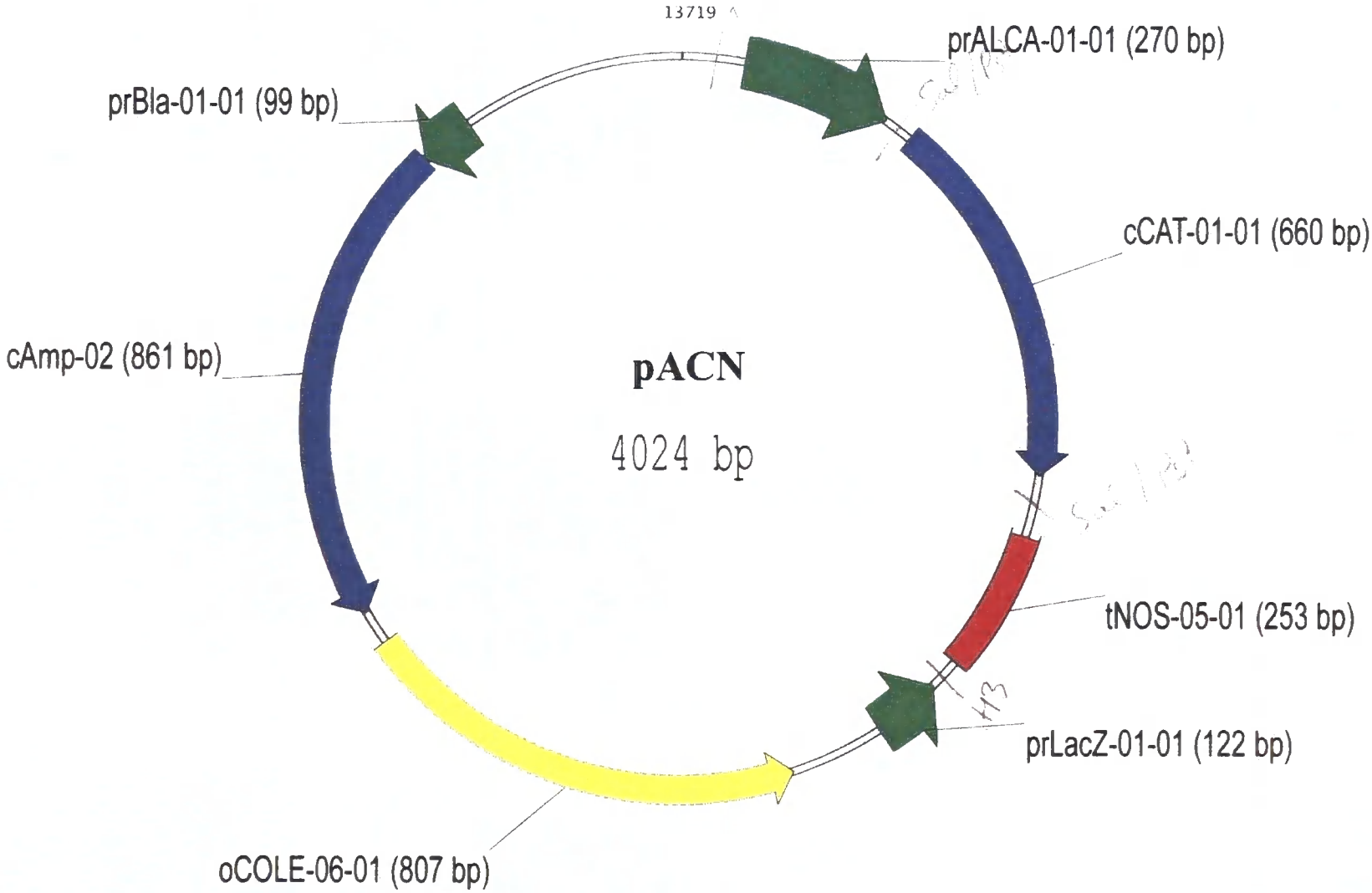


5' MCS



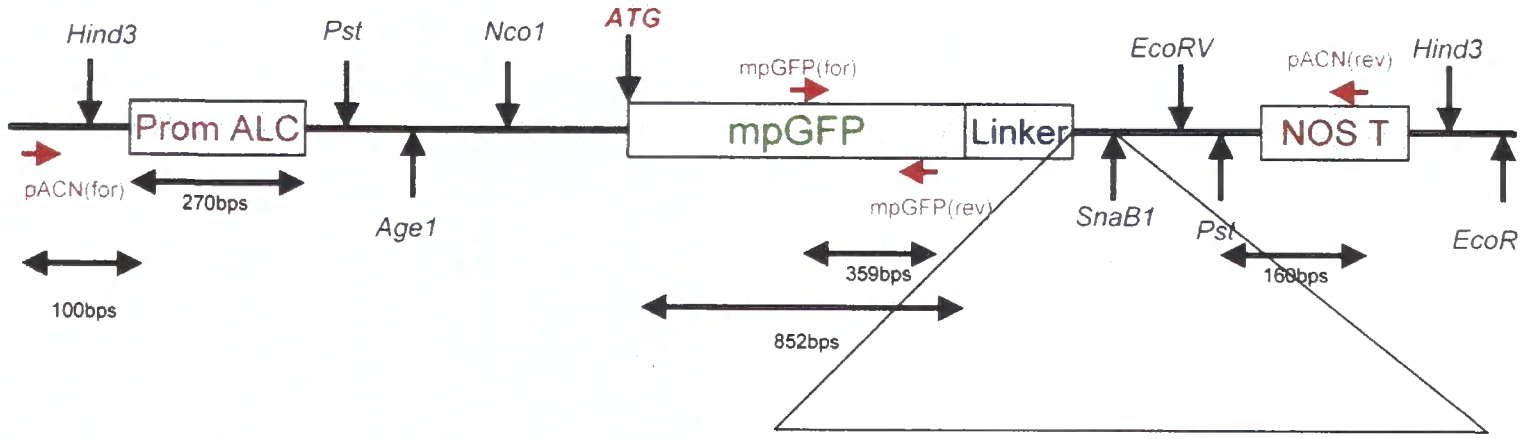
N-terminal MCS

NB stop codon in frame between SnaB1 and Spe1 sites.

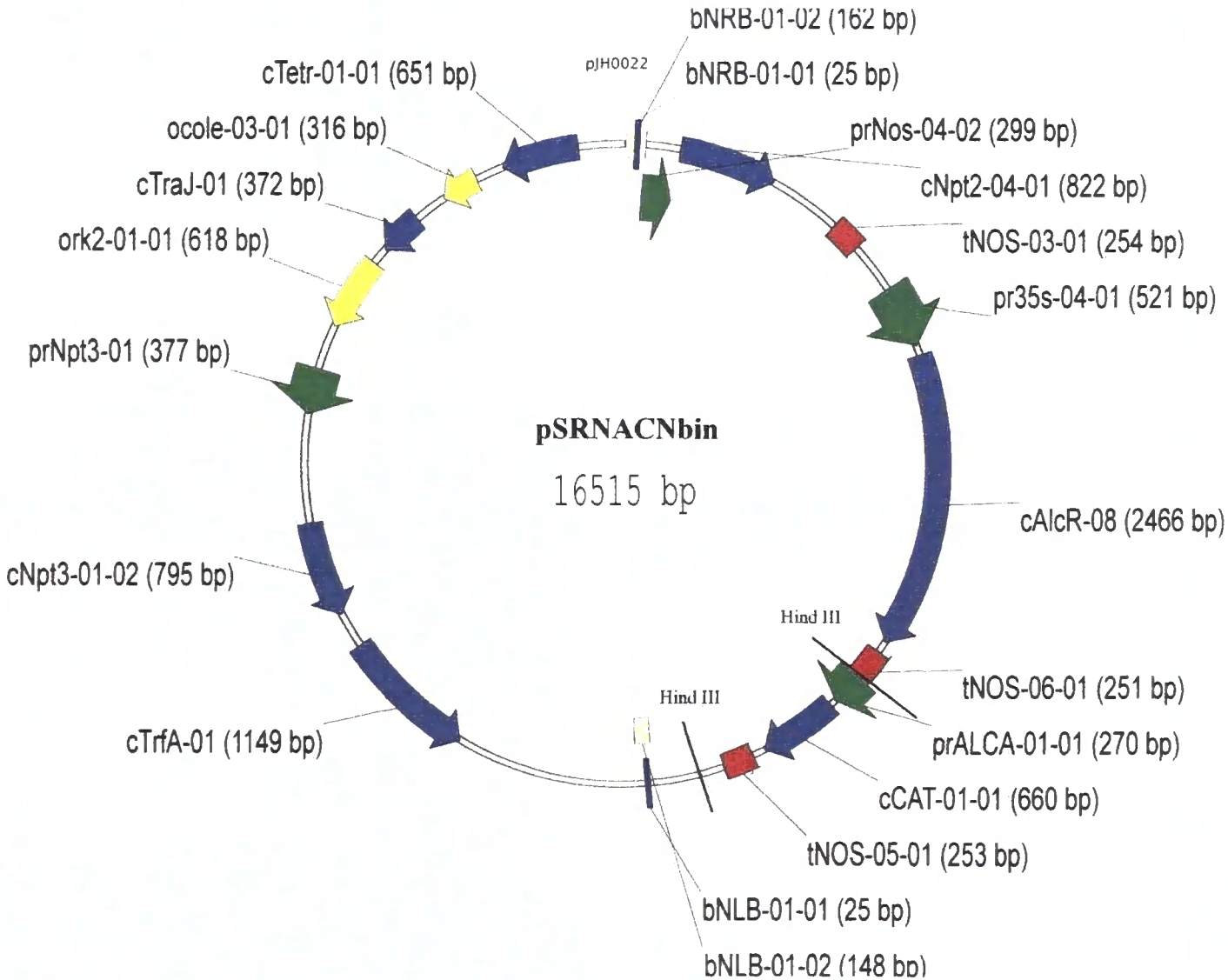

13719
prALCA-01-01 (270 bp)
prBla-01-01 (99 bp)
cCAT-01-01 (660 bp)
cAmp-02 (861 bp)
pACN
4024 bp
tNOS-05-01 (253 bp)
prLacZ-01-01 (122 bp)
oCOLE-06-01 (807 bp)

pACN:GFP(N)

This construct is derived from pACN(Pst) and comprises the ALCA promoter (270bps) driving the mpGFP from pMP-Nlink GFP (Mags 2006). Following insertion of a C-terminal sequence(at the SnaB1 site)the whole insert can only be moved as a Hind3 fragment into pSRNACNbin, therefore if a Hind3 site is present in the gene to be linked to the GFP then this construct should not be used and the gene inserted directly into pSRNACNbin:GFP. All inserts should be cloned into the SnaB1 site (blunt) to be upstream of the STOP codon (TAA). Check that the insert is in frame!

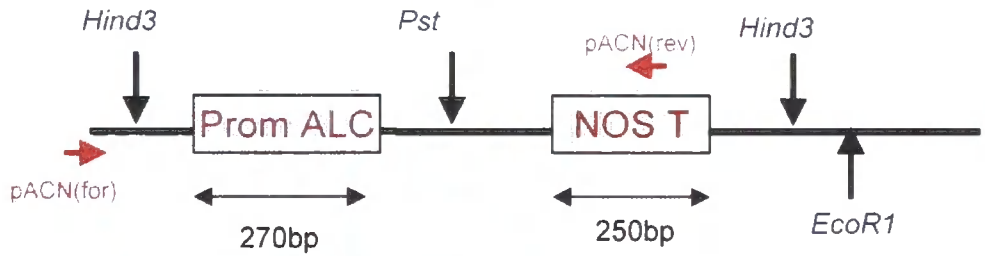


GCA.CTG.TAC.ATA.TGC.GAT.CGT.ACG.TAA.CTAGTT
(SnaB1) (STOP)


bNRB-01-02 (162 bp)
pJH0022
bNRB-01-01 (25 bp)
cTetr-01-01 (651 bp)
prNos-04-02 (299 bp)
ocole-03-01 (316 bp)
cNpt2-04-01 (822 bp)
cTraJ-01 (372 bp)
tNOS-03-01 (254 bp)
ork2-01-01 (618 bp)
pr35s-04-01 (521 bp)
prNpt3-01 (377 bp)
pSRNACNbin
16515 bp
cAlcR-08 (2466 bp)
cNpt3-01-02 (795 bp)
Hind III
tNOS-06-01 (251 bp)
Hind III
prALCA-01-01 (270 bp)
cTrfA-01 (1149 bp)
cCAT-01-01 (660 bp)
tNOS-05-01 (253 bp)
bNLB-01-01 (25 bp)
bNLB-01-02 (148 bp)

pACN (Pst)

This construct is derived from pACN and comprises the ALCA promoter (270bps) and the Nos terminator
The only site available is Pst. The cassette can be removed as a H3 fragment into pSRNACNbin(-CAT).

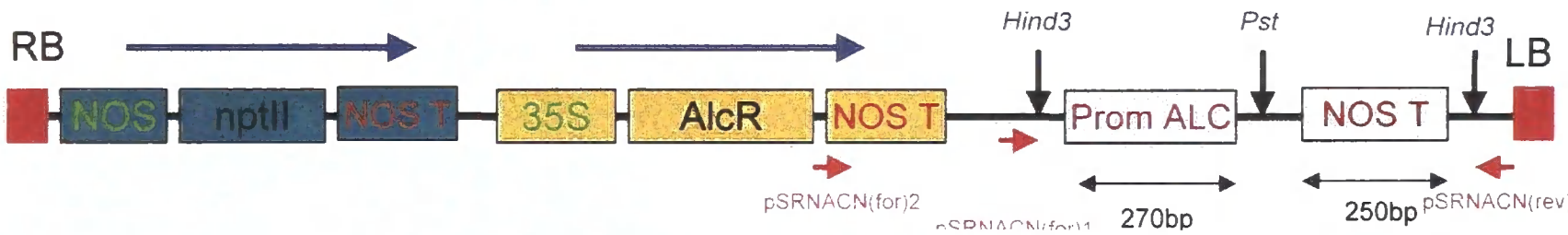


pUC based
Amp^r

pSRNACNbin(-CAT)

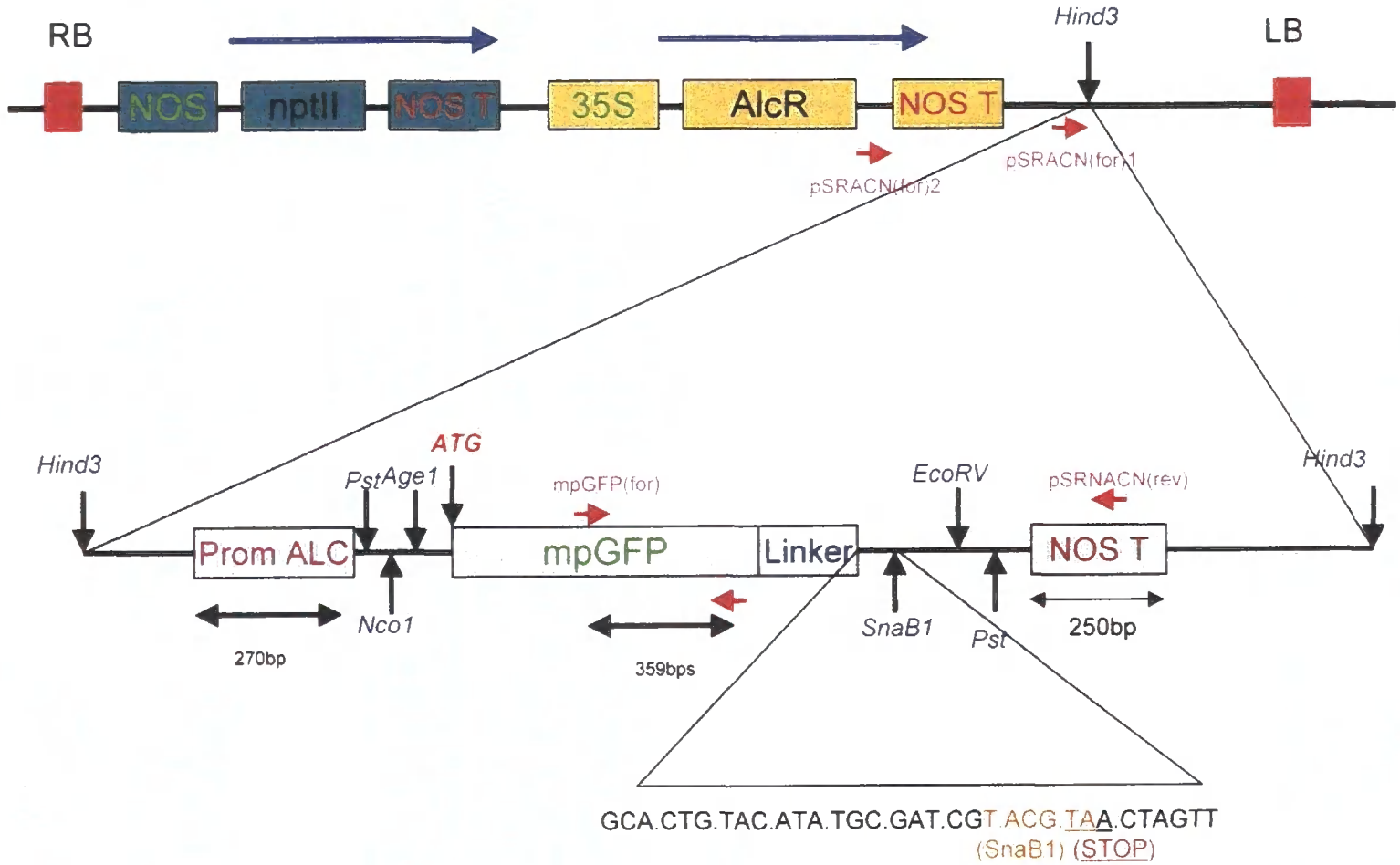
#267.

This construct is derived from pSRNACNbin, and is based on BIN19 (Kan^r)



pAI(bin)NGFP

#268



6.3. *Appendix C – Protocols*

- Expand High Fidelity PCR System

- RNeasy Plant Mini Protocol

- Wizard® Plus SV Minipreps DNA Purification System - Promega

- High Pure PCR Product Purification Kit - Roche

For general laboratory use.
FOR *IN VITRO* USE ONLY.

Expand High Fidelity PCR System

eoxynucleoside-triphosphate: DNA deoxynucleotidyltransferase, E.C. 2.7.7.7

at. No. 1 732 641 100 U for approx. 40 reactions
at. No. 1 732 650 500 U (2 × 250 U) for approx. 200 reactions
at. No. 1 759 078 2500 U (10 × 250 U) for approx. 1000 reactions

Version 4, Feb. 2003
Stable at −15 to −25° C

oduct overview

ick content

	Vial	Content
1	Expand High Fidelity Enzyme mix	• 30 µl (100 U pack size) • 2× 72 µl (500 U pack size) • 10× 72 µl (2500 U pack size) Enzyme storage buffer: 20 mM Tris-HCl, pH 7.5 (25°C), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.5% Nonidet P40 (v/v), 0.5% Tween 20 (v/v), 50% glycerol (v/v)
2	Expand High Fidelity buffer, 10× conc. with 15 mM $MgCl_2$	• 1 ml (100 U pack size) • 2×1 ml (500 U pack size) • 10× 1 ml (2500 U pack size)
3	Expand High Fidelity buffer, 10× conc. without $MgCl_2$	• 1 ml (100 U pack size) • 1 ml (500 U pack size) • 10× 1 ml (2500 U pack size)
4	$MgCl_2$ stock solution: 25 mM	• 1 ml (100 U pack size) • 1 ml (500 U pack size) • 10× 1 ml (2500 U pack size)

oduct scription

Expand High Fidelity PCR System is composed of a unique enzyme mix containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity. This powerful polymerase mixture is designed to generate PCR products of high yield, high fidelity and high specificity from all types of DNA (1). Expand High Fidelity PCR System is especially optimized to efficiently amplify DNA fragments up to 5 kb. PCR is possible up to 9 kb with yield diminishing as DNA fragment length increases.

For the generation of longer PCR products, the Expand Long Template PCR System, which is optimized for the amplification of DNA fragments ranging from 3 kb to 27 kb in length, is recommended.

Due to the inherent 3'-5' exonuclease or „proofreading" activity of Tgo DNA polymerase, the fidelity of DNA synthesis with Expand High Fidelity PCR System (error rate approx. 4.8×10^{-6}) shows a 3-fold increase compared to Taq DNA polymerase (error rate approx. 1.6×10^{-5}).

orage and ability

Stable at -15 to -25°C until the control date printed on the label.

plications

- Polymerase Chain Reaction (PCR)
- DNA labeling reactions

Enzyme properties

Volume activity	3.5 U/µl
Error rate*	approx. 4.8×10^{-6}
Optimal enzyme concentration	varies from 0.5–5 U per 50 µl reaction
Standard enzyme concentration	2.6 U (0.75 µl) per 50 µl reaction
Optimal elongation temperature	72°C. For PCR products >3 kb the optimal elongation temperature is 68°C.
Optimal Mg^{2+} concentration	varies from 1.5 – 4 mM (as $MgCl_2$)
Standard Mg^{2+} concentration	1.5 mM (as $MgCl_2$) when using 200 µM dNTP each
PCR product size	up to 5 kb
PCR cloning	TA-cloning
Repair of mismatched primers at 3' end	yes, due to the 3'-5' exonuclease activity of the proofreading polymerase
Incorporation of modified nucleotides	accepts modified nucleotides like DIG-dUTP, biotin-dUTP, fluorescein-dUTP**
Prevention of carry-over contamination	no***

* Relative fidelity determined by the lacI assay (2).

** For generating probes for Southern analysis the concentration of modified dUTP should be 50 µM (with 150 µM dTTP). When using fluorescein-dUTP the $MgCl_2$ concentration should be increased to 4 mM. For ELISA based detection systems a concentration of 10 µM modified dUTP is sufficient.

*** Unlabeled dUTP (instead of dTTP) is a poor substrate for the Expand enzyme mix. Therefore it is not recommended to use the Expand enzyme mix in combination with UNG carry over prevention.

Standard PCR procedure

General considerations

The optimal conditions (incubation times and temperatures, concentrations of enzyme, template DNA, Mg^{2+}) depend on the system used and have to be determined individually. In particular, the Mg^{2+} concentration and the amount of Expand enzyme mix used per assay should be titrated for optimal efficiency of DNA synthesis.

As a starting point, use the following guidelines:

- Optimal enzyme concentration: 0.5 – 5 U/50 µl. The recommended starting concentration is 2.6 U (0.75 µl).
- Optimal Mg^{2+} concentration can vary from 1.5 – 4 mM. The recommended starting concentration is 1.5 mM when using 200 µM dNTP (each).
- dNTP concentration: always use balanced solutions of all four dNTP. The final concentration of each dNTP should be between 50 and 500 µM; the most commonly used concentration is 200 µM. Increase concentrations of Mg^{2+} when increasing the concentration of dNTP.

- The optimal buffer for the template DNA is either simply sterile double-distilled water or 5-10 mM Tris (pH 7-8). Avoid dissolving the template in TE buffer because EDTA chelates Mg^{2+}.
- Usually it is not necessary to add additives. Nevertheless in some cases improvements can be achieved by using up to 100 µg/ml bovine serum albumin (BSA), 0.1% Tween 20 (v/v) or 1-2% DMSO.

eparation of action mixes

For a larger number of reactions, we recommend that you prepare two reaction mixes. This circumvents the need of „Hot Start" and avoids that the 3'-5' exonuclease activity of the proofreading polymerase partially degrades primers and template during the reaction set-up.

It is also recommended to prepare a Master Mix for setting up multiple reactions. The Master Mix typically contains all of the components needed for all PCR tests to be performed at a volume 10% greater than that required for the total number of PCR assays.

Step	Action
1	Briefly vortex and centrifuge all reagents before starting.
2	• Prepare two mixes of reagents in sterile microfuge tubes (on ice): • **Mix 1** (for one reaction)

Reagent	Volume	Final conc.
sterile double-dist. water	add up to 25 µl	
Deoxynucleotide mix (10 mM of each dNTP)	1 µl	200 µM of each dNTP
Upstream primer	variable	300 nM
Downstream primer	variable	300 nM
Template DNA	variable	0.1 - 250 ng[a]
Final volume	**25 µl**	

• **Mix 2** (for one reaction)

Reagent	Volume	Final conc.
sterile double-dist. water	19.25 µl	
Expand High Fidelity buffer, 10× conc. with 15 mM $MgCl_2$	5 µl	1× (1.5 mM $MgCl_2$)
Expand High Fidelity enzyme mix	0.75 µl	2.6 U/ reaction
Final volume	**25 µl**	

Note: When titrating the Mg^{2+} concentration use the Expand High Fidelity buffer, 10× conc. without $MgCl_2$ and the $MgCl_2$ stock solution (25 mM).

3	• Combine Mix 1 and Mix 2 in a thin-walled PCR tube (on ice). • Gently vortex the mixture to produce a homogeneous reaction, then centrifuge briefly to collect sample at the bottom of the tube. ***Note:*** Overlay the reaction carefully with mineral oil if required by your type of thermal cycler.

[a] e.g. human genomic DNA template: 10 ng-250 ng; plasmid DNA template: 0.1 ng - 15 ng.

Thermal cycling

Place samples in the thermal cycler, and start cycling using the thermal profile below. The gradually increasing extension time ensures a higher yield of amplification products.

Note: The elongation step should be performed at **68°C** when PCR products longer than 3 kb are amplified.

	Temp.	Time	Cycle No.
Initial denaturation	94°C	2 min	1 x
Denaturation Annealing Elongation	94°C 45°C-65°C[b] 72°C or 68°C[c]	15 s 30 s 45 s - 8 min[d]	10 x
Denaturation Annealing Elongation	94°C 45°C-65°C[b] 72°C[c]	15 s 30 s 45 s - 8 min[d] + 5 s cycle elongation for each successive cycle[e]	15 - 20 x
Final elongation	72°C	7 min	1 x
Cooling	4°C	unlimited time	

[b] Optimal annealing temperature depends on the melting temperature of the primers and on the system used.

[c] For PCR products up to 3 kb elongation temperature should be at 72°C; for PCR products larger than 3 kb elongation temperature should be at 68°C.

[d] Elongation time depends on fragment length: 45 s for up to 0.75 kb, 1 min for 1.5 kb, 2 min for 3 kb, 4 min for 6 kb, 8 min for 10 kb.

[e] For example, cycle no. 11 is 5 s longer than cycle 10, cycle no. 12 is 10 s longer than cycle 10, cycle no. 13 is 15 s longer than cycle 10, etc.

Note: The thermal profiles were developed for the Applied Biosystems GeneAmp PCR System 9600. Other thermal cyclers may require different profiles.

Trouble shooting

Little or no PCR product

Possible cause	Recommendation
Difficult template e.g. GC-rich templates	• Use the GC-RICH PCR System. • Perform initial denaturation step at 95°C for 3 - 5 min.
Poor DNA template quality	Check quality and concentration of template: • Analyze an aliquot on an agarose gel to check for possible degradation. • Make a control reaction on template with an established primer pair or PCR system. • Check or repeat purification of template.
Enzyme concentration too low	Increase the amount of enzyme mix in 0.5 U steps.
$MgCl_2$ concentration too low	Increase the $MgCl_2$ concentration in steps of 0.25 mM. (1.5 mM $MgCl_2$ is the minimal concentration.)
Cycle conditions not optimal	• Reduce annealing temperature. • Increase cycle number. • Make sure that the final elongation step was carried out.
Primer design not optimal	Design alternative primers.
Primer concentration not optimal	• Both primers must have the same concentration. • Titrate primer concentration (0.2 - 0.6 µM).
Annealing temperature too high	• Reduce annealing temperature (minimal annealing temperature is 45°C). • Determine the optimal annealing temperature by touch-down PCR.

continued on next page

otice to urchaser

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to order, solve technical queries, find product information, or contact your local sales representative.

www.roche-applied-science.com/pack-insert/1732641a.pdf

Roche Diagnostics GmbH
Roche Applied Science
Nonnenwald 2
82372 Penzberg
Germany

RNeasy Plant Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues, and Filamentous Fungi

Important notes before starting

- Total RNA isolation from plant cells and tissues and filamentous fungi requires the RNeasy Plant Mini Kit and cannot be performed with the RNeasy Mini Kit alone.
- A maximum of 100 mg plant material or 1 x 10_7 cells can be used per preparation. See "How much starting material can I use?" (page 12) for more information.
- If using RNeasy for the first time, please read "Disruption and homogenization of starting materials" (page 16).
- If preparing RNA for the first time, please read Appendix A (page 54).
- The RNeasy Plant Mini Kit provides two different lysis buffers, Buffer RLT and Buffer RLC, which contain guanidine isothiocyanate (GITC) or guanidine hydrochloride (GuHCl), respectively. In most cases Buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of GITC. However, depending on the amount and type of secondary metabolites in some tissues (such as milky endosperm of maize or mycelia of filamentous fungi), GITC can cause solidification of the sample, making extraction of RNA impossible. In these cases Buffer RLC should be used.
- Buffer RPE is supplied as a concentrate. Before using for the first time add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT or Buffer RLC before use (see page 20). Add 10 µl β-ME per 1 ml of Buffer RLT or Buffer RLC. The solution is stable for 1 month.
- The time between harvesting of tissue and freezing should be minimized. Once tissue is frozen, do not allow to thaw.
- After disruption, all steps of the RNeasy protocol should be performed at 20 to 25°C. During the procedure, work quickly.
- All centrifugation steps should be performed in a microcentrifuge at 20 to 25°C.

1. Grind sample under liquid nitrogen to a fine powder using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube, and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Continue immediately with step 2.

Note: Incomplete grinding of the starting material will lead to reduced RNA yields.

2. Add 450 µl of either Buffer RLT or Buffer RLC (see notes above) to a maximum of 100 mg of tissue powder. Vortex vigorously.

A short (1–3 min) incubation at 56°C may help to disrupt tissue. However for samples with high starch content, incubation at elevated temperatures should be omitted to prevent swelling of the starting material.

Note: Ensure β-ME is added to Buffer RLT or Buffer RLC before use (see "Important notes before starting").

3. Apply lysate to the QIAshredder spin column (lilac) sitting in a 2-ml collection tube, and centrifuge for 2 min at maximum speed. Transfer flow-through fraction from QIAshredder to a new tube (not supplied) without disturbing the cell-debris pellet in the collection tube.

It may be necessary to cut the end off the pipet tip to apply the lysate to the QIAshredder spin column. This centrifugation through QIAshredder removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIAshredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet while transferring the lysate to a new tube (not supplied).

4. Add 0.5 volumes (usually 225 µl) ethanol (96–100%) to the cleared lysate and mix well by pipetting.

If some lysate is lost during homogenization, reduce volume of ethanol proportionally. A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

*See optional step listed at the end of this protocol.

5. Apply sample (usually 675 µl), including any precipitate which may have formed, onto an RNeasy mini spin column (pink) sitting in a 2-ml collection tube. Centrifuge for 15 sec at ≥8000 x g (≥10,000 rpm).

If the volume of the mixture exceeds 700 µl, load aliquots successively onto the

RNeasy column and centrifuge as above. Reuse the same collection tube but discard flow-through* after each step. Reuse the collection tube in step 6.
* Flow-through contains Buffer RLT and is therefore not compatible with bleach.
RNeasy Mini Handbook 05/99 **47**

6. Pipet 700 µl Buffer RW1 onto the RNeasy column, and centrifuge for 15 sec at ≥8000 x g (≥10,000 rpm) to wash.
Discard flow-through* and collection tube.
7. Transfer RNeasy column into a new 2-ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at ≥8000 x g (≥10,000 rpm).
Discard flow-through and reuse the collection tube in step 8.
Note: Ensure ethanol is added to Buffer RPE before use (see "Important notes before starting").
8. Add 500 µl Buffer RPE to the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane. Continue directly with step 9, or to eliminate any chance of possible Buffer RPE carryover, continue first with step 8a.
It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. This spin ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.
Note: Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.
8a. (Optional): Place the RNeasy spin column in a new 2-ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
9. Transfer RNeasy column into a new 1.5-ml collection tube (supplied), and pipet 30–50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute. Repeat if the expected RNA yield is >20 µg.
If a second elution step is performed, elute into the same collection tube using another 30–50 µl RNase-free water.
* Flow-through contains Buffer RW1 and is therefore not compatible with bleach

*** DNase Step (Optional)**

a. Add 10µl DNase solution from Qiagen RNase-Free DNase Set to 70µl buffer RDD from same and mix gently without vortexing.
b. Add the 80µl DNase mix to the membrane taking care to avoid any of the mix sticking to the sides.
c. Incubate at room temperature for 15 minutes.

Wizard® *Plus* SV Minipreps DNA Purification System

INSTRUCTIONS FOR USE OF PRODUCTS A1330, A1340, A1460 AND A1470.

Quick PROTOCOL

Centrifugation Protocol

Production of Cleared Lysate

1. Pellet 1–10ml overnight culture for 5 minutes.
2. Thoroughly resuspend pellet with 250µl Cell Resuspension Solution.
3. Add 250µl Cell Lysis Solution to each sample; invert 4 times to mix.
4. Add 10µl Alkaline Protease Solution; invert 4 times to mix. Incubate 5 minutes at room temperature.
5. Add 350µl Neutralization Solution; invert 4 times to mix.
6. Centrifuge at top speed for 10 minutes at room temperature.

Binding of Plasmid DNA

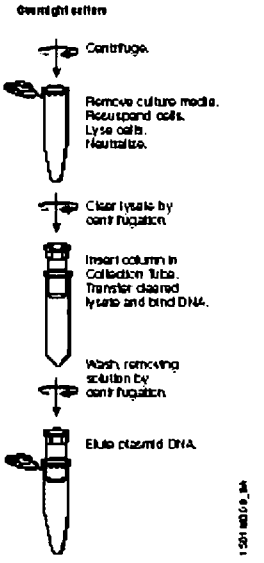
7. Insert Spin Column into Collection Tube.
8. Decant cleared lysate into Spin Column.
9. Centrifuge at top speed for 1 minute at room temperature. Discard flowthrough and reinsert Column into Collection Tube.

Washing

10. Add 750µl Wash Solution (ethanol added). Centrifuge at top speed for 1 minute. Discard flowthrough and reinsert column into Collection Tube.
11. Repeat Step 10 with 250µl Wash Solution.
12. Centrifuge at top speed for 2 minutes at room temperature.

Elution

13. Transfer Spin Column to a sterile 1.5ml microcentrifuge tube.
14. Add 100µl of Nuclease-Free Water to the Spin Column. Centrifuge at top speed for 1 minute at room temperature.
15. Discard column and store DNA at –20°C or below.



Additional product information is available in Technical Bulletin #TB225 available upon request from Promega or online at www.promega.com

ORDERING/TECHNICAL INFORMATION:
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High Pure PCR Product Purification Kit - Roche

2.3 Purification of PCR Products in Solution after Amplification
In the following protocol the purification of PCR products in solution after amplification is described.
L To process a larger sample (>100 _l), either increase proportionally the amount of Binding Buffer (Step 1), or divide the larger sample into several 100 _l aliquots and process each as a separate sample.
_ After PCR is complete, adjust total volume for each PCR tube (reaction components + DNA product) to 100 _l:
• Add 500 _l Binding Buffer to each 100 _l PCR tube.
N Mineral oil or wax do not need to be removed from the PCR solution before adding the Binding Buffer.
• Mix sample (Binding Buffer + PCR solution) well.
_ • Insert one High Pure Filter Tube into one Collection Tube.
• Transfer the sample from step 1 using a pipette to the upper reservoir of the Filter Tube.
• Centrifuge 30 - 60 s at maximum speed in a standard table top centrifuge at +15 to +25°C.
_ • Disconnect the Filter Tube, and discard the flowthrough solution.
• Reconnect the Filter Tube to the same Collection Tube.
_ • Add 500 _l Wash Buffer to the upper reservoir.
• Centrifuge 1 min at maximum speed (as above).
_ • Discard the flowthrough solution.
• Recombine the Filter Tube with the same Collection Tube.
• Add 200 _l Wash Buffer.
• Centrifuge 1 min at maximum speed (as above).
N This second 200 _l wash step ensures optimal purity and complete removal of Wash Buffer from the glass fibers.
_ • Discard the flowthrough solution and Collection Tube.
• Reconnect the Filter Tube to a clean 1.5 ml microcentrifuge tube.
_ • Add 50 - 100 _l Elution Buffer to the upper reservoir of the Filter Tube.
• Centrifuge 1 min at maximum speed.
N Do not use water for elution since alkaline pH is required for optimal yield.
_ The microcentrifuge tube now contains the purified DNA.
N When subsequent OD_{260} determination is planned, centrifuge the eluate for more than 1 min at maximum speed to remove residual glass fibers from the eluate, because they may disturb absorbance measurement. Use an aliquot of the supernatant to determine concentration.
L Either use the eluted DNA directly or store the eluted DNA at +2 to +8°C or _15 to _25°C for later analysis.

2.4 Purification of DNA Fragments from Agarose Gel
In the following table the purification procedure for DNA from a 100 mg agarose gel slice is described:
_ Isolate DNA band of interest electrophoretically as follows.
• Load PCR reaction mixture on a 0.8 - 2% agarose gel.
• Use 1 × TAE or 1 × TBE as running buffer.
• Electrophorese until DNA band of interest is isolated from adjacent contaminating fragments
_ Identify bands by staining gel with ethidium bromide or SYBR Green I Nucleic Acid Gel Stain*.
N Wear gloves, ethidium bromide is a known potent carcinogen.
_ Cut desired DNA band from gel using an ethanol-cleaned scalpel or razor blade.
N Minimize gel volume by visualizing DNA and cutting the smallest possible gel slice on a UV light box.
_ • Place excised agarose gel slice in a sterile 1.5 ml microcentrifuge tube.
• Determine gel mass by first pre-weighting the tube, and then reweighting the tube with the excised gel slice.

_ Add 300 _l Binding Buffer for every 100 mg agarose gel slice to the microcentrifuge tube.
_ • Dissolve agarose gel slice in order to release the DNA:
• Vortex the microcentrifuge tube 15 - 30 s to resuspend the gel slice in the Binding Buffer.
• Incubate the suspension for 10 min at 56°C.
• Vortex the tube briefly every 2 - 3 min during incubation.
_ After the agarose gel slice is completely dissolved:
• Add 150 _l isopropanol for every 100 mg agarose gel slice to the tube.
• Vortex thoroughly.
_ • Insert one High Pure Filter Tube into one Collection Tube.
• Pipette the entire contents of the microcentrifuge tube into the upper reservoir of the Filter Tube.
N Do not exceed 700 _l total volume. If mixture is > 700 _l, split the volume and use two separate Filter Tubes for each portion.
Centrifuge 30 - 60 s at maximum speed in a standard table top centrifuge at +15 to +25°C.
• Discard the flowthrough solution.
• Reconnect Filter Tube with the same Collection Tube.
_ • Add 500 _l Wash Buffer to the upper reservoir.
• Centrifuge 1 min at maximum speed (as above).
_ • Discard the flowthrough solution.
• Recombine Filter Tube with the same Collection Tube.
• Add 200 _l Wash Buffer.
• Centrifuge 1 min at maximum speed.
L This second 200 _l wash step ensures optimal purity and complete removal of Wash Buffer from the glass fibers.
• Discard the flowthrough solution and Collection Tube.
• Recombine Filter Tube with a clean 1.5 ml microcentrifuge tube.
_ • Add 50 - 100 _l Elution Buffer to the upper reservoir of the Filter Tube.
• Centrifuge 1 min at maximum speed.
_ The microcentrifuge tube now contains the purified DNA.
N When subsequent OD_{260} determination is planned, centrifuge the eluate for more than 1 min at maximum speed to remove residual glass fibers from the eluate, because they may disturb absorbance measurement. Use an aliquot of the supernatant to determine concentration.
L Either use the eluted DNA directly or store the eluted DNA at +2 to +8°C or _15 to _25°C for later analysis.

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