The role of the POLARIS peptide in ethylene signalling and root development in Arabidopsis thaliana

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Abstract

The plant hormones ethylene, auxin and cytokinin have a pivotal role in plant growth, including differential cell elongation and division, tissue patterning, root development and apical hook formation. The *POLARIS (PLS)* gene in *Arabidopsis thaliana* is critical for correct signalling and crosstalk between these hormones and encodes a 36 amino acid PLS peptide which acts to negatively regulate the ethylene signalling pathway, subsequently mediating root growth and development. PLS is expressed in the tips of primary and lateral roots, and it has been previously shown to bind to the ethylene receptor ETR1 in yeast and onion cells. ETR1 has been previously characterised and was shown to be localised to the endoplasmic reticulum (ER) membrane, requiring a copper ion for correct ethylene binding function and transduction of the ethylene signal.

In addition to previous work revealing the ethylene-mediated downregulation of the *PLS* gene, work in this thesis demonstrates that the expression and localisation of the PLS peptide are regulated by ethylene in the *A. thaliana* root. It is revealed that the PLS peptide localises to the ER in root cells, where it interacts with the *A. thaliana* ETR1 protein. Evidence is presented which highlights the importance of copper ions in the role of the PLS peptide. The PLS N-terminus is essential for correct peptide activity in *A. thaliana* seedlings and notably requires the presence of two cysteine residues that have the potential to coordinate a metal ion. Interestingly, the PLS/ETR1 interaction is evidently enhanced in the presence of copper ions. Moreover, the loss-of-function *pls* mutant exhibits altered responses to copper perturbations and there is strong evidence that the PLS peptide can coordinate copper ions *in vitro*. Therefore, it is proposed here that the PLS peptide regulates copper ion availability to the ETR1 receptor protein at the ER, mediating ethylene receptor function and downstream ethylene responses, and consequently acting to regulate root development and growth.

Declaration

I confirm that the original research described in this thesis is my own work.

However, in the event that work was carried out by others, appropriate credit has been given within this thesis wherever reference has been made to such work. It should be noted that the *pPLS::PLS:GFP* and *pPLS::GFP* constructs and the subsequent stably-transformed *Arabidopsis* lines used for localisation studies in Chapter 4 were made by Dr. Saher Mehdi (Durham University) prior to this project. The pBIN2 vector containing *RFP:HDEL* used in Chapter 4 was a kind gift from Dr. Pengwei Wang (Durham University). Also in Chapter 4, the *pB7WG2-SH-GFP* construct was created by Piers Hemsley (Durham University at that time). The protein extraction, co-immunoprecipitation experiments, and western blotting in Chapter 5 were performed out by Dr. Beatriz Orosa Puente (Durham University). The copper binding assays using the PLS peptide were carried out by Dr. Andrew Foster (Durham University).

No material contained in this thesis has been submitted for the award of a higher degree elsewhere.

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Statement of Copyright

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List of Abbreviations

5-FAM	5-Carboxyfluorescein
ACC	1-aminocyclopropane-1-carboxylic acid
AO	acridine orange
APS	ammonium persulphate
BCA	bicinchoninic acid
BCS	bathocuproine disulphonic acid
CLSM	confocal laser scanning microscopy
d.a.g	days (of plant growth) after germination
DCM	dichloromethane
dH_2O	distilled water
DIPEA	*N,N*-diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid) – Ellman's reagent
EB	ethidium bromide
EBD	ethylene binding domain (in an ethylene receptor protein)
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
Fmoc	9-fluorenylmethoxycarbonyl (amino acid protecting group)
GFP	green fluorescent protein
GOF	gain-of-function (mutant)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
KOH	potassium hydroxide

LB	lysogeny broth (bacterial medium)
LOF	loss-of-function (mutant)
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MeCN	acetonitrile
MeOH	methanol
NaCl	sodium chloride
ORF	open reading frame (DNA)
PCR	polymerase chain reaction
PI	propidium iodide
PLS/*PLS*/*pls*	POLARIS peptide/gene/loss-of-function mutant
PyBOP®	(benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
QC	quiescent centre
RFP	red fluorescent protein
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPPS	Solid-phase peptide synthesis
TEMED	*N,N,N',N'*-tetramethylethylenediamine
TFA	trifluoroacetic acid
TIPS	triisopropylsilane

Chapter 1 . Introduction

1.1 *Arabidopsis thaliana* – studying root development in a model system

The plant hormones auxin, ethylene, cytokinin, abscisic acid, gibberellin and brassinosteroid act within complex hormone signalling networks to mediate responses to stress, pathogens and environmental stimuli, and are vital for the correct growth and development of plant tissues throughout the lifetime of the plant.

Previous work has shown that a small plant peptide POLARIS plays a role in hormone signalling in *Arabidopsis thaliana* roots (Casson et al., 2002; Chilley et al., 2006; Liu et al., 2010). Gene expression studies have found that the *POLARIS* gene is regulated by a number of different plant hormones, and appears to be a regulatory element in several pathways leading to growth of the root. The role of the POLARIS peptide needed to be elucidated further in *Arabidopsis thaliana* (henceforth referred to as *Arabidopsis*) to determine its cellular localisation, interacting partners, key protein domains and detailed gene expression.

Arabidopsis is an annual flowering dicot belonging to the *Brassicaceae* family. Its short generation time and ease of growth have led to *Arabidopsis* becoming popular as a plant model organism in research. Most importantly, it has a small sequenced genome (~135 Mbp), fully published in 2000 (*Arabidopsis* Genome, 2000), enabling routine genetic modification and gene expression investigations. The annotated chromosome sequences have helped to characterize many similar genes from other plant species, with particular value for functional gene analysis in commercial crop species (Meinke et al., 1998).

The *Arabidopsis* embryo has been well characterized. Knowledge of key embryogenesis events such as early cell divisions and gene expression provide a platform for studying dicot embryo development, although it must be noted that the *Arabidopsis* embryo is atypical of dicots in general (Chandler et al., 2008). The *Arabidopsis* root is derived from one of two embryonic daughter cells, formed by a division of the zygote, and regulated by gene patterning (De Smet and Jurgens, 2007). Plant hormones are important factors in developmental and growth responses to environmental stimuli. Although single hormone pathways have been studied in isolation, elucidating the mechanisms of signal transduction

and gene expression, it is apparent that developmental outputs are modified by a network of hormonal interactions (Benkova and Hejatko, 2009).

1.1.1 Architecture of the *Arabidopsis thaliana* embryo

Embryogenesis in plants generates a basic body organization, as opposed to a small version of the final adult body. Plant postembryonic development is subject to environmental regulation and continues from two separate stem cell systems (shoot and root; (Fonseka et al., 2013). Plants form polarized axes during embryogenesis, upon which organ patterning and cell differentiation can occur. During *Arabidopsis* embryogenesis, establishment of an apical-basal axis allows populations of stem cells to accumulate, from which the shoot and root originate (Smith and Long, 2010). Secondary axes are derived from this primary axis, and are the basis of all lateral organs, such as lateral roots, side shoots and leaves, whilst the radial axis is perpendicular to the primary axis and represents the concentric layers of tissues in the root (De Smet and Jurgens, 2007).

Patterning in the *Arabidopsis* embryo gives rise to founder cells for the primary root. The apical-basal axis of polarity correlates with the asymmetric division of the zygote, generating two daughter cells that differ in size, gene expression, and fate (De Smet and Jurgens, 2007). The apical cell engenders almost the entire embryo, whereas the basal cell produces a few extra-embryonic cells. Through perpendicular shifts during three rounds of cell divisions, the apical daughter cell becomes an eight-cell embryo with four regions with different developmental fates (Fonseka et al., 2013). The apical embryo domain continues to become the shoot meristem and cotyledons whilst the central embryo will predominantly become the hypocotyl and root (Laux et al., 2004). The uppermost cell from the basal daughter cell, the hypophysis, adopts an embryonic fate and thus initiates root meristem formation (Weijers and Jurgens, 2005), eventually giving rise to the quiescent centre (QC) and the columella root cap (Scheres et al., 1994). The fourth embryonic domain consists of extra suspensor cells which provide a connection to the mother tissue.

The eight-cell embryo undergoes cell division and enters several sequential embryo phases: the 16-cell embryo, early and late globular stages, a transition stage into a heart stage embryo, and then maturity into the shoot, hypocotyl and root tissues (Laux et al., 2004). A condensed sequence is illustrated in Figure 1-1. The phytohormone auxin (discussed in more detail later) plays a crucial role in embryonic patterning and the creation of the apical-basal axis.

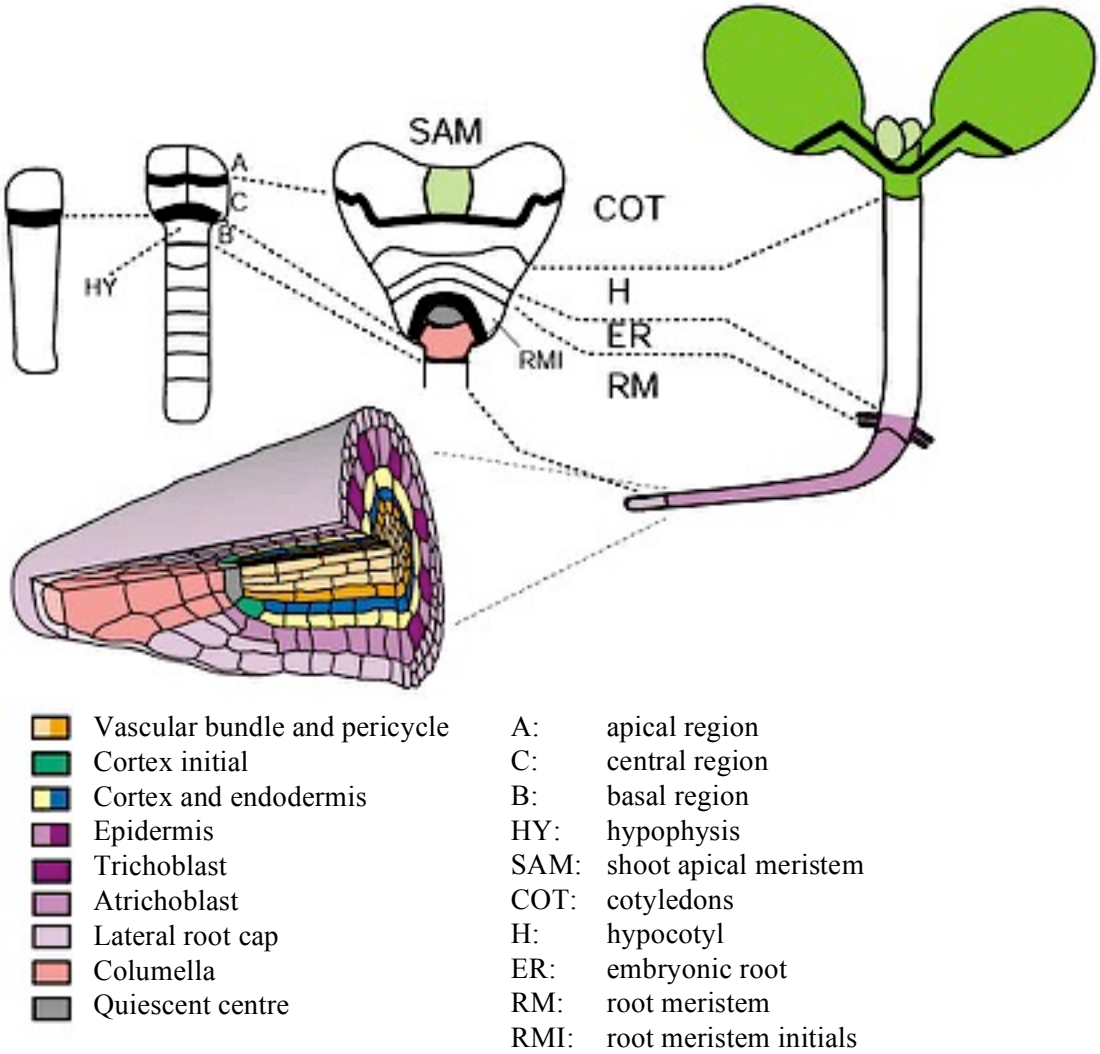


Figure 1-1. The embryonic origin of the *Arabidopsis* root. From left to right: the first asymmetric zygote division, eight-cell embryo, heart stage embryo, and the resulting seedling. The root meristem region is enlarged showing the radial patterning of different root cell types (Scheres et al., 2002).

1.1.2 Architecture and growth of the *Arabidopsis* root

Along the longitudinal axis (Figure 1-2), the root meristem forms a distal root tip, including a stem cell niche, columella and lateral root cap, a proximal meristem with a population of rapidly dividing cells, an elongation zone, where cells moving out of the root meristem undergo rapid highly-polarized longitudinal expansion (where the 'growth' of the root occurs) and finally a differentiation zone in which cells mature and become fully differentiated (Dolan et al., 1993). This root meristem organization is completed during postembryonic development, creating a balance of the rate of generation of new cells, and the differentiation of daughter cells, giving a meristem of stable size (Dolan et al., 1993; Dello Ioio et al., 2007).

There are four radially symmetrical distinct cell files in a transverse root section (Figure 1-1): epidermis is the most external layer, then cortex, endodermis and the pericycle in the centre, surrounding the vascular tissue (phloem, xylem and procambium) (Scheres et al., 1995). The vascular tissue and pericycle together are termed the stele.

Four types of stem cell initials at the root tip undergo stereotyped divisions to generate all other cell types in the root, which then differentiate and expand (Dolan et al., 1993). As root growth is continuous throughout the life of the plant, the root continues to have all its developmental stages present at all times. Patterning is not confined to embryogenesis, but continues to produce new structures like lateral roots along the growing primary root and initiates secondary body axes (De Smet and Jurgens, 2007).

Root cell files clearly originate from their own initials, which divide asymmetrically to produce self-renewing cells and daughter cells. The epidermal/lateral root cap initials generate the epidermis and the lateral root cap on the outside of the root tip. The columella, the central root cap, has its own set of initials, the cortex and endodermis are created from the cortex/endodermis initials and the vascular tissue and the pericycle also have their own initials. All root initial cells are in physical contact with the four mitotically relatively inactive cells which make up the quiescent centre (QC) and remain under the influence of a short-range signal from the QC to prevent differentiation and maintain their stem cell status (Dolan et al., 1993; vandenBerg et al., 1997; van den Berg et al., 1998).

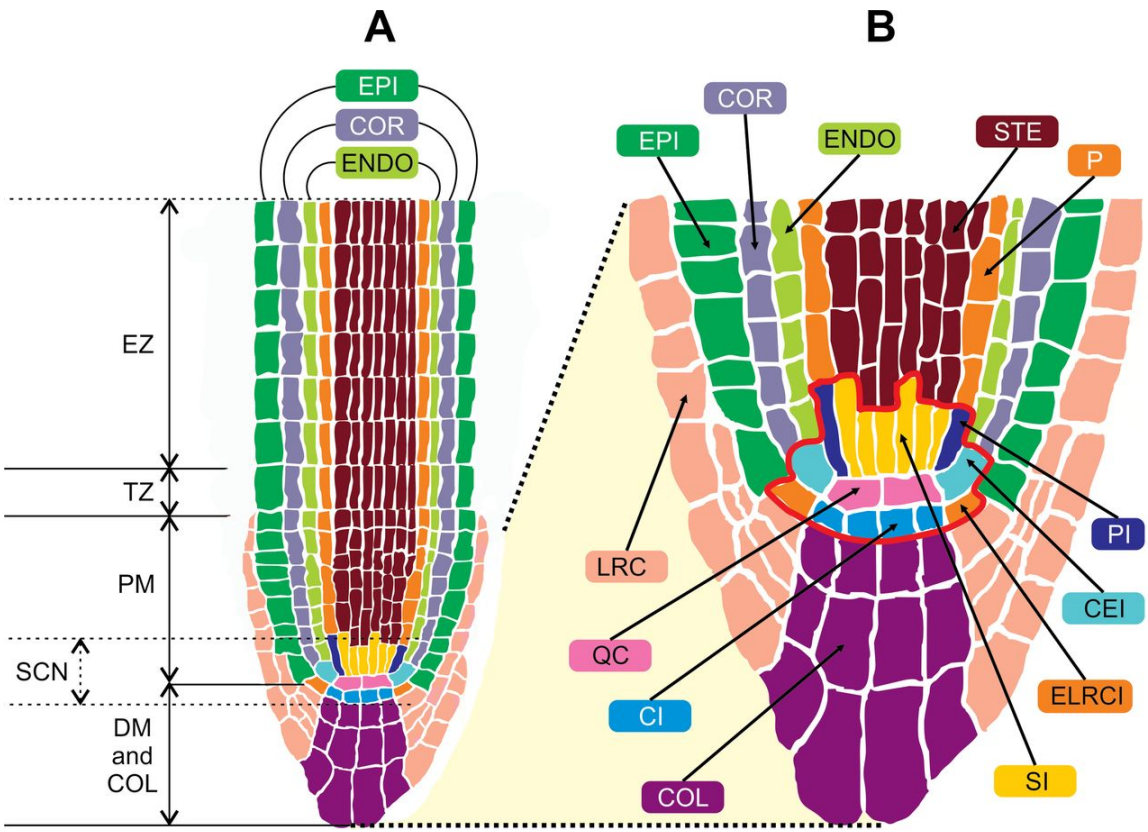


Figure 1-2. Structure of the *Arabidopsis* root. (A) Schematic longitudinal section of the *Arabidopsis* root. There are three distinct developmental zones: the meristematic zone (MZ), the transition zone (TZ), and the elongation zone (EZ). The meristematic zone can be divided into the distal meristem (DM) and the proximal meristem (PM). In the meristematic zone, there is a 'stem cell niche' (SCN) that consists of the QC and initials (stem cells). (B) Longitudinal section of the *Arabidopsis* root tip. The area enclosed with the red line shows the SCN. Around the QC, there are four initials (root stem cells). QC, quiescent centre (pink); CEI, cortex/endodermis initials (light teal); ELRCI, epidermis/lateral root cap initials (orange); CI, columella initials (sky blue); SI, stele initials (yellow); LRC, lateral root cap (peach); EPI, epidermis (green); COR, cortex (lilac); ENDO, endodermis (light green); STE, stele (dark brown). (Lee et al., 2013).

1.1.3 Gene expression in the root

Organization of the root meristem along the longitudinal axis is to a significant extent under the control of the plant hormone auxin. An auxin gradient is established along the root, with a maximum close to the QC, providing developmental positional information. A family of auxin-related *PLETHORA (PLT)* (*APETALA2*-like transcription factor) genes are master regulators of cell fate in the root and are expressed in the root meristem throughout embryo development, with *PLT* expression following the auxin gradient along the meristem and producing the auxin maximum in the stem cell niche. High *PLT* activity promotes stem cell identity and maintenance, whereas low activity encourages mitotic activity of stem cell daughters, with even lower levels needed for cell differentiation (Aida et al., 2004; Galinha et al., 2007).

In tandem with the *PLT* genes, the SHORTROOT/SCARECROW (SHR/SCR) pathway acts to regulate radial patterning in the root, and the two pathways combine to regulate the specificity and function of the stem cell niche (Benkova and Hejatko, 2009). Plants with the *shr* and *scr* mutations are unable to divide the cortex/endodermis initial daughter cell, and therefore form a single layer of ground tissue, instead of two (Benfey et al., 1993; Scheres et al., 1995).

1.2 Auxin and development

Communication between cells is important for axis formation and patterning. Local signalling, such as secreted peptide ligands and transcription factors transported between cells, and long-range signalling by small non-peptide molecules or phytohormones help to convey patterning information throughout the embryo (De Smet and Jurgens, 2007). As touched upon in the previous section, the phytohormone auxin plays a crucial role in the patterning of the embryo and root.

Auxin, or indole-3-acetic acid (IAA), regulates a broad range of root developmental mechanisms: the whole process of root organogenesis has been shown to be regulated in some part by auxin, from a critical role right at the beginning in the establishment of root polarity (Friml et al., 2003; Weijers et al., 2006) and positioning and formation of the stem cell niche (Sabatini et al., 1999; Blilou et al., 2005), to the maintenance of cell division in the meristem (Beemster and Baskin, 2000; Dello Ioio et al., 2007; Galinha et al., 2007;

Stepanova et al., 2008) and the elongation and differentiation of cells leaving the meristematic zone (Rahman et al., 2007). Auxin also has roles in responding to light and gravity, control in shoot apices, initiation of new root meristems and vascular tissue patterning (Hardtke and Berleth, 1998).

Auxin is synthesized in the aerial regions of the plant and is subsequently transported down to the root, acting as a signal to coordinate development of plant tissue, such as the growth of new leaves with the initiation of new roots; and there is another site of auxin synthesis in the root (Stepanova et al., 2005; 2008). Auxin can either be a versatile intercellular messenger, or confer fundamental cell patterning information, determining cell differentiation and the position and size of existing tissues throughout the life of the plant (Berleth and Sachs, 2001).

A key process in the regulatory capacity of auxin is the auxin gradient established along the longitudinal axis of the root meristem (Sabatini et al., 1999; Friml et al., 2002; Benkova et al., 2003). Root-generated auxin contributes to the maintenance of the gradients and auxin maxima required for normal root development (Ljung et al., 2005). A local auxin maximum forms just distal to the QC in the root meristem and is required for distal position-dependent specification (Scheres et al., 2002). Shifts in the localization of this maximum correlates with shifts in the pattern of meristematic cell fates. Regularly spaced auxin signals in the root act to programme pericycle cells associated with xylem poles to become lateral root primordia, in a root region near the meristem termed the ‘zone of competence’ (De Smet et al., 2007). Further into lateral root primordium development, the auxin flow shifts, changing the growth axis of the main root, and auxin starts to be transported to the new root tip (Benkova et al., 2003; Sauer et al., 2006).

Auxin is transported within the root by the polar auxin transport (PAT) pathway, involving the AUX/LAX (AUXIN/LIKE AUX1) auxin influx proteins (Bennett et al., 1996; Ljung et al., 2001), the PIN-FORMED (PIN) auxin efflux protein family (Friml et al., 2002; Friml et al., 2003; Blilou et al., 2005; Weijers et al., 2005; Petrasek et al., 2006), and some members of the multi-drug-resistant/P-glycoprotein (MDR/PGP) subfamily of ATP-binding cassette (ABC) proteins (Blakeslee et al., 2007). If auxin transport is disrupted, plants show substantial defects in the patterning and development of the root meristem (Sabatini et al., 1999; Friml et al., 2002; Blilou et al., 2005).

Polar auxin transport creates auxin gradients in the root via the asymmetric membrane localisations of the PIN auxin efflux proteins and AUX family influx proteins (Wisniewska et al., 2006). The polar localisation of PIN proteins to cell membranes can be rapidly modulated in response to external or developmental cues. PINs are cycled to and from plasma membrane locations by reversible phosphorylation targeting by the serine/threonine protein kinase PINOID (PID) and protein phosphatase 2A (PP2A) and continuous GNOM ARF GEF-dependent endosomal trafficking (Geldner et al., 2003). *GNOM* encodes a **G**DP/GTP **e**xchange **f**actor for small G proteins of the ARF class (ARF-GEF; (Steinmann et al., 1999). Auxin itself can regulate PIN and PID expression, and therefore mediates PIN polarity (Benjamins et al., 2001; Sauer et al., 2006).

Large gene families have been found to be involved in auxin signal transduction, providing a network of sufficient complexity to allow auxin to promote numerous independent downstream signalling messages (Kim et al., 1997; Guilfoyle et al., 1998). The auxin signal is communicated throughout the root via TIR/AFB (TRANSPORT INHIBITOR RESPONSE/AUXIN BINDING PROTEIN) auxin receptors in the F-box protein family (Dharmasiri et al., 2005), over 29 AUX/LAX negative regulators (Overvoorde et al., 2005), and over 23 ARF (AUXIN RESPONSE FACTORS) transcription factors (Okushima et al., 2005), which activate the expression of downstream genes. In root development, the specific pair of IAA12/BDL (BODENLOS) and ARF5/MP (MONOPTEROS) transcription factors are involved in auxin signalling and establishing the root pole in early embryogenesis (Hamann et al., 2002).

Both the auxin/PLT and SHR/SCR pathways have links with other plant hormonal pathways and there is a network of hormone signalling that contributes to root development. One of the direct targets of SHR is involved in brassinosteroid synthesis (Shimada et al., 2003), and another in gibberellin signalling (Levesque et al., 2006). The hormone cytokinin acts antagonistically to auxin to regulate cell division and differentiation in the root (Dello Ioio et al., 2007). Another key hormone, ethylene, has a key role in the regulation of root growth and participates in the regulation of QC cell division to maintain the stem cell niche (Ortega-Martinez et al., 2007). Ethylene plays a large part in every aspect of the work described in this thesis and is discussed in detail below.

1.3 Ethylene and development

The phytohormone ethylene (ethene, C_2H_4) can diffuse into many plant tissues and is involved in a wide range of complex developmental and regulatory processes. Throughout the lifetime of the plant, ethylene has roles in growth, apical hook formation, seed germination, organ senescence, abscission, fruit ripening, gravitropism and response to stresses (Abeles et al., 1992). Plants sense ethylene from their environments, but can also synthesise it themselves from derivatives of the amino acid methionine during the Yang cycle (Adams and Yang, 1979). The rate-limiting step is the conversion of *S*-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (ACS), before ACC is converted to ethylene (Kende, 1993).

In this project, attention was focussed on the role of ethylene in plant root development. *Arabidopsis thaliana* seedlings grown in the dark and in the presence of ethylene display a characteristic "triple response" phenotype and have short, hairy roots, a shortened and expanded hypocotyl, and an exaggerated apical hook (Knight et al., 1910; Ecker, 1995; Johnson and Ecker, 1998). The native triple response is induced by greater environmental ethylene levels or increased ethylene biosynthesis, promoted by physical obstruction to the seedling (Goeschl et al., 1966).

Ethylene-mediated modulation of developmental processes is closely linked with the reciprocal regulatory action of the plant hormone auxin. Ethylene primarily affects root growth by inhibiting the rapid expansion of cells exiting the root meristem via an auxin-dependent mechanism (Le et al., 2001; Ruzicka et al., 2007; Swarup et al., 2007). Ethylene has a role in the maintenance of the quiescent centre and its stem cell niche by regulating the balance of stem cell proliferation and quiescence. Plants with high ethylene responses display supernumerary cell divisions in the quiescent centre, and vice versa in mutants with a loss of ethylene signalling (Ortega-Martinez et al., 2007). Ethylene has also been shown to be required for correct epidermal cell patterning (Cao et al., 1999).

1.4 Crosstalk and feedback mechanisms between auxin and ethylene

There is a large amount of evidence for the relationship and crosstalk between just auxin and ethylene, regardless of the inputs of the other plant hormones. At the simplest level, ethylene can reduce auxin responses and transport (Morgan and Gausman, 1966; Suttle, 1988; Haver et al., 2002). Evidence for the cooperation of auxin and ethylene has been well documented in the regulation of hypocotyl elongation (Vandenbussche et al., 2003), root hair growth and differentiation (Pitts et al., 1998), apical hook formation (Li et al., 2004), root gravitropism (Buer et al., 2006), and root growth (Rahman et al., 2001). Ethylene plays a role in root meristem maintenance, either directly or mediated by auxin (Ortega-Martinez et al., 2007).

Several auxin-related genes are mediated by ethylene in their relevant tissues including the auxin biosynthesis genes *ASA1/WEI2/TIR7 (ANTHRANILATE SYNTHASE α1/WEAK ETHYLENE INSENSITIVE2/TRANSPORT INHIBITOR RESPONSE7), ASB1/WEI7 (ANTHRANILATE SYNTHASE β1/WEI7),* and *TAA1/SAV3/WEI8 (TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/SHADE AVOIDANCE3/WEI8;* (Stepanova et al., 2005; Stepanova et al., 2008)*,* the auxin transport genes *PIN1, PIN2, PIN4, AUX1* and some of the auxin response genes, *ARF2* and *ARF19* (Li et al., 2004).

Mutations in the *Arabidopsis* genes *ASA1* and *ASA2*, encoding the alpha subunit of the anthranilate synthase enzyme that synthesizes an auxin precursor, confer ethylene insensitive root growth phenotypes (Stepanova et al., 2005). These auxin components are downstream of the ethylene signal transduction pathway, suggesting that auxin biosynthesis, signalling and transport are required for the ethylene inhibition of root growth (Roman et al., 1995; Stepanova et al., 2005).

A model was proposed in which ethylene stimulates auxin biosynthesis and increases the auxin transport capacity of the plant, by regulating transport component transcription, including the upregulation of *PIN1, PIN2* and *AUX1* genes (Ruzicka et al., 2007). The higher levels of auxin are transported to the root elongation zone, mediated by AUX1 and PIN2, where its accumulation induces local responses that inhibit cell elongation and therefore hinder overall root growth (Ruzicka et al., 2007; Stepanova et al., 2007; Swarup et al., 2007).

Both auxin and ethylene have intertwining roles with another plant hormone, cytokinin. Rapid expansion of cells in the root transition zone appears to be under the control of at

least auxin, ethylene and cytokinin, and there is evidence of feedback control mechanisms between the hormones; auxin regulates both cytokinin biosynthesis (Nordstrom et al., 2004), and ethylene biosynthesis (Tsuchisaka and Theologis, 2004). Auxin biosynthesis is also partially inhibited by cytokinin (Nordstrom et al., 2004)

Cytokinin can negatively regulate PIN protein levels (Ruzicka et al., 2009), while repressing auxin biosynthesis and promoting ethylene responses (Nordstrom et al., 2004; Chandler et al., 2008; Liu et al., 2010). Cytokinin also has the capacity to modulate auxin transport by transcriptional regulation of the *PIN* genes (Ruzicka et al., 2009), and can regulate expression of genes involved in the auxin signalling pathway (*SHY2-2/IAA3, AXR3/IAA17* or *SAUR-AC1*) (Rashotte et al., 2005).

1.5 The ethylene signalling pathway

Many experiments described in this thesis will refer to components of the ethylene signalling pathway. The proteins, signal transduction mechanisms and ethylene signalling mutant plant lines are discussed in more detail in this section.

At least two classes of ethylene binding site were discovered and characterized in plants in the late 1970s and early 1980s, revealing a capacity for high affinity ethylene binding in cell membrane compartments in a variety of plant species (Sisler, 1979; Sisler and Filka, 1979; Evans et al., 1982b; Evans et al., 1982a; Sanders et al., 1991).

Studies found that nanomolar concentrations of ethylene were sufficient to promote ethylene-related physiological responses (Abeles et al., 1992), indicating the existence of high-affinity receptors, and components involved in the ethylene signalling pathway started to be identified by screening for mutant seedlings displaying ethylene-related growth defects (Bleecker et al., 1988; Guzman and Ecker, 1990; Kieber et al., 1993; Roman et al., 1995).

One such example is the identification of a mutated form of the *ETO1 (ETHYLENE-OVERPRODUCER1)* gene thanks to the increased triple response in the *eto1* mutant plants (Guzman and Ecker, 1990). *eto1* seedlings overproduce ethylene, revealing that the associated protein must play a role in this pathway. The ETO1 protein is a ubiquitin E3 ligase which negatively regulates the activity of the ethylene biosynthesis gene ACS5 (section 1.3; (Wang et al., 2004) with a key role in regulating ethylene production.

To react to the internally synthesised or environmental levels of ethylene, the plant needs to recognise ethylene molecules and respond accordingly.

1.5.1 The ethylene receptor family in *Arabidopsis*

1.5.1.1 Structure

The effects of ethylene in *Arabidopsis* are mediated by a family of five receptor protein isoforms (Figure 1-3): ETR1, ETR2, EIN4, ERS1 and ERS2 (ETHYLENE RESPONSE1, ETHYLENE RESPONSE2, ETHYLENE INSENSITIVE4, ETHYLENE RESPONSE SENSOR1 and ETHYLENE RESPONSE SENSOR2; (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998).

The five proteins share 57-79% sequence homology with each other, with the N-terminal region having greatest similarity, suggesting possible conserved ethylene binding activity in this domain (Hua and Meyerowitz, 1998), with all five receptors capable of binding ethylene (O'Malley et al., 2005).

The *Arabidopsis* receptors contain N-terminal transmembrane domains, followed by a GAF (c**G**MP-specific phosphodiesterases, **a**denylyl cyclases, **f**ormate hydrogen lyase transcriptional activator) domain and a protein kinase domain at the cytoplasmic C-terminus, sharing some sequence similarity with bacterial two-component histidine kinase regulators (Chang et al., 1993). The structures of the receptor proteins differ most at the C-terminus: ETR1, ETR2 and EIN4 have both a putative histidine (His) kinase domain and a receiver domain whereas ERS1 and ERS2 lack the receiver domain (Hua and Meyerowitz, 1998). The receptors are generally grouped into two subfamilies according to their His kinase activity: ETR1 and ERS1 (subfamily I) show His kinase activity *in vitro* (Gamble et al., 1998; Moussatche and Klee, 2004), whereas ETR2, ERS2 and EIN4 (subfamily II) contain degenerate His kinase domains but have Ser/Thr kinase activity *in vitro* (Hua et al., 1998; Sakai et al., 1998), although ERS1 is unusual and exhibits both kinase activities (Moussatche and Klee, 2004). At the gene sequence level, intron positions are conserved between receptors in the same subfamily, but not between the two subfamilies (Hua et al., 1998).

The role of the His kinase domain in receptor function is disputed. Some evidence showed that ETR1 required the presence of the C-terminal His kinase domain in order to function and it may have a role in receptor inactivation when ethylene binds (Qu and Schaller,

2004) but this domain was not found to be required for transduction of the ethylene binding signal (Wang et al., 2003). The His kinase domain may have roles in other mechanisms. *Arabidopsis* ETR1 His kinase activity appears to modulate growth (Qu and Schaller, 2004; Cho and Yoo, 2007) and ethylene sensitivity, along with the receiver domain (Qu and Schaller, 2004). ETR1, ETR2 and EIN4 receptors, all containing the receiver domain, were revealed to play a more important role in the recovery from ethylene-mediated growth inhibition than ERS1 and ERS2 (Binder et al., 2004b).

The five receptors exhibit some differences at the N-terminus. Subfamily I have three transmembrane (TM) domains but subfamily II have four (Kendrick and Chang, 2008), with the extra TM domain possibly functioning as a localisation signalling sequence (Wang et al., 2006).

The receptors form homodimers at their N-termini, stabilised via two disulphide bonds between conserved cysteine residues (Schaller et al., 1995; Hall et al., 2000), although the disulphide bonds are not required for ethylene binding (Chen et al., 2010), or for functional receptor proteins (Xie et al., 2006).

Evidence suggests that the GAF domain is also important for receptor dimersation and may have a role in communicating between different receptor types in large heteromeric receptor complexes (Gao et al., 2008) as all five receptors have been shown to be present in higher order multimeric protein complexes (Chen et al., 2010), allowing the ethylene signal to be propagated and amplified by lateral interactions. Receptors appear to interact with each other via non-covalent interactions in a number of surprising combinations, including the subfamily I ETR1 receptors seeming to preferentially complex with subfamily II receptors over ERS1 (Gao et al., 2008), as well as the ability of the truncated N-terminus of ETR1 (amino acids 1-349) to mediate ethylene signalling by interacting with native receptors and modulating their activity (Xie et al., 2006).

This higher order clustering may help explain why *Arabidopsis* expresses five receptors which appear to perform similar roles and show functional redundancy (Hua and Meyerowitz, 1998). Ethylene treatment is able to affect the interactions and the composition of the complexes (Gao et al., 2008), indicating that varying growth conditions can promote the formation of different receptor complexes which could participate in non-overlapping downstream signalling pathways (Chen et al., 2010). Furthermore, RNA *in situ* hybridisation data suggest that the receptor genes are differentially expressed in some

tissues (Hua et al., 1998). This could explain how plants can detect variable concentrations of ethylene, ranging from <1 nl/l to 1000 µl/l, and mediate differential signal outputs (Grefen et al., 2008), allowing for fine control over the sensitivity of the ethylene response in tissue development (Hua and Meyerowitz, 1998).

The family of redundant receptors may also have been retained if the proteins have other ethylene-pathway-independent roles in the plant (Hua and Meyerowitz, 1998) and there is a hypothesised model in which the ethylene receptors have overlapping but distinct roles in mediating ethylene signalling (Binder, 2008). ETR1 also appears to have an ethylene-independent role in promoting cell elongation (Hua and Meyerowitz, 1998).

In addition, the five receptors are differentially regulated by ethylene at the transcriptional level: RNA levels of *ETR1* and *EIN4* are not significantly regulated by ethylene, whereas *ERS1, ERS2* and *ETR2* are upregulated upon the presence of ethylene (Hua et al., 1998). This could be a method of regulating downstream ethylene responses by producing a greater number of receptor proteins to repress the ethylene responses, whilst ethylene itself is activating the same pathway. However, although the abundance of the *ETR2* transcript was increased by ethylene, the actual receptor protein levels were decreased (Chen et al., 2007). Similar observations were found in tomato during fruit ripening: although transcript levels increase as fruit ripening proceeds; the tomato LeETR3, LeETR4 and LeETR6 receptor protein abundance declines (Kevany et al., 2007). Both studies found that ethylene perception is required for the breakdown of the ethylene receptors in these cases, and inhibitors of 26S proteasome function block this degradation (Chen et al., 2007; Kevany et al., 2007).

Increased turnover of the receptor proteins could mediate ethylene sensitivity. The receptors have non-overlapping roles in ethylene signalling, so differential control of receptor levels provides another level of control of ethylene responses. This seems to be the case with LeETR4 and LeETR6, which have roles in fruit ripening and are controlled post-transcriptionally (Tieman et al., 2000; Kevany et al., 2007).

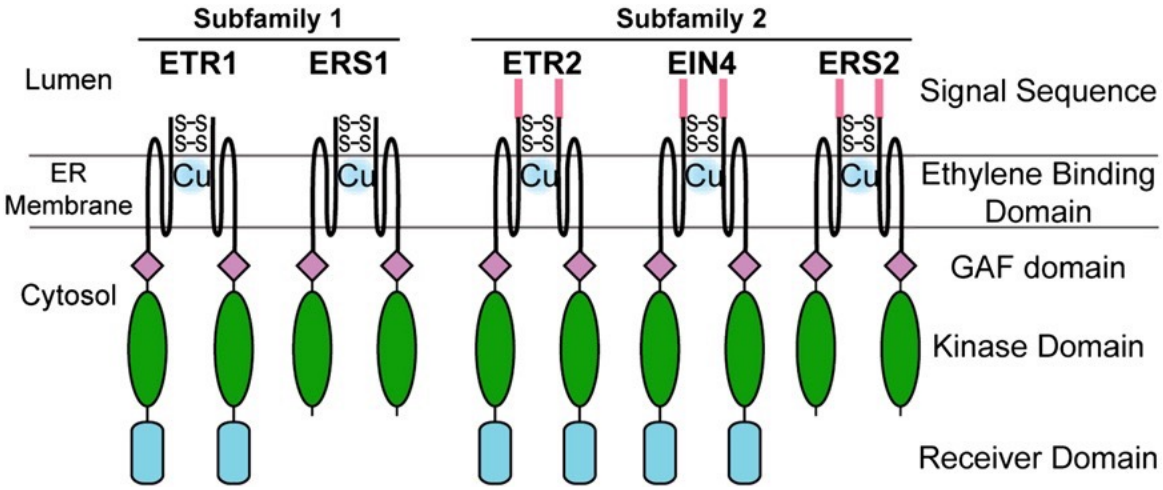


Figure 1-3. The ethylene receptor family in *Arabidopsis.* The ethylene receptors fall into two subfamilies. Each receptor monomer has three transmembrane domains in the endoplasmic reticulum membrane, followed by a GAF domain, histidine kinase domain and, in three members of the family, a receiver domain. The subfamily II receptors also contain an N-terminal signal sequence. Receptor monomers form dimers, stabilised by two disulphide bonds at the N-terminus, creating an ethylene binding pocket and a region that can coordinate copper ions (Lacey and Binder, 2014).

There is evidence that the subfamily I receptors play a more dominant role in ethylene perception and signalling, with the subfamily II receptors having a cooperative role to enhance signalling (Binder and Bleecker, 2003). Loss-of-function (LOF) mutations in one of the subfamily I receptors cause a slight increase in ethylene sensitivity and are predominantly compensated for by the other subfamily I receptor, whereas loss of both members results in a strong constitutive ethylene response phenotype (Qu et al., 2007). Equally, the phenotype of a severe double subfamily I mutant *etr1-7;ers1-2* can only be rescued by subfamily I receptors (Wang et al., 2003). LOF mutations in subfamily II receptors produce plants with phenotypes indistinguishable from the wild type and subfamily II receptors may be dependent on subfamily I for their function (Qu et al., 2007).

Specific gain-of-function (GOF) mutations in any of the five receptor proteins confer dominant ethylene insensitivity in the plant, demonstrating all contribute to ethylene signalling (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998; Hall et al., 1999). The original GOF *etr1* mutant alleles (*etr1-1* to *etr1-4*) are dominant and exhibit reduced ethylene binding capacity (saturable ethylene binding in *etr1-1* decreased to one fifth of that measured in wild type plants; (Bleecker et al., 1988) which results from missense mutations in the hydrophobic N-terminal ethylene binding domain (Chang et al., 1993). The dominant phenotypes of all four receptor mutations are similar to that of *etr1* mutants, with only slight differences between mutant alleles (which may result from different expression levels of those genes; (Hua and Meyerowitz, 1998). Due to these dominant characteristics, it was only when loss-of-function (LOF) alleles were identified that the mode of action of the receptors could be established. *etr1* LOF mutants (*etr1-5, etr1-6, etr1-7, etr1-8)* have triple responses similar to that of the wild type, with greatly reduced hypocotyl lengths at high ethylene concentrations, showing ethylene sensitivity like that of the wild type (Hua and Meyerowitz, 1998). Double, triple and quadruple LOF receptor mutants show constitutive ethylene responses, revealing that the receptors function as negative regulators of the ethylene signalling pathway (Hua and Meyerowitz, 1998).

The idea that the receptors form multimeric complexes may explain the dominant GOF phenotypes of the single receptor mutation. A mutant dominant-active receptor could maintain the signalling state of other native receptors (see section 1.5.3 for details of

receptor signalling states), resulting in continued repression of ethylene responses (Binder, 2008).

1.5.1.2 Subcellular localisation

All five receptor proteins localise to the endoplasmic reticulum in *Nicotiana benthamiana* (tobacco) and *Arabidopsis* (Chen et al., 2002; Grefen et al., 2008) via their N-terminal transmembrane (TM) domains, with their localisation remaining unchanged upon ethylene binding.

Arabidopsis ETR1 has also been shown to localise to the Golgi apparatus (Dong et al., 2008) which may be as a result of a continuum between the two organelles (Hawes and Satiat-Jeunemaitre, 2005). The Golgi apparatus is required for some ethylene-mediated processes; cell wall synthesis components are produced in the Golgi (Lerouxel et al., 2006) and are required for cell elongation and expansion, often regulated by ethylene, which could require local ethylene recognition by ETR1.

Examples of ethylene receptor proteins from other species have been found at various cell locations. The tobacco NTHK1 (Nicotiana tabacum histidine kinase-1) ethylene receptor (subfamily II) appears to localise at the plasma membrane (PM; (Xie et al., 2003), unpublished work by Klee and Tieman (University of Florida) suggests that the tomato NEVER-RIPE receptor (subfamily I) may also localise to the PM (Dong et al., 2008), while the melon ethylene receptor CmERS1 (subfamily I) has been localised to the endoplasmic reticulum (Ma et al., 2006).

1.5.2 The ethylene receptor proteins require a copper cofactor

The structural requirements for the biological activity of olefin molecules (also known as alkenes and characterised by a double carbon-carbon bond at the alpha position) suggested that plant cells would require a transition metal ion, such as copper or zinc, in order to recognise ethylene, one of the simplest olefins (Burg and Burg, 1967). A consideration of the organometallic chemistry of the biologically important transition metals alongside investigations into Cu^{+}-monoolefin complexes revealed that the coordination chemistry of copper ions was consistent with the proposed role of the metal ion in ethylene binding, with an indication that Cu^{+} was required rather than Cu^{2+} (Thompson et al., 1983). More

recently, all five ethylene receptors were found to require one copper ion per receptor homodimer for correct function (Rodriguez et al., 1999; McDaniel and Binder, 2012).

Further evidence for the requirement of a copper ion was provided by the identification of a P-type ATPase copper transporter RAN1 (RESISTANT TO ANTAGONIST1), with homology to the human Menkes/Wilson Disease-related copper-transporting P-type ATPase and the yeast copper transporter Ccc2p (Hirayama et al., 1999). The RAN1 copper transporter acts upstream of the ethylene receptors and is predicted to be involved with ethylene receptor biogenesis (Binder et al., 2010). LOF *ran1* mutant plants lack ethylene binding activity, even though the ETR1 receptor is found at normal protein levels, and have a severe constitutive ethylene response phenotype similar to that observed when mutants have multiple non-functional ethylene receptors (Woeste and Kieber, 2000; Binder et al., 2010). In a yeast cell system lacking the RAN1 yeast homologue Ccc2, the ability of ETR1 to bind ethylene is restored by the addition of copper ions (Binder et al., 2010). Flooding the receptor's environment with copper ions may have bypassed the need for copper delivery by RAN1.

It has been suggested that RAN1 resides in the Golgi apparatus membrane or post-Golgi compartments, trafficking copper ions into the membrane systems for incorporation into receptor proteins (Dunkley et al., 2006). RAN1 has not yet been found in the endoplasmic reticulum (ER) membrane, which raises questions about how the copper cofactor is delivered to the ER-localised receptors, perhaps suggesting that the receptors are transported back to the ER by retrograde trafficking after receiving their copper ions. Alternatively, once copper ions have been transported into membrane compartments by RAN1, copper delivery into the ethylene receptor proteins may not be tightly controlled (Binder et al., 2010).

Copper resides in group 11 in the periodic table. The group 11 metals silver and gold can also support ethylene binding, and silver ions have been known for decades to block the transduction of ethylene signalling in plants (Beyer, 1976). Silver can replace copper in the receptor binding pocket, but prevents the transmission of the signal onto downstream proteins (Binder, 2008), although the effects of silver are mostly dependent on ETR1, and ETR1 alone is sufficient for the effects of silver, suggesting there might be functional differences between the receptor ethylene binding domains (McDaniel and Binder, 2012). Gold ions also support ethylene binding but do not block ethylene signalling like silver, instead affecting seedlings independently of ethylene signalling (Binder et al., 2007).

1.5.3 The mechanism of ethylene binding

The ethylene-binding domain in ETR1 is contained within the N-terminal 128 amino acids which constitute the hydrophobic transmembrane (TM) domain (Rodriguez et al., 1999). Mutations in the majority of the highly conserved amino acid residues in the midregions of TM helices I and II result in dominant ethylene insensitivity, with each group of residues located along a single helical face, indicating that these two surfaces are required for the ethylene/copper binding pocket (Wang et al., 2006). The third TM helix has less of a role in actual ethylene binding but is predicted to aid binding by stabilising the binding pocket (Wang et al., 2006).

The receptors act upstream of the rest of the ethylene signal transduction pathway (Kieber et al., 1993). In the absence of ethylene the receptors rest in their 'active' state, negatively regulating the ethylene signalling pathway and repressing the induction of ethylene responses (Chen et al., 2005). Genetic data support the 'inverse-agonist' model of ethylene binding, in which ethylene molecules bind to the 'active' receptors and induce their inactivation, thus relieving receptor inhibition on the pathway and causing the promotion of transcriptional responses to ethylene (Hua and Meyerowitz, 1998; Bleecker, 1999).

The model is supported by mutations in the ethylene binding sites which render the receptors incapable of binding ethylene and produce plants with dominant ethylene insensitivity, as the receptor is maintained in its 'on' or 'active' state. For example, the Cys-65 residue is crucial for coordinating a copper ion (Rodriguez et al., 1999). Cys-65 is mutated in the *etr1-1* GOF mutant, which cannot bind ethylene, and exhibits no ethylene responses (Bleecker et al., 1988).

Currently, a three-state model of ethylene binding is acknowledged (Figure 1-4). In the first signalling state (state 1), the receptor does not bind ethylene and continues its activation of interacting proteins, thus maintaining its inhibition on the downstream ethylene signalling pathway. Upon ethylene binding, the receptor enters an intermediate state (state 2) and continues inhibitory signalling and protein interactions, although it is likely to be in an unstable condition. While ethylene is bound, this more unstable state is in equilibrium with the more stable ethylene-bound state (state 3), in which receptor signalling has ceased to occur, relieving the inhibition on the downstream pathway. In two of the three states, ethylene is found bound to the receptor, but binding is predicted to shift the equilibrium towards state 3 (Wang et al., 2006; Binder, 2008; Binder et al., 2010).

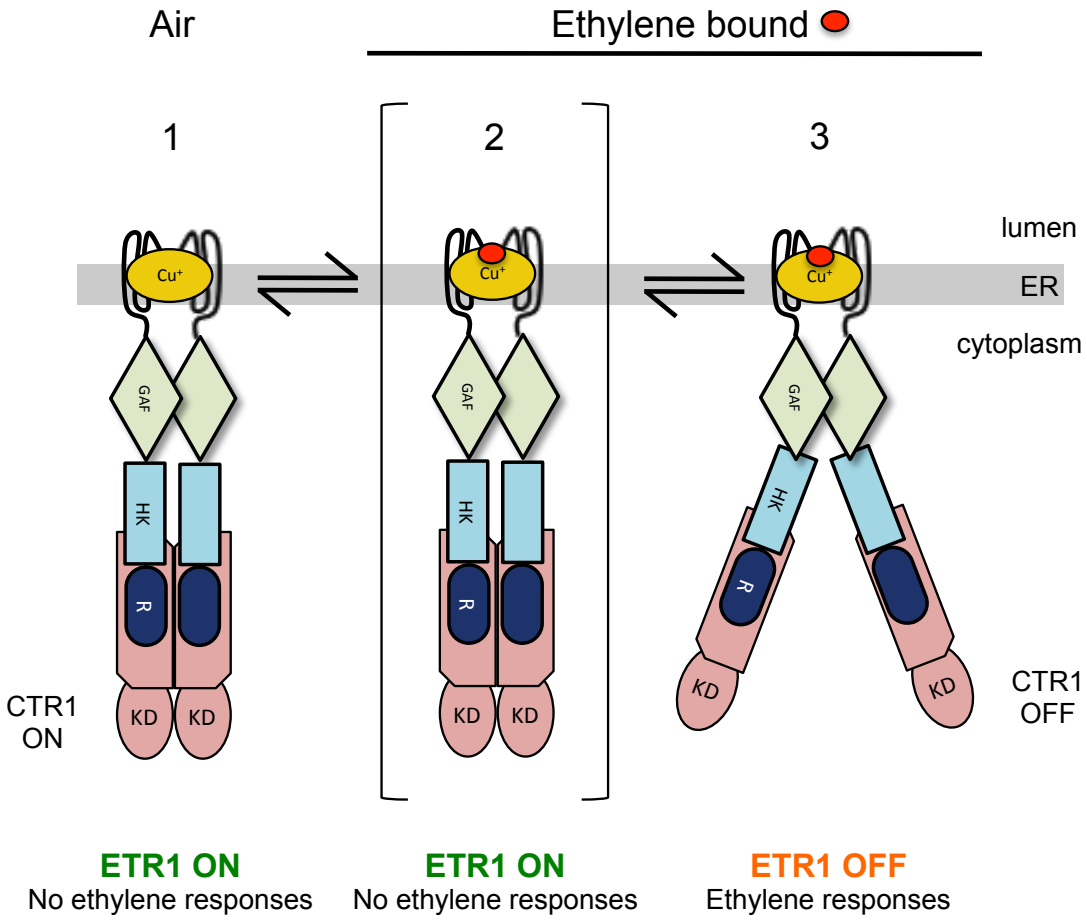


Figure 1-4. A three-state model for ethylene binding. The ethylene receptor ETR1 is shown as a homodimer anchored in the endoplasmic reticulum membrane by the N-terminal helical transmembrane domains which form the binding domain for both ethylene (red) and copper (yellow). The C-terminus of the receptor protein (green, pale blue, navy), containing the GAF and histidine kinase domains, extends into the cytoplasm. In air, the ETR1 receptor is 'active/on' (state 1) and it interacts with the CTR1 protein (pink) via its cytoplasmic C-terminus, acting to inhibit ethylene responses. When ethylene binds, the receptor enters an unstable intermediate state (2) in which the receptor is still active and inhibits ethylene responses. State 2 is in equilibrium with state 3, in which ethylene binding causes inactivation of both ETR1 and CTR1 and allows the downstream ethylene signalling pathway to continue. Adapted from Wang et al., 2006; Resnick et al., 2008; Lacey and Binder, 2014.

The ability of plants to detect and respond to ethylene over a wide range of concentrations may reflect differing ethylene affinities for each receptor isoform (O'Malley et al., 2005). Clustered receptor dimers could produce an amplification response to ethylene binding, wherein the occupancy state of one dimer can alter the signalling states of the receptors nearby (Bray et al., 1998; Duke and Bray, 1999; Shimizu et al., 2003), thus causing an amplified signalling response from a low ethylene concentration.

The receptor-clustering model has been used to explain why a truncated *etr1-1* protein confers high ethylene insensitivity (Gamble et al., 2002); the mutated (truncated) receptor is able to convert adjacent receptors to their "active" states, therefore with reduced ethylene responses, even when ethylene is present.

This three-state model could account for the more severe constitutive response phenotype observed in receptor null mutations, for example subfamily I double mutants *etr1-9;ers1-3* and *etr1-7;ers1-3* (Qu et al., 2007), compared to the more mild phenotypes seen in wild type plants saturated with ethylene (Hua and Meyerowitz, 1998; Hall and Bleecker, 2003; Wang et al., 2003). A small number of ethylene-bound receptors in the wild type plants are in the intermediate state and are still signalling, thus partially negatively regulating ethylene responses, while the rest of the receptors are rendered 'inactive' in state 3 and are allowing downstream ethylene signalling. In contrast, the null receptor plants have constitutive ethylene responses as they lack receptors in the intermediate state (Binder, 2008).

1.5.4 RTE1 negatively regulates the ethylene signalling pathway

RTE1 (REVERSION-TO-ETHYLENE SENSITIVITY 1) is a novel membrane protein which functions as a positive regulator of the ETR1 receptor protein, and therefore negatively regulates ethylene responses (Dong et al., 2008). It was first identified via the LOF mutant *rte1* and the RTE1 protein can restore ethylene sensitivity to the *etr1-2* mutant (Resnick et al., 2006*)*. A similar gene (*GREEN-RIPE*) has been identified in tomato and is also involved in ethylene responses, including ethylene-dependent fruit ripening (Barry and Giovannoni, 2006). The role in ethylene signalling is the only known function for RTE1, although homologues have been identified in other plants, metazoans and some fungi (Zhou et al., 2007). *Arabidopsis* contains a second *RTE1* gene called *RTE1-*

HOMOLOGUE (RTH), although *RTH* does not seem to share the same role in ethylene signalling and it does not interact with ETR1 (C. Chang unpublished data).

RTE1 is globally expressed in all developmental stages (Zhou et al., 2007) in similar tissues to ETR1; the apical hook, root tip and root hairs (Dong et al., 2008). It is predicted to comprise two to four transmembrane domains and co-localises with ETR1 at ER and Golgi membranes (Dong et al., 2008). The RTE1 C-terminus is essential for function and its action is dependent on the presence of the ETR1 N-terminus, appearing to be ETR1 specific (Zhou et al., 2007; Rivarola et al., 2009). Some studies suggest RTE1 may interact weakly with truncated forms of ERS1 (Dong et al., 2008), although this might have been a result of ERS1 complexing with ETR1.

Transcription of the *RTE1* gene produces mRNA transcripts of two lengths with the longer fragment coding for RTE1. The shorter transcript may have a role in regulating RTE1 or might be a separate gene (Zhou et al., 2007). *RTE1* transcript levels are increased by the presence of ethylene, accumulating ~2.5 hours after ethylene treatment, and are reduced by the inhibition of ethylene signalling, suggesting the presence of an ethylene- or ethylene signalling-related feedback signalling mechanism (Zhou et al., 2007). No change in RTE1 subcellular localization is observed under ACC treatment (Dong et al., 2008).

RTE1 may regulate ETR1 by modulating the transition of the receptor between its inactive and active states (Rivarola et al., 2009) and may be essential for ETR1 stability during this transmission (Zhou et al., 2007). Ethylene binding upregulates *RTE1* expression, thus conceivably synthesising enough RTE1 via a feedback mechanism to help the receptor revert back to its non-ethylene-bound 'active' state, especially as turnover of the bound ethylene-ETR1 complex does not seem to be mediated by *ETR1* transcription or degradation of the complex (Hua and Meyerowitz, 1998). Studies did note however that the upregulation of *RTE1* by ethylene seems to be too modest to significantly affect ethylene responses, so RTE1 may be more involved in the fine tuning of responses (Zhou et al., 2007).

1.5.5 Transduction of the ethylene signal

Perception of the hormone ethylene by the receptors needs to be signalled to the nucleus in order to regulate downstream ethylene gene responses (refer to Figure 1-5 and Figure 1-6, pages 29/30, for a model of the ethylene signalling pathway).

Many residues in the ethylene-binding domain cause ethylene insensitivity when mutated. However, these residues are not involved in the binding of the ethylene molecule itself and ethylene binding is not impaired when they are mutated. The residues are predicted to be involved in receptor conformational changes and signal transduction to the cytoplasmic receptor domain for transmission to downstream signalling components. This implies that any steric changes required for signal transmission in the ETR1 receptor are small enough to avoid affecting the ethylene-binding pocket. As many of the conserved residues in the ethylene binding domain are crucial for function even without actually binding ethylene, it suggests that the conserved general function of the binding domain is to control the conformation of an attached signalling domain (Wang et al., 2006).

The nature of conformational changes within the receptor could include α-helical translation, pistoning, pivoting, and rotating perpendicular to the membrane (Matthews et al., 2006). As the receptors function as dimers, conformational changes could also include intramolecular realignments between helices within a monomer, realignments between monomers in the dimer pair or intermolecular alterations in the high molecular weight multimeric complexes (Binder, 2008).

Residues which are crucial for receptor structure and signal transduction are located at the bottom of helices I and III, and may form a domain that is essential for 'switching off' the receptor. There may also be a domain which is responsible for the 'switching on' of the receptor, as two mutations at the cytoplasmic end of helix III confer a LOF phenotype, suggesting these residues are important for maintaining the receptor in its "active" state (no ethylene bound, interaction with CTR1; (Wang et al., 2006). The ETR1 receiver domain has also been implicated in receptor inactivation when ethylene binds (Qu and Schaller, 2004).

The receptors bind and activate the CTR1 protein in the absence of ethylene, which inhibits transduction of downstream signalling. Binding of ethylene molecules causes the inactivation of the ethylene receptor-CTR1 complex. Ethylene therefore inhibits an inhibitory step in the pathway, leading to ethylene responses. This induces the remainder of the pathway and the subsequent ethylene responses via a proposed mitogen-activated protein kinase (MAPK) cascade (Chang, 2003; Ouaked et al., 2003).

The following sections provide more information about key proteins in the ethylene signalling pathway, acting to modulate downstream ethylene responses in the presence or absence of ethylene.

1.5.6 CTR1 acts downstream of ethylene detection

The ethylene receptors regulate activity of CTR1, a mitogen-activated protein kinase kinase kinase (MAPKKK) with homology to eukaryotic Raf-like mitogen-activated Ser/Thr protein kinases, and so named because of the **c**onstitutive **t**riple **r**esponse observed in *Arabidopsis ctr1* mutants (Kieber et al., 1993).

In the absence of ethylene, CTR1 acts as a negative regulator of ethylene signalling transduction (Kieber et al., 1993). The N-terminus of CTR1 can interact with all five ethylene receptors resulting in the recruitment of CTR1 to the ER membrane and possibly to the Golgi apparatus (Gao et al., 2003), despite the protein lacking any predicted transmembrane domains (Huang et al., 2003). Membrane association might also localise CTR1 in the vicinity of other ethylene pathway regulatory elements. Some studies report a stronger physical interaction between CTR1 and the subfamily I receptors, compared to that between CTR1 and subfamily II, which could explain the greater importance of subfamily I in ethylene signalling (Qu et al., 2007).

CTR1 is a serine-threonine kinase, with its kinase activity essential for interaction with the ethylene receptors (Huang et al., 2003), suggesting the existence of a protein kinase cascade in the ethylene signalling pathway. Interaction of CTR1 with either ETR1 or ERS1 requires a functional receptor His kinase domain, but receptor His kinase activity is not necessary (Gao et al., 2003). It has been speculated that perhaps subfamily II receptors, lacking the functional His kinase domain, require subfamily I receptors to pass the signal onto the CTR1 protein via the previously mentioned higher-order protein complexes (Zhong et al., 2008). The active CTR1 kinase domain forms a homodimer, whereas the inactive form remains as a monomer, which has led to proposals that the interaction of CTR1 dimers with receptor dimers may help formation of receptor protein complexes (Mayerhofer et al., 2012). The active dimer may also provide a mechanism for CTR1 regulation, in which conformational changes in the receptor proteins upon ethylene binding are transmitted to CTR1, converting active dimers into inactive monomers.

Ethylene binding inhibits the ethylene receptors, leading to reduced CTR1 activity, which in turn leads to the release of the inhibition on the downstream ethylene signalling

pathway, resulting in the transcription of ethylene responsive genes (Lacey and Binder, 2014). Inactivation of the receptor-CTR1 complexes could activate a protein kinase cascade involving MKK9 and MPK3/6, controlling downstream protein turnover (e.g. EIN3) by altering its phosphorylation state (Yoo et al., 2008). CTR1 has been shown to interact with and directly phosphorylate the cytosolic C-terminal domain of EIN2, the next downstream protein in the ethylene signalling pathway (Ju et al., 2012).

Arabidopsis CTR1 protein is not transcriptionally regulated by the presence of ethylene, although levels of CTR1 protein are increased suggesting a post-transcriptional method of regulating protein abundance, and ethylene treatment promotes CTR1 association with the membrane compartments (Gao et al., 2003).

We cannot be completely sure of the role of CTR1 as the biochemical nature of the signal from the ethylene receptors themselves is as yet unknown and there are layers of complexity to receptor function. For example, the ETR1 N-terminus can signal independently of CTR1, possibly via the GAF domain and other receptors (Xie et al., 2012). There is a possibility that RTE1 and the N-terminal domain of ETR1 can work together to mediate ethylene signalling through a CTR1-independent pathway, as demonstrated by the ability of co-expressed RTE1 and ETR1 residues 1-349 to confer ethylene insensitivity upon the constitutive ethylene response *ctr1-1* mutant (Qiu et al., 2012). The CTR1-independent role for the ETR1 N-terminus has also been noted in mutants lacking CTR1 as there is still a receptor-mediated ethylene response output (Zhang et al., 2014).

The receptors might also interact with other proteins. ETR1 can interact with several histidine phosphotransfer proteins (AHP1, 2 and 3; Urao et al., 2000) and is hypothesised to regulate other cellular responses via *Arabidopsis* response regulators (ARRs; (Hass et al., 2004). Histidine kinase 5 from *Arabidopsis* is also able to modulate ethylene responses in the root, although not in the hypocotyl (Iwama et al., 2007).

The *ctr1* mutant adult plant shows constitutive ethylene responses even in the absence of ethylene and its physiology resembles wild type plants grown in high ethylene concentrations (Kieber et al., 1993). Studies on *ctr1* demonstrated that its LOF mutation is epistatic to the dominant alleles of the ethylene receptor mutants revealing that all five receptors can act through CTR1 (Hua and Meyerowitz, 1998).

The quadruple LOF receptor mutants have more severe ethylene responses than seen in the *ctr1* mutant, and double mutants between the dominant receptor alleles and *ctr1* are bigger and healthier than *ctr1*. This provides more evidence for the existence of a CTR1-independent ethylene-signalling pathway, and some receptors may have functions that are independent of ethylene signalling altogether, for example ETR1 appears to have an ethylene-independent function in regulating cell elongation (Hua and Meyerowitz, 1998).

1.5.7 EIN2 links ethylene detection with gene regulation

The protein ETHYLENE INSENSITIVE2 (EIN2) resides downstream of CTR1 in the signalling pathway (Alonso et al., 1999), possibly after a mitogen-activated protein kinase (MAPK) cascade (Chang, 2003; Ouaked et al., 2003). However, LOF MAPK6 plants show normal responses to ethylene, so the involvement of a MAPK signalling cascade is still uncertain (Ecker, 2004).

EIN2 is localised to the ER membrane via its N-terminus and can interact with the receptor proteins, suggesting a level of receptor regulation of EIN2 (Bisson et al., 2009; Bisson and Groth, 2010). The hydrophobic N-terminal domain of EIN2 shows some similarity to the Natural Resistance-Associated Macrophage Protein (NRAMP) metal ion transporter family and has a unique C-terminal domain, which may be regulated by its own N-terminus. The loss of EIN2 function results in complete ethylene insensitivity, suggesting it is required to activate downstream components of the pathway (Alonso et al., 1999).

EIN2 is targeted for 26S proteasome-dependent ubiquitin-mediated degradation by two F-box proteins ETP1 and ETP2. In the absence of ethylene, the levels of F-box proteins are high, which prevents EIN2 protein levels from increasing. In the presence of ethylene, the levels of ETP1 and 2 are reduced, leading to EIN2 accumulation. The *ETP* and *EIN2* genes are not transcriptionally regulated by ethylene, but ethylene does induce the downregulation of ETP protein levels (Qiao et al., 2009).

A putative COP9 (constitutive photomorphogenesis 9) signalosome component EER5 (enhanced ethylene response protein 5) can physically interact with the EIN2 C-terminus and may be involved in protein turnover, contributing to the resetting of the signalling pathway (Christians et al., 2008).

The inactivation of ETR1 and CTR1 proteins by ethylene binding allows EIN2 to activate EIN3 and EIN3-like transcription factors. The C-terminus of EIN2, containing a functional

nuclear localisation signal, is cleaved in the presence of ethylene and upon regulation by CTR1, and is transported into the nucleus where it acts to stabilise EIN3 and activate ethylene responses (Wen et al., 2012).

EIN2 was identified in a screen for strong ethylene insensitive mutants, with the LOF *ein2* mutation rendering plants completely insensitive to ethylene (Guzman and Ecker, 1990).

1.5.8 EIN3 transcription factors mediate ethylene responses

Signals coming through the ethylene signalling pathway converge on the EIN3 (ETHYLENE INSENSITIVE 3) and EIL (EIN3-LIKE) transcription factors, which appear to mediate the primary output of the signalling pathway. *EIN3* belongs to a plant-specific transcription factor family with five additional *EIL* genes (Chao et al., 1997).

In the absence of ethylene, two F-box proteins, EBF1 and EBF2, directly interact with EIN3 and mediate its proteasomal degradation (Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004) ensuring protein levels remain low in the absence of ethylene. When ethylene is present, accumulation of EIN3 is dependent upon the activity of EIN2 (Guo and Ecker, 2003). It is not known how EIN2 regulates EIN3, although another MAP kinase cascade has been proposed (Yoo et al., 2008). EIN3 accumulates in the nucleus and helps mediate the expression of hundreds of genes, with almost all ethylene-mediated gene transcription regulated by this transcription factor family (Alonso et al., 2003)

Like the ETP proteins regulating EIN2, the EBF F-box proteins are regulated by the presence of ethylene. In the case of EIN3, ethylene downregulates the levels of EBF1 and 2 to allow the transcription factors to accumulate in the nucleus and modulate gene expression (Qiao et al., 2009). EBF2 is itself a target of EIN3, creating a sensitive feedback mechanism (Konishi and Yanagisawa, 2008).

Mutations in the *EIN3* gene cause partial ethylene insensitivity, but not full insensitivity, as the small family of EIN3 and EIL proteins are somewhat functionally redundant, whilst overexpression of *EIN3* confers constitutive ethylene responses (Hua and Meyerowitz, 1998). EIN3 directly regulates the *ERF1* gene, which in turn modulates expression of other ethylene-responsive genes (Solano et al., 1998).

As with many components in this signalling pathway, there is another layer of complexity to the role of the EIN3/EIFL family. Some ethylene responses seem to be regulated without requiring transcriptional changes. Immediate plant growth regulation via ethylene responses appears to be EIN3/EIL1 independent, although some other members of the family may regulate this pathway (Binder et al., 2004a).

1.5.9 ERF transcription factors

The most well-known direct gene targets of EIN3 is the *ERF* family of APETALA2 (AP2)-domain containing transcription factors (Stepanova and Alonso, 2009). There are 122 *ERF* family genes in *Arabidopsis* which code for transcriptional regulators involved in a wide range of developmental and physiological processes. The *ERF* family is part of the *AP2/ERF* superfamily, defined by the AP2/ERF domain comprising 60-70 amino acids and which is involved in DNA binding (Nakano et al., 2006). The ERF family can be divided into two further subfamilies: ERF and CBF/DREB (Sakuma et al., 2002), revealing roles for ethylene in response to cold and drought.

The ERF domain was identified as a conserved motif in four DNA-binding proteins from tobacco: ethylene-responsive element-binding proteins 1, 2, 3 and 4 (EREB1, 2, 3 and 4 but now renamed ERF1, 2, 3 and 4). This motif binds a GCC box, a DNA element involved in the ethylene-responsive transcription of genes (Ohmetakagi and Shinshi, 1995).

The *ERF1* promoter contains two inverted repeat sequences that are recognised by an EIN3 dimer, a sequence which is also present in the promoters of other ethylene responsive genes e.g. *GST1* (Solano et al., 1998).

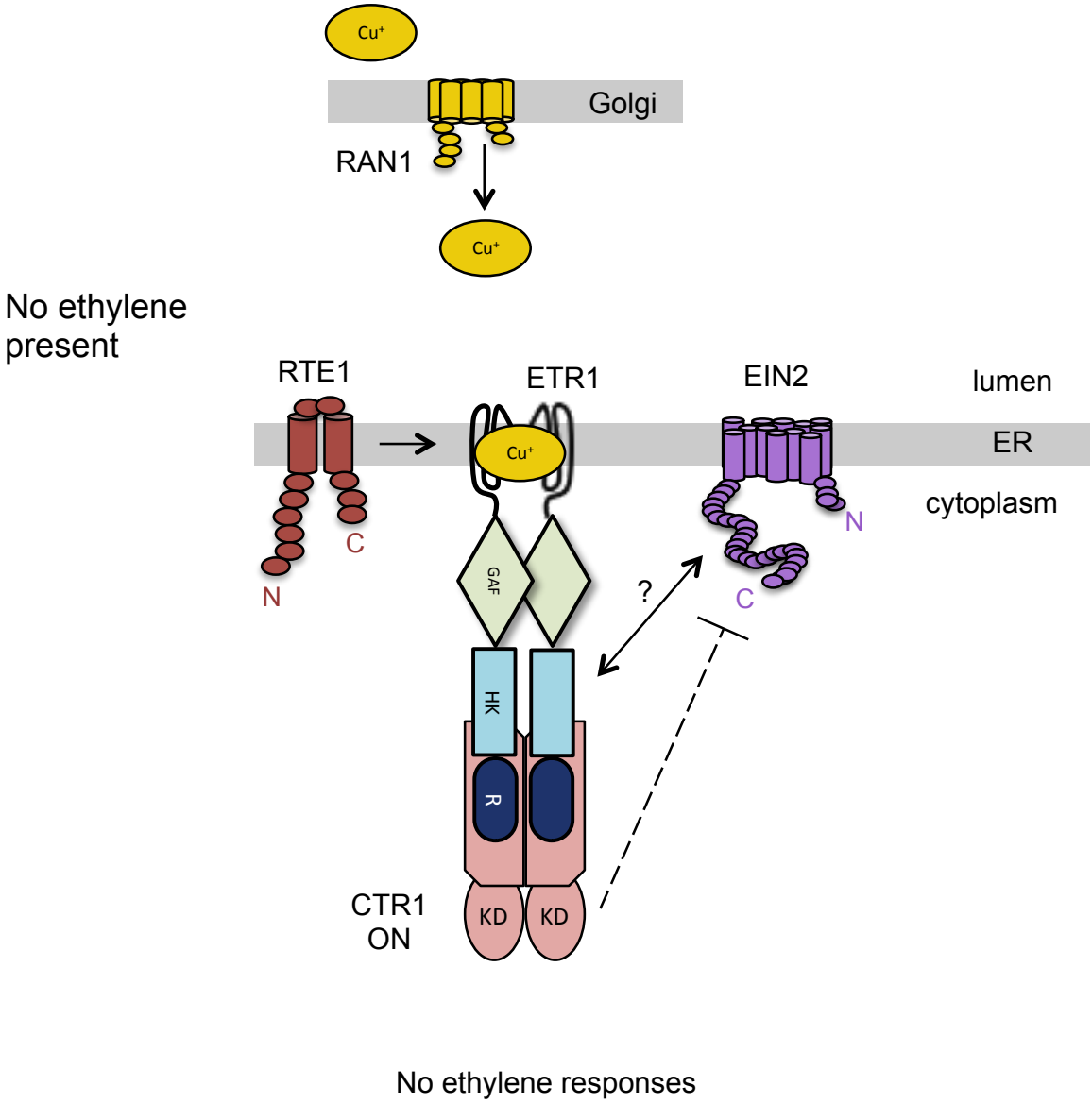


Figure 1-5. The inhibition of the ethylene signalling pathway in the absence of ethylene. The copper transporter RAN1 (yellow) transports copper ions across the Golgi apparatus membrane. The copper ions are delivered to the ethylene binding domain of the endoplasmic reticulum-localised ethylene receptor ETR1 (green/blue/navy) by an unknown mechanism. In the absence of ethylene, ETR1 is active which interacts with CTR1 (pink) via the histidine kinase domain (blue/navy) and maintains CTR1 activity. This interaction inhibits EIN2 (purple) and stops further transduction of the ethylene signal. There may also be some direct regulation of EIN2 by ETR1 (question mark). Downstream ethylene responses are inhibited. The membrane protein RTE1 (brown) is reported to help maintain the active state of ETR1. (Figure adapted from (Ju and Chang, 2012).

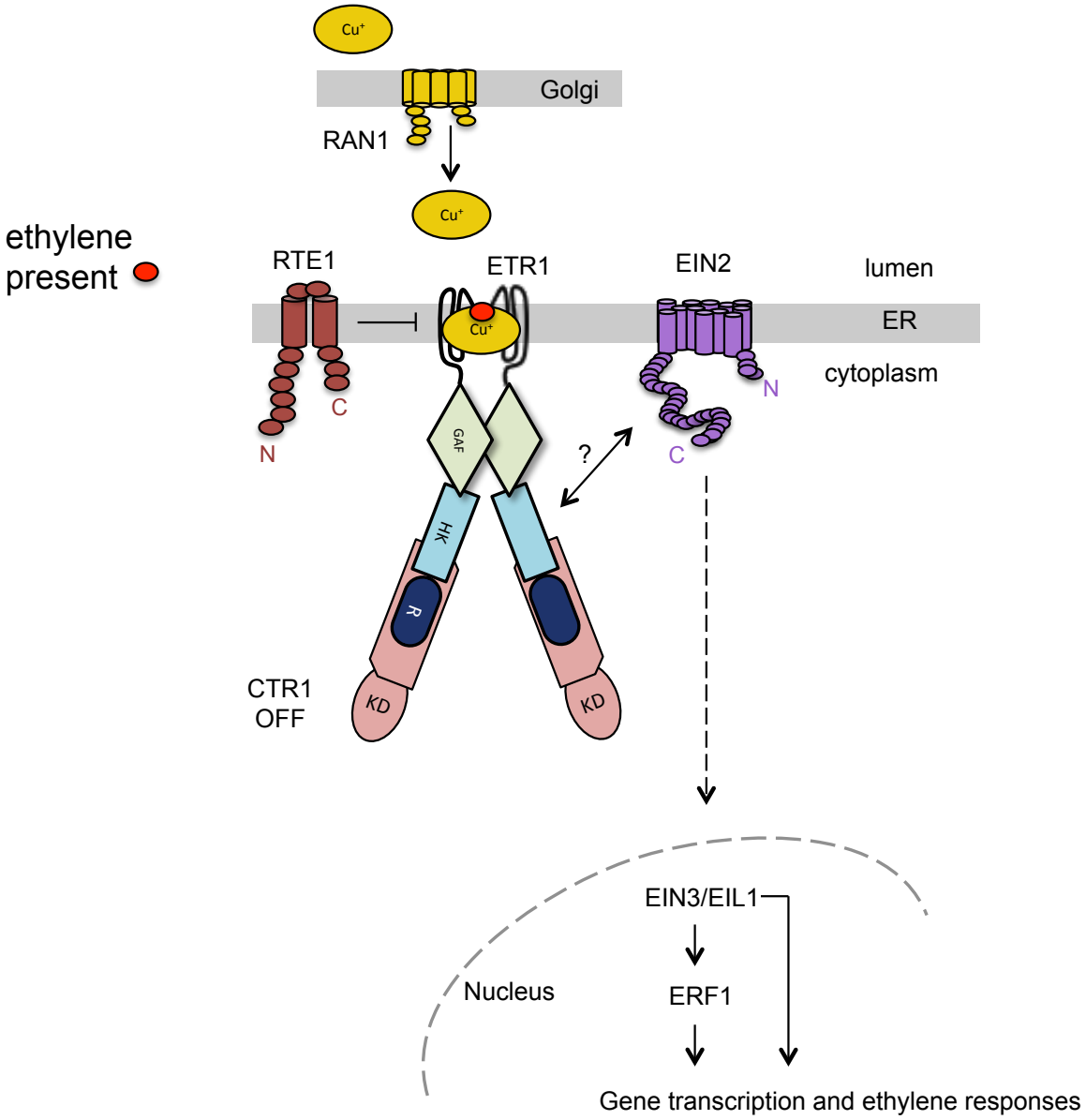


Figure 1-6. Response of the ethylene signalling pathway to the presence of ethylene. An ethylene molecule (red) binds to the ethylene binding domain in the N-terminal transmembrane domains of the ethylene receptor ETR1. Ethylene binding requires the presence of a copper ion. This results in a conformational change in ETR1, causing CTR1 (pink) to become inactive, and thus releases the previous inhibition on EIN2 (purple). The ethylene signalling pathway can continue and promotes the transcription of ethylene related genes, leading to ethylene-mediated responses.

1.6 The POLARIS peptide

POLARIS (PLS) plays a small part in a network of hormone crosstalk events between the plant hormones ethylene, auxin and cytokinin, with the addition of other branches of the network involving abscisic acid among other hormones. This network of hormone interactions ultimately results in the correct patterning and development of the root and aerial vasculature, and suitable responses of the plant as a whole to its environment. Mutation of the *PLS* gene results in an enhanced ethylene-response phenotype, defective auxin transport and alters sensitivity of microtubules to inhibitors (Chilley et al., 2006).

The *PLS* gene was identified in the *Arabidopsis* C24 wild type background by promoter trapping using a single-copy *β-glucuronidase (GUS*) T-DNA insertion sequence (line AtEM101), revealing *PLS* gene expression is primarily in the embryonic and seedling root (Figure 1-7 A), with low expression in vascular tissues in the aerial parts of the plant (Casson et al., 2002). *pPLS::GUS* activity is predominantly exhibited in the embryonic root from the heart stage and in the tips of primary and lateral roots in the seedling (Topping et al., 1994), rather than older regions of the root (Casson et al., 2002). The mutant was named *polaris* because it is expressed in a polar pattern in the *Arabidopsis* embryo (Topping et al., 1994).

AtEM101 seedlings homozygous for the T-DNA insertion were backcrossed to the wild type to reveal segregating mutants. GUS positive homozygous *pls* seedlings showed a short-root phenotype when grown in the light, and reduced vascularization in the leaves (Casson et al., 2002). Fourteen days post-germination, the length of the *pls* mutant primary root is ~50% of the length of the wild type, with the cells of the *pls* root meristem and primary root cortex observed to be significantly shorter and more radially expanded than in the wild type. The reduction in axial cell elongation in *pls* would account somewhat for the decreased root length in the mutant, with the added contribution of *pls* meristematic cells dividing less frequently than in the wild type (Casson et al., 2002).

Seedlings that are heterozygous for the T-DNA insertion have an intermediate primary root length (Figure 1-7 B), between those of the *pls* mutant and the wild type, as well as intermediate levels of leaf venation, indicating that the *pls* mutation is semi-dominant (Casson et al., 2002). Typically, *pls* mutants also produced a greater number of anchor roots at the root-hypocotyl junction than wild type seedlings (Casson et al., 2002).

The T-DNA had inserted into a small open reading frame (ORF) of 108bp in a 755 base pair region between two larger genes (Figure 1-7 E). The upstream gene (located 420bp upstream of the *PLS* ORF) encodes an unknown protein of 92 amino acids, designated *GENE X*. The other gene, *BRI1*, places *PLS* at the bottom of *Arabidopsis* chromosome 4 (Li and Chory, 1997). Although originally thought to be an exon of one of the neighbouring genes, *PLS* was found to be separately transcribed. 3' rapid amplification of cDNA ends (RACE) of *GENE X* showed that its transcription is terminated with a polyadenylation sequence 356bp upstream of the T-DNA insertion site in the *PLS* ORF, and RNA gel blot analysis revealed two distinct transcripts (Casson et al., 2002). The disrupted open reading frame was found to be within a short, auxin-inducible transcript of approximately 500 nucleotides, and was predicted to encode a small peptide of 36 amino acid residues (Casson et al., 2002).

Transcription of the *PLS* RNA transcript is initiated by sequences upstream of the *GENE X* polyadenylation site. Two transcription sites were found, ~95 nucleotides apart, with start site 2 (~23bp upstream of *GENE X* poly(A) site) used more frequently than start site 1 (~117bp upstream of poly(A) site), although start site 1 appears to produce a more abundant transcript. Start site 2 produces a shorter transcript, but the *PLS* transcript was shown to have a variable 3' end, producing a transcript between 427 and 606 nucleotides long, depending on the transcription start and polyadenylation sites used (Casson et al., 2002).

Sequencing of the *PLS* locus revealed that the T-DNA was inserted into the 25th codon (Leu) of a 108 nucleotide ORF encoding the predicted PLS polypeptide, comprising 36 amino acids with an expected molecular mass of 4.6kD. The *pls* mutation can be partially complemented by the *PLS* cDNA, demonstrating that the ORF produces a functional protein. Analysis of the sequence revealed no significant homology with known proteins, although the *Arabidopsis* Columbia (Col-0) wild type allele is identical in sequence (Casson et al., 2002).

Amino acid structural analysis was used to predict the secondary structure of the peptide. The PLS N-terminus is predicted to form two β-sheets, whereas the C-terminal 12 amino acids can potentially form an α-helical structure. Between the two β-sheets, there are three arginine residues, which may form a turn region in the amino acid backbone, or be a potential cleavage site. The second β-sheet contains a repeated SIS amino acid residue motif, which, combined with the Arg residues, could be a cAMP- and cGMP-dependent

protein kinase phosphorylation site. The C-terminal KLFKLF repeated motif, indicative of an α-helix, would present the three lysine residues on the same face of the helix, creating an amphipathic helix with both hydrophobic and charged faces. This region could be involved in protein-protein interactions, particularly with the presence of the leucine residues providing the potential for a leucine zipper motif (Casson et al., 2002).

Structural knowledge of the PLS peptide is currently only predicted as PLS has not been isolated from *Arabidopsis* plants. Antibodies were raised previously to the N-terminal 18 amino acids but failed to detect the peptide (P. Chilley, 2006; S. Mehdi 2009, unpublished data). However, PLS was since detected in cell extracts by proteomic analysis, bound to the metallopeptidase Aminopeptidase M1 (APM1) (Angus Murphy and Wendy Peer, personal communication). APM1 is a membrane protein which catalyses the cleavage of amino acids from the N-terminus of peptide substrates and has roles in the regulation of auxin transport and meiosis, among other processes (Murphy et al., 2002; Peer et al., 2009). If PLS can interact with APM1 it provides information about the structure and function of the peptide. Firstly, the PLS peptide may be capable of binding to other proteins, possibly via the predicted leucine zipper motif at the C-terminus. Secondly, the peptide may undergo cleavage by a protein modification enzyme like APM1, which reinforces the presence of the predicted cleavage site near the N-terminus of the peptide.

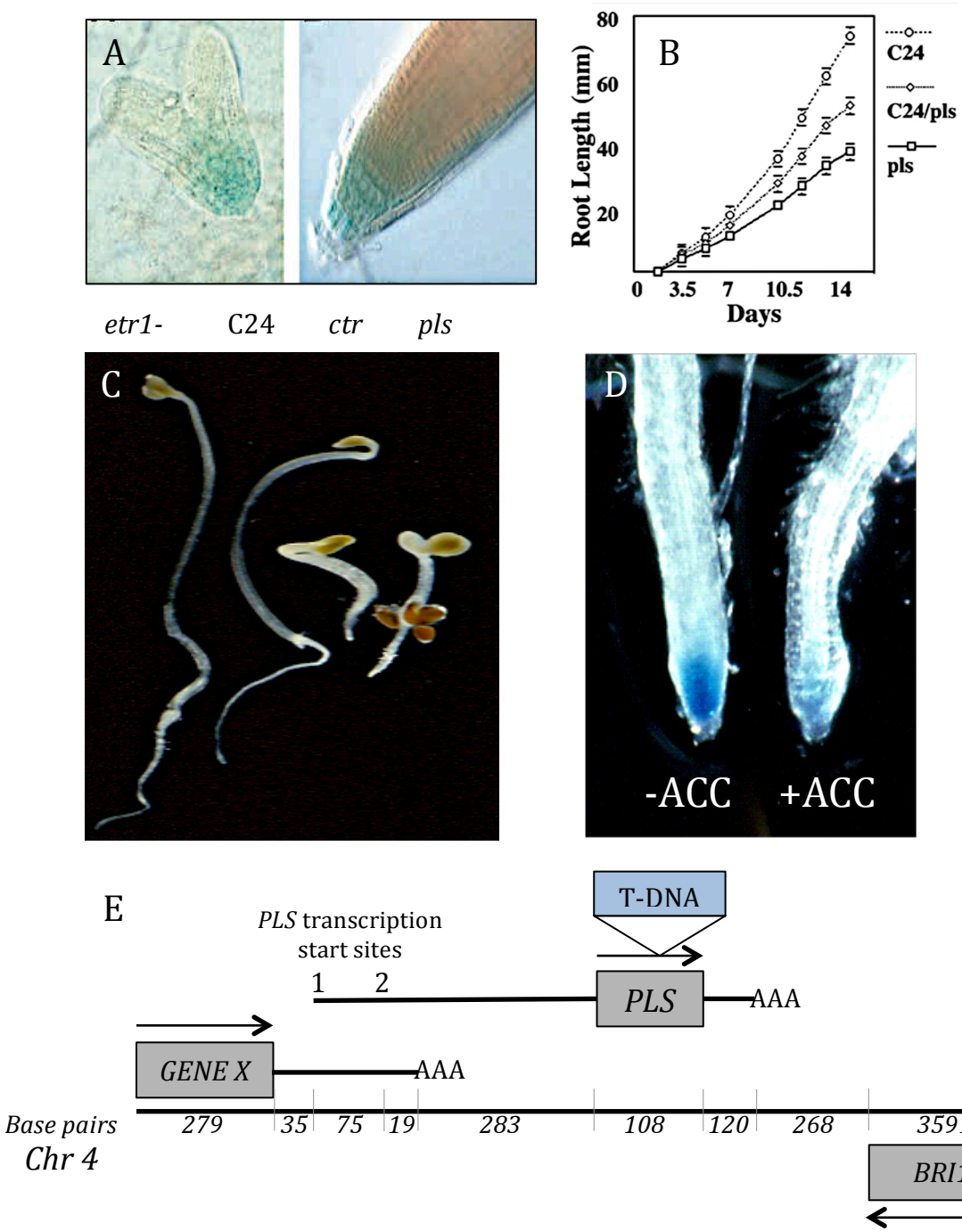


Figure 1-7. Characteristics of the *pls* mutant and the *PLS* gene. A. The *PLS* promoter powers the T-DNA-inserted *GUS* gene in the *Arabidopsis* embryo and root tips. B. C24/*pls* heterozygous seedlings show an intermediate root-length phenotype. C. Dark-grown phenotype of *pls* compared to wild type C24, the ethylene insensitive mutant *etr1-1* and the constitutive ethylene response mutant *ctr1*. D. Application of the ethylene precursor ACC results in downregulation of the *PLS* gene. E. Location of the *PLS* locus on chromosome 4 in *Arabidopsis;* distance between features is indicated in base pairs. Gene open reading frames (ORF) are depicted as grey boxes, untranslated transcript regions are lines, and arrows show the direction of the gene. 'AAA' is a polyadenylation site; the *PLS* poly(A) site commonly used is 120bp after the end of the ORF. *Gene X* (At4G39404) is a predicted gene with no known function; *BRI1* (At4G39400) is involved in brassinosteroid signalling. The *PLS* T-DNA insertion site is located 75bp from the start of the 108bp *PLS* open reading frame (grey). (Panels A and B: Casson et al., 2002; C and D: Chilley et al., 2006).

1.6.1 POLARIS negatively regulates ethylene responses whilst ethylene negatively regulates *PLS* expression

The *PLS* gene is required for the correct operation of the ethylene signalling pathway and the PLS peptide negatively regulates ethylene responses (Chilley et al., 2006). *pls* seedlings exhibit enhanced ethylene signalling, demonstrated by the triple response phenotype (Figure 1-7 C), and upregulation of the ethylene-mediated genes *ERF10* and *GSTF2*, demonstrating that the functional gene is required to negatively regulate ethylene responses. Genetic or pharmacological inhibition of ethylene signalling restores the defective *pls* phenotype (short roots, reduced auxin transport and low auxin accumulation) to wild type levels, whilst overexpression of the *PLS* gene reduces the inhibitory effects of exogenous ACC (the ethylene precursor) on primary root growth, further supporting the role of PLS in an inhibitory role in ethylene signalling (Chilley et al., 2006). As previously mentioned, C24/*pls* heterozygous plants show an intermediate root length (Casson et al., 2002). This semi-dominant effect suggests that *PLS* has a dose-dependent effect in suppressing ethylene responses.

Studies on the cause of the enhanced ethylene signalling in *pls* provided clues about the function of the PLS peptide in *Arabidopsis.* The enhanced ethylene responses in *pls* are not due to an increase in ethylene biosynthesis, revealing that PLS must have a role further downstream in the ethylene-signalling pathway (Chilley et al., 2006). Secondly, the shorter length of the *pls* primary root can be restored to that of the wild type by using silver ions to inhibit ethylene responses. As previously discussed (section 1.5.2), silver ions can be coordinated in the copper ion binding site of the receptor ethylene binding domains, allowing ethylene molecules to be recognised, but inhibiting any signal transduction from the receptor proteins and thus reducing plant ethylene responses (Binder, 2008). The fact that silver ions can still exert this influence over ethylene receptor function, despite the loss of the PLS function in *pls*, demonstrates that the PLS peptide does not have a role downstream of the ethylene receptors or the ethylene/copper binding site where silver is coordinated.

Furthermore, the ethylene insensitive *etr1-1* mutant can rescue the *pls* phenotype, but *pls* cannot suppress the *etr1-1* mutation (Chilley et al., 2006). The failure of *pls* to suppress *etr1-1* reveals that the absence of the PLS peptide does not affect the inability of *etr1-1* to bind ethylene, due to a mutation resulting in defective copper binding in the *etr1-1* EBD

(Rodriguez et al., 1999), and that the PLS peptide does not act downstream of the ethylene receptor ETR1.

However, the role of the *PLS* gene is more complex than suggested by this evidence. The CTR1 protein acts downstream of the ethylene receptors (and PLS) and therefore the *ctr1-1* mutation could be expected to suppress all mutations in the *PLS* gene. In reality, the *ctr1-1* mutation (with constitutive ethylene responses and a strong ethylene phenotype involving short roots) can only incompletely suppress the overexpressed *PLSOx* phenotype (displaying reduced ethylene responses and longer roots; Chilley et al., 2006), suggesting that PLS has additional roles in the modulation of root growth. For example, light-grown PLSOx/*ctr1* double mutants have longer roots than the same seedlings grown in the dark, indicating there may be a light-sensitive regulatory mechanism involved with PLS function (Chilley et al., 2006).

The regulation of the *PLS* gene adds greater complexity to the role of PLS. When grown in the presence of ACC, the original EM101 and *pPLS::GUS* lines show a decrease in *PLS* promoter activity in the root tip (Figure 1-7 D), with reduced *PLS* transcript (Chilley et al., 2006), revealing that ethylene, and/or downstream ethylene signalling, negatively regulate *PLS* transcription in a feedback mechanism.

Beyond the genetic work which placed PLS action at the level of the ETR1 receptor, the protein interaction was studied between PLS and ETR1. Yeast 2-hybrid and Bimolecular Fluorescence Complementation (BiFC) assays revealed that the PLS peptide can physically interact with ETR1 (Mehdi, 2009), with PLS purportedly regulating ethylene binding by changing the receptor protein conformation, or by some other unknown mechanism.

1.6.2 Auxin positively regulates *PLS* expression and *PLS* is required for normal auxin responses

In addition to the ethylene defects, the *pls* mutant was found to display both a low auxin phenotype and reduced responses to auxin, suggesting the role and regulation of *PLS* and the encoded peptide are not solely determined by ethylene.

The *PLS* gene transcript is rapidly upregulated by the presence of exogenous auxin and *pls* has reduced auxin levels, therefore exhibiting subdued auxin responses (Casson et al., 2002; Chilley et al., 2006). The free IAA (auxin) content of *pls* was found to be up to 70%

lower than in the wild type, the mutant lacks the auxin maximum found in the *Arabidopsis* wild type root tip, and *pls* has a greatly decreased ability to transport auxin, showing only ~24% of the transport measured in wild type plants (Chilley at al., 2006). Likewise, transgenic *PLS* overexpressing lines show a higher free IAA concentration than the *pls* mutant (Chilley et al., 2006). It was observed that the growth-inhibitory effects of auxin on root length were lessened in the *pls* mutant, with low auxin concentrations leading to longer roots in *pls* (Casson et al., 2002). The presence of a functional *PLS* gene is thus essential for correct auxin transport, accumulation and root growth.

The localisation of expression and the sequence of the *PLS* promoter provide further evidence that *PLS* expression is strongly linked to auxin levels. The *PLS* promoter contains TGTCTC-like putative auxin-responsive elements (AuxREs; (Ulmasov et al., 1997), illustrating that *PLS* transcription can be regulated by auxin. *PLS* promoter activity is strongest in the root tip, and this site has a relatively high auxin concentration and/or sensitivity, and *PLS* is expressed in the columella initial cells, similar to the auxin-inducible DR5 promoter (Sabatini et al., 1999). *PLS* is also expressed strongly at the site of lateral root formation (Topping and Lindsey, 1997), which is induced by auxin (Celenza et al., 1995; Tian and Reed, 1999).

Correct *PLS* gene patterning is also dependent on GNOM activity (Topping and Lindsey, 1997), a protein which is essential for correct PIN protein localization and therefore auxin distribution (Steinmann et al., 1999; Geldner et al., 2003).

The reduced auxin responses and altered root architecture observed in the *pls* mutant can be partially explained by the enhanced ethylene responses in the mutant. The root phenotype of the *pls* mutant, displaying reduced cell elongation and increased cell width, can be induced by exposition or sensitivity to several hormones, including ethylene (Abeles et al., 1992) or auxin (Ljung et al., 2001). The increased ethylene responses in *pls* suppress the auxin-mediated initiation of new lateral roots (Chilley et al., 2006). In addition, *pls* mutants with low free auxin levels were crossed with the ethylene insensitive mutant *etr1-1* and the resulting plants showed free auxin levels similar to those of the wild type. Auxin transport in these double mutants was also restored to ~85% of wild type levels, and lateral root numbers were rescued to ~80% of the wild type (Chilley et al., 2006). The addition of ACC to *pls* mutants fails to promote auxin accumulation in the root tip and *pls* has a defective *WEI2* gene expression - WEI2 enzyme is required for ACC-mediated auxin synthesis (Mehdi, 2009; Stepanova et al., 2005; 2008). Enhanced ethylene

responses have been shown to upregulate the PIN auxin transport proteins (Ruzicka et al., 2007), and *pls* has upregulated *PIN1* and *PIN2* genes (Mehdi, 2009; Liu et al., 2013).

However, modelling of PLS action has revealed that the dependence of auxin concentration on ethylene signalling can be flexible, with increasing ethylene responses able to promote both increases and decreases in auxin concentration (Liu et al., 2010). PLS can also independently affect auxin biosynthesis and auxin transport, possibly still caused by upstream ethylene responses (Liu et al., 2010), with an increase in the rate of auxin biosynthesis predicted to be associated with a PLS-induced decrease in the concentration of the hormone cytokinin.

1.6.2.1 Cytokinin adds an additional layer of complexity

In contrast to its reduced responses to auxin, the *pls* mutant shows hyperresponsiveness to the plant hormone cytokinin (Casson et al., 2002).

Cytokinins have a negative role in root growth, are important regulators of cell division and appear to be synthesized in root tips (Benkova and Hejatko, 2009). Cytokinin exerts its control on the rate of cell differentiation, resulting in shortening of the meristematic zone (Dello Ioio et al., 2007) and reduction of the relative elongation rate of roots, somewhat resembling typical ethylene-induced inhibition of growth (Beemster and Baskin, 2000), whereas decreased levels of cytokinin produce an enhanced root meristem and increased root growth (Werner et al., 2003; Yang et al., 2003). Cytokinins can act antagonistically with auxin in developmental contexts such as lateral root formation, but can also act synergistically, as in ethylene biosynthesis (Vogel et al., 1998).

The *pls* mutant exhibits increased responsiveness to cytokinin, possibly due to the upregulation of a cytokinin-inducible gene *ARR5/IBC6* (Casson et al., 2002). The short-root *pls* seedlings have enhanced responses to exogenous cytokinin, producing plants with even shorter roots, and *PLS* overexpressing plants grow significantly longer roots than wild type, suggesting that the *PLS* gene can negatively regulate cytokinin responses and has a role in partially suppressing the growth-inhibitory effects of cytokinin (Casson et al., 2002). The *PLS* gene is therefore required for correct auxin-cytokinin homeostasis to modulate root growth and leaf vascular patterning (Casson et al., 2002).

1.6.3 The role of POLARIS

Expression and patterning of *PLS* in the root is predominantly mediated by an antagonistic relationship between ethylene and auxin signalling. Auxin localization to the root tip promotes *PLS* expression, whilst ethylene signalling represses expression. The complicated role of PLS in ethylene signalling, in conjunction with evidence of the relationship between PLS and auxin and cytokinin signalling, suggests that PLS could be a point of crosstalk between hormone pathways leading to root development and part of a complex feedback mechanism.

PLS negatively regulates ethylene responses, and the subsequent effects on root cell division and expansion, via downstream mechanisms involving auxin signalling and microtubule cytoskeleton dynamics (Chilley et al., 2006). *PLS* is required for both root elongation and lateral root formation, in the latter case via ethylene-mediated control of auxin transport to the pericycle (Chilley et al., 2006). *PLS* transcription is activated at the root tip by the auxin maximum, required in that region for correct cell division (Sabatini et al., 1999; Ljung et al., 2001; Friml et al., 2002; Blilou et al., 2005). PLS acts here as a negative regulator of ethylene signalling, which would act to inhibit cell division and expansion, and therefore root growth (Souter et al., 2004). This model could account for the suppression of the inductive effects of auxin and cytokinin on ethylene biosynthesis at the root tip, a site of cytokinin synthesis (Vogel et al., 1998).

It is perhaps not surprising that the auxin-linked *PLS* has an association with cytokinin modulation too. Cytokinin and auxin biosynthesis are dependent on each other, and disruption of one affects the other. An increase in auxin concentration produces a decrease in the cytokinin level, and auxin biosynthesis is partially inhibited by cytokinin (Nordstrom et al., 2004). Separately, cytokinin may modulate the local auxin gradient and expression of PIN proteins during lateral root development (Laplaze et al., 2007; Kuderova et al., 2008).

A flexible regulatory loop between auxin, ethylene, cytokinin and *PLS* is formed via the modulation of *PLS* expression (Liu et al., 2010). The effects of this hormone network on root growth and development appear to be mediated via PLS regulation of ethylene signalling. The evidence points to the PLS peptide mediating ethylene signalling at the level of the ethylene receptors. Ethylene suppresses *PLS* transcription in the root to allow ethylene signalling to take place (Chilley et al., 2006), and the peptide is capable of binding to the ethylene receptor ETR1 *in vitro* and *in vivo* (Mehdi, 2009).

The mechanism of PLS peptide action is under investigation. It has been hypothesised that the PLS peptide could bind to the ethylene receptor protein and activate the conversion of the inactive ethylene-bound receptor protein in state 3, which promotes ethylene signalling and the subsequent ethylene responses, to the active receptor (state 1) which does not have ethylene bound and thus inhibits the pathway (see section 1.5.3). Alternatively, PLS might inhibit the conversion of the active state (1) to the inactive state (3), and therefore inhibit the downstream transduction of the ethylene signal. PLS may also target the ethylene-bound receptor proteins for degradation, so fewer receptors are present to bind ethylene and generate responses. Alternatively, the peptide could inhibit receptor-CTR1 interaction to suppress signal transduction beyond the CTR1 protein.

1.7 Aims and objectives

The project aimed to elucidate the mechanism by which the POLARIS peptide regulates ethylene signalling, and thus affects ethylene-mediated root growth.

Investigations began with the PLS peptide itself. The sequences of the *PLS* gene and the PLS peptide were used to identify homologues of PLS in other plant species. Structural studies were undertaken to identify key domains and crucial residues within the 36-residue PLS peptide; bioinformatics tools were recruited to predict secondary and tertiary structures, whilst chemical synthesis of full length and truncated versions of PLS produced a number of peptides which were employed for structure/function relationship studies.

In order to determine the mode of action of the peptide, it was important to research the location of PLS expression in the *Arabidopsis* root as a whole and at the subcellular level. Localisation studies on the PLS peptide were undertaken using a PLS-GFP fusion protein and previously characterised cell organelle markers. The expression of the PLS-GFP protein was studied in response to ethylene treatment, revealing information about the relationship between PLS and ethylene signalling.

Finally, a variety of techniques were employed to study the mechanism of the PLS peptide. Quantification of the gene expression of the *PLS* and ethylene receptor *ETR1* genes upon ethylene treatment enabled investigation into relationships between the two genes at the transcriptional level. A previously reported physical interaction between the PLS and ETR1 proteins was further analysed *in vivo* using antibody-based co-immunoprecipitation techniques. In addition, the role of copper ions was investigated with respect to the PLS peptide through subjecting *Arabidopsis* seedlings to excess or depleted copper, and analysing the copper-binding capacity of the PLS peptide. Bioinformatics tools were used to predict plausible 3D structures of the peptide in light of new evidence.

The role of the PLS peptide in ethylene signalling is subsequently discussed in the context of the work in this thesis and the wider literature. Several models for the action of PLS have been proposed, aiming to enhance our understanding of the mechanism by which the PLS peptide regulates plant ethylene responses.

Chapter 2 . Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in the following experiments were obtained from Sigma Aldrich (Poole, UK) and Fisher Scientific Ltd (Loughborough, UK) unless otherwise stated.

2.1.2 Plant lines

Wild type *A. thaliana:* Col-0, C24

Ethylene-related mutants: EM101 *pls, etr1-1, etr1-9, ctr1, eto1*

Transgenic fluorescent lines: *pPLS::PLS:GFP, pPLS::GFP, p35S::SH-GFP, p35S::ETR1:RFP, p35S::PLS:GFP*

Transgenic tagged lines: *p35S::ETR1:HA*

Progeny of crossing: *pPLS:PLS-GFP* x *etr1-9*

Nicotiana benthamiana

2.1.3 Bacterial Strains

OneShot® TOP10 chemically competent *Escherichia coli (E. coli)* or OneShot® Mach1™ T1 Phage-Resistant chemically competent *E. coli* (Invitrogen™, Thermo Fisher Scientific, UK) were used for all Gateway cloning work. The *Agrobacterium tumefaciens* strain GV3 101 was used for stable *Arabidopsis thaliana* transformations and transient expression in *Nicotiana benthamiana*.

2.2 Plant Tissue Culture

2.2.1 Seed Sterilisation

Aliquots of seeds were placed in sterile 1.5 ml microcentrifuge tubes in a laminar flow cabinet and treated with 70% v/v ethanol for 30-60 seconds to partially de-wax the testa. The tubes were then filled with 20% v/v commercial bleach for 15 minutes, with occasional mixing by inversion. The seeds were washed five times with sterile milliQ filtered water and retained at 4°C for at least three days to encourage and synchronise germination (stratification). Seeds were stored in this manner for up to one month.

2.2.2 Culture Media

2.2.2.1 Liquid ½ MS10

Seedlings were grown in sterile ½ MS10: 2.2 g/l Murashige and Skoog medium (Sigma Aldrich; (Murashige and Skoog, 1962)), 10 g/l sucrose, at pH 5.7 (adjusted using 1M KOH). Media was autoclaved at 121°C for 20 minutes.

2.2.2.2 ½ MS10 with agar

8 g/litre agar was added to the above media recipe before autoclaving.

2.2.2.3 ½ MS10 with phytagel

2.5 g/litre was added to the above media recipe before autoclaving.

2.2.3 Plant Growth Methods

2.2.3.1 Agar and phytagel

Seedlings were distributed onto solid sterile ½ MS10 media in 90mm petri dishes (Sarstedt, Leicester, UK). Lids were sealed using Micropore^{TM} (3M, Minnesota, USA) tape, and seedlings were grown at 21°C, under a 16 hour photoperiod.

2.2.3.2 Liquid 1/2MS10

Liquid media (1 ml/well) was added into sterile 24-well plates (Sarstedt). For hormone and peptide assays, one seedling was grown in each well, at 21°C, under a 16 hour photoperiod.

2.2.3.3 Soil-based Growth

Seedlings were grown in a 5:1 mixture of Gem multipurpose compost and horticultural silver sand (both from LBS Horticulture Ltd., Lancashire UK) into 24-well trays and grown at 21°C, with a 16 hour photoperiod.

All compost was treated with “Intercept” systemic insecticide (Levinton Horticulture Ltd., UK), at a concentration of 60 mg/24-well tray.

2.2.4 Arabidopsis Seed Collection

Arabidopsis seeds were collected using the ARACON container system (Beta Tech, Gent, Belgium). ARACON bases were placed over the rosettes of the Arabidopsis plants as soon as they started to produce flowering stems. ARACON tubes fitted into the bases and contained each plant, preventing cross-pollination and enabling seed to be collected. After

drying the plants, seed was collected, sieved, and left to dry in a petri dish for two weeks. Seeds were transferred to microcentrifuge tubes and stored at room temperature.

2.2.5 Cross-pollination of *Arabidopsis thaliana* plants

Arabidopsis plants from one transgenic line were cross-pollinated using pollen from a different line. Plants from the parent lines were grown in soil for approximately two weeks. Open flowers and any siliques were removed from the plant to be pollinated. Large unopened buds were opened with fine forceps, and the stamens removed. An open flower from the second *Arabidopsis* plant line was removed just below the petals to reveal the anthers, and was brushed against the stigma to transfer the pollen. After leaving overnight, a second pollination was carried out to ensure the flower was pollinated. Maturing siliques were closely observed, so they could be removed from the plant prior to dropping seed.

2.2.6 Hormones

2.2.6.1 1-Aminocyclopropane-1-carboxylic acid (ACC)

ACC is an ethylene precursor molecule and is converted to ethylene by plants when supplied in plant media. ACC was stored at -20°C as 10 mM stock: 10.1 mg in 10 ml sdH_2O, and used at concentration required.

2.3 Bacterial Culture Conditions

2.3.1 LB Media

Transformed *E. coli* were grown on LB Media plates: 10 g/l Tryptone, 5 g/l yeast extract and 10 g/l NaCl, pH 7 adjusted using 1M KOH. Media was autoclaved at 121°C for 20 minutes. For solid LB media, 10 g/l agar was added before autoclaving. Antibiotic was added as appropriate once autoclaved.

2.3.2 Overnight Bacterial Growth

Bacteria used for transformations were spread onto sterile LB agar plates containing the appropriate antibiotic, and grown overnight at 37ºC.

Bacterial cultures were grown overnight in 5 ml sterile LB media containing the appropriate antibiotic, at 37ºC with shaking at 220 rpm.

2.3.3 Antibiotics

Antibiotic	Stock Solution Concentration	Working Concentration
Gentamycin	50 mg/ml in water	10-50 μg/ml
Kanamycin	50 mg/ml in water	50 μg/ml
Rifampicin	20 mg/ml in methanol	100 μg/ml

2.4 Nucleic Acid Isolation

2.4.1 Genomic DNA Extraction (Edward's Prep Method)

The Edward's Prep method produces low quality DNA for PCR applications not requiring pure DNA (Edwards et al., 1991). ≤100 mg plant tissue was frozen in a sterile 1.5 ml microcentrifuge tube and ground in liquid nitrogen with a micropestle. 400 μl extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added, the solution was vortexed and the sample was centrifuged at 15000x *g* for 4 minutes (1 minute in the original protocol). 300 μl of the supernatant was transferred to a new microcentrifuge tube. 300 μl isopropanol was added to precipitate genomic DNA; solutions were left at room temperature for 5 minutes and centrifuged at 15000x *g* for 5 minutes. Supernatant was removed and discarded from the visible pellet. In an addition to the original protocol (which vacuum dried the pellet), 200 μl 70% ethanol was added without resuspending to wash off salts, and samples were centrifuged at 15000x *g* for 5 minutes. Ethanol was removed, and pellet was allowed to dry on the bench. DNA was resuspended in 30 μl sterile water and stored at -20°C.

2.4.2 RNA Extraction and cDNA Synthesis

2.4.2.1 Plant Tissue

2.4.2.1.1 *Arabidopsis thaliana* seedlings

Seedlings (seven to ten days after germination) were transferred into a sterile microcentrifuge tube up to a fresh weight of 100 mg. Seedlings were frozen in liquid nitrogen and ground in the microcentrifuge tubes over dry ice, using a micropestle.

2.4.2.2 RNA Extraction

RNA was extracted from plant tissue samples using the Spectrum™ Plant Total RNA Kit from Sigma Aldrich.

A maximum of 100 mg ground plant tissue was placed into an RNase-free, liquid-nitrogen-cooled microcentrifuge tube without allowing the tissue to thaw. 500 µl of the Lysis Solution (containing 10 µl/ml β-mercaptoethanol) was added immediately and the tube contents were vortexed vigorously for at least 30 seconds. The tubes were incubated at 56°C for 5 minutes. Cellular debris was pelleted by centrifugation at 15000x *g* for 3 minutes. The supernatant was pipetted into the filtration column and centrifuged for 1 minute at maximum speed. 500 µl of Binding Solution was added to the flow through lysate and mixed thoroughly, then 700 µl of the mixture was transferred to a binding column and centrifuged for 1 minute at 15000x *g*. This was repeated for the remainder of the mixture.

The On-Column DNase Digestion Set (Sigma Aldrich) was used to remove DNA from the RNA preparations. The column was washed before treatment with 500 µl of Wash Solution 1 and centrifuged for 1 minute at 15000x *g*. 10 µl of DNase enzyme was added to 70 µl of DNase buffer, pipetted onto the centre of the binding column and incubated at room temperature for 15 minutes. 500 µl Wash Solution 1 was put through the column again and centrifuged at 15000x *g*.

The column was washed twice with 500 µl Wash Solution 2, centrifuging as before for 30 seconds and discarding the flow through liquid after each wash. The column was dried for 1 minute at 15000x *g*. RNA was eluted into a new 2 ml tube using 50 µl of Elution Solution, room temperature incubation for 1 minute, and 1 minute centrifugation at 15000x *g*. RNA was quantified (section 2.4.2.4), used for cDNA synthesis and finally frozen at -80°C for long-term storage.

2.4.2.3 cDNA Synthesis

cDNA was synthesised from RNA (above) using the Superscript® III First-Strand Synthesis System (Invitrogen).

2.5 µg sample RNA, 1 µl Oligo dT 20 (50 µM) and 1 µl dNTPs (10 µM) were placed into a 0.5 ml PCR tube, and the volume made up to 8 µl using sterile water. The samples were incubated at 65°C for 5 minutes, before being placed on ice for at least 1 minute.

The cDNA synthesis mix (below) was added to each tube, and the contents centrifuged briefly to collect the reaction mixture (20 µl total).

cDNA synthesis mix for 1 reaction:

5X RT Buffer	4 µl
25 mM MgCl2	4 µl
0.1 DTT	2 µl
RNaseIN	1 µl
Superscript III	1 µl
Total	**12 µl**

The reactions were incubated at 50°C for 50 minutes, then 85°C for 5 minutes before being put on ice for a minute, then centrifuged briefly to collect the samples at the bottom of the tubes. To remove the RNA template, 1 µl RNase H enzyme was added before incubating at 37°C for 20 minutes.

The cDNA samples were diluted 1 in 4 for PCR using milliQ H_2O.

2.4.2.4 Spectrophotometric Analysis of DNA and RNA

Nucleic acid concentration was determined using a NanoDrop (Wilmington, Delaware, USA) ND-1000 Spectrophotometer, set at 230 nm, and Nanodrop ND-1000 V3.5.2. software.

2.5 Polymerase Chain Reaction

2.5.1 Primers

Appendix V contains a complete list of primers used throughout this project.

2.5.2 Standard PCR

Standard PCR reactions used MyTaq™ DNA Polymerase (Bioline, London, UK) and a G-Storm GS1 Thermal Cycler (Somerton , Somerset, UK).

4 µl 10X MyTaq Reaction Buffer, 0.1µl MyTaq™ DNA Polymerase and 14.4 µl sterile H_2O were mixed per reaction.

Standard volumes of 0.5 µl template DNA and 0.5 µl of each primer (at 20 pmol/µl) were added per PCR tube to give a total reaction volume of 20 µl. The concentration of DNA

added to the reaction varied between 20 pg and 4 ng, dependant on the quality of the DNA and primers, and the difficulty of the template.

Thermal cycling temperatures:

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	95	1 minute	1
Denaturation	95	10 seconds	25-40 cycles
Annealing	*	10 seconds	
Extension	72	15 seconds	
Final Extension	72	1 minute	1
Refridgerate	4	Hold	1

*modified to tailor to annealing temperatures of specific primers – usually 2-5°C below the lower Tm of the primer pair.

2.5.3 Q5 Hot-Start High Fidelity DNA Polymerase Enzyme PCR

Q5 Hot-Start High Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA) was used to produce high quality PCR products for subsequent cloning. The enzyme has 3' to 5' exonuclease (proofreading) activity to minimise replication errors.

10 µl 5x Q5 reaction buffer with $MgSO_4$, 1 µl 10 mM dNTPs, 0.5 µl Q5 Hot-Start High Fidelity DNA Polymerase and 30.5 µl sterile H_2O were mixed together per reaction. 1 ng to 1 µg genomic DNA template, or 1 pg to 1 ng plasmid DNA template, and 2.5 µl of each primer (20 pmol/µl) were added to each PCR tube for a total reaction volume of 50 µl.

Thermal cycling temperatures:

Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	98	30 seconds	
Denaturation	98	10 seconds	30 cycles
Annealing	*	20 seconds	
Extension	72	** minutes	
Final Extension	72	2 minutes	
Soak	4	Hold	

* Q5 annealing temperatures (Ta = Tm_lower+3°C) (NEB Tm calculator)

** Extension time depends on size of desired PCR product – 20-30 seconds/kb (up to 40 seconds/kb for cDNA/long complex templates)

2.5.4 Quantitative PCR (qPCR)

Gene expression was measured by qPCR. Total plant cDNA was synthesised from total extracted sample RNA and gene expression was quantified using a SensiFAST™ SYBR® No-ROX Kit (Bioline) for qPCR. 20µl reactions were set up on ice, and in triplicate for each cDNA sample as below:

For/Rev Primer (20 pmol/µl)	0.23 µl each
cDNA (synthesised from 0.5 µg RNA)	0.5 µl
SYBR	10 µl
sdH_2O	9 µl

qPCR was run on a Rotor-gene Q (Qiagen/Corbett Rotor-gene 6000) with Rotor-gene 6 software (Corbett Life Science, St. Neots, Cambridgeshire, UK), under standard PCR cycling conditions (annealing temperature depending on primer T_a values) and between 30 and 50 cycles, depending on transcript abundance.

The PCR reaction mixture includes a SYBR® Green fluorescent dye which absorbs light at 488 nm and emits fluorescence at 520 nm, in the green region of the spectrum, when intercalated with double-stranded (ds) DNA (Invitrogen). At the end of each PCR cycle (of denaturing, annealing and extending), the dye associates with the newly formed strands of dsDNA and the emitted fluorescence is measured. The amount of fluorescence detected is

proportional to how much dsDNA is present in the reaction, which is ultimately dependent upon the amount of RNA transcript, derived from the gene of interest, that was present in the plant at the time of RNA extraction.

For each cDNA sample, transcript abundance of a gene of interest is quantified in relation to a stably-expressed internal reference 'housekeeping' gene (primer list in Appendix V). This allows the determination of fold-differences in the expression of the gene of interest which can be attributed to the treatment of the sample, and corrects variation between samples such as differences in the quality or quantity of the extracted RNA. The data was analysed by comparative quantitation using Rotor-gene Software. Each experiment contained three biological samples. The mean transcript abundance was calculated from the relative transcript abundance from each biological sample and represented graphically. Error bars show the upper and lower limit of the standard error of the mean.

2.5.5 Colony PCR

Colony PCR was used to detect a plasmid of interest in *E. coli* colonies after a transformation reaction into competent cells. Individual post-transformation *E. coli* colonies, grown overnight, were picked from LB agar plates using autoclaved sterile cocktail sticks and dipped into a standard PCR mix (section 2.5.2), including an appropriate primer pair producing a PCR product of approximately 500-600 bp (Appendix V). Thermal cycling conditions were the same as for standard PCR, with an additional 5 minutes initial denaturation step at the start to lyse bacterial cells. PCR reactions showing a high concentration of product on a 2% gel (section 2.6) were prepared for sequencing.

2.5.6 Purification of PCR Products

DNA fragments produced by PCR reactions were purified using the QIAquick PCR Purification Kit (Qiagen, Manchester, UK).

5 volumes of Buffer PB (containing 1:250 volume pH Indicator I) were mixed with 1 volume of the PCR sample. If the colour if the mixture was orange or violet, 10 µl 3M sodium acetate was added to turn the solution yellow. The sample was passed through a QIAquick spin column by centrifugation at 16000x *g* for 30-60 seconds. The flow-through liquid was discarded and the sample washed with 750 µl Buffer PE, centrifuging as before. The flow-through was discarded and the spin column was dried by centrifugation for an additional minute. The QIAquick column was transferred to a clean 1.5 ml microcentrifuge tube and DNA was eluted with 50 µl Buffer EB and centrifugation as before.

2.6 Agarose Gel Electrophoresis

Upon completion of a PCR reaction, DNA samples were separated by size using gel electrophoresis to identify PCR products. Agarose gels were prepared by dissolving either 1% or 2% (w/v) Agarose Multi-Purpose (Bioline) in 1x TAE Buffer (diluted 1 in 10 from 10x TAE Buffer: 242 g Tris, 37.2 g $Na_2EDTA.2H_2O$, 57.1 ml glacial acetic acid, in 5 litres) in a microwave. After cooling, 0.1 µg/ml of ethidium bromide was added before the solution was poured into a gel tray and left to set at room temperature. Samples were mixed with 1 in 4 volume 10x loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 0.25% acridine orange (w/v), 25% Ficoll (type 400) in water). Hyperladders I and IV (Bioline) were used as molecular markers. Gels were run in a gel tank containing 1x TAE, at 90V. Gels were imaged using a Syngene Bio Imaging System with GeneSnap software.

2.6.1 Gel Extraction of PCR Product

The S.N.A.P.™ Gel Purification Kit (Invitrogen) was used for the rapid purification of DNA fragments (100bp-6kb) from agarose gels.

DNA PCR products were separated on an agarose gel with Hyperladder I. The band was excised using a razor blade and placed into a 1.5 ml microcentrifuge tube. The gel slice was weighed, and 1 mg was assumed to be equal to 1 µl volume for the duration of the procedure. 2.5x the volume (of the gel slice) of 6.6 M sodium iodide was added and vortexed to mix well. The solution was incubated at 50°C until the agarose was melted. 1.5x the volume (of the gel slice and the sodium iodide combined) of binding buffer was added and vortexed.

The total mixture was put through the S.N.A.P. Purification Column and Vial three times, each time centrifuging at 3000x *g* for 30 seconds. Flow through was discarded and the column washed twice with 1x final wash. Column was dried by 1 minute centrifugation at >10000x g. DNA was eluted into a new sterile microcentrifuge tube with 40 µl sterile water. DNA concentration was determined using a Nanodrop ND-1000 Spectrophotometer, set at 230nm, and Nanodrop ND-1000 V3.5.2. software.

2.7 Creating Transgenic Plants

2.7.1 PCR product into entry vector

The gene of interest was amplified by PCR (section 2.5.3) from wild type *Arabidopsis* cDNA (section 2.4.2.3), the product size checked by gel electrophoresis (section 2.6), and purified (section 2.6.1). To create protein fusions to fluorescent or protein tags at the C-terminus of the gene of interest, the reverse primer was designed so it did not include the gene stop codon.

The kanamycin-resistant pENTR™/D-TOPO® vector (Life Technologies, California, USA) was used as an entry vector for the Gateway cloning system. The pENTR™/D-TOPO® vector provides Gateway *attL* sites on either side of the insertion region, for subsequent use with Gateway binary vectors (Appendix VI for vector details).

A 0.5:1 to 2:1 molar ratio of PCR product:TOPO vector was used in the TOPO cloning reaction. 7 µl fresh PCR product, 1.5 µl salt solution, 0.5 µl sterile H_2O and 1 µl of the pENTR™/D-TOPO® vector were mixed gently and incubated for 5 minutes at room temperature, before being placed on ice.

2.7.2 Transformation of chemically-competent *E. coli* cells

Vectors were transformed into OneShot® Mach1™ T1 Phage-Resistant chemically competent *E. coli* (Invitrogen™). 2 µl of the TOPO cloning reaction was added to each vial of the One Shot® TOP10 *E. coli* (thawed on ice) and mixed gently, without pipetting. The vials were incubated on ice for 5 minutes and heat-shocked for 30 seconds at 42°C without shaking. The vials were immediately transferred to ice, 250 µl LB medium was added and the tubes were shaken horizontally (200 rpm) at 37°C for 1 hour. 50-200 µl from each transformation were spread onto selective LB agar plates containing kanamycin and incubated overnight at 37°C. Colonies grown overnight were picked for analysis using colony PCR (section 2.5.5).

Control cloning and transformation reactions were carried out as detailed in the Life Technologies pENTR™ Directional TOPO Cloning Kit Manual.

2.7.3 Plasmid Purification

Colony PCR (section 2.5.5), using colony PCR primers (Appendix V), and gel electrophoresis (section 2.6) were used to identify bacterial colonies containing the entry

vector from 2.7.1. Colonies positive for the entry vector were grown overnight in 5 ml liquid LB media (section 2.3.2) containing the appropriate antibiotic.

Plasmid DNA was purified from the *E. coli* overnight cultures using the Wizard®Plus SV Minipreps DNA Purification System Kit (Promega, Southampton, UK).

5 ml of each bacterial culture was harvested by centrifugation for 10 minutes at 3000x *g*. The supernatant was discarded, and the pellet was resuspended by vortexing in 250 µl cell resuspension solution (CRA). The resuspended pellet was transferred to a sterile microcentrifuge tube and the cells were lysed by adding 250 µl cell lysis solution (CLA). The contents were mixed by inverting the tube four times and then incubated at room temperature for up to 5 minutes until the cell suspension cleared.

To inactivate endonucleases and other proteases release during the cell lysis step, 10 µl of alkaline protease solution was added, the tube was inverted four times to mix the contents and then incubated at room temperature for no longer than 5 minutes. 350 µl of neutralisation solution was added to each tube, the contents mixed by inverting four times, and the bacterial lysate was centrifuged at maximum speed (≥14000x *g*) in a microcentrifuge for 10 minutes at room temperature.

850 µl of the resulting cleared lysate was transferred to a prepared spin column. The supernatant was centrifuged at ≥14000x *g* for 1 minute at room temperature, before dismantling the spin column and discarding the flow through. With the spin column reassembled, 750 µl of column wash solution (previously diluted with 95% EtOH) was added to the column, and then centrifuged at ≥14000x *g* for 1 minute at room temperature. The flow through was discarded and the column was reinserted into the tube. The column wash was repeated with 250 µl of column wash solution, and the apparatus was centrifuged again for 2 minutes at room temperature.

The spin column was transferred to a sterile microcentrifuge tube and the plasmid DNA was eluted with 50 µl nuclease-free water in the centre of the spin column, and centrifuged at ≥14000x *g* for 1 minute. The purified plasmid DNA was stored at -20°C.

2.7.4 Glycerol Stocks

Bacterial cultures grown overnight were prepared for long-term storage by mixing the culture with glycerol.

1 ml of overnight bacterial culture (section 2.3.2) was mixed 2:1 with 60% glycerol, frozen in liquid nitrogen and stored at -80°C.

2.7.5 DNA Sequencing of plasmids

To check whether the purified plasmid from section 2.7.3 contains the correct sequence, plasmid samples were submitted for DNA sequencing.

The Durham University DNA Sequencing Service provided all DNA sequencing, using an Applied Biosystems 3730 DNA Analyser. The resulting DNA sequences were analysed using ApE (A Plasmid Editor, M Wayne Davis).

2.7.6 Linearizing pENTR™/D-TOPO® using Restriction Enzymes

Entry vectors containing the correct sequence of the gene of interest were used in the next steps of Gateway cloning.

Subsequent Gateway cloning steps are made more efficient if the entry vector is linearized beforehand. Some binary Gateway vectors also contain a kanamycin resistance gene for growth in *E. coli*, akin to the resistance gene in the pENTR™/D-TOPO® entry vector. The restriction enzyme PvuI was used to cut the kanamycin resistance gene in the entry vector, thus ensuring that any bacterial cells that take up the entry vector cannot survive in the presence of the antibiotic. PvuI only cuts once in the entry vector, importantly outside the *ETR1* or *PLS* gene sequences and the Gateway cassette, and produces linear vector DNA. A different restriction enzyme may have to be used if cloning other genes.

0.5 μg of pENTR™/D-TOPO® plasmid was mixed with 2 μl Buffer 3, 0.5 μl PvuI enzyme and 0.2 μl BSA (100 μg/μl) (New England Biolabs). The reaction was made up to 20 μl with sdH_2O and incubated for 1 hour at 37°C.

The whole reaction was analysed by agarose gel electrophoresis (section 2.6) and the plasmid was gel purified (section 2.6.1) to avoid any contaminating DNA.

2.7.7 Gateway LR Reaction

The LR reaction mobilises the gene of interest from the pENTR™/D-TOPO® entry vector, into a larger destination (binary) vector which can be introduced into plants. An LR Clonase II enzyme catalyses a recombination reaction between the *att*L sites either side of the gene in pENTR™/D-TOPO® vector and the *att*R sites in the destination vector, inserting

the gene of interest and associated resistance genes into the destination vector, creating an expression vector.

150 ng of the purified linear entry vector pENTR™/D-TOPO® (see above) and 75 ng of the destination vector were incubated for 1 hour at 25°C with 2 µl LR Clonase II enzyme (Life Technologies), in a total reaction volume of 12 µl using TE buffer. The reaction was stopped with 1 µl Proteinase K solution (2 µg/µl), incubated at 37°C for 10 minutes.

Newly-formed expression vectors were transformed into chemically competent *E. coli* (section 2.7.2), plated onto LB agar plates with the appropriate antibiotic and grown overnight at 37°C.

To check the presence of the gene of interest, colony PCR was performed on individual colonies (section 2.5.5). Sections 2.7.3 to 2.7.5 were repeated for the expression vectors.

2.7.8 Transformation of *Agrobacterium tumefaciens*

Expression vectors were transformed into the *Agrobacterium tumefaciens* strain GV3 101 for subsequent introduction into *Arabidopsis* plants.

1 µg of expression vector DNA was added to 250 µl of competent GV3 101 glycerol. Cells were incubated for 5 minutes on ice, 5 minutes in liquid nitrogen and 5 minutes at 37°C. 1 ml of liquid LB media was added and cells were shaken at 220 rpm for 2 hours at 30°C to recover.

Cells were pelleted in a microcentrifuge for 5 minutes at 3000x *g*. 1 ml of supernatant was removed, the cells were resuspended in the remaining liquid and spread onto fresh LB agar plates containing 50 µg/ml rifampicin, 25 µg/ml gentamycin and expression plasmid antibiotic selection. Plates were incubated for two to three days at 30°C. Colony PCR (section 2.5.5) was performed to check presence of plasmid, with an additional step to prepare the plasmid DNA from the *A. tumefaciens* cells. Before adding the bacterial colony to the PCR reaction, a small amount of the colony was picked from the agar plate and suspended in 10 µl of sterile H_2O. This solution was microwaved at full power for 2 minutes, then 0.5 µl of the solution was added to the PCR reaction. This method helps to break open the bacterial cells to allow access to the plasmid DNA inside.

2.7.9 Floral Dipping of *Arabidopsis thaliana* plants as a method of *Agrobacterium tumefaciens*-mediated stable gene expression

This protocol is a modified version of that described by (Bechtold et al., 1993). *Arabidopsis* plants of six to eight weeks in age were used in floral dipping. In the weeks before the dipping procedure, the plant stems were cut back several times to the rosette to promote the growth of more inflorescences. Open flowers and any young siliques were removed at two to three days before the dipping procedure, and again on the day of dipping itself.

A 5 ml liquid LB culture (containing 50 µg/ml rifampicin, 25 µg/ml gentamycin and appropriate selection for the transformation vector being used) was inoculated with a single GV3 101 *A. tumefaciens* colony (created and identified by section 2.7.8) and grown for 24 hours at 30°C with shaking at 220 rpm. This culture was used to inoculate 200 ml liquid LB (with the same antibiotic selection), and grown for 24 hours at 30°C with shaking. The culture was pelleted by centrifugation and resuspended in 1L of fresh 5% (w/v) sucrose solution. Finally, Silwett L-77 (Lehle Seeds, Texas, USA) was added to a final concentration of 0.05% (v/v).

Prepared *Arabidopsis* plants were submerged into the above solution containing the *A. tumefaciens* and stirred gently for 20 seconds. Following dipping, the transformed plants were placed in a plastic bag and left in a shaded position overnight to maintain humidity. The next day, the plants were removed and returned to normal growth conditions in the greenhouse. The same dipping procedure was repeated seven days after the first dipping and seed was periodically extracted and stored after three to four weeks.

2.7.10 Identification of *Agrobacterium*-transformed *Arabidopsis thaliana* plants

2.7.10.1 Soil selection

Dry seeds collected from plants transformed with a Basta herbicide-resistant construct were spread thinly on wet, intercept-treated soil. Trays were placed at 4°C for four days to promote germination, then moved into a long day photoperiod at 21°C. Seedlings were sprayed with 1:2500 Basta™ herbicide (Bayer CropScience, Cambridge, UK) seven and ten days after germination. Seedlings with Basta resistance are visibly larger after seven to ten days, and were transferred into soil for seed.

2.7.10.2 Agar Plate Selection

All agar plates used with antibiotics or other selective compounds were additionally supplemented with 200 μg/ml augmentin to avoid growth of residual *A. tumefaciens* still associated with the seed from the transformation process. The relevant selective treatment for the transformed construct was also added to these agar plates.

½ MS10 agar plates were supplemented with glufosinate-ammonium (Honeywell Riedel-de Haën, Seelze, Germany) to a final concentration of 10 μg/ml to select for seedlings with Basta resistance where it would be useful to check such seedlings for functional fluorescence during selection. After sterilisation and stratification (section 2.2.1), seedlings were grown at 21°C in the dark for two days before being transferred to a long day photoperiod for up to two weeks. Resistant seedlings are green and progress to the four-leaf stage, where they are transferred to soil. Non-resistant seedlings show an elongated hypocotyl and yellow cotyledons.

½ MS10 agar plates were supplemented with hygromycin to a final concentration of 20 μg/ml to select for seedlings containing hygromycin resistance.

Plants transformed with fluorescently-tagged genes were additionally screened for emitted green or red fluorescence on a Leica M165FC epifluorescence microscope (blue light for GFP, green light for RFP).

2.8 Transformation of *Arabidopsis pPLS:PLS-GFP* with organelle markers

The POLARIS-GFP fusion line *pPLS::PLS:GFP* was transformed with a kanamycin resistant pBIN2 vector containing the *p35S* promoter and DNA encoding for an RFP protein with an endoplasmic reticulum-localising HDEL motif, kindly provided by Pengwei Wang (Durham University). The construct was transformed into *A. tumefaciens* GV3 101 (section 2.7.8) and stably introduced into the *pPLS::PLS:GFP* plants (section 2.7.9). Successful transformants were selected by screening for emitted red fluorescence on a Leica M165FC epifluorescence microscope.

The trans-Golgi apparatus marker construct *pFGC-ST:mCherry* (Appendix VI) was obtained from NASC/ABRC and transformed into plants expressing *pPLS::PLS:GFP* via *A. tumefaciens* (sections 2.7.8 and 2.7.9). Successful transformants were identified by differential growth on ½ MS10 agar media containing glufosinate-ammonium: plants

expressing the construct were identifiable after ten days by the correct growth of leaves and roots, and mCherry (red) expression was confirmed as above.

2.9 Transient expression and purification of PLS-GFP and ETR1-HA, and PLS/ETR1 co-immunoprecipitation

2.9.1 Constructs

To investigate the interaction between the PLS peptide and the ethylene receptor ETR1, two DNA constructs were created by Gateway cloning (sections 2.7.1 to 2.7.8). The 105-nucleotide *PLS* gene (without the stop codon) was inserted into the pEarlyGate103 (pEG103) destination vector, containing the *p35S* promoter and a C-terminal GFP tag, producing a vector containing the *p35S:PLS:GFP* DNA (Appendix VI). The ETR1 cDNA was inserted into the pEarlyGate301 (pEG301) vector to create a *p35S::ETR1:HA* construct, producing an ETR1 protein with a C-terminal HA tag (Appendix VI).

2.9.2 Infiltration into *Nicotiana benthamiana*

The transient expression of constructs in *N. benthamiana* (tobacco) leaves was based on the protocol stated in (Brandizzi et al., 2002). Competent *A. tumefaciens* GV3101 cells were transformed with the desired plasmid containing the fragment of interest (as described in 2.2.2.2). Individual colonies were used to inoculate liquid LB cultures containing 25 μg/ml gentamicin, 25 μg/ml rifampicin and the specific antibiotic required to select for the desired plasmid. The liquid cultures were grown at 30°C for 14-16 hours with shaking at 220 rpm. Additionally, liquid cultures of GV3101 containing the p19 protein that is encoded by the tomato bushy plant virus were also prepared in order to suppress post-transcriptional gene silencing (Voinnet et al., 2003). The overnight cultures were grown until an OD_{600} of approximately 0.6 was reached, and then centrifuged at 3000x *g* for 5 minutes. These cells were then twice washed with 2 ml of an infiltration buffer containing 10 mM $MgCl_2.6H_20$, 200 μM acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone (Fluka)) and 10 mM MES (2-(N-Morpholino)-ethanesulfonic acid, Melford) pH 6.5, resuspended in 1 ml of the same solution and subsequently incubated at room temperature for 3-5 hours. Prior to infiltration, each construct was mixed with p19 and infiltration buffer in a 1:1.2:1.8 ratio.

Several small cuts were made with a scalpel on the abaxial surface of the *N benthamiana* leaves, and were subsequently injected with each of the constructs using a syringe. The

plants were approximately seven to ten weeks old; the chosen leaves were healthy and of length 3-6 cm, and three to four leaves were infiltrated with each construct.

2.9.3 Protein extraction and PLS/ETR1 co-immunoprecipitation

Total protein was extracted from the infiltrated leaves of *N. benthamiana* plants three days after infiltration for co-immunoprecipitation (Co-IP/pull-down) experiments to investigate the interaction between PLS and ETR1 proteins.

1.5 g of leaf tissue was harvested from each *A. tumefaciens* construct infiltration event, frozen with liquid nitrogen and ground gently using a mortar and pestle. The homogenate was transferred to a pre-cooled microcentrifuge tube. 2 ml of extraction buffer was added (20 mM sodium phosphate pH 7.4, 100 mM NaCl, 80 mM KCl, 1% glycerol, 0.1 % Triton, 10 mM DTT, plus 1 mini protease inhibitor cocktail tablet (Roche, Switzerland) per 20 ml of extraction buffer, and the extra addition of either 2 mM EDTA or 0.5 µM $CuSO_4$ for binding studies), and the solution was ground further and votexed until the homogenate was smooth. The solution was centrifuged for 12 minutes at 14000x *g*, 4°C.

ChromoTek (Planegg, Germany) anti-GFP beads were used to immunoprecipitate the PLS-GFP protein, and Miltenyi Biotec (Surrey, UK) anti-HA beads for the HA-tagged ETR1.

25 µl bead slurry was resuspended in 500 µl ice-cold dilution buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) and centrifuged for 2 minutes at 2500x *g* at 4°C. the supernatant was discarded and the beads were washed twice more with 500 µl ice-cold dilution buffer.

The supernatant from the protein sample extraction from *N. benthamiana* plants was mixed with 50 µl GFP or HA beads and incubated for 30 minutes at 4°C, mixing every 2 minutes. The mixture was centrifuged at 2500x *g* for 2 minutes at 4°C, washed twice with 500 µl ice-cold dilution buffer, and the beads were transferred to a new microcentrifuge tube.

The target protein was eluted with the addition of 100 µl 2x SDS sample buffer (120 mM Tris pH 6.8, 50 mM 4% (w/v) SDS, 20% (v/v) glycerol) and the sample was boiled for 10 minutes at 95°C to dissociate immunocomplexes from the beads. The mixture was centrifuged for at 2500x *g* for 2 minutes at 4°C to separate the beads, and the supernatant was transferred to a new microcentrifuge tube. The supernatant was used in SDS-PAGE analysis.

To investigate the specificity of the PLS-ETR1 interaction, 5 nM or 25 nM of synthetic full length PLS peptide (synthesis protocol set out in 2.11) was added to the *N. benthamiana* leaves 30 minutes prior to harvesting. The synthetic peptide was added to compete with the transiently expressed PLS peptide.

2.9.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate protein fragments. The complexed proteins from the pull-down assay were analysed on 10-12% acrylamide gels.

Firstly, the resolving gel was prepared by adding the chosen amount of acrylamide (ProtoGel, 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide solution, National Diagnostics) to the resolving buffer (0.1% (w/v) SDS, 375mM Tris, polymerized via the addition of 0.1% (v/v) ammonium persulphate solution (APS) and finally set by the addition of 1.4 µl/ml TEMED (NNN'N'-Tetramethylethylenediamine). The stacking gel was then prepared again by adding the appropriate amount of acrylamide to the stacking buffer (consisting of 0.1% w/v SDS, 125 mM Tris). Polymerization was activated by adding 0.1% (v/v) APS and set using 4 µl/ml TEMED.

SDS-PAGE gels were run in a tank containing an electrode buffer (25 mM Tris, 0.1% (v/v) glycerol, 190 mM glycine, diluted 1:10 with dH2O) at 90V for approximately 90 minutes. 6 µl PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoFisher Scientific) was loaded as a protein size marker, displaying coloured bands at 10, 15, 25, 35, 55 70, 100, 130 and 250 kDa.

2.9.5 Western Blotting

Following electrophoresis, the SDS gels were first washed in 1x transfer buffer (0.04% (w/v) SDS, 20% (v/v) methanol, 38 mM glycine, 48 mM Tris) for 5 minutes. The proteins were then transferred overnight onto nitrocellulose membranes (Whatman, GE Healthcare Life Sciences, Buckinghamshire, UK) in a 5 litre tank containing transfer buffer at 30 V.

The nitrocellulose membranes were incubated in milk buffer (5% (w/v) dried skimmed milk powder (Tesco), 150 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween 20, pH 7.4) for 20 minutes to block non-specific protein binding. Following this treatment, the membranes were incubated with primary antibody for 2.5 hours (GFP [Abcam, Cambridge]: rabbit, 1:10000; HA [Roche], rat, 1:3000). Excess primary antibody was then removed by washing three times in 2x TBST (150 mM NaCl, 10 mM Tris, 0.1% (v/v) Tween 20, pH 7.4); for 2 minutes, 5 minutes and 10 minutes. Membranes were incubated in milk buffer

made with 1x TBST (5% (w/v) dried skimmed milk powder, 75 mM NaCl, 5 mM Tris, 0.1% (v/v) Tween 20, pH 7.4) for 10 minutes, and subsequently incubated for 1 hour with the ECL peroxidase-labelled anti-rabbit or anti-rat IgG secondary antibody, diluted 1:20000 in milk buffer. Excess secondary antibody was removed again by washing three times in 1x TBST, as with the primary antibody. In order to visualize the probed blot, the membrane was incubated with ECL Western Blotting Detection Reagent immediately prior to imaging. The horseradish peroxidase conjugated to the secondary antibody was detected by using X-ray film, which was subsequently developed in a dark room.

2.10 Confocal Laser Scanning Microscopy (CSLM)

CSLM images were obtained by a Leica SP5 TCS confocal microscope using 40X and 63X oil immersion lenses. Excitation of fluorophores was performed by the specified lasers: 405 nm UV (for ethidium bromide), 488 nm 20mW Argon (for all GFP, 5-FAM and acridine orange experiments), 543 nm 1.2mW HeNe (for propidium iodide, RFP and mCherry fluorophores) and 594 nm 2mW HeNe (for ER Tracker™).

For standard photomultiplier tubes, a laser power of 21% and a smart gain of 800-1000 mV were used, depending on the intensity of the fluorescence. The HyD detector was used at 70 to 120%.

2.10.1 Seedlings expressing GFP

pPLS::PLS:GFP, pPLS::GFP and *p35S::GFP* seedlings were grown for seven days on phytagel ½ MS10 media (section 2.2.2.3), approximately 25 mm of the root tip was removed and mounted in dH_2O on a microscope slide, and a 1.5 mm cover slip was placed on top. The cover slip was secured to the microscope slide using Micropore^{TM} tape (3M).

pPLS::PLS:GFP seedlings treated with ACC (section 2.2.6.1) were grown for seven days on phytagel ½ MS10 media, then transferred into liquid ½ MS10 media (2.2.3.2) containing either 1 or 10 μM ACC for 2 or 24 hours. Root tips were mounted as above.

Seedlings were imaged using laser settings at 21% power, 488 nm 20 mW, 1013 V gain; detected at 519 nm.

2.10.2 Synthetic 5-FAM-PLS(N1) Treatment

Seedlings were grown for 10 days from seed according to sections 2.12.2 and 2.12.2.1, in growth media supplemented with 50nM and 100nM fluorescent 5-FAM-PLS(N1) peptide. Root tips were mounted as above and imaged by CLSM at ten d.a.g. using identical laser and brightness settings: 488 nm argon, 20 mW at 30%, HyD 5 detector at 110.0 at a maximum of 518 nm.

2.10.3 Root Staining

2.10.3.1 Propidium Iodide

Whole *Arabidopsis* seedlings were incubated in 10 mg/l propidium iodide solution for 90 seconds. Seedlings were transferred to dH_2O for the same amount of time and the root tip was removed and mounted as before on a microscope slide in dH_2O.

Root tips were imaged by laser settings at 543 nm HeNe 1.2 mW, detected by HyD:70 at a maximum of 636 nm.

2.10.3.2 ER-Tracker™

Seven-day-old seedlings were removed from ½ MS10 phytagel media and placed into liquid ½ MS10 media containing 1 µM ER Tracker™ Red (BOPIDY® TR Glibenclamide) (Life Techonologies). Seedlings were incubated in the dye for 30 minutes in the dark before root tips were mounted in the same solution.

ER Tracker™ dye was excited at 594 nm by HeNe 2 mW laser, detected by HyD:58 at a maximum of 615 nm.

2.10.3.3 Acridine Orange/Ethidium Bromide

As described in Kasibhatla et al. (2006).

Acridine orange (AO)/ethidium bromide (EB) staining was used to visualise cells which have lost membrane integrity due to DMSO treatment.

Plants were grown in liquid ½ MS10 containing 0, 0.5 or 1% DMSO by volume, root tips were removed and stained for 2 minutes in a 1x solution of AO/EB (10 µl of 100x stock solution in 990 µl PBS buffer, pH 7.4).

Stock solution: 50 mg ethidium bromide (5 ml of 10 mg/ml solution into 50 ml H_2O), 15 mg acridine orange, in 1 ml 95% ethanol, 49 ml dH_2O, stored at -20°C, no light.

Root tips were mounted in water or staining media, the cover slip was secured around each edge by nail varnish, and the roots were imaged by CLSM. AO emits light at 525 nm, EB at 605 nm. Healthy cell membranes are stained with acridine orange (green), damaged membranes are stained inside with ethidium bromide (orange).

2.11 *In vitro* Peptide Synthesis

2.11.1 Automated Fmoc Solid Phase Peptide Synthesis (SPPS)

Peptides were synthesised by SPPS using an automated microwave method and carried out on a CEM Liberty1 single-channel peptide synthesiser with a Discovery microwave unit. Reactions were performed in a 30ml PTFE reaction vessel with microwave heating and agitation by bubbling nitrogen.

Reagents were sourced from Novabiochem, Sigma Aldrich, CEM Corp and AGCT Bioproducts.

2.11.1.1 Resin

Peptide synthesis extended from the C-terminus, bound to 2-chlorotrityl chloride resin (0.122 mmol scale, Novabiochem) using Fmoc protected amino acids. The first amino acid residue at the C-terminus (histidine) was coupled manually by mixing 76 mg (1 eq.) Fmoc-His(trt)-OH, 0.09 ml (4 eq.) N,N-diisopropylethylamine (DIPEA), 1 ml dichloromethane (DCM) and 1 ml dimethylformamide (DMF) until the amino acid powder had dissolved. The mixture was added to 0.1 g resin and stirred gently for 120 minutes at room temperature. The resin was washed with 3x DCM/MeOH/DIPEA (17:2:1), 3x DCM, 2x DMF and 2x DCM. Peptides that do not have histidine at the C-terminus were synthesised by the automated microwave method from the start.

2.11.1.2 Amino Acid Coupling

Amino acid coupling reactions were performed using Fmoc-protected amino acids present in a 5-fold excess (2 M concentration), activator (10 eq, 0.5 M HOBt in DMF) and activator base (0.8 M DIC in DMSO). For double and triple couplings the reaction vessel was drained after each coupling cycle and fresh reagents were added.

Before synthesis, a room temperature preactivation period of 1 to 2 hours was used. Microwave-assisted couplings were performed at 0.10 mmol scale for 10 minutes at 75°C at 25W power unless otherwise stated. Cys and His residues were coupled at low temperature (10 minutes at room temperature followed by 10 minutes at 50°C, 25W). Arg

residues were double coupled, firstly by 45 minutes at room temperature plus 5 minutes at 75°C (25W), and second by the standard microwave conditions above. Fmoc group removal was carried out by two piperidine solution treatments (20% piperidine in DMF) in succession: 5 minutes then 10 minutes.

2.11.1.3 Cleavage

Peptide cleavage from resin was carried out in 3 ml 95% TFA in dH_2O/TIPS (2.85 ml TFA, 0.15 ml dH_2O, 0.15 ml triisopropylsilyl) for 3 hours with gentle stirring. Solution was filtered through a fritted reaction vessel into pre-chilled diethylether (-20°C) to precipitate before centrifugation at 4500 rpm for 15 minutes at room temperature. Supernatant was discarded; pellet was resuspended in chilled ether and centrifuged as before.

Peptide was dissolved in H_2O with a small volume of MeCN and lyophilized to produce a powder using a Sciquip Christ Alpha 2-4 LSC freeze dryer.

2.11.2 Preparative High-Performance Liquid Chromatography (HPLC)

Peptide products were analysed and purified by HPLC at 280 nm. 25-50 mg of freeze-dried peptide sample was dissolved in 1 ml 1:1 H_2O:MeCN and injected onto a Speck and Burke Analytical C18 Column (5.0 μm, 10.0 x 250 mm) attached to a PerkinElmer (Massachusetts, USA) Series 200 LC Pump and 785A UV/Vis Detector. Separation was achieved by gradient elution of 10-80% solvent B (solvent A = 0.08% TFA in H2O; solvent B = 0.08% TFA in ACN) over 60 minutes, followed by 80-100% B over 10 minutes, with a flow rate of 2 ml/min. Selected peptide fractions were lyophilized and a mass assigned using MALDI-TOF MS.

2.11.3 Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Peptide sequences were identified using MALDI-TOF MS, using an Autoflex II ToF/ToF mass spectrometer (Bruker Daltonik GmBH, Germany) equipped with a 337 nm nitrogen laser. A few mg of freeze-dried peptide were dissolved in 1:1 deionized water/MeCN. Sample solution (1 mg/ml) was mixed with matrix solution (α-cyano-4-hydroxy-cinnamic acid at ~50 mg/ml) in ratio of 1:9. 1 μl of this solution was spotted onto a metal target and placed into the MALDI ion source. Reflectron mode was used for molecules with m/z < 4000. MS data was processed using FlexAnalysis 2.0 (Bruker Daltonik GmBH). MALDI spectra for the synthetic peptides are displayed in Appendix II.

2.12 Plant Root Length Assays

2.12.1 DMSO Assays

Col-0 wild type and *pls* mutant seedlings were sterilised and stratified (section 2.2.1). Seeds were placed onto 90 mm square (Sarstedt) ½ MS10 agar plates containing varying concentrations of DMSO (0.01, 0.1, 1, 2, 4, 5, 6, 8 and 10% DMSO, with 10% H_2O/0% DMSO control). The seedlings were grown vertically at 22°C, under a 16 hour photoperiod and root growth was measured every day at noon for ten days.

The plates were scanned using an Epson Expression 1680Pro flatbed scanner and root length from time of transferral was measured using the 'Measure' tool on ImageJ.

2.12.2 Liquid Media Assays

Assays to introduce synthetic peptides or copper ions into seedlings were based on similar peptide-feeding experiments (Matsuzaki et al., 2010).

The following assays were all performed using stratified *Arabidopsis* seeds (section 2.2.1), placed individually into 1 ml liquid ½ MS10 plant media (section 2.2.2.1), and grown in 24-well tissue culture plates (Sarstedt) with 1 ml of media per well. The treatment added to the liquid media differs in each case.

Seedlings were grown for ten days post germination, arranged on acetate, scanned to create a digital image and root lengths of seedlings were measured using ImageJ. Statistical analysis was performed using the Real Statistics Resource Pack software (Release 3.8) in Excel. Copyright (2013 – 2015) Charles Zaiontz. www.real-statistics.com.

2.12.2.1 Synthetic Peptides

Purified freeze-dried peptide was dissolved in 1 ml DMSO to create a 500 μM stock solution.

Peptide stock solution was added to liquid ½ MS10 plant media containing 0.1% DMSO to make a final peptide concentration of 50 or 100 nM (or 10, 25, 50 and 100 nM for dose-dependent assays).

2.12.2.2 Copper Sulphate

1 mM copper sulphate ($CuSO_4$) solution was filter sterilised and added to autoclaved liquid ½ MS10 plant media to create final $CuSO_4$ concentrations: 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μM.

2.12.2.3 Copper Chelator

The copper chelator bathocuproine disulfonic acid (BCS) was added to liquid media to produce final concentrations of 0, 10, 50, 100, 250 and 500 μM.

2.13 Copper Binding Assays with the POLARIS Peptide

The capability of the PLS peptide to bind copper ions was investigated by adding reduced Cu^+, also known as Cu(I), to synthetic PLS peptide and recording the resulting changes in absorbance and fluorescence.

2.13.1 Estimating Peptide Concentration and Quantifying Thiol Groups

1.3 mg of freeze-dried synthetic PLS peptide (Cambridge Research Biochemicals, Billingham) was dissolved in 1 ml DMSO. An aliquot was added to aqueous buffer (10 mM HEPES pH7, 20 mM NaCl, 8mM KCl) and the UV-vis spectrum was recorded at 280 nm. From the absorbance and the ProtParam estimated extinction coefficient of 2980 M^{-1} cm^{-1}, the concentration was estimated to be 395 μM.

Reduced thiol groups on the cysteine residues were quantified by reacting 50 μl of the peptide-DMSO solution with Ellman's reagent (Ellman, 1959; Riddles et al., 1979).

2.13.2 Titration with Cu^+

50 μl of the PLS stock solution was added to 950 μl buffer (10 mM HEPES pH7, 20 mM NaCl, 8mM KCl) giving a final PLS concentration of 19.6 μM. The diluted PLS solution was titrated with 0, 10, 20, 30 and 40 μM reduced Cu^+ in an anaerobic glove box (Belle Technology, Weymouth, UK). After each subsequent addition of Cu^+, the solution was mixed thoroughly and the absorbance (Lambda 35 UV/Vis spectrophotometer, PerkinElmer, Massachusetts, USA) and fluorescence (Cary Eclipse Fluorescence spectrophotometer, Agilent, California, USA) spectra were recorded.

2.13.3 Titration with Cu^+ in the presence of BCS and BCA copper chelators

In a glove box, as above, PLS peptide prepared as above was titrated with 0, 20, 40, 60, 80 and 100 μM Cu^+ in the presence of 140 μM BCA and the absorbance was recorded at 562 nm. 140 μM BCA was also titrated with the same Cu^+ concentrations in the absence of PLS, and both with and without 50 μl/ml DMSO.

PLS was also titrated with the same Cu^+ concentrations in the presence of 120 μM BCS, and the absorbance recorded at 483 nm.

Chapter 3 . Structure and Function Relationships of POLARIS

3.1 Introduction

The experimental work in this chapter aims to investigate homologues of *POLARIS* in other species, predict secondary and tertiary structure of the peptide, and deduce whether specific domains of the PLS peptide are required for its function whilst identifying key amino acid residues.

The *POLARIS* (*PLS*) gene was identified in *Arabidopsis thaliana* by insertional mutagenesis using a promoter trap method, resulting in a T-DNA insertion (containing a promoterless *gusA* gene) interrupting the *PLS* coding sequence (Topping et al., 1994). Homozygous transgenic lines have a short-root phenotype, designated the *pls* mutant. The T-DNA had inserted into the small open reading frame (ORF) of 108bp, which was predicted to code for a 36-amino acid POLARIS (PLS) peptide (Casson et al., 2002). Heterozygote seedlings have an intermediate root length between those of the wild type and the *pls* mutant, promoting further investigation of a dose-dependent root growth response to the PLS peptide.

There is little information about the structure of the POLARIS peptide. The first part of this chapter examines the predicted functional domains and potential structures of the peptide using a bioinformatics approach.

The *pls* mutant is partially complemented by *PLS* cDNA, suggesting that *PLS* transcription is necessary for gene function (Casson et al., 2002), but attempts to isolate the PLS peptide have proved unsuccessful, possibly due to its small size or high hydrophobicity. To avoid this issue, synthetic PLS peptide has been produced and has been used in this chapter to investigate the responses of wild type C24 and the *pls* mutant to PLS.

3.2 The structure of the POLARIS peptide

Structural characteristics of amino acid residues can be analysed by Jalview (Waterhouse et al., 2009) to give information about the peptide domains. The PLS C-terminus contains amino acids which are likely to form a hydrophobic region (Figure 3-1, top) with a helical structure (Figure 3-1, bottom).

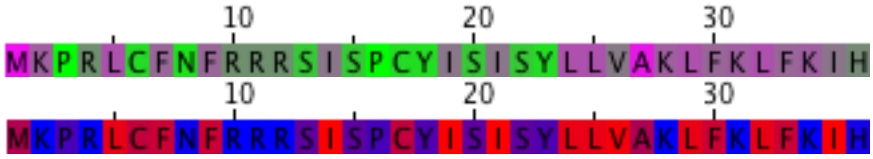
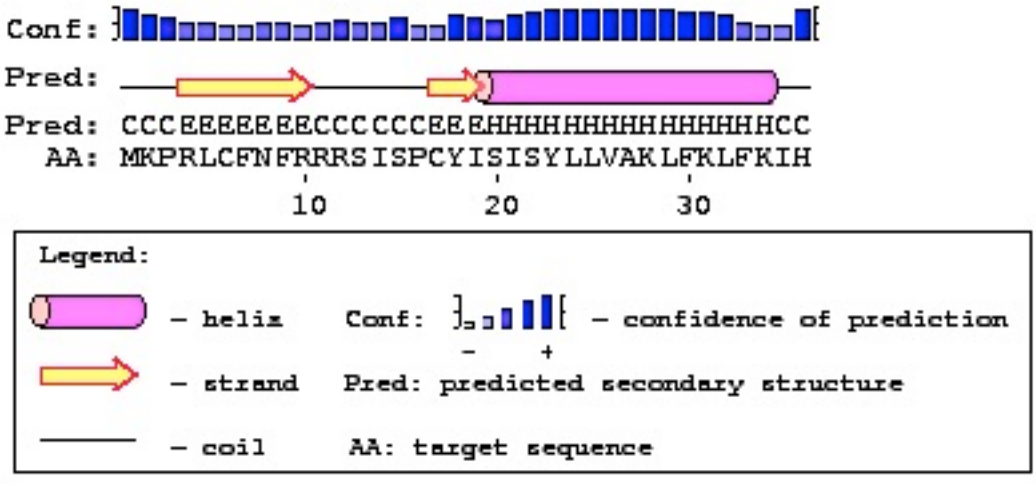


Figure 3-1. Predicted regions of secondary structure in the 36 residue POLARIS peptide derived from amino acid characteristics. Top; purple indicates hydrophobic residues. Bottom; red shows the likelihood of an amino acid to be present in a helix.

The C-terminus is predominantly helical, with a pattern of hydrophobic valine (V), leucine (L) and phenylalanine (F) residues promoting the formation of a helix. The presence of these hydrophobic amino acids suggests this region of the peptide may be involved in interactions with other hydrophobic region in membranes or other proteins.

The N-terminus is predicted to contain beta-sheet regions between amino acid residues 4-11 and 13-24, with some bioinformatics tools predicting a turn in the vicinity of the three arginine (R) residues at positions 10-12 (Figure 3-2 B).

A



B

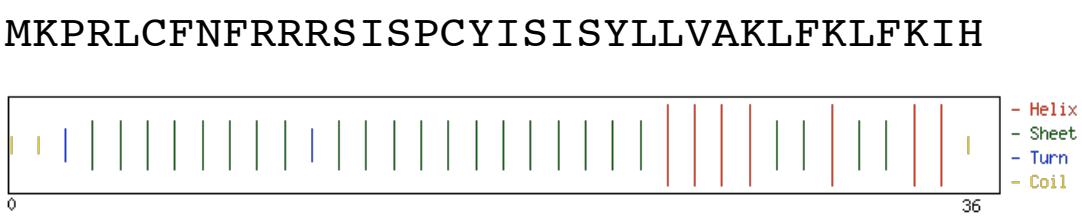


Figure 3-2. Secondary structure predictions for the POLARIS peptide. A. The PSIPRED Protein Sequence Analysis Workbench, UCL, (Jones, 1999). B. Chou and Fasman Secondary Structure Prediction Server (Chou and Fasman, 1974b, a).

3.2.1.1 POLARIS peptide structure in 3D

The predicted secondary structures were used to propose several approximate 3D structures for the PLS peptide (Figure 3-3). Panels A-E represent the five most likely structures for PLS in descending order of quality (with A being the highest quality model).

The models were produced by RaptorX (Peng and Xu, 2011b, a; Kaellberg et al., 2012; Ma et al., 2013); a protein structure prediction server which helps to predict structures of protein sequences without close homologues in the Protein Data Bank (PDB) (usually recruited as a prediction tool by other structure prediction servers). The PDB does not hold any information about the crystal structure of PLS, therefore modelling software cannot increase the quality of the model due to a lack of structural information from homologous proteins. The RaptorX server assesses the quality of the predicted models using a number of measures. Each model is assigned a score (falling between 0 [worst] and the number of amino acids in the protein [best]), an unnormalized Global Distance Test (uGDT) to

estimate the modelling error (a uGDT >50 is a good indicator of quality), and a p-value to predict the likelihood of a model being worse than the best of a set of randomly-generated models for the protein in question. P-values of less than 10^{-3} are a good indicator of quality. The quality scores for the five predicted PLS models are shown in Table 3-1.

Table 3-1. Quality scores for POLARIS predicted structure models.

Model	**Score**	**uGDT**	**p-value**
Threshold for good quality	***Up to 36***	***>50***	***<10^{-3}***
Model A	22	27	1.72e-02
Model B	21	24	2.32e-02
Model C	19	21	3.37e-02
Model D	19	21	3.65e-02
Model E	19	20	3.33e-02

The quality scores for the five PLS models are below the threshold to be considered 'good quality' models. This is partly due to the small size of the peptide; the longer the sequence, the more confident RaptorX can be in its predictions. The predicted models will be taken into consideration when analysing the structure and function of the PLS peptide, but we cannot infer too much structural or functional knowledge from predicted structures in the absence of experimental evidence.

Nevertheless, the predicted models are consistent with the secondary structure predictions mentioned previously (Figure 3-2). The C-terminus is uniformly predicted to be alpha-helical, and the N-terminus contains beta-sheets or unstructured regions. All five models predict that the three arginine residues at positions 10-12 create a loop region in the protein backbone, which appears to create a pocket-like area which could be important for peptide function.

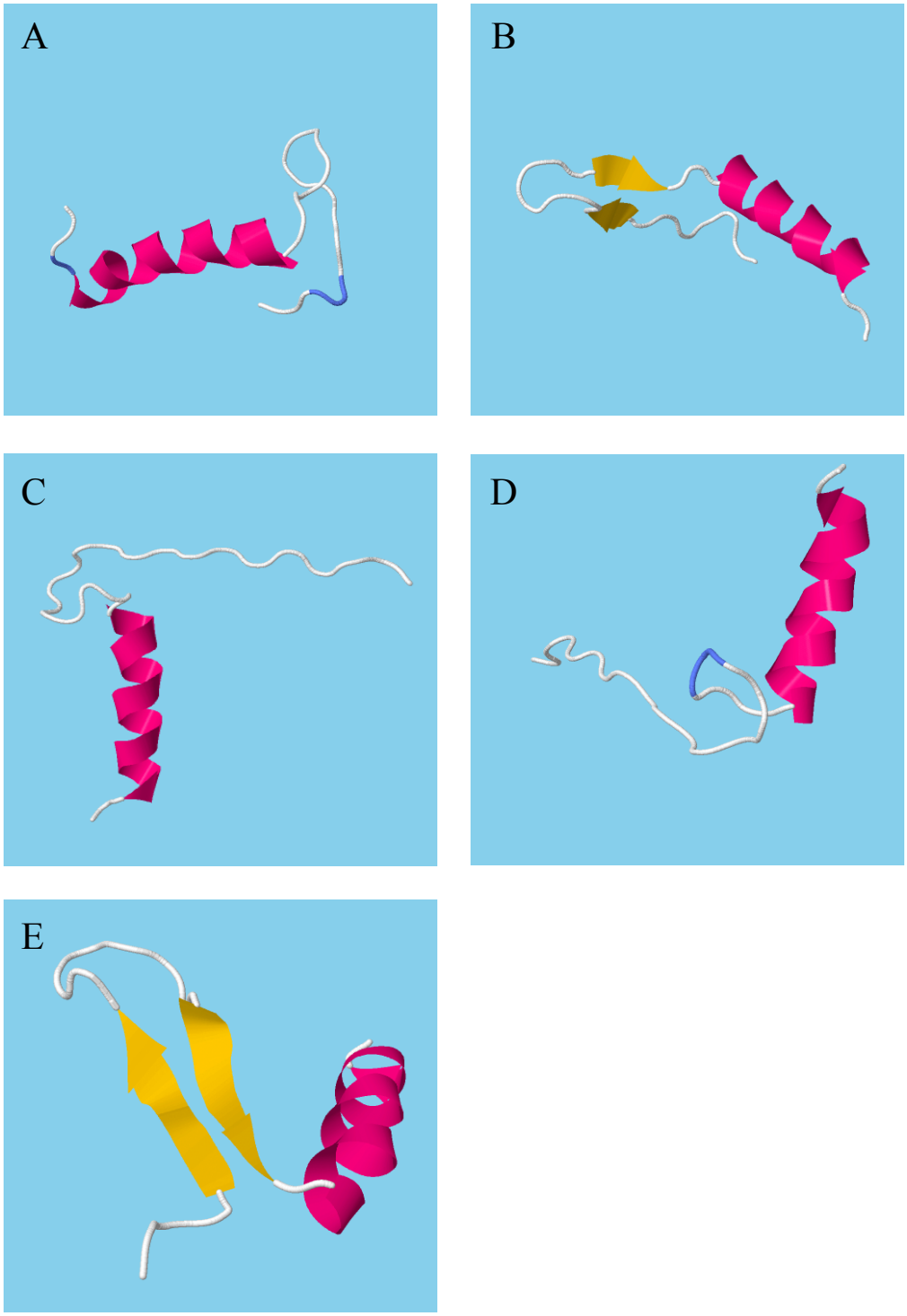


Figure 3-3. Predicted tertiary structures of the POLARIS peptide generated by the RaptorX server. A-E: five predicted 3D structures of PLS, ranked from best (A) to worst (E) quality, judged by the RaptorX quality scores (refer to **Table 3-1**). Alpha helices are shown in pink and beta-sheet structures are indicated by yellow arrows. Regions of amino acid residues which may form turn structures are marked in blue.

3.3 Identifying homologues of POLARIS in other species

The PLS peptide was identified in *Arabidopsis*, and evidence points to the peptide having a role in mediating ethylene signalling, and additionally contributing to signalling pathways involving other plant hormones, for example auxin. Ethylene has been identified as a key phytohormone throughout the plant kingdom, contributing patterning, growth and cell death information to regulate a huge range of plant processes (Schaller and Kieber, 2002).

In *Arabidopsis*, PLS appears to play a key role in mediating ethylene responses and could be expected to be present in many other plant species. To investigate whether the *PLS* gene may exist in other plants, a BLAST search for homologues in a number of plant species was undertaken, with resulting sequences aligned using CLUSTALX and viewed in Jalview (Waterhouse et al., 2009).

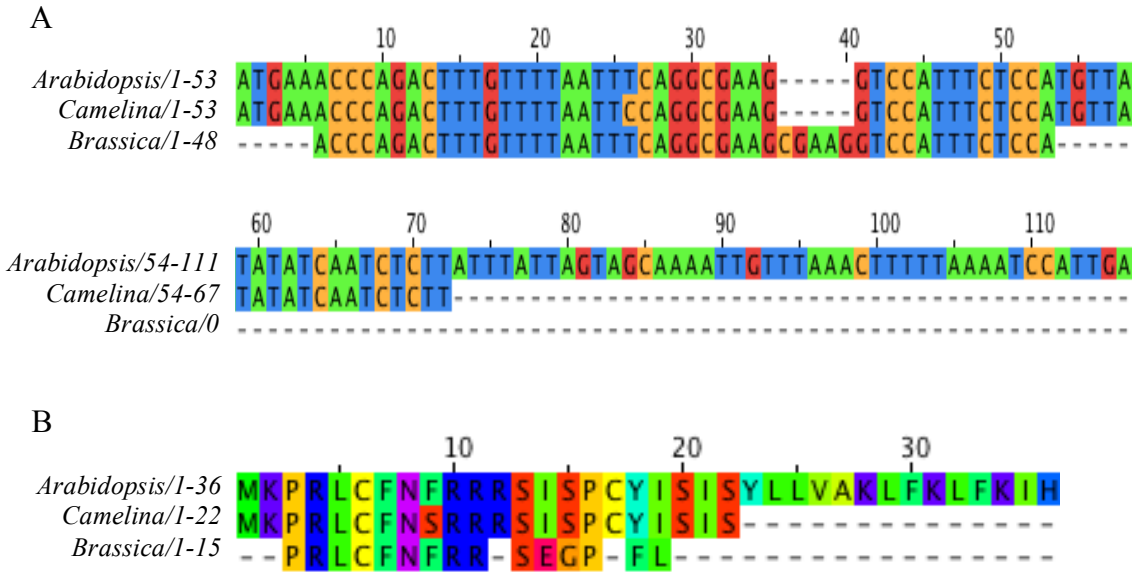


Figure 3-4. Homology between the *PLS* nucleotide sequence (A) and the PLS peptide amino acid sequence (B) in *Arabidopsis thaliana, Camelina sativa* and the partial sequence identified in *Brassica rapa* sp. pekinensis.

Two species were identified in having partial sequence similarity to the *PLS* gene (Figure 3-4): *Camelina sativa* (a relative of *Arabidopsis,* from the same family; *Brassicaceae*) and *Brassica rapa* subsp. pekinensis (chinese cabbage).

The *C. sativa* PLS is identical to the N-terminal 22 amino acids of the *Arabidopsis* PLS except for one substitution; phenylalanine to serine at position nine. The fragment of sequence identified in *B. rapa* has less homology with *Arabidopsis* PLS, but still includes 11 identical amino acids over the 16 N-terminal residues in PLS, with other positions substituted for amino acids with similar properties; for example isoleucine changed to leucine, both hydrophobic and non-polar, at position 19. Figure 3-5 illustrates the homology between the amino acid sequences identified in *Arabidopsis, C. sativa* and *B. rapa* with highlighted conserved residues.

Figure 3-5. Conserved residues between the PLS homologues. The amino acid sequences in *Arabidopsis thaliana, Camelina sativa* and the partial sequence identified in *Brassica rapa* sp. pekinensis illustrating the degree of homology between the three (Jalview)

The *B. rapa* sp. pekinensis sequence is part of a larger 150 nucleotide open reading frame sequence located at position 10847319 - 10847469 of the *B. rapa* chromosome 1 (Altschul et al., 1997). The translated nucleotide sequence of this larger ORF shows some homology to the alpha-helical C-terminal region of the *Arabidopsis* PLS peptide (Figure 3-6).

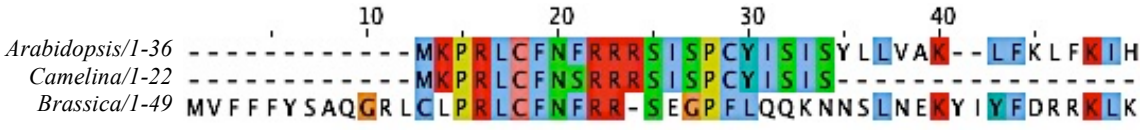


Figure 3-6. Homology between the PLS amino acid sequences in *Arabidopsis thaliana, Camelina sativa* and the translated 150 nucleotide ORF identified in *Brassica rapa* sp. pekinensis.

It was considered that the sequence identified in *B. rapa* may be more conserved within other plant species than *Arabidopsis* PLS. The 150 nucleotide ORF from *B. rapa* was used to identify other possible homologues to this protein via an NCBI Blast search (Altschul et al., 1997); Figure 3-7).

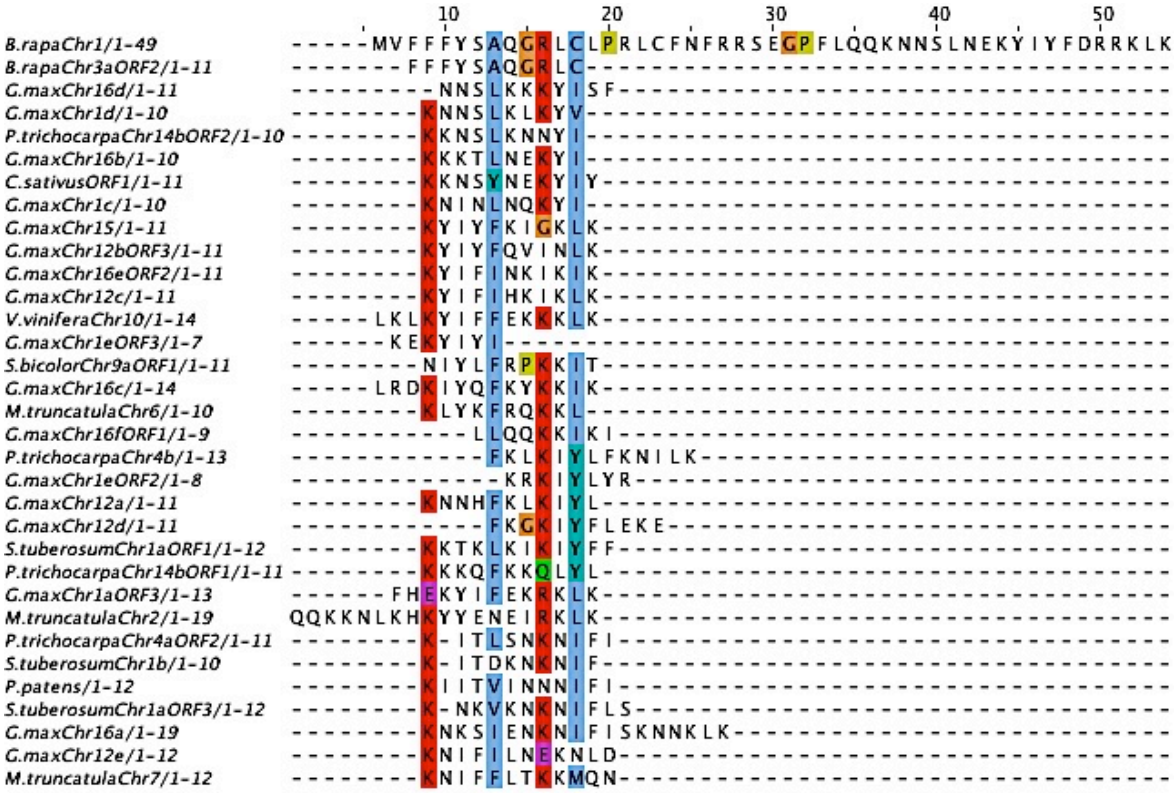


Figure 3-7. Conservation of sequence fragments identified as homologous to the *Brassica rapa* 50 amino acid sequence. *Glycine max* = soybean, *Sorghum bicolor* = sorghum, *Vitis vinefera* = grape, *Populus trichocarpa* = poplar, *Medicago truncatula* = small legume, *Solanum tuberosum* = potato, *Physcomitrella patens* = moss, *Crocus sativus* = crocus.

There were no sequences identified in the Blast search with much homology to the *B. rapa* amino acid sequence. Only fragments of proteins were reported, most of which contained one or two key lysine residues, and a partially conserved residue (position 13, Figure 3-7) with one each of alanine, valine, leucine, isoleucine or phenylalanine (all of which are non-polar, hydrophobic amino acids). There is a wider variety of amino acids at position 18, with some sequences containing hydrophobic residues, and some with hydrophilic tyrosine. Sequences related to the key area of homology between the *Arabidopsis, C. sativa* and *B. rapa* PLS sequences (Figure 3-6, positions 13-30 in the *B. rapa* protein) were not found by this search.

3.4 Investigating the functional domains of POLARIS

Previous attempts to isolate the PLS peptide from *Arabidopsis,* using antibody-based methods designed to the N-terminus, have been unsuccessful (P. Chilley, S. Mehdi; unpublished data), possibly due to the small size of the peptide or hypothesised post-translational modifications. Nonetheless, wild type *PLS* cDNA can complement the *pls* short root mutant phenotype (Casson et al., 2002), suggesting that *PLS* gene transcription and mRNA translation are required for peptide function, so the PLS peptide should be present in plant tissue. Protein isolation difficulties are compounded by very low expression of the *PLS* gene, predominantly in root tips.

To investigate how the amino acid sequence and the concentration of PLS affects root length, the full length LS peptide, and truncated sections of the peptide, were chemically synthesised using Fmoc solid-phase peptide synthesis (SPPS), and subsequently introduced into *Arabidopsis* plants.

3.4.1 POLARIS domains containing cysteine increase root length

Truncations of PLS (Figure 3-8) were synthesised by SPPS (Materials and Methods, 2.11) to investigate whether specific domains of the PLS peptide are required for its function.

PLS(FL)	MKPRLCFNFRRRSISPCYISISYLLVAKLFKLFKIH
PLS(C1)	YLLVAKLFKLFKIH
PLS(C2)	SISPCYISISYLLVAKLFKLFKIH
PLS(N1)	MKPRLCFNFRRRSISPCYISIS
PLS(N2)	MKPRLCFNF

Figure 3-8. Amino acid sequences of the full length and truncated peptides synthesised by SPPS. Appendix II contains MALDI-TOF MS spectra of synthetic peptides.

C24 wild type and *pls* seedlings were grown for ten days in liquid ½ MS10 hydroponic growth medium supplemented with either no peptide, one of the four truncations (Figure 3-8), or full length PLS(FL). Peptides were dissolved in DMSO and added to the plant medium to create a final concentration of 50 nM (Materials and Methods, 2.12.2.1).

Additional DMSO was included, if required, up to 0.01% DMSO by volume (DMSO concentration optimisation is outlined in Appendix I). Roots were measured using ImageJ.

The primary root length of C24 seedlings does not change significantly with the addition of full length PLS or any of the truncated peptides (Figure 3-9) (ANOVA, $F_{(5,121)} = 1.6$, $p = 0.16$). In contrast, the *pls* mutant is significantly affected by the addition of PLS peptides (ANOVA, $F_{(5,112)} = 6.13$, $p = 4.65E\text{-}5$), with the addition of the PLS(N1) truncation and the full length PLS peptide significantly increasing the primary root length of the *pls* seedlings (Tukey's test; post-hoc analysis was undertaken using Tukey HSD [honest significant difference] tests to identify differences between the mean in each treatment group of the *pls* mutant compared to the control mean). In the absence of peptide, the *pls* mutant had primary roots that measured on average 27.0 mm; approximately 75% of the C24 wild type root length, at 36.7 mm. After treatment with the PLS(N1) peptide truncation, the average length of the *pls* primary root had increased to 31.9 mm; i.e. 89% of the length of the C24 roots (37.1 mm on average). When treated with the full length PLS peptide, the *pls* mutant root length increased to an average of 33.6 mm, compared with the C24 average length of 35.5 mm.

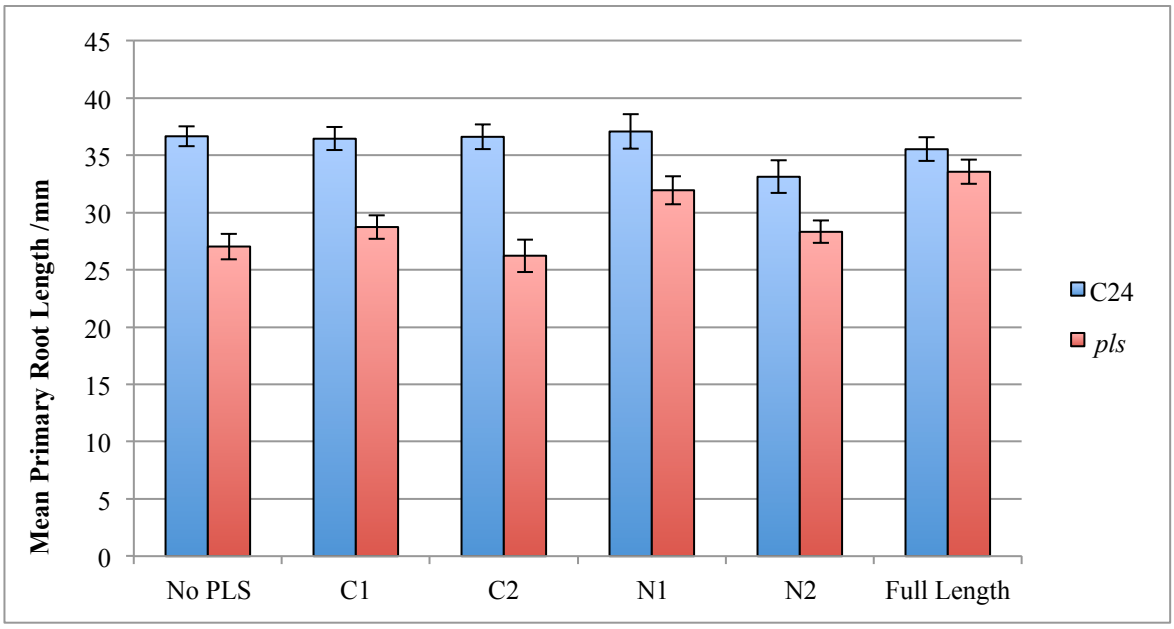


Figure 3-9. Mean primary root length of C24 and *pls* seedlings treated with full length PLS and peptide truncations. Stratified seeds were grown in individual wells for ten days after germination under a 16 hour photoperiod at 21°C, in 1 ml liquid ½ MS10 growth medium supplemented with no peptide, truncations C1, C2, N1, N2, or full length PLS. Growth media contained a final concentration of 50 nM peptide, with 0.01% DMSO by volume. Root length was measured at ten d.a.g. using ImageJ. Error bars show ± 1 standard error, statistical analysis was performed using the Real Statistics Resource Pack software (Release 3.8) in Excel. $N \leq 21$.

3.4.2 The POLARIS peptide has a dose-dependent role

It was observed previously that *Arabidopsis* plants with a heterozygous C24/*pls* genotype showed an intermediate root length between that of the homozygous lines: C24 wild type and short-root *pls* (Casson et al., 2002). This suggested that the role of PLS in ethylene signalling-mediated root growth might be dependent on the peptide concentration within the plant. To investigate the relationship between peptide dose and root length, C24 wild type and *pls* mutant seedlings were treated with full length synthetic PLS peptide (Cambridge Research Biochemicals, Billingham) over a range of concentrations.

Stratified seeds (Materials and Methods, 2.2.1) were grown for ten days post-germination in hydroponic culture medium (Materials and Methods, 2.2.2.1) containing a range of PLS peptide concentrations (0, 10, 25, 50 and 100 nM) and 0.01% DMSO by volume (Materials and Methods, 2.12.2.1).

After ten days, the length of the *pls* mutant roots showed rescue to that of C24 wild type seedlings when grown in the presence of 10 nM or 50 nM PLS peptide, and *pls* root length was longer than the C24 seedlings when treated with 100 nM PLS (Figure 3-10).

C24 primary root length increased from 65.8 mm, with no PLS added, to 70.5 mm when treated with 50 nM PLS, before decreasing to 63.6 mm with 100 nM PLS. In contrast, the primary root length of the *pls* mutant seedlings had a much larger increase in length, from 61.1 mm after the control treatment up to 70.1 mm when treated with 100 nM PLS.

The mean primary root length of C24 seedlings did not change significantly across all peptide treatments (ANOVA, $F_{5, 121} = 1.6$, $p > 0.1$). In contrast, treating *pls* seedlings with PLS peptide did have a significant effect upon primary root length (ANOVA, $F_{5, 112} = 6.13$, $p = 4.65E\text{-}5$). Tukey's HSD test showed that the 10, 50 and 100 nM PLS treatments differed significantly from the 0 nM control treatment at the 0.05 level of significance. The treatment of the PLS peptide has therefore rescued the *pls* mutant primary root length to that of the wild type.

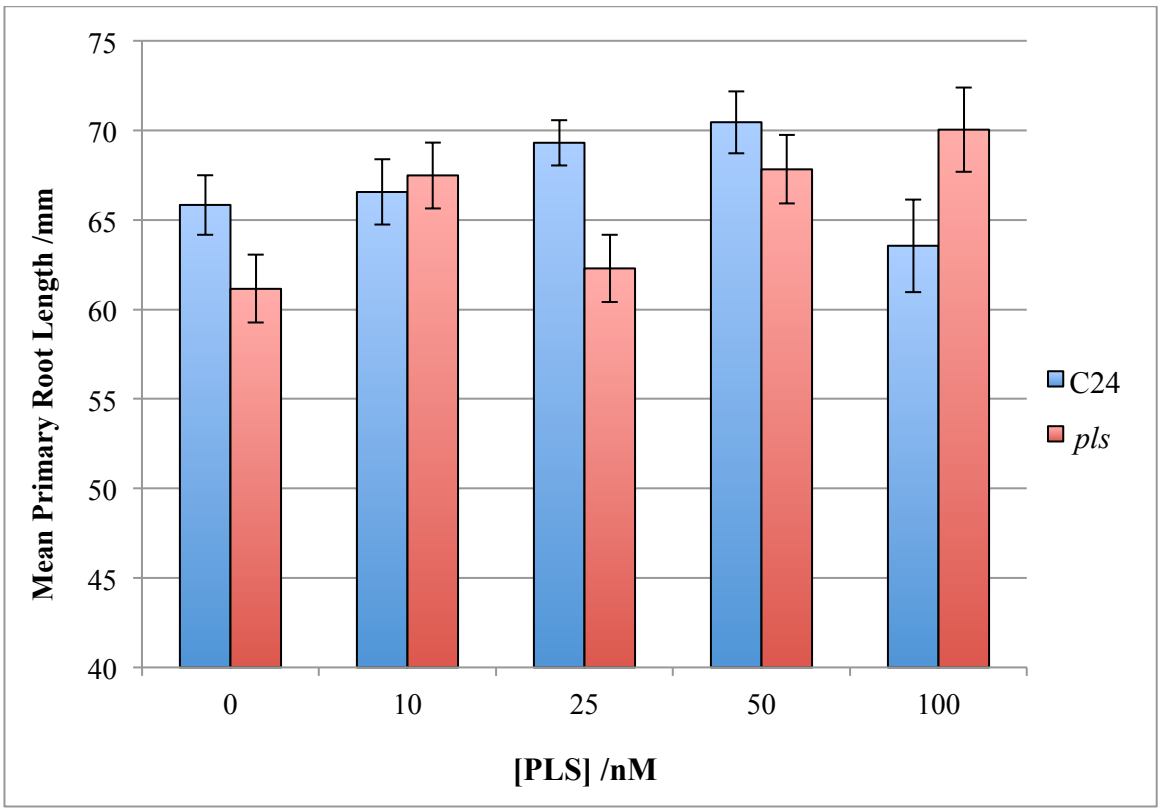


Figure 3-10. Primary root length response of C24 wild type and *pls* mutant seedlings after treatment with increasing concentrations of POLARIS peptide. Stratified seeds were grown in individual wells for ten days after germination under a 16 hour photoperiod at 21°C, in 1 ml liquid ½ MS10 growth medium supplemented with full length synthetic PLS peptide (Cambridge Research Biochemicals) dissolved in DMSO. DMSO was added to growth medium solutions to a final volume of 0.01%. Root lengths were measured using ImageJ software. Error bars show ± 1 standard error, statistical analysis was performed as before. $N \leq 22$.

There is a difference in the response of C24 and *pls* to treatment with 100 nM PLS; *pls* seedlings continue to grow longer primary roots, whereas C24 roots are significantly shorter. It was shown previously that more PLS peptide in *PLS* overexpressing transgenic lines produces longer primary roots, and it was expected that this effect would be observed in both the wild type and mutant lines. It may be that higher concentrations of additional PLS have a toxic effect on seedling growth, but any such effect should be evident in both plant lines, rather than just the wild type. The PLS peptide may be playing another role in the regulation of plant growth. The C24 seedlings would already be expressing wild type levels of PLS peptide in addition to the exogenous supply, so perhaps the addition of synthetic PLS pushed the peptide levels too high for an optimum growth response. In the *pls* mutant, the addition of PLS would partially or fully restore wild type PLS levels, revealing the increased root length effect.

3.4.3 Fluorescently-tagged POLARIS N1 truncation is taken up by root cells

Introducing the synthetic PLS to seedlings by way of the plant medium raises questions about the extent of uptake of these large molecules by the plant roots.

PLS truncation N1 (Figure 3-8) was tagged at the N-terminus with a 5-carboxyfluorescein (5-FAM) fluorescent tag to allow peptide uptake to be detectable. The 5-FAM molecule contains a carboxylic acid moiety that can be attached to the N-terminal primary amine and is excited at 488 nm. The remainder of the molecule is planar, with four 6-carbon rings and a chemical formula of $C_{21}H_{12}O_7$. Due to the large size of 5-FAM, it was attached to the shorter PLS(N1) peptide rather than full length PLS to avoid the possibility that the latter construct may be too big for root uptake.

The primary root length of C24 and *pls* seedlings was recorded after growth for ten days after germination in hydroponic ½ MS10 culture medium (Materials and Methods, 2.2.2.1) supplemented with either 0, 50 or 100 nM 5-FAM-PLS(N1) peptide (Materials and Methods, 2.12.2 and 2.12.2.1). A random sample of seedlings was taken from the peptide growth assay after ten days and used for confocal fluorescence microscopy (Figure 3-11; Material and Methods, 2.10 and 2.10.2). The root lengths of the remaining seedlings were measured. The laser and gain settings remained constant for all fluorescent images, revealing that while untreated seedlings showed some autofluorescence, those treated with the 5-FAM-PLS(N1) peptide displayed brighter cytoplasmic fluorescence in cells in the root.

C24 and *pls* seedlings treated with 5-FAM-PLS(N1) both showed a significant response in root length (Figure 3-12; C24:ANOVA, $F_{2, 39} = 3.23$, p = 2.55E-05. *pls:*ANOVA, $F_{2, 32} = 3.29$, p = 3.45E-4). Post-hoc Tukey's HSD tests for C24 showed that only treatment with 50nM 5-FAM-PLS(N1) was different to the control treatment at the 0.05 level of significance. In *pls* however, both peptide treatments were significant at the 0.05 level. The unchanged length of the C24 wild type roots after treatment with 100 nM 5-FAM-PLS(N1) correlates with the same observation from the 100 nM treatment with PLS(FL) (Figure 3-10).

The combined evidence from the fluorescence and root length studies reveals that tagged PLS(N1) peptide is taken up by the seedlings' roots, and demonstrates an activity in the root, leading to increased mean primary root length.

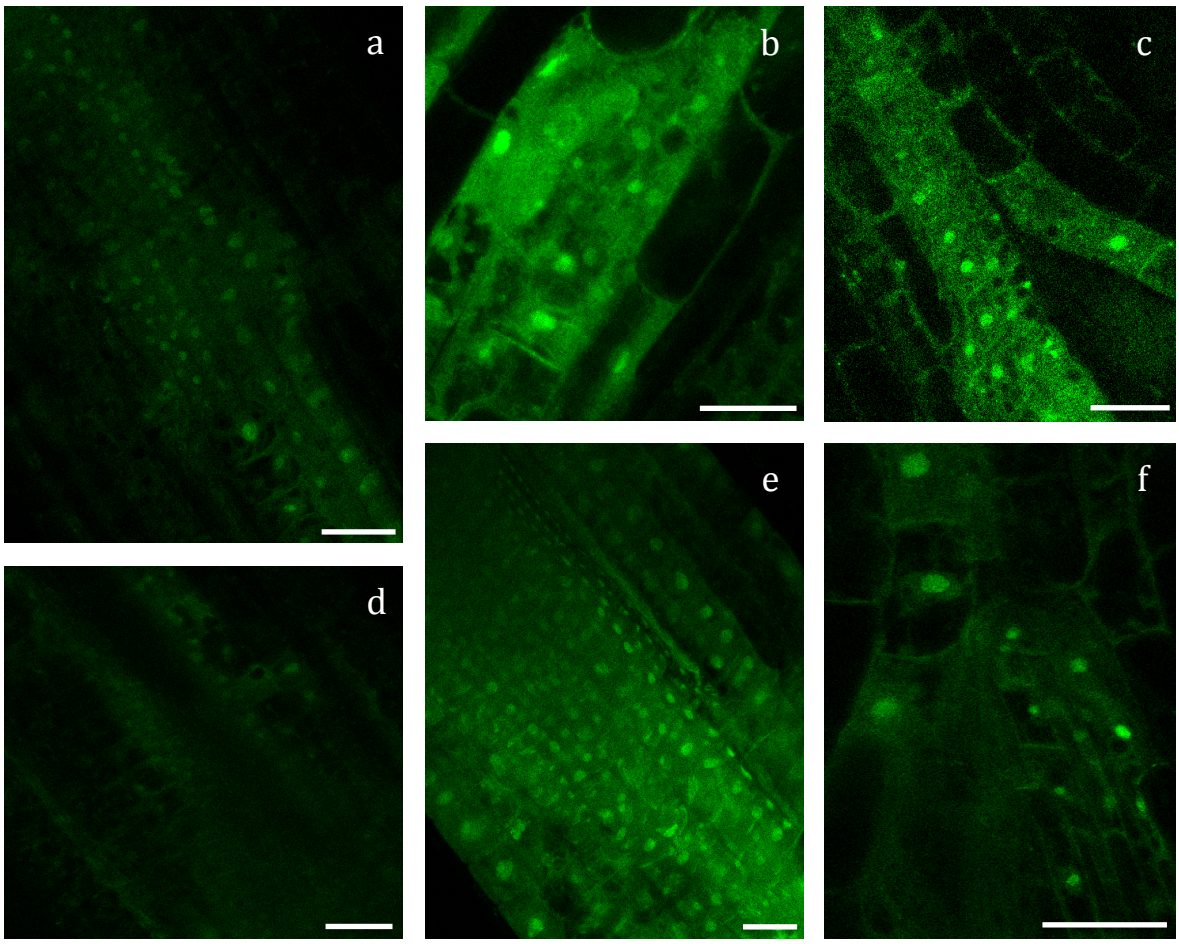


Figure 3-11. 5-FAM-PLS(N1) is observed in C24 and *pls* roots. C24 and *pls* seedlings were grown for ten days in liquid plant media supplemented with 5-FAM-PLS(N1) fluorescently-tagged peptide. Top panels show the C24 root elongation zone after treatment with no peptide (a), 50nM peptide (b) and 100nM peptide (c). Bottom panels show *pls* roots exposed to no peptide (d), 50nM (e) and 100nM peptide (f). Images were obtained by CLSM at ten d.a.g. using identical laser and brightness settings: 488 nm argon, 20 mW at 30%, HyD 5 detector at 110.0. Images were obtained from one biological replicate. n = 8. Images are representative of the data set as a whole.

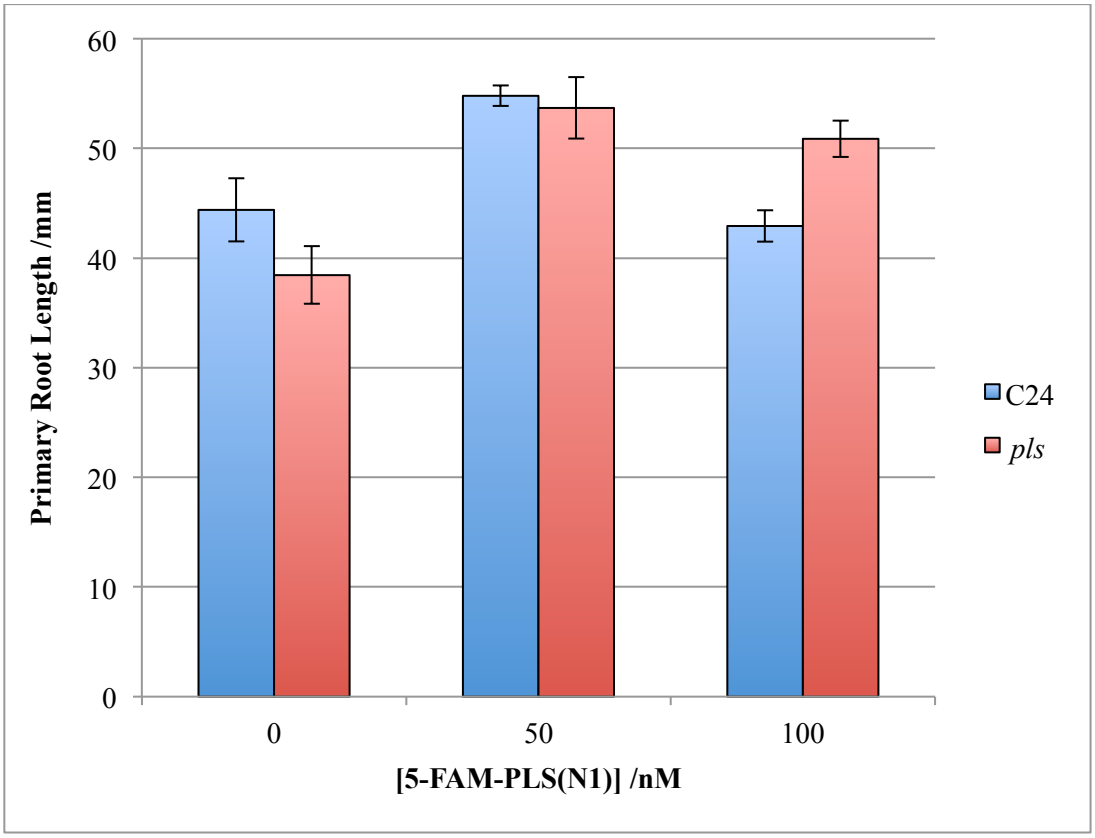


Figure 3-12. Primary root length of C24 and *pls* seedlings after treatment with fluorescently-tagged 5-FAM-PLS(N1) peptide. Seedlings were grown as before with plant media supplemented with PLS(N1), tagged at the N-terminus with a carboxyfluorescein molecule (5-FAM). Peptide was added to the media to make a final concentration of 0, 50 or 100nM, with 0.01% DMSO by volume. Roots were measured at ten days after germination using ImageJ. Error bars show ± 1 standard error. $n \leq 17$.

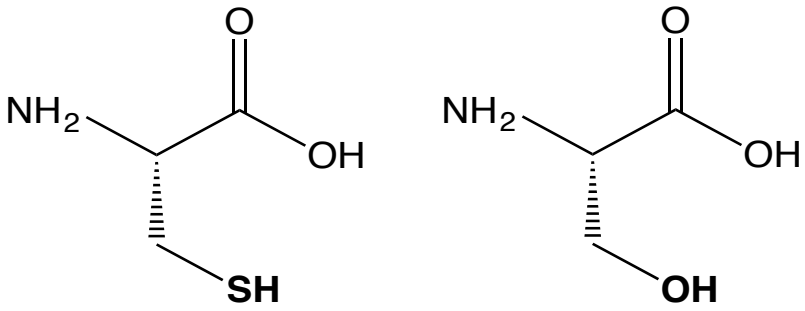
3.4.4 Full length PLS peptide with substituted cysteine residues is inactive

There are two versions of the PLS peptide that produce longer *Arabidopsis* roots: the full length peptide, and the PLS(N1) truncation (Figure 3-9). PLS(N1) shares its N-terminal nine amino acids with PLS(N2), and some of its C-terminus with PLS(C2) (Figure 3-13). Neither the N2 nor the C2 peptide however were found to have the capacity to increase root length. One characteristic that is only shared by the full length PLS and PLS(N1) peptides is the presence of both cysteine residues, Cys-6 and Cys-17. To investigate a possible functional role for the cysteine residues, a mutated full length peptide, henceforth identified as PLS(FL)*C6S,C17S,* was synthesised by SPPS, in which both cysteine amino acids were replaced with serine (Figure 3-13).

PLS(FL)	MKPRLCFNFRRRSISPCYISISYLLVAKLFKLFKIH
PLS(C1)	YLLVAKLFKLFKIH
PLS(C2)	SISPCYISISYLLVAKLFKLFKIH
PLS(N1)	MKPRLCFNFRRRSISPCYISIS
PLS(N2)	MKPRLCFNF
PLS(FL)*C6S,C17S*	MKPRLSFNFRRRSISPSYISISYLLVAKLFKLFKIH

Figure 3-13. Amino acid sequences of the five PLS truncations and PLS(FL)*C6S,C17S*: the full length peptide with cysteine residues replaced with serine (underlined).

The amino acids cysteine and serine have similar structures (Figure 3-14), with the amine and carboxylic acid groups attached to the alpha carbon, and a CH_2-xH side chain. Cysteine residues contain a redox-active sulphur atom which can undergo oxidation and reduction, and can be involved in creating disulphide bonds (Sevier and Kaiser, 2002), interacting with other proteins or coordinating metal ions (Crabtree, 1994). Serine has a simple OH group in place of the sulphur and therefore does not have the same functionality.



Cysteine Serine

Figure 3-14. Chemical structures of the amino acids cysteine and serine.

C24 wild type and *pls* seedlings were grown for ten days in liquid ½ MS10 growth media (Materials and Methods, 2.2.2.1) supplemented with no peptide, PLS(FL)*C6S,C17S* or unmodified PLS(FL). Peptides were dissolved in DMSO and added to the plant media to

create a final concentration of 100 nM (Materials and Methods, 2.12.2.1). Additional DMSO was included, if required, up to 0.01% DMSO by volume. Roots were measured using ImageJ.

The PLS peptide with the two cysteine residues replaced with serine (PLS(FL)*C6S,C17S*) did not show the same activity in *Arabidopsis* roots as the unmodified PLS(FL) (Figure 3-15).

The mean primary root length in C24 seedlings did not change significantly when treated with either the mutated peptide PLS(FL)*C6S,C17S*, or the natural PLS(FL) (ANOVA, $F_{2, 58} = 2.42$, $p = >0.05$). However, the *pls* seedlings were significantly affected (ANOVA, $F_{2, 51} = 9.48$, $p = 3.15E\text{-}4$). Further testing by Tukey's HSD method revealed that only the full length PLS peptide significantly affected the mean *pls* root length while the mutated PLS(FL)*C6S,C17S* peptide did not have a significant effect.

The presence of the two cysteine residues is therefore required for PLS peptide function.

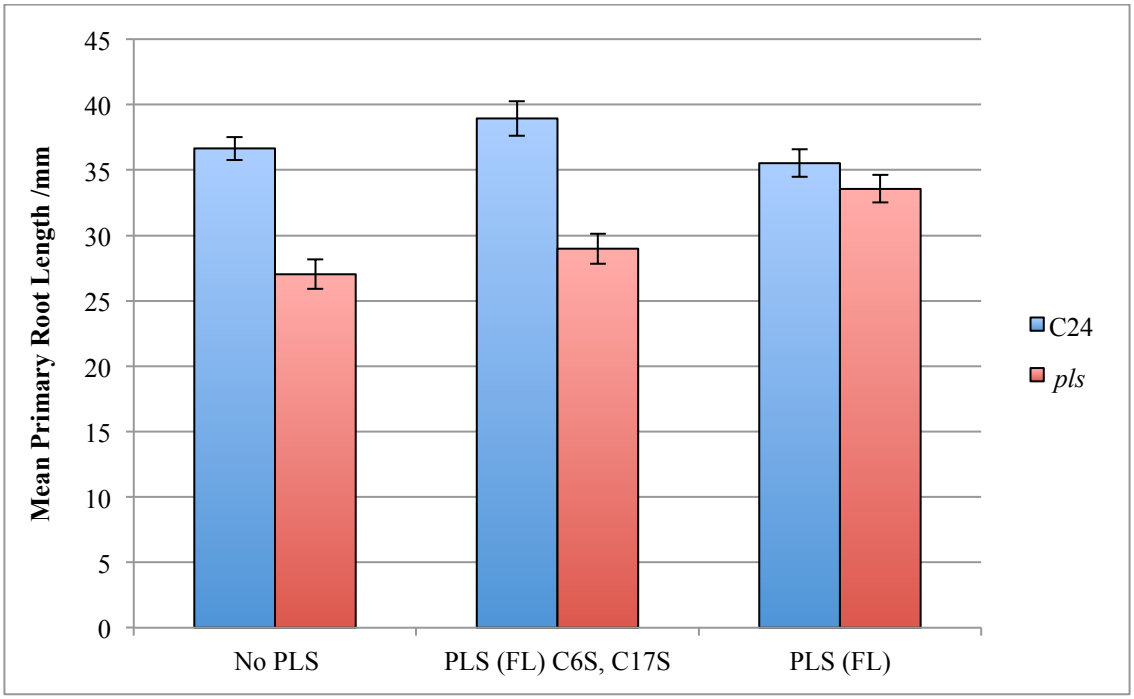


Figure 3-15. PLS(FL)*C6S,C17S* does not increase *Arabidopsis* mean root length. Seedlings were grown for 10 days in liquid ½ MS10 media supplemented with no peptide, mutated PLS(FL)*C6S,C17S* or unmodified full length PLS. Growth media contained a final concentration of 100 nM peptide, with 0.01% DMSO by volume. Root length was measured at ten d.a.g. using ImageJ. Error bars show ± 1 standard error. $n \leq 21$.

3.4.5 *Camelina sativa* PLS homologue in *Arabidopis thaliana*

The 22-amino acid peptide identified in *C. sativa* has clear homology to the N-terminus of the *Arabidopsis* PLS peptide, similar to the PLS(N1) peptide truncation that partially rescues the *pls* short root length. The peptide has only one amino acid substitution; phenylalanine to serine (underlined) at position nine (Figure 3-16), but it still contains the two cysteine residues shown to be important for PLS activity.

The *C. sativa* PLS homologue was synthesised by SPPS (Cambridge Research Biochemicals, Billingham) to investigate its functionality in *Arabidopsis*.

A. thaliana Full Length	MKPRLCFNFRRRSISPCYISISYLLVAKLFKLFKIH
A. thaliana PLS(N1)	MKPRLCFNFRRRSISPCYISIS
C. sativa PLS	MKPRLCFNSRRRSISPCYISIS

Figure 3-16. Amino acid sequences of the *Arabidopsis thaliana* full length PLS and PLS(N1), and the *Camelina sativa* PLS homologue.

C24 and *pls* seedlings were stratified and grown as before (Materials and Methods, 2.2.1 and 2.2.3.2) for ten days in ½ MS10 plant media supplemented with no peptide, or 100 nM of either full length *Arabidopsis* PLS or *C. sativa* PLS, plus 0.01% DMSO. Primary root lengths were measured using ImageJ.

Application of neither the *A. thaliana* nor *C. sativa* PLS peptide to C24 wild type seedlings had no effect on the mean primary root length (Figure 3-17), from 49.5 mm with no peptide, to 45.3 mm and 46.1 mm respectively after treatment (ANOVA, $F_{2, 44} = 1.74$, $p = 0.19$), similar to the previous observation in Figure 3-10.

However, both peptide treatments caused a significant increase in the primary root length of the *pls* mutant (Figure 3-17; ANOVA, $F_{2, 41} = 6.86$, $p = 0.003$; Tukey's HSD test), from 39.5 mm in untreated *pls* plants, to 45.5 mm in the presence of *A. thaliana* PLS and 45.9 mm after treatment with the *C. sativa* homologue.

The activity of the *C. sativa* PLS peptide in *Arabidopsis* reveals that there may be a conserved function of PLS in other plant species, and that the Phe residue in the *Arabidopsis* peptide is not crucial for PLS activity.

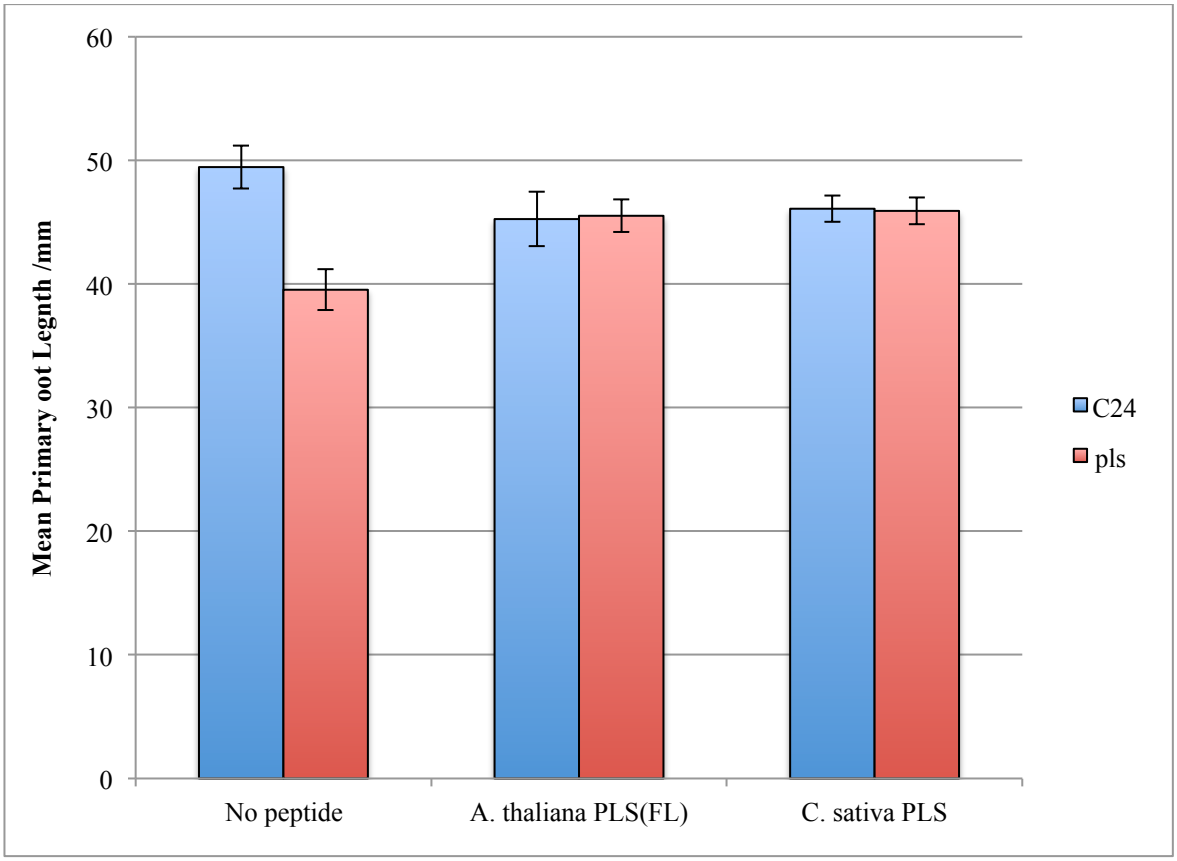


Figure 3-17. *Camelina sativa* PLS increases the length of the *Arabidopsis pls* mutant root. Seedlings were grown for ten days as before in the presence of 100 nM *C. sativa* or *A. thaliana* PLS peptides, or with no peptide present. Primary root length was measured using ImageJ. Error bars show ± 1 standard error. N ≤ 22.

3.5 Conclusion

The PLS peptide has a predicted beta-sheet N-terminus and an alpha-helical C-terminus with a potential turn region containing three arginine residues. The full length synthetic peptide (PLS(FL)) can increase the primary root length in the short-root, loss-of-function *polaris (pls)* mutant at the highest concentrations used. The rescue of root length in *pls* is also observed after treatment with the synthetic peptide comprising of the N-terminal 22 amino acids, designated PLS(N1), but not with the other PLS truncations tested.

The sequences of the PLS(FL) and PLS(N1) peptides both contain two cysteine residues. Replacing both cysteine residues with serine in the full length sequence produced an inactive 36 amino acid synthetic peptide, revealing the cysteine residues are important for PLS function.

Few homologues of PLS have been identified in plant species other than *Arabidopsis.* A 22-amino acid peptide has been found in *Camelina sativa* with 95% homology with the N-terminus of *Arabidopsis* PLS. A synthetic version of the *C. sativa* PLS peptide increases *pls* mutant root length, revealing that there may be functional homologues of PLS in other plant species.

It has been shown that the N-terminus of the PLS peptide can regulate root length in *Arabidopsis.* Experimental work in the next chapter will focus on the localisation of the peptide in the *Arabidopsis* root and its subcellular localisation to cell organelles to help determine the role of PLS in root growth.

Chapter 4 . POLARIS Localisation

4.1 Introduction

The *Arabidopsis thaliana* POLARIS (PLS) peptide has been shown to increase the primary root length of the *pls* short-root mutant, as described in Chapter 3. The response of root length to the addition of varying concentrations of the full length PLS peptide reveals that there is a dose-dependent response of root growth to PLS, also demonstrated by genetic analysis that showed the heterozygous *pls* mutant has an intermediate primary root length compared to wild type and the homozygous mutant (Casson et al., 2002). The experiments in this chapter were designed to investigate where the PLS peptide is localised in the root in order to perform its regulation of root length and the response of the peptide to the addition of the phytohormone ethylene.

The *PLS* promoter has been shown previously to drive expression of a *GUS* gene at the tips of the primary and lateral roots, and in the embryonic root meristem (Casson et al., 2002). The work in this chapter concentrates on the localisation of a PLS peptide-GFP fusion protein, under the control of its endogenous promoter (Mehdi, 2009) in seedling root tissues and at the sub-cellular level.

Evidence suggests that the PLS peptide acts alongside the ethylene receptor protein ETR1 (Mehdi, 2009). The endoplasmic reticulum is reported to harbour membrane-associated ethylene receptors (Chen et al., 2002) so PLS protein localisation was investigated in association with cellular membrane structures.

The transcription of the *PLS* gene is negatively regulated by ethylene and addition of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) causes downregulation of *pPLS* promoter activity (Chilley et al., 2006). Using the PLS-GFP fusion protein, experiments were undertaken to establish the effect of ethylene on both the translation of the *PLS:GFP* transcript and the localisation of the PLS peptide at whole-root and sub-cellular levels under perturbed ethylene conditions.

4.2 Localisation studies

To investigate the location of the POLARIS peptide in the *Arabidopsis* root, a DNA construct containing a 1.5kb section of the *PLS* promoter powering the 108 nucleotide *PLS* ORF was inserted into the pMDC107 binary vector (Appendix VI) and introduced into the

Col-0 *Arabidopsis* wild type ecotype (construct and transgenic line created by S. Mehdi, 2009). The pMDC107 plasmid contains a green fluorescent protein (*GFP*) nucleotide sequence, ultimately producing a C-terminal PLS-GFP fusion protein under the control of the endogenous *PLS* promoter (the resulting transgenic plants are henceforth referred to as *pPLS::PLS:GFP*). This enabled studies to be undertaken on PLS peptide localisation, with the PLS-GFP protein expressed at endogenous *PLS* levels.

The following microscopy experiments were undertaken on seedlings grown under the same conditions unless otherwise stated: plants were stratified and grown under standard growth conditions on phytagel plant medium (Materials and Methods, 2.2.1, 2.2.3.1) for seven days after germination (d.a.g) before being used for imaging experiments using confocal laser scanning microscopy (CLSM) on a Leica TCS SP5 confocal microscope (Materials and Methods 2.10).

To provide a comparison to the expression and localisation of *pPLS::PLS:GFP,* a control *Arabidopsis* plant line was included, which was transformed with a pB7WG2 plasmid expressing a GFP protein driven by the constitutive 35S Cauliflower Mosiac Virus (CaMV) promoter from the vector backbone (created by Dr. Piers Hemsley, Durham University). This *GFP* sequence is strongly expressed throughout the plant, and produces cytoplasmic GFP protein. It must be noted that the N-terminus of the resulting GFP protein is fused to two small protein tags, Strep and HA, but the SH-GFP protein has been previously independently observed to behave as free cytoplasmic GFP.

4.2.1 POLARIS localisation in the root

Previous *PLS* expression studies have focused on transgenic *Arabidopsis* lines expressing a *GUS* gene driven by the *PLS* promoter (Casson et al., 2002). The PLS-GFP translated fusion protein can improve our understanding of how PLS acts upon the ethylene signalling pathway by providing location and targeting information about the protein itself.

The PLS-GFP fusion protein, expressed under the control of the endogenous *pPLS* promoter, is localised to the tips of primary roots (Figure 4-1). Root cell architecture was illustrated by treating the seedlings with the fluorescent dye propidium iodide (PI) before imaging the root (Materials and Methods, 2.10.3.1). PI is a DNA stain, but in healthy root tissue, membranes are impermeable to PI, and the stain can be used to visualise the cell walls (Sullivan and Kay, 1999), thus giving cellular context to a complementary fluorescent protein, for example GFP.

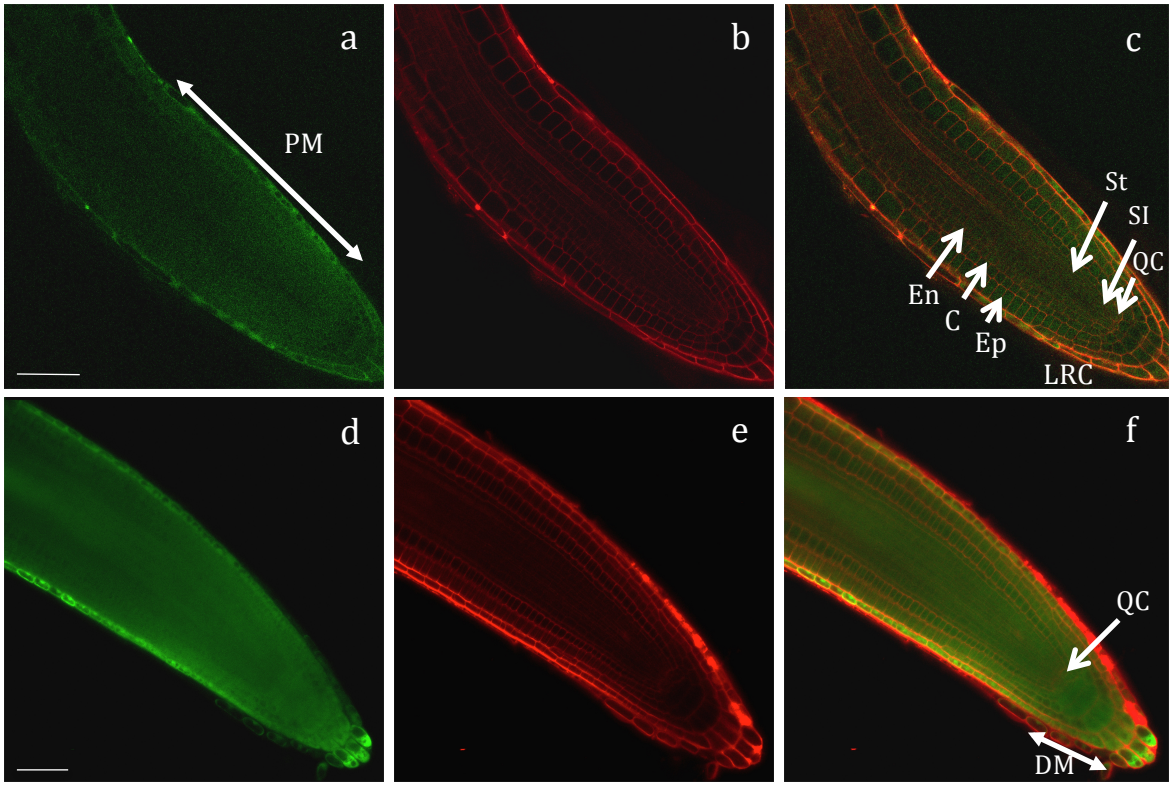


Figure 4-1. *pPLS::PLS:GFP* expression in the *Arabidopsis thaliana* root tip. Seedlings expressing *pPLS::PLS:GFP* (a) and *35S::SH:GFP* (d) were grown for seven days, treated with 10 mg/l propidium iodide (b,e) for 90 seconds and imaged by CLSM using a 40X lens. Panels c and f show the merged images. Proximal meristem (PM), distal meristem (DM), quiescent centre (QC), stele initials (SI), endodermis (En), cortex (C), epidermis (Ep) and lateral root cap (LRC) are indicated. Laser settings maintained at 21% power, 488 nm 20 mW, 1013 V gain (GFP); 543 nm HeNe 1.2 mW, HyD:70 (PI). Refer to Appendix III for brightness contrasts. Scale bars = 50µm. Four separate biological replicates were each grown according to section 2.2.3.1. At least 6 roots were imaged from each replicate. The images are representative of the whole data set.

The PLS-GFP fusion expression is powered by the weak *PLS* promoter, and therefore shows a reduced fluorescent signal when compared to the strongly expressed *p35S* promoter (see Appendix III for comparative brightness). PLS-GFP can be seen at the root tip, behind the quiescent centre (QC), with some expression in the stele initials (SI) and extending back into the stele (St), but predominantly localised in the endodermis (En), cortex (C) and epidermis (Ep) cell files. The construct shows low expression in the distal meristem, between the extreme apex of the root tip and the QC. There is also some expression in the lateral root cap (LRC) which appears brighter than in other areas, but this may be a result of these areas of tissue being thinner on the outside of the root so GFP fluorescence within these cells is detected more easily.

PLS-GFP expression extends through the proximal meristematic zone (PM), into the elongation zone but expression appears reduced by the upper limit of the PM, approximately 250 μm behind the QC.

In contrast, strongly expressed GFP under the control of the *p35S* promoter is located throughout the cell files, including the distal meristem (DM) and the stele.

4.2.2 Sub-cellular localisation of POLARIS

CLSM was used to investigate the localisation of the PLS-GFP fusion protein in the root cells of the *pPLS::PLS:GFP* line (Figure 4-2).

PLS-GFP is located in the nuclei of root transition zone epidermal cells and resides in membranous structures (arrows) surrounding the nucleus (Figure 4-2 (a, series d-i)). The fusion protein was also observed in aggregates (arrows) localised to predicted endomembrane structures (Figure 4-2 (b)). Unattached GFP protein powered by the *p35S* promoter also localises to the nuclei of epidermal cells, but has a much more diffuse cytoplasmic localisation than the PLS-GFP protein (Figure 4-2 (c)). This suggests that the small PLS peptide (36 amino acids) is able to target the larger GFP protein (244 amino acids) to specific cellular features.

Experiments were subsequently undertaken to identify the membrane compartments using the *pPLS::PLS:GFP* line.

Images are taken from observations made over three biological replicates, with at least 8 roots studied in each replicate. The images below are representative of the whole data set.

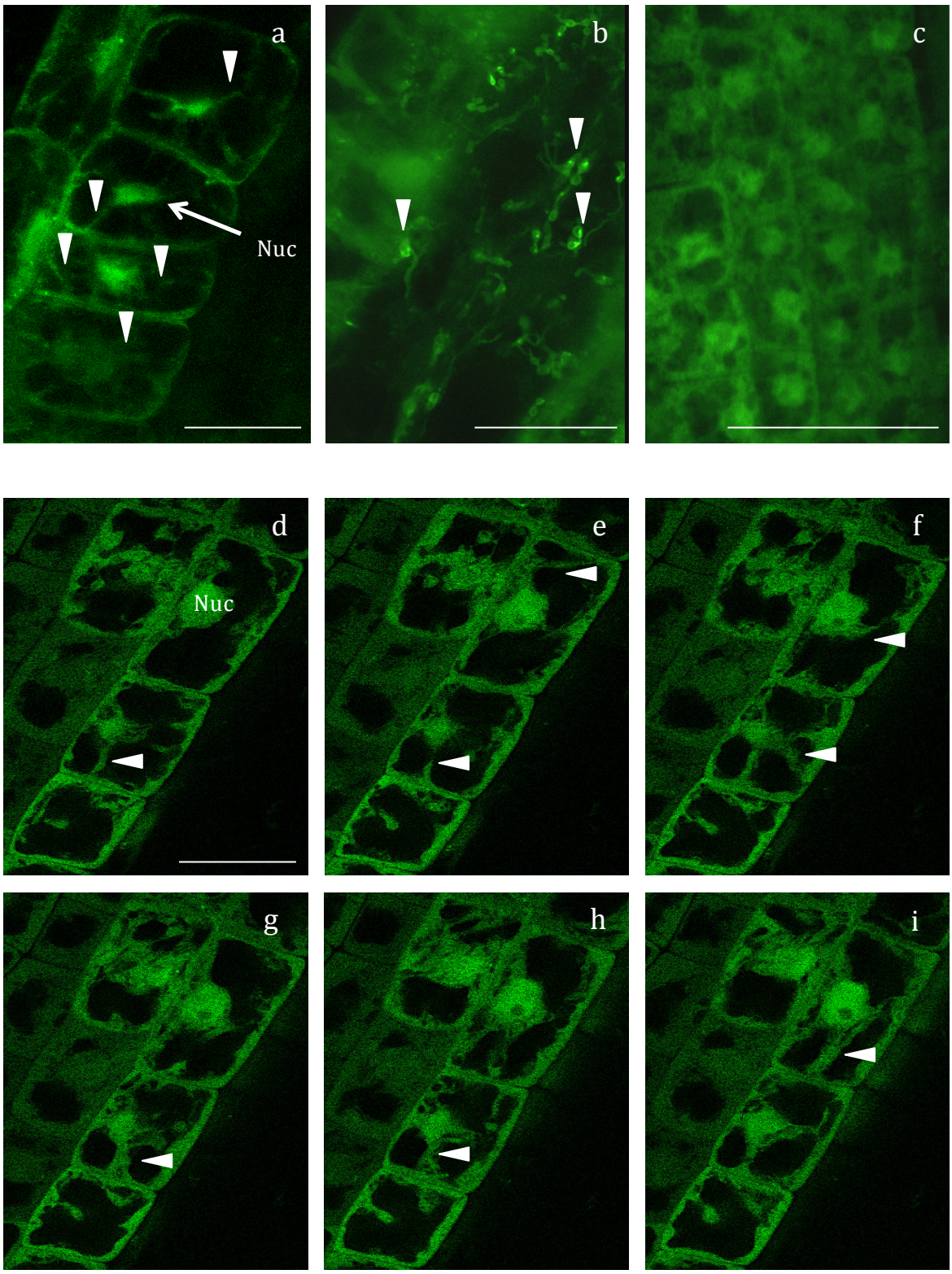


Figure 4-2. Subcellular localisation of *pPLS::PLS:GFP* and *p35S::GFP* in *Arabidopsis thaliana* root epidermal cells from the root transition zone. Transgenic GFP seedlings were grown for ten days and the root tips imaged by CLSM. The PLS-GFP protein localises to the nucleus (Nuc) and membranous structures (a; arrows) and endomembrane-type structures can be observed (b; arrows). Free GFP (c) is more diffuse throughout the cytoplasm. Series d – i shows consecutive frames from a z-stack, illustrating the localisation of PLS-GFP to membrane structures (arrows) around the nuclei. Laser settings: 21% 488 nm argon, 20 mW, 980 V gain. Scale bars = 25 µm.

4.2.3 POLARIS localises to the endoplasmic reticulum

The PLS peptide has been shown to play a role in the regulation of ethylene signalling in *Arabidopsis*. Previous studies have deduced that PLS acts at the level of the ethylene receptors; the *pls* mutant does not have an ethylene biosynthesis defect and it cannot rescue downstream ethylene mutants such as *ctr1-1* (Chilley et al., 2006).

The family of ethylene receptor proteins reside in the endoplasmic reticulum (ER) membrane (Chen et al., 2002), and some more recent evidence suggests the receptor proteins are found in the Golgi apparatus membrane too (Dong et al., 2008). Yeast 2-hybrid and Biomolecular Fluorescence Complementation (BiFC) experiments have shown that the PLS peptide and the ethylene receptor protein ETR1 can interact (Mehdi, 2009) and the PLS peptide has a putative ER retention signal (Casson et al., 2002), so PLS might be expected to be located nearby in these membrane organelles. The localisation of the PLS-GFP fusion protein was explored in relation to previously-characterised fluorescent ER and Golgi apparatus markers.

4.2.3.1 Endoplasmic reticulum dye

Plants expressing *pPLS::PLS:GFP* were grown as before (Materials and Methods, 2.2.3.1) and treated with a fluorescently-active dye which localises to the endoplasmic reticulum, ER-Tracker™ Red (BODIPY® TR Glibenclamide) (Life Technologies; Materials and Methods, 2.10.3.2).

Confocal microscopy shows that the PLS-GFP fusion protein colocalises with the ER-Tracker™ dye in *Arabidopsis* root epidermal cells (Figure 4-3 (c); arrows), shown by the yellow colour. PLS-GFP can also be observed in the nuclei (green).

To investigate whether the PLS peptide is targeting the GFP to the ER, or whether the larger GFP protein would localise there regardless, ER-Tracker™ dye was also applied to plants expressing a *pPLS::GFP* construct (S. Mehdi 2009), producing an independent GFP protein powered by the *PLS* promoter in the pMDC107 vector backbone. GFP protein in the *pPLS::GFP* seedlings appear to form small aggregates of protein, manifesting as green punctae (Figure 4-3(d)). When these roots are treated with ER-Tracker™ (Figure 4-3(e)), they do not show the same degree of colocalisation (Figure 4-3(f)) to membranous structures as seen with the *pPLS::PLS:GFP* construct, i.e. less yellow fluorescence.

Seedlings expressing the *p35S::SH:GFP* construct were also treated with ER-Tracker™ (Figure 4-3(g-i)). The cytoplasmic GFP in these root cells did not localise with the ER-Tracker™ dye (no yellow colour), suggesting the presence of the PLS peptide in the *pPLS::PLS:GFP* plant lines is responsible for targeting the GFP to the ER.

The constitutively expressed GFP under the *35S* promoter (Figure 4-3(g)) does not show the same punctate-like GFP pattern seen in the *pPLS::GFP* lines (d).

4.2.3.2 ER-localised RFP

The PLS-GFP fusion protein colocalises with the ER-Tracker™ dye, suggesting it resides in or near the endoplasmic reticulum, or is associated in some way with the ER membrane.

The PLS-GFP protein and ER-Tracker™ demonstrated a strong degree of colocalisation, therefore new transgenic plant lines were created to investigate this relationship by co-expressing PLS-GFP and an ER-localised fluorescent protein in the same cells.

A construct, *p35S::RFP:HDEL,* containing an endoplasmic reticulum-targeted red fluorescent protein (RFP; a gift from Dr. Pengwei Wang, Durham University) was introduced into the *pPLS::PLS:GFP Arabidopsis* plant line by *A. tumefaciens* transformation (Materials and Methods, 2.7.8 and 2.7.9).

Selection of the T1 plants containing the *p35S::RFP:HDEL* construct was performed by fluorescence screening using epifluorescence microscopy (Materials and Methods, 2.8); successful transformants emitted red fluorescence throughout the seedling upon excitation with green light. Seed was collected from the transformants for imaging.

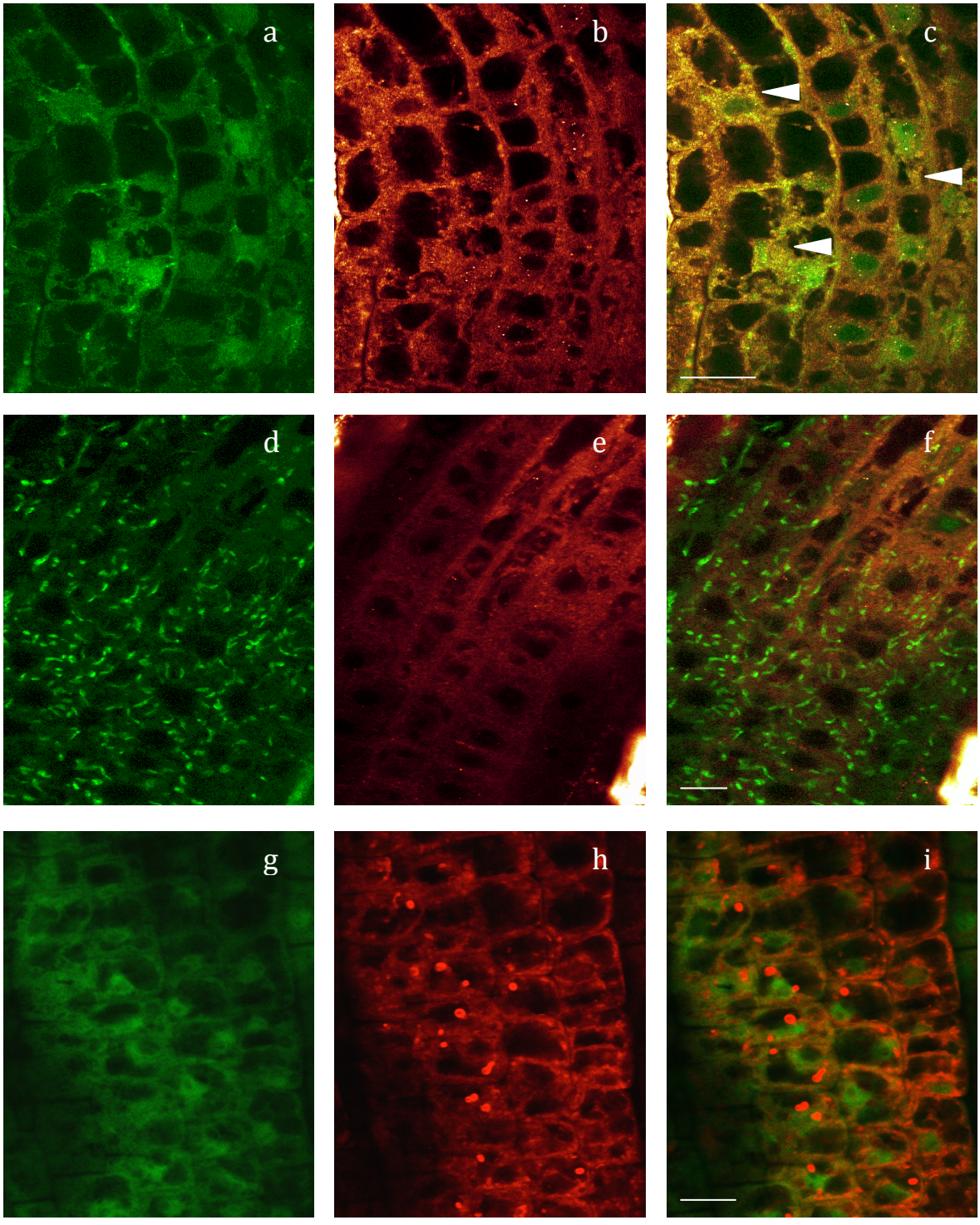


Figure 4-3. *pPLS::PLS:GFP* (a), *pPLS::GFP* (d) and *35S::SH:GFP* (g) show differing degrees of colocalisation with ER Tracker™ dye. Transgenic seedlings were grown for seven days, treated for 30 minutes with 1µM ER Tracker™ (b,e,h) and imaged by CLSM. Merged images (c,f,i) display yellow features if fluorophores co-localise. Arrows show colocalisation between PLS-GFP and ER Tracker™. Laser settings: 21% 488 nm 20 mW, 851 V gain (green); 594 nm HeNe 2 mW, HyD:58 (red). Scale bars = 25µm (c), 10µm (f and i).

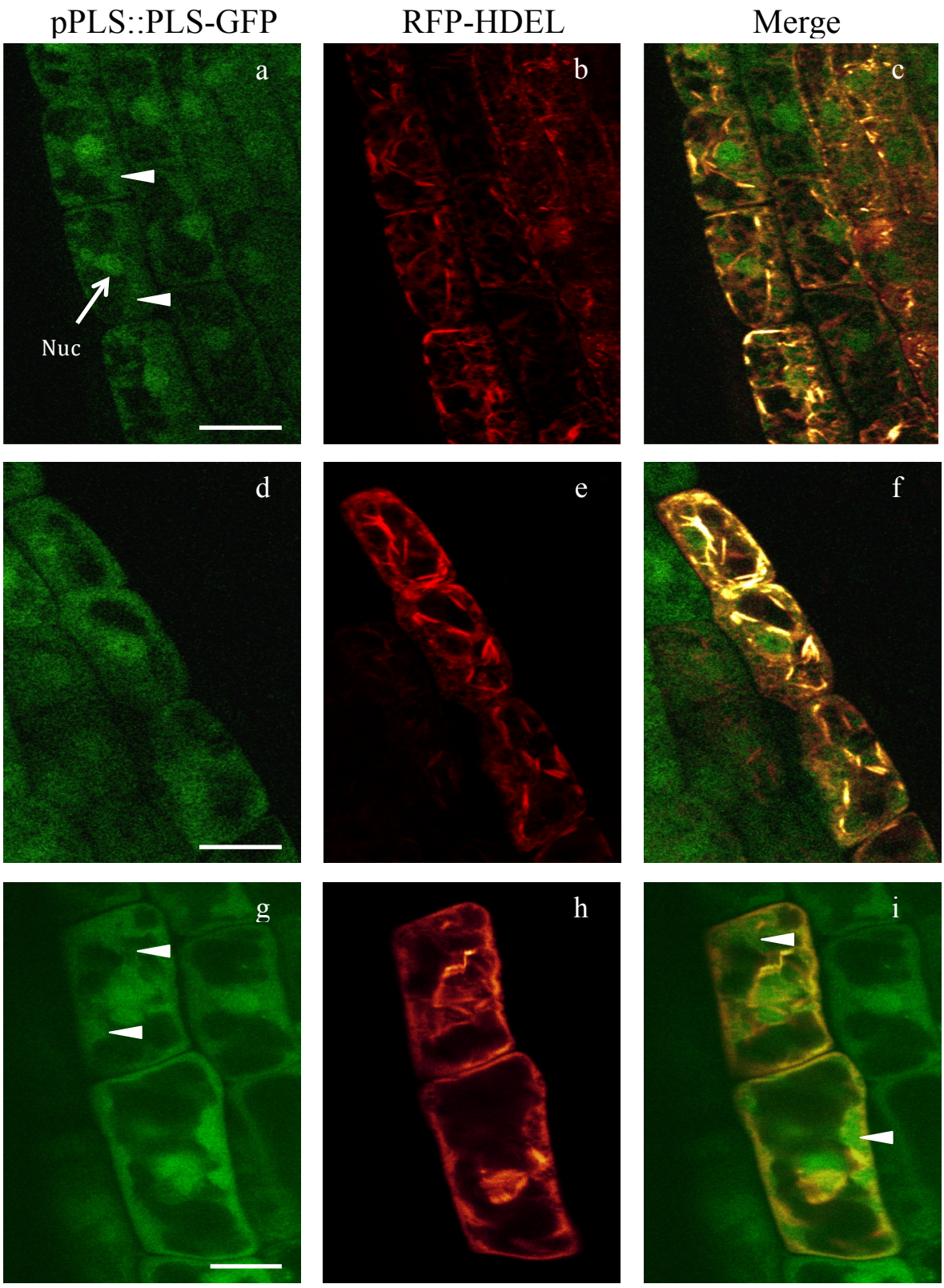


Figure 4-4. PLS-GFP colocalises with the endoplasmic reticulum marker RFP-HDEL. Transgenic *pPLS::PLS:GFP* (green; a,d,g) seedlings containing the *p35S::RFP:HDEL* construct (red; b,e,h) were imaged by CLSM at seven days after germination, showing co-localisation (yellow; c,f,i) of the PLS-GFP and RFP-HDEL proteins in root epidermal cells (three separate root examples). Arrows depict membranous PLS-GFP. Laser settings: 21% 488 nm 20 mW, 1034 V (green); 543 nm 1.2 mW, HyD:124 (red). Scale bars = 10 µm.

pPLS::PLS:GFP;p35S::RFP:HDEL seedlings show colocalisation of PLS protein and the endoplasmic reticulum marker (Figure 4-4). The PLS-GFP fusion protein can be seen in the epidermal cell nuclei (Nuc) and in nearby membranous structures (Figure 4-4 a,d,g; arrows in panels a and g). When the location of PLS-GFP is merged with that of the RFP-HDEL protein (Figure 4-4 b,e,h), strong colocalisation is apparent (yellow) around the nuclei (Figure 4-4 c,f,i),. However, not all the PLS-GFP colocalises with the RFP, illustrated by the arrows in in panel (i); suggesting some of the PLS-GFP protein resides in the cytosol, as well as showing a clear nuclear localisation.

4.2.4 POLARIS does not localise to the Golgi apparatus

There is some evidence that the ethylene receptors do not only reside in the membrane of the endoplasmic reticulum, but also in the Golgi apparatus (Dong et al., 2008). Therefore, to investigate whether the PLS peptide may also reside in this compartment, the localisation of the PLS-GFP protein was imaged together with a fluorescent mCherry-tagged protein marker for the Golgi apparatus; *Arabidopsis thaliana* SULFOTRANSFERASE 1 (ST1). ST1 is a trans-Golgi-localised brassinosteroid and flavenoid sulfotransferase protein which adds sulfuryl groups to the appropriate molecules (Bauer and Papenbrock, 2002).

The construct *pFGC-ST:mCherry* (Appendix VI) was obtained from NASC/ABRC and transformed into *pPLS::PLS:GFP* plants via *A. tumefaciens* (Materials and Methods, 2.7.8 and 2.7.9). Successful transformants were identified by differential growth on ½ MS10 agar media containing glufosinate-ammonium (Materials and Methods, 2.8): plants expressing the construct were identifiable after ten days by the correct growth of leaves and roots. The seed of these plants was grown for seven days and used for imaging.

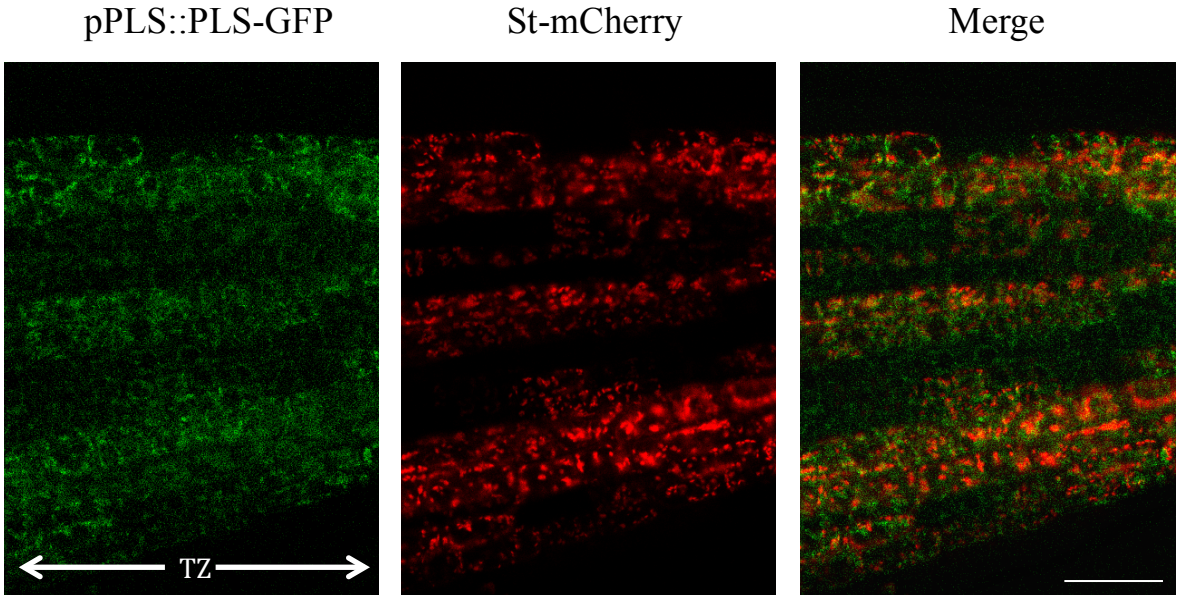


Figure 4-5. The PLS-GFP protein does not colocalise with the Golgi apparatus marker ST-mCherry. Transgenic seedlings expressing both *pPLS::PLS:GFP* (green) and the *p35S::ST-mCherry* construct (red) in root transition zone (TZ) epidermal cells were imaged by CLSM at seven days after germination, showing that the PLS-GFP and ST-mCherry proteins do not colocalise (absence of yellow colour in merged image). Laser settings: 21% 488 nm 20 mW, 960 V gain (green); 543 nm HeNe 1.2 mW, HyD:86 (red). Scale bar = 25 μm.

Colocalisation imaging experiments were performed on *Arabidopsis* root epidermal cells located in the transition zone up to ~250 μm away from the root quiescent centre, which expressed both the *pPLS::PLS:GFP* and the Golgi apparatus-localised *p35S::ST:mCherry* fluorescent constructs (Figure 4-5). Although *PLS* expression was found to be weaker in these zones than in the meristematic zone (see Figure 1-2, page 5 for root zones), the more expanded cells found further away from the tip allowed for clearer imaging of the fluorescent protein localisations. The PLS-GFP and ST-mCherry fusion proteins are not observed in the same location in the epidermal cells, illustrated by the absence of yellow when the two channels are merged in Figure 4-5, suggesting the PLS peptide is not localised to the Golgi apparatus.

4.3 Response of POLARIS to ethylene perturbations

The whole-root and subcellular localisation of PLS provides a system to investigate whether the localisation of the PLS peptide differs in the presence of ethylene, which is possible if PLS plays a role in ethylene responses. Studies have reported previously that the addition of ethylene represses the expression of a *GUS* gene powered by the *PLS* promoter (Chilley et al., 2006). The response of the PLS-GFP fusion protein to ethylene in the following experiments expands upon this work.

4.3.1 POLARIS expression is reduced upon ethylene treatment

Plants expressing the PLS-GFP fusion protein were treated with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) to simulate an increase in ethylene in the plant environment. Plants convert methionine to ethylene via the intermediates of *S*-adenosylmethionine and ACC (Adams and Yang, 1979). The last step is a rate-limiting conversion of ACC to ethylene, catalysed by ACC oxidase (Kende, 1989). Providing plants with ACC will result in its conversion into ethylene via the pathway above, and the water-soluble molecule enables straightforward ethylene treatment without having to grow plants in ethylene-containing gas chambers.

Seedlings were grown on phytagel ½ MS10 growth media (Materials and Methods, 2.2.2.3) for either six or seven days, then transferred into liquid ½ MS10 media for 24 hours or 2 hours respectively before imaging with CLSM (Materials and Methods, 2.10). The liquid media contained 0, 1 or 10 μM ACC (Materials and Methods, 2.2.6.1).

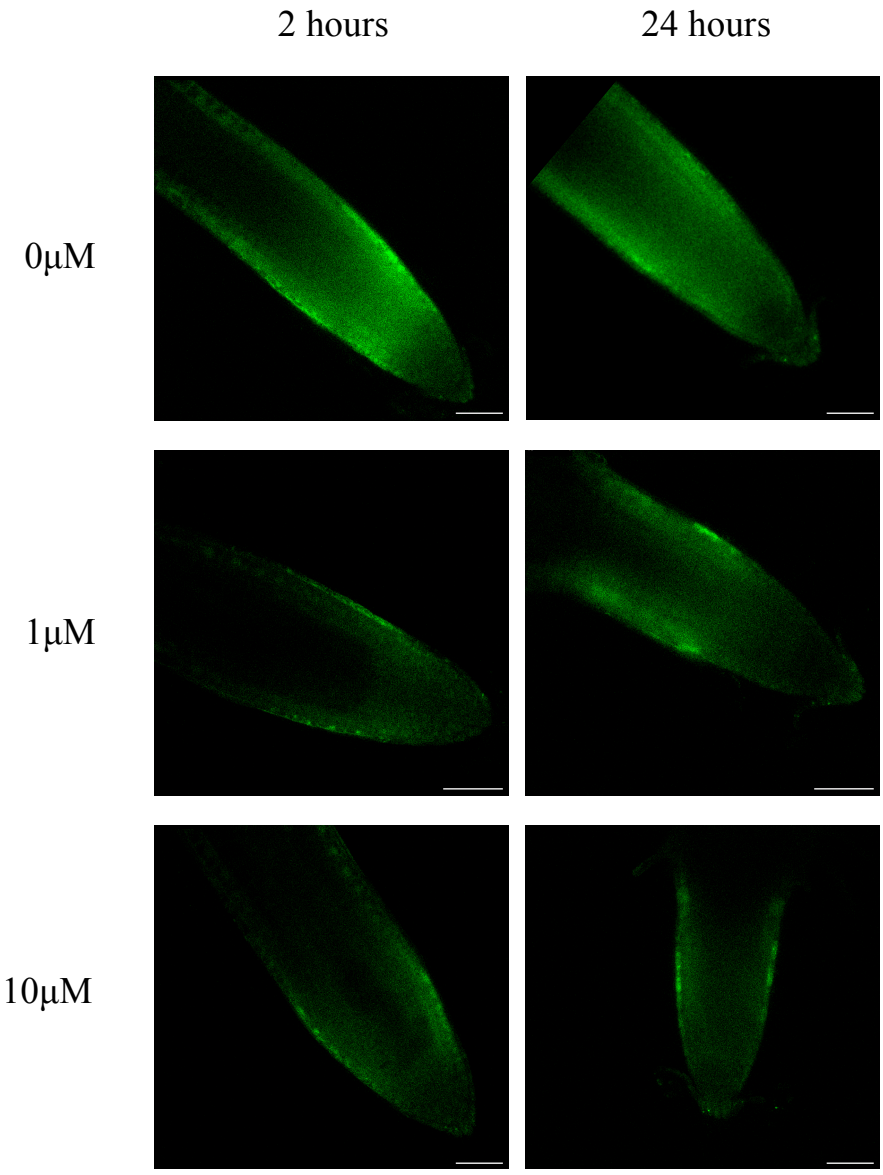


Figure 4-6. Expression of the PLS-GFP fusion protein is downregulated in the root tip after ethylene treatment. Transgenic seedlings were treated with 0, 1 or 10 μM ACC for either 2 or 24 hours and imaged by CLSM at seven d.a.g. Laser settings were maintained at 21% 488 nm 20 mW, 970 V gain. Scale bars = 50 μm.

ACC treatment of seven day old seedlings expressing the *pPLS::PLS:GFP* construct caused downregulation of the transgene and a decrease in the PLS-GFP protein synthesis (Figure 4-6). Control seedlings were grown under the same conditions and transferred into liquid media without ACC present for 2 or 24 hours. The time difference in the liquid media did not seem to affect the expression of PLS-GFP. Seedlings treated with 1 or 10 μM ACC, at both time intervals, showed a decrease in fluorescence, with PLS-GFP expression almost vanishing from the root tip and expressed predominantly in the epidermal cells along the elongation zone. The epidermal cells expressing PLS-GFP in the treated roots seem to be located further back from the root tip (a more proximal position) in the roots treated for 24 hours.

The ACC treatment for both time periods and both ACC concentrations appear to downregulate the expression of PLS-GFP to a similar extent, with the fluorescence emitted by the treated roots appearing much the same. To investigate this further, the fluorescence of each root was quantified using ImageJ, and the mean fluorescence intensity was calculated and plotted (Figure 4-7). There were at least 17 individual primary roots measured for each treatment group.

Seedlings grown for either 2 or 24 hours in the absence of ACC showed no change in the level of fluorescence intensity, measured at 87.8 and 86.8 respectively (Student's *t* test, p = 0.45). Seedlings treated with ACC for 2 hours showed a greater decrease in GFP fluorescence (39.2 and 35.5) than those treated for 24 hours (54.7 and 54.9), but this reduction appears to be unaffected by the concentration of ACC in the media. Qualitative observations from Figure 4-6 appear to suggest there is not much difference in the fluorescence intensity between the ACC treated roots, whereas quantification of fluorescence does seem to reveal a difference, illustrated in Figure 4-7. Therefore, in order to investigate this further, the means of each group were analysed for their statistical relevance.

After 2 hours of treatment with either 1 μM or 10 μM ACC, PLS-GFP fluorescence had decreased significantly compared to the control treatment (ANOVA, $F_{(2,58)} = 14.8$, p = 6.39E-06). Post-hoc analysis using Tukey's HSD test revealed that the means of both treatments are significantly different to the 2-hour control with no ACC added. They are

not, however, different to each other, suggesting that ACC concentrations of both 1 μM and 10 μM have the same effect on PLS-GFP expression over a 2 hour treatment.

Similar statistical tests were performed on the seedlings treated for 24 hours. The expression of PLS-GFP reportedly underwent a significant change when treated with ACC (ANOVA, $F_{(2,65)} = 3.78$, $p = 0.0278$). Although this p value falls under the 0.05 significance threshold, it is much larger than the 6.39E-06 p value obtained from the 2-hour data. Furthermore, Tukey's HSD test revealed that neither ACC treatment (1 or 10 μM) produced a significant change in the expression of PLS-GFP over the 24 hour period.

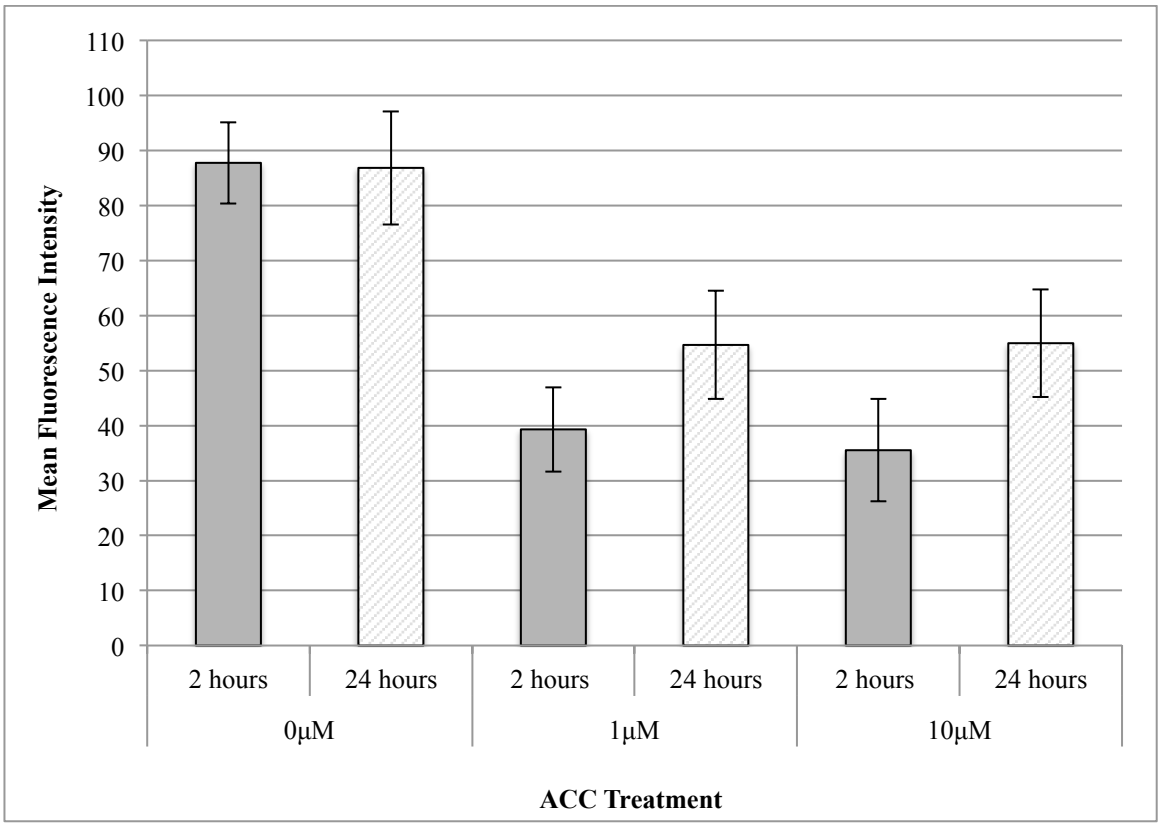


Figure 4-7. Quantification of PLS-GFP fluorescence after ACC treatment, detected in seedlings expressing the *pPLS::PLS:GFP* construct. Seedling root tips at seven d.a.g were imaged by CLSM after treatment with 0, 1 or 10 μM ACC for either 2 or 24 hours. Fluorescence intensity in each root was measured using ImageJ and the mean intensity for each treatment was calculated. Error bars show ± 1 standard error. N ≥ 17.

Further study was undertaken to investigate whether the location of PLS-GFP expression in the root tip was altered by ACC treatment. The data set examined in Figures 4-6 and 4-7 was analysed for the upper (proximal) and lower (distal) limits of PLS-GFP expression after treatment with 0, 1 and 10 μM ACC for either 2 or 24 hours (Figure 4-8).

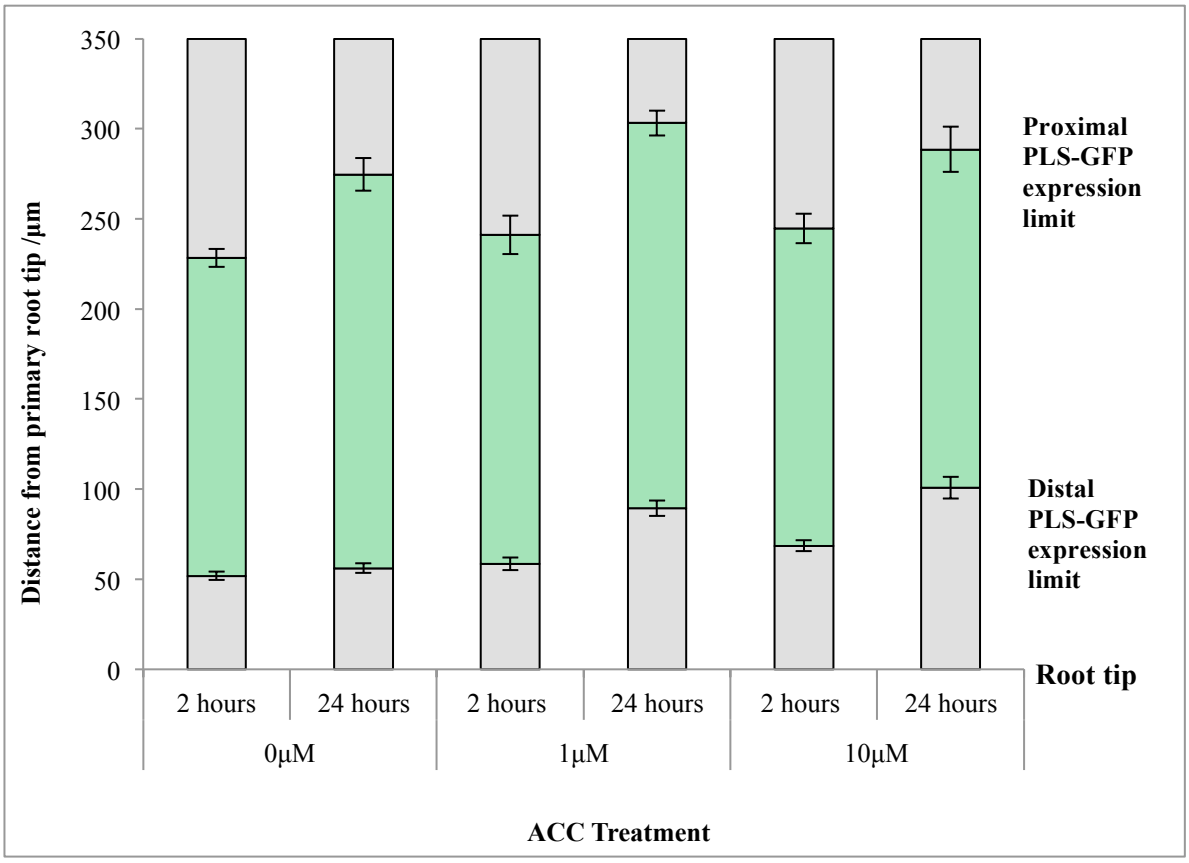


Figure 4-8. The extent of PLS-GFP expression after ACC treatment in the primary root. Seedlings expressing the PLS-GFP fusion protein were grown for seven days and treated with 0, 1 or 10 µM ACC for either 2 or 24 hours. Each bar represents the primary root under the specified treatment, from the root tip at 0 µm to 350 µm back from the tip, and the mean distal and proximal limits of PLS-GFP expression after each treatment. Error bars show ± 1 standard error. N ≥17.

Treating the seedlings with ACC for 24 hours caused the upper (proximal) limit of PLS-GFP expression to be found at a more proximal location, further away from the root tip. More specifically, after 24 hours of treatment with 0 µM, 1 µM and 10 µM ACC, PLS-GFP was seen to be expressed approximately 46 µm, 75 µm and 60 µm further away from the root tip respectively at the proximal limit, compared to the 2 hour treatment with 0 µM ACC.

However, only the 24-hour treatments with ACC present (1 and 10 µM) resulted in the distal PLS-GFP expression limit also becoming more proximal, by approximately 33 µm and 44 µm respectively, which was not observed in the control (0 µM) treatment (ANOVA, $F_{(2,65)} = 34.3$, $p = 5.67E\text{-}10$, Tukey's HSD test).

Seedlings treated for only 2 hours with any of the three ACC concentrations seemingly did not show a difference in the location of PLS-GFP expression at the proximal limit. However, treatment with 10 μM ACC for 2 hours did cause PLS-GFP expression to move away from the root tip by approximately 17 μm at the distal limit (ANOVA, $F_{(2,65)} = 8.08$, $p = 8.65E\text{-}4$, Tukey's HSD test), whereas no change was observed after treatment with 1 μM ACC for 2 hours.

These results suggest that PLS-GFP expression is modulated within 2 hours of increasing ethylene signalling by ACC, but the location of PLS-GFP expression responds to ethylene over a longer time period. Analysis of the fluorescence intensity of PLS-GFP expression after ACC treatment (Figure 4-7) revealed that treatment with ACC for just 2 hours caused a significant decrease in gene expression. However, the location of PLS-GFP expression after 2 hours does not change unless the ACC concentration is high, and then expression is observed to become more proximal near the root tip.
In contrast, the decrease in PLS-GFP expression after 24 hours of ACC treatment appeared to be not significant, suggesting some recovery of expression, but the PLS-GFP localisation analysis revealed that PLS-GFP expression has moved further away from the root tip.

Further work was planned using the ACC-treated roots to investigate whether increased ethylene levels promoted a change in the subcellular localisation of the PLS-GFP fusion protein. Unfortunately, due to the resulting decrease in PLS-GFP expression upon ACC treatment, which was further reduced from an already low endogenous *pPLS* promoter expression level, the confocal laser scanning microscopy facilities available could not detect any subcellular detail in the root cells.

4.3.2 *POLARIS* is downregulated in the presence of non-functional ETR1

Experiments were undertaken to investigate whether the absence of active ETR1 ethylene receptor protein affects the localisation of the PLS peptide.

The PLS peptide can interact with the ETR1 protein in yeast 2-hybrid and BiFC experiments (Mehdi, 2009), but it has not been studied whether the PLS peptide can interact with the other four receptor proteins. Removing functional ETR1 protein from the

plants may further our understanding about whether PLS is recruited solely to the functional membrane-bound ETR1, or whether PLS localisation is ETR1-independent.

To create a plant line in which the localisation of PLS can be studied in the absence of ETR1, plants expressing the *pPLS::PLS:GFP* construct were crossed with the single loss-of-function (LOF) mutant *etr1-9*. LOF mutations within the ethylene receptor family do not generally show a strong phenotype due to the functional redundancy between the five receptors (Hua and Meyerowitz, 1998). Indeed, *etr1-9* shows ethylene responses similar to those seen in the wild type, although it is slightly hypersensitive to ethylene in the dark, illustrated by a shorter hypocotyl (Qu et al., 2007).

etr1-9 is a T-DNA insertion mutant allele, with the T-DNA being inserted at the start of the fourth exon of the *ETR1* gene. Northern blot analysis confirmed the insertion as a LOF mutation as neither the full-length *ETR1* transcript or ETR1 protein could be detected. There is increased transcription of a truncated *ETR1* transcript, prior to the T-DNA insertion site, possibly due to a feedback mechanism in an attempt to compensate for the lack of ETR1 receptor protein. The location of the T-DNA insertion would produce a protein with an intact N-terminal ethylene-binding transmembrane domain, but a disrupted C-terminal domain, which is acknowledged to be important for downstream signalling via CTR1 (Gao et al., 2003; Qu et al., 2007).

etr1-9 mutant plants (female) were crossed (Materials and Methods, 2.2.5) with the *pPLS::PLS:GFP* fluorescent line (male). The resulting heterozygote F1 seeds were screened for GFP fluorescence (as in section 2.8) before being grown to the F2 generation. F2 plants were genotyped by PCR for homozygote loss of function *ETR1* DNA by ensuring that there was no full length *ETR1* gene present.

Homozygote seeds were investigated for a change in GFP fluorescence and PLS-GFP fusion protein localisation. Although all F1 and F2 generation heterozygotes synthesised the PLS-GFP protein, F2 *etr1*-9 *x pPLS::PLS:GFP* homozygote seedlings showed no fluorescence and were no use for PLS localisation experiments.

qPCR experiments were carried out (Materials and Methods, 2.5.4) to determine whether production of *PLS:GFP* transcript was affected in the homozygous lines, and thus whether altered gene expression could explain the absence of fluorescence in the F2 generation. cDNA was tested from the *etr1*-9*xpPLS::PLS:GFP* plants that were homozygous for the *etr1-9* mutation, a heterozygote line, and the *pPLS::PLS:GFP* and *etr1-9* parent lines,

using primers positioned from the *PLS* gene into the *GFP* DNA in the *pPLS::PLS:GFP* construct. Transcript abundance was quantified relative to the housekeeping gene PP2C using primers listed in Appendix V.

Each qPCR reaction was performed in triplicate. Figure 4-9 shows the mean relative expression for each plant line, calculated from the values obtained from three biological replicates, each grown separately under the conditions indicated in section 2.2.3.1.

Relative expression of the *PLS:GFP* transcript decreased approximately six-fold in both the heterozygous crossed line (with wild type *ETR1* from the *pPLS::PLS:GFP* parent plant and an *etr1-9* mutant allele) and two independent lines of crossed plants homozygous for the *etr1-9* mutation (A4 and C1) in comparison to the *pPLS::PLS:GFP* parent line (Figure 4-9). This reduction of an already weakly-expressed *PLS:GFP* DNA construct may explain why the PLS-GFP protein could not be detected by CLSM for PLS localisation studies.

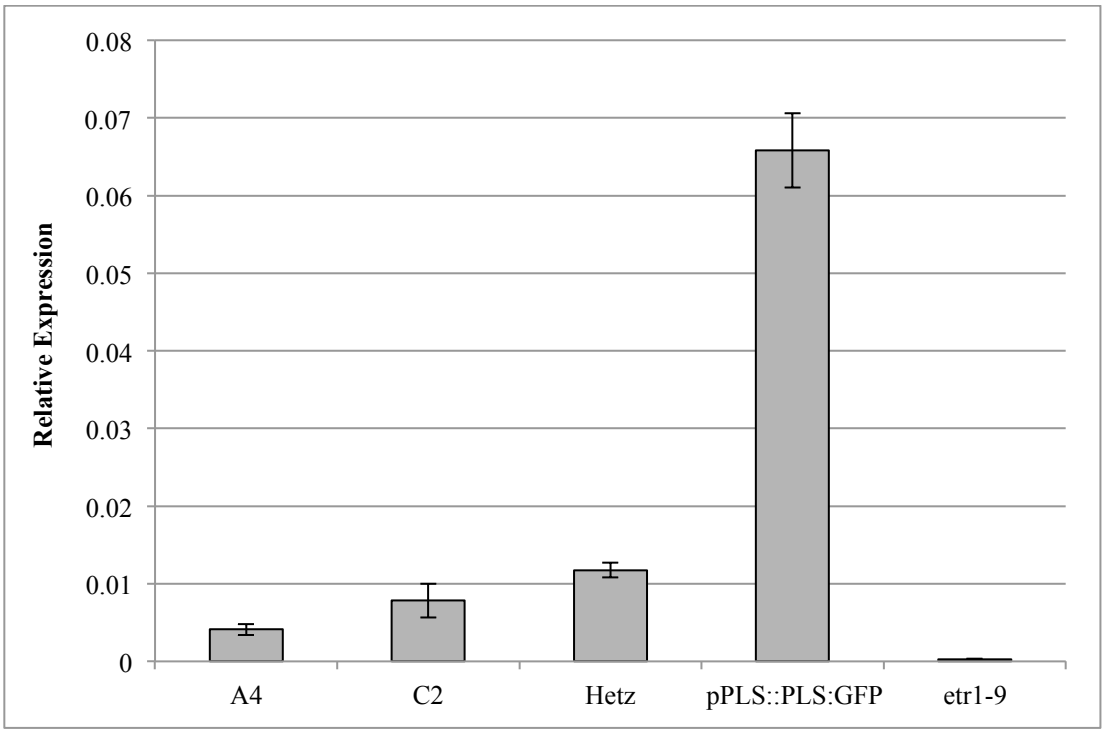


Figure 4-9. Relative expression of the *PLS:GFP* cDNA in homozygous *etr1-9xpPLS::PLS:GFP* crossed plants. Relative expression of the *PLS:GFP* transcript was measured by qPCR in the A4 and C2 homozygous *etr1-9xpPLS::PLS:GFP* lines, a heterozygous *etr1-9xpPLS::PLS:GFP* line, and the *pPLS::PLS:GFP* and *etr1-9* parent plant lines. Error bars are ±1 standard error of the mean of quantified transcript measured in the three biological replicates.

Conclusion

The PLS peptide was found to be localised to the nuclei and surrounding membranous structures in cells located at the *Arabidopsis* root tip, predominantly behind the quiescent centre. Colocalisation with chemical and genetic markers suggest the peptide localises to the ER, in which the ethylene receptor proteins are found, but does not localise to the Golgi apparatus.

The expression of a PLS-GFP fusion protein was found to be considerably reduced under two separate ethylene-modulation experiments. It was previously reported that the activity of the *PLS* promoter is repressed upon ethylene treatment (Chilley et al., 2006). Experiments in this chapter showed that the level of the PLS-GFP fusion protein is reduced in *Arabidopsis* roots after treatment for two hours with the ethylene precursor molecule ACC, indicating that both the gene and the peptide are regulated by ethylene. Treating *Arabidopsis* seedlings with ACC for 24 hours caused a change in the location of the root cells expressing the PLS-GFP protein, with the pattern of PLS-GFP expression becoming more proximal. Secondly, *etr1-9* plants lacking a functional ETR1 ethylene receptor protein were found to have considerably reduced expression of the *PLS:GFP* construct.

The information that PLS resides in or near the ER lends more weight to the idea that PLS interacts with the ethylene receptor ETR1. The next chapter will attempt to identify the role of the PLS peptide in ethylene signalling, and its relationship with ETR1.

Chapter 5 . The Role of the POLARIS Peptide

5.1 Introduction

The work in this chapter aims to increase our understanding of the relationship between POLARIS and ETR1, and attempts to define a role for POLARIS in the ethylene signalling pathway.

POLARIS was predicted to act upon the ethylene signalling pathway at the level of the receptor proteins. The *pls* mutant has increased ethylene responses, but this is not a result of increased ethylene biosynthesis, or of downstream effects beyond the ethylene receptors (Chilley et al., 2006).

The expression of the *PLS* gene is negatively regulated by ethylene (Chilley et al., 2006). Part of this chapter focuses on how the expression of both the *PLS* and *ETR1* genes are modified by the presence of ethylene in a range of wild type and ethylene-signalling mutant backgrounds, to develop a greater understanding of the role of the PLS peptide in the ethylene signalling pathway.

Confocal microscopy presented in Chapter 4 of this thesis revealed that the POLARIS peptide localises to the endoplasmic reticulum, in which the ethylene receptor ETR1 is an integral membrane protein. Furthermore, it has been previously shown via Yeast 2-hybrid and BiFC complementation assays in onion cells that the ethylene receptor ETR1 and the POLARIS peptide can physically interact *in vitro* and *in vivo* respectively (Mehdi, 2009). In the work described in this Chapter, co-immunoprecipitation experiments were performed in *N. benthamiana* to further examine the possible interaction between PLS and ETR1, and copper binding experiments were carried out.

ETR1 requires a copper ion for activity (Rodriguez et al., 1999), and the PLS peptide was shown to be inactive when the potential metal ion-coordinating cysteine residues were removed (Chapter 3). It can be hypothesised that PLS might consequently have a role in copper ion delivery or other interaction to ensure correct receptor function. Therefore, the mechanism by which PLS may act upon ETR1 has been explored by investigating the capability of the peptide to bind copper ions and the response of the loss-of-function *pls* mutant to the presence and absence of copper.

5.2 Expression relationships between *POLARIS* and *ETR1*

To gain some understanding of how PLS might regulate ethylene signalling, *ETR1* ethylene receptor and *PLS* gene expression was quantified after seedlings were treated with the ethylene precursor molecule ACC to simulate increasing ethylene concentrations.

Gene expression was measured by quantitative PCR (qPCR) (Materials and Methods, 2.5.4). The resulting information about the levels of gene expression under changing conditions can provide a better understanding about the regulation of plant processes.

Arabidopsis seedlings from the Col-0 and C24 wild type backgrounds, the *pls* mutant, the ethylene overproducing mutant *eto1 (ethylene overproducer1)* and the ethylene-insensitive receptor mutant *etr1-1 (ethylene resistant1)* were grown in liquid media containing 0, 0.01, 0.1, 1 or 10 μM ACC (the ethylene precursor 1-aminocyclopropane-1-carboxylic acid; Materials and Methods, 2.2.3.2, 2.2.6.1). At seven days after germination (d.a.g.), RNA was extracted from each plant line at each ACC concentration, providing a template for cDNA synthesis, which was subsequently used as a template for qPCR. The qPCR study focused on three genes which, together, provide information about the expression and regulation of ethylene signalling: *ERF1,* an ethylene responsive transcription factor which illustrates the level of ethylene response occurring in the plants; *PLS*, with primers designed to bind either side of the tDNA insertion location in the *pls* mutant; and *ETR1,* to investigate whether the transcription of this ethylene receptor is regulated or altered by the changing levels of PLS peptide and ethylene.

To determine the changes in gene expression, the measurement of each target gene needs to be compared to a stably expressed reference gene, chosen for its invariable expression throughout the conditions during the experiment. This comparison helps to minimise small changes in the expression of the target genes, which may be due to differences in the cDNA samples rather than altered endogenous gene expression. Several candidates for reference genes were identified and tested for their stability in the seven plant lines and five ACC treatments. The most stable was found to be *PROTEIN PHOSPHATASE 2C* (*PP2C*), a serine/threonine protein kinase with a role in signal transduction pathways (Rodriguez, 1998). The relative expression of the reference gene is set to 1, with target gene expression measured in relation to 1 in all cases.

Three technical replicates were performed for each qPCR reaction, and the whole experiment was repeated using cDNA obtained from three different samples of

Arabidopsis seedlings grown under identical conditions. The following figures illustrate mean gene expression, relative to the housekeeping gene, calculated from the gene expression values from the three biological samples. The error bars show the upper and lower limits of the standard error of the mean.

Before investigating how *PLS* and *ETR1* gene expression responds to the presence of ethylene, it was important to obtain a level of context with regard to ethylene responses. To measure the response of the ethylene signalling pathway to exogenous ACC, primers were designed for the *ETHYLENE-RESPONSE-FACTOR1 (ERF1)* gene. *ERF1* encodes an APETALA2 (AP2)-domain-containing transcription factor, which is upregulated by the EIN3 and EIN3-like transcription factors situated downstream of ethylene-receptor binding. Upon ethylene binding, the ethylene signalling cascade causes these transcription factors to accumulate in the nucleus and promote the transcription of ethylene-responsive genes (Stepanova and Alonso, 2005).

The expression of *ERF1* was quantified after treatment with the five concentrations of ACC: 0, 0.01, 0.1, 1 and 10 μM (Figure 5-1). As ACC concentration increases, the wild type Col-0 and C24 plant lines show a similar responsive increase in the expression of *ERF1*. The *pls* mutant shows a two-fold increase in *ERF1* expression compared to C24 at all ACC concentrations tested, and the ethylene overproducer *eto1* also expresses *ERF1* to higher relative levels than the wild type plants. Both *pls* and *eto1* however have a higher level of *ERF1* expression in the untreated samples, an expected observation considering the higher levels of ethylene signalling in these two mutants: *pls* lacks the negative regulation of the PLS peptide upon the ethylene signalling pathway (Chilley et al., 2006), and *eto1* produces higher levels of ethylene due to elevated activity of the ACC synthase (ACS) enzyme which catalyses the rate-limiting step in ethylene biosynthesis (Woeste et al., 1999).

The ethylene-resistant gain-of-function mutant *etr1-1* (Bleecker et al., 1988) has constitutively active ETR1 ethylene receptor proteins which maintain their inhibition of the downstream ethylene signalling pathway and prevent ethylene responses. In wild type plants, the inhibitory effect is removed upon ethylene binding to the receptor proteins, promoting downstream responses. The removal of inhibition does not occur in *etr1-1* plants, a result of a point mutation in the N-terminal ethylene-binding domain, so the receptors are unable to bind ethylene (Hall et al., 1999). The absence of downstream

ethylene responses is illustrated in Figure 5-1, with no detectable *ERF1* expression, even in the presence of ACC.

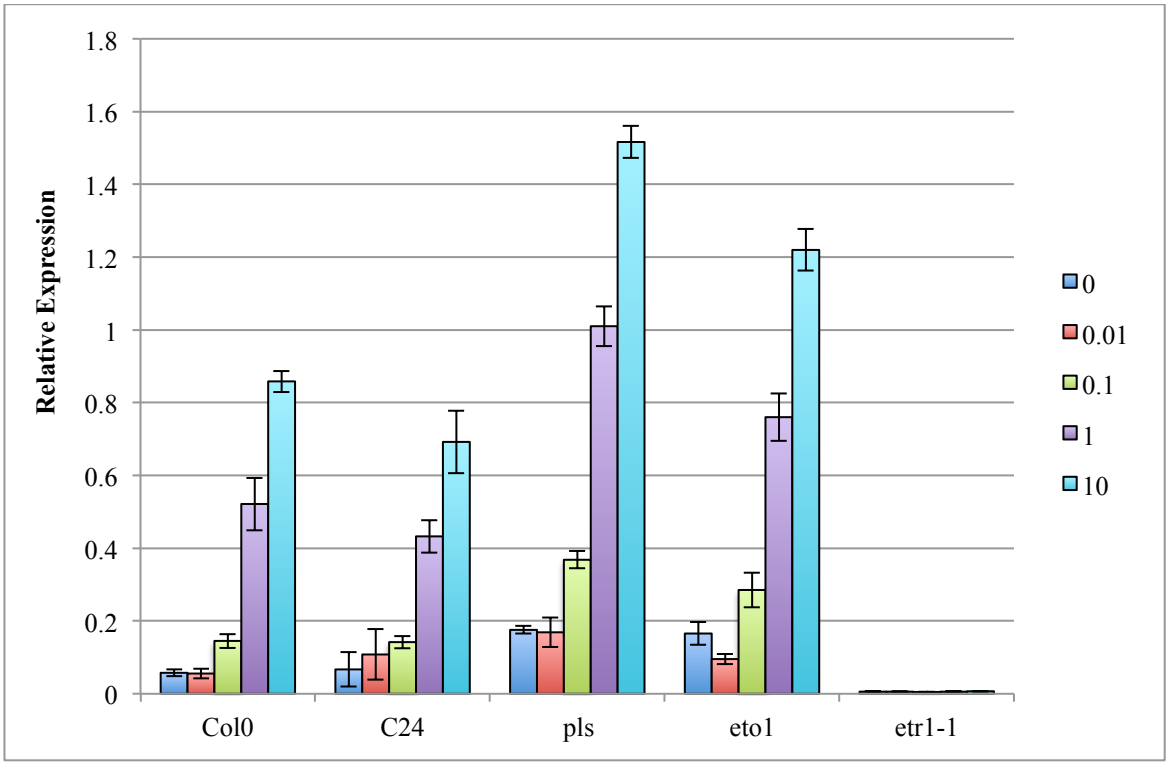


Figure 5-1. Relative expression of the *ERF1* ethylene-inducible gene after ACC treatment in wild type and ethylene mutant plant lines. Col-0, C24, *pls, eto1* and *etr1-1* seedlings were treated with 0, 0.01, 0.1, 1 or 10 µM ACC for seven d.a.g and quantitative PCR was carried out on the resulting cDNA. *ERF1* transcript levels were measured alongside a reference gene transcript *PP2C.* Error bars are ±1 standard error of the mean of the biological replicates.

Expression of the *PLS* gene was investigated first in the Col-0 and C24 wild type lines and the *pls* mutant line over the five increasing ACC concentrations (Figure 5-3). *PLS* primers showed the expected knock down of the *PLS* gene in the loss-of-function *pls* mutant (third set of data in Figure 5-3), as the reverse primer is complementary to a region of the *PLS* gene disrupted by the tDNA insertion in the mutant background (primer pair B (blue) in Figure 5-2).

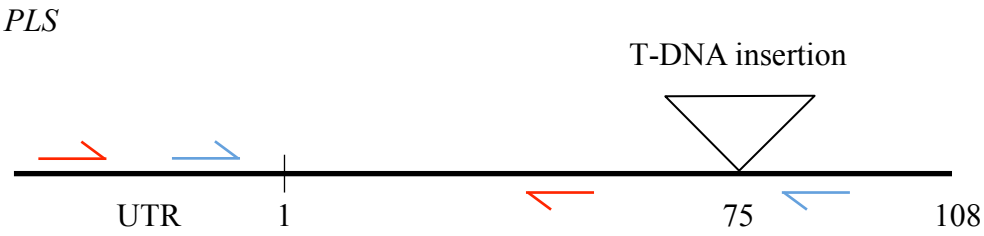


Figure 5-2. Position of the two primer pairs in the *PLS* gene, used for qPCR on the *pls* mutant after treatment with ACC. Primer pair A (red) produces a 130bp product in both the wild type and *pls* mutant plants. Primer pair B (blue) will not produce a detectable DNA product in the *pls* mutant, due to the tDNA insertion interrupting the transcript, but will produce a 132bp product in the wild type. Transcript lengths above are not to scale.

As ACC concentration increases, *PLS* expression decreases in the C24 background (ANOVA, $F_{(4,10)} = 11.5$, $p = 0.0014$) but not significantly in Col-0 (ANOVA, $F_{(4,10)} = 2.15$, $p = 0.14$), with similar *PLS* expression levels in both Col-0 and C24 plants after treatment with the highest ACC concentrations. The *PLS* gene has notably low endogenous expression (Casson et al., 2002), illustrated as a relative expression of only 0.01 in the untreated C24 sample, compared to a set relative expression of 1 for the *PP2C* reference gene.

The fourth set of expression data (labelled 'pls pre-tDNA') in Figure 5-3 was derived using an alternative pair of PCR primers for the *PLS* gene, and shows the relative expression of a truncated section of the *PLS* gene transcript in the *pls* mutant. The primers (primer pair A (red) in Figure 5-2) are located in a section of the *PLS* gene transcript that is not affected by the tDNA insertion in the mutant, and are able to produce a measurable PCR product.

The presence of ethylene in the C24 wild type causes *PLS* expression to drop. A decrease in expression can also be seen in the 'pls pre-tDNA' mutant data (NB *pls* was created in the C24 background), but at much greater relative expression levels: the expression of the N-terminus of *PLS* is approximately three times greater in *pls* than in C24. This suggests that the *pls* mutant plants are upregulating the *PLS* gene, even without the addition of exogenous ACC, in an attempt to control ethylene signalling levels (observed to be notably higher in *pls* in Figure 5-1).

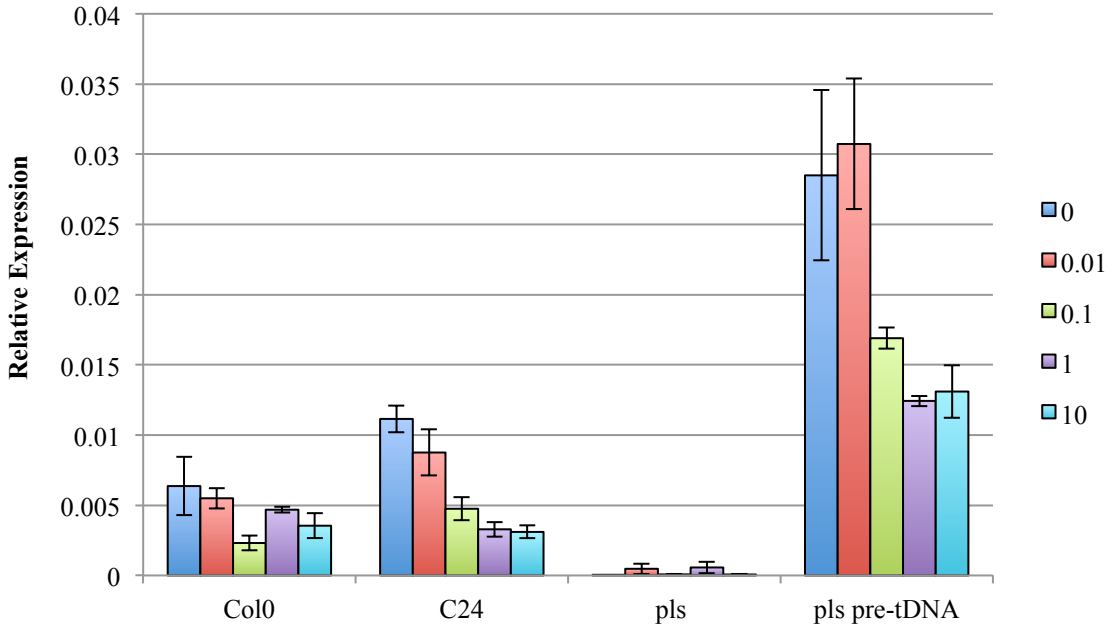


Figure 5-3. *PLS* gene expression under ACC treatment. Col-0, C24 and *pls* seedlings were treated with 0, 0.01, 0.1, 1 or 10 µM ACC for seven d.a.g and quantitative PCR was carried out on the resulting cDNA. *PLS* transcript levels were measured alongside a reference gene transcript *PP2C*. Comparison is made between the *PLS* expression in Col-0 and C24 wild types and the *pls* mutant, in which the reverse qPCR primer is located beyond the tDNA insertion in the *pls* mutant. In the fourth set of bars, a second set of *PLS* primers were used, located before the tDNA insertion, to detect a truncated and inactive *PLS* transcript. Error bars show ±1 standard error.

PLS gene expression was then investigated in the ethylene signalling mutants *eto1* and *etr1-1* (Figure 5-4) using *PLS* primer pair B in Figure 5-2. The relative expression of *PLS* in Col-0, C24 and *pls* are the same as in Figure 5-3.

There is a slight change in *PLS* expression in the ethylene overproducing mutant *eto1* as ACC concentration is increased (ANOVA, $F_{(4,10)} = 3.78$, $p = 0.04$). However, *PLS* levels remained between 0.0017 and 0.0043, with only *eto1* plants treated with 10 µM ACC showing an increase in gene expression (Tukey's HSD test).

The expression of *PLS* in the ethylene-resistant *etr1-1* mutant is similar to that seen in its Col-0 wild type plant background; *PLS* expression decreases upon addition of ethylene, from a relative expression of 0.0058 with no extra ethylene to 0.0022 after treatment with 10 µM ACC. The reduced ethylene responses in *etr1-1* appear not to affect *PLS* expression with respect to the Col-0 wild type.

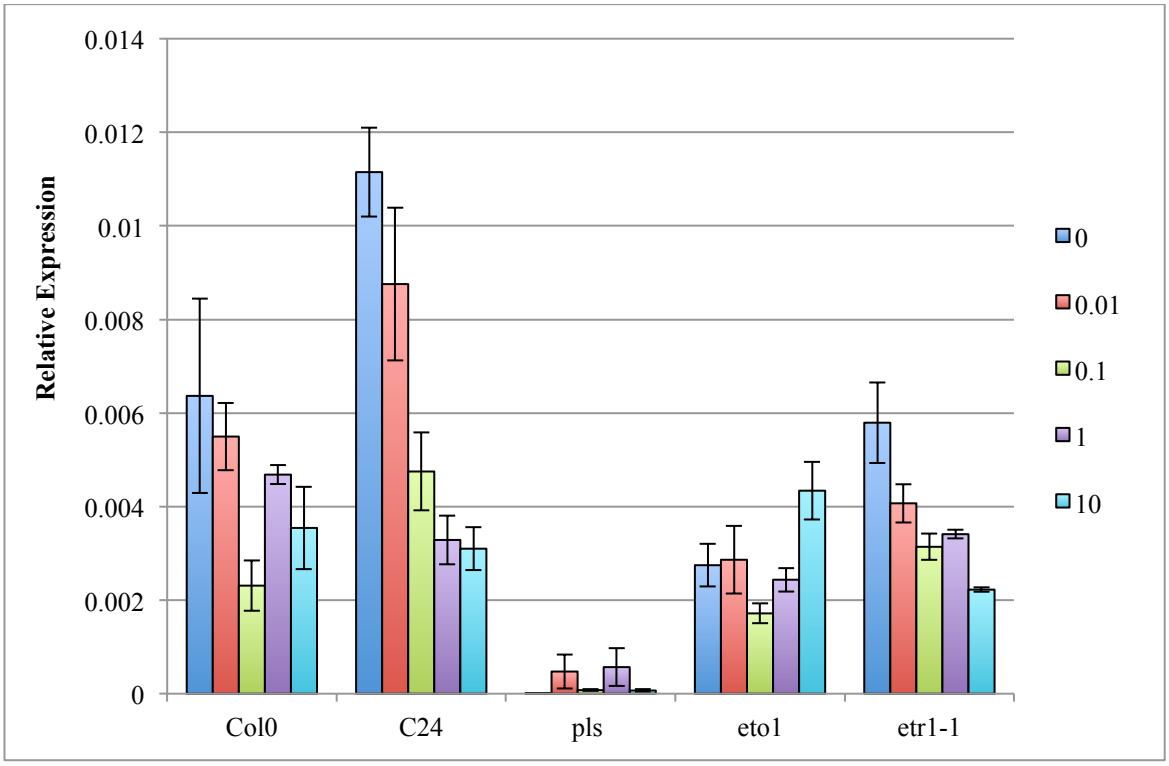


Figure 5-4. Relative expression of the *PLS* gene in wild type and ethylene mutants after ACC treatment. RNA was extracted from plants after treatment with 0, 0.01, 0.1, 1 or 10 µM ACC for seven d.a.g, total plant cDNA synthesised and used as templates in quantitative PCR, measuring the expression of the POLARIS peptide *PLS* gene with reference to the stable gene *PP2C*. Error bars are ±1 standard error.

The expression of the ethylene receptor gene *ETR1* was also quantified to investigate the relationship between *PLS* and *ETR1* transcription, and plant ethylene levels (Figure 5-5). There are various possible explanations for the role of PLS in ethylene signalling. For example, the PLS peptide may negatively regulate ethylene signalling by decreasing ETR1 protein production, thus providing fewer ethylene binding sites and fewer ethylene responses. Alternatively the peptide could have a role in receptor degradation and turnover, or in providing copper as part of receptor complex synthesis or function.

PLS does not seem to have a role in modulating *ETR1* mRNA transcription or degradation as the steady state level of the *ETR1* transcript did not alter over the range of ACC concentrations in either C24 or *pls* (ANOVA, $F_{(4,10)} = 1.45$, $p = 0.29$, and $F_{(4,10)} = 1.89$, $p = 0.19$ respectively). There was no significant difference in *ETR1* expression between the

C24 wild type and the *pls* mutant (in the C24 background), although both showed approximately five times the relative expression compared to the levels in Col-0.

eto1 showed no change in *ETR1* expression as ACC concentration increased (ANOVA, $F_{(3,8)} = 3.46$, $p = 0.07$), but then exhibited a huge reduction in *ETR1* expression after treatment with 10 µM ACC, with effects similar to Col-0 *ETR1* levels at the highest ACC concentration.

Untreated *etr1-1* gain-of-function mutant plants again had a similar level of *ETR1* expression to Col-0. After treatment with all ACC concentrations, the relative expression of *ETR1* increased four-fold, with some statistical difference found between the treatments (ANOVA, $F_{(3,8)} = 5.6$, $p = 0.035$), due to the higher expression in plants treated with 0.1 µM ACC.

These data suggest that the transcription of *ETR1* is not affected by the concentration of ethylene present, the level of ethylene responses in the plant, or by the expression of *PLS*.

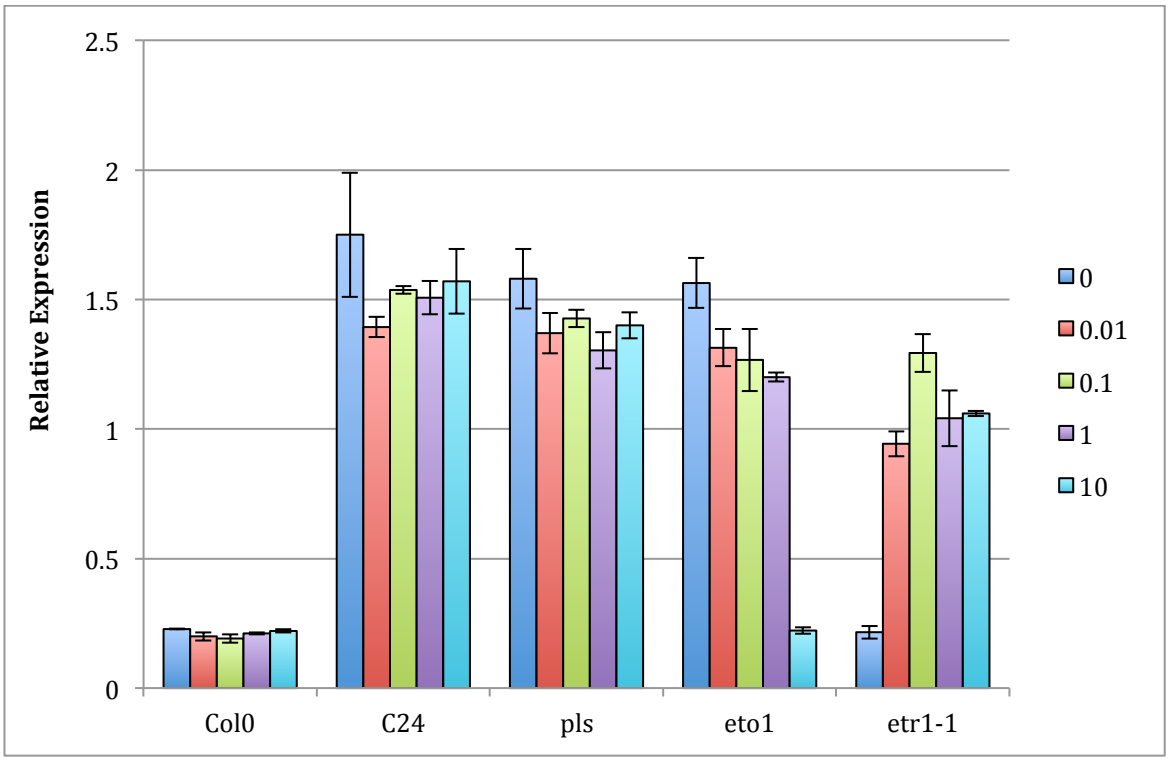


Figure 5-5. Relative expression of the *ETR1* ethylene receptor gene after ACC treatment in wild type and ethylene mutants. RNA was extracted from plants after treatment with 0, 0.01, 0.1, 1 or 10 µM ACC for seven days, total plant cDNA synthesised and used as templates in quantitative PCR, measuring the expression of the ethylene receptor *ETR1* gene with reference to the stable gene *PP2C*. Error bars are ±1 standard error.

5.3 PLS binding to ETR1

The physical interaction between the ETR1 receptor protein and the PLS peptide has been investigated previously by a GAL4 yeast two-hybrid (Y2H) assay and by Bimolecular Fluorescence Complementation (BiFC) in onion cells (Mehdi, 2009). The Y2H work suggested that PLS can physically interact with ETR1, with further evidence for the interaction in plant cells provided by the BiFC experiment, in which two halves of a yellow fluorescent protein (YFP) are fused separately to PLS and ETR1, producing a complete and fluorescing YFP protein when PLS and ETR1 proteins reside within 100 Å of each other (Hu et al., 2002; Kerppola, 2008).

This work suggested these two proteins have the capability to interact in yeast and onion cells. Further work was required to investigate this proposed interaction between PLS and ETR1 in *Arabidopsis.*

5.3.1 PLS and ETR1 localisation

Two fluorescent fusion constructs were created to investigate the subcellular localisation and potential colocalisation between PLS and ETR1 by CLSM on root tip cells and further microscopy techniques such as FRET-FLIM (Fluorescence Resonance Energy Transfer/Fluorescence Lifetime Imaging; (Wallrabe and Periasamy, 2005). FRET-FLIM uses the overlapping excitation spectra of two fluorophores (e.g. GFP and RFP) on potentially interacting partners. If the two fluorophores are within a few nanometers i.e. the two fusion proteins are close enough or interacting, excitation of one fluorophore (donor) will transfer energy to the other (acceptor), which results in the acceptor emitting a fluorescence photon and the fluorescence lifetime of the donor fluorophore molecule decreases. This technique was employed to distinguish between PLS and ETR1 simply existing in the same cellular membranes, and a real interaction between the two proteins.

The *pPLS::PLS:GFP* line, used extensively in Chapter 4, was transformed with a red fluorescent protein (*RFP*)-tagged *ETR1* construct to create *pPLS::PLS:GFP; p35S::ETR1:RFP* fluorescent plants.

Total plant RNA was isolated from Col-0 10 day old *Arabidopsis* seedlings. Total plant cDNA was synthesised according to the methods in section 2.4.2.3. The *ETR1* gene cDNA sequence was amplified by PCR from the total cDNA template, using primers that did not include the stop codon of the gene. The amplified *ETR1* PCR product was inserted into the

hygromycin-resistant pMDC83 Gateway expression vector (Appendix VI), which contains a double *p35S* promoter and a C-terminal RFP tag (Materials and Methods, 2.7, for vector creation). The expression vector was transformed into *pPLS::PLS:GFP* by floral dipping (Materials and Methods, 2.7.9).

Successful transformants were selectively grown on hygromycin agar plates, and the presence of the construct was confirmed by PCR on extracted genomic DNA (Figure 5-6, top; Materials and Methods, 2.4.1). The GFP fluorescence of the *pPLS::PLS:GFP* line could be detected by screening with an epifluorescence microscope in the T1 generation, but no RFP fluorescence could be detected in these positive transformants.

Further tests were carried out to understand whether the *ETR1:RFP* gene was present but not transcribed, or perhaps transcribed but failing to produce a fluorescently-active fusion protein. RNA was extracted from positive transformants and used as a template for total plant cDNA synthesis (Materials and Methods, 2.4.2). The cDNA was tested by PCR to see whether there was a *ETR1:RFP* gene transcript present in the plants (Materials and Methods, 2.5.2).

An *ETR1:RFP* DNA fragment was amplified by PCR from total plant cDNA, revealing all 3 transformed plants were expressing the *ETR1:RFP* RNA transcript (Figure 5-6, bottom). This suggests that there is an issue occurring after gene transcription, involving failed translation of the protein, or production of a non-functional, perhaps misfolded, fusion protein.

The fluorescent plant line was not tested further to investigate whether an ETR1-RFP fusion protein of the correct size was synthesised. The interaction between PLS and ETR1 was studied using alternative techniques.

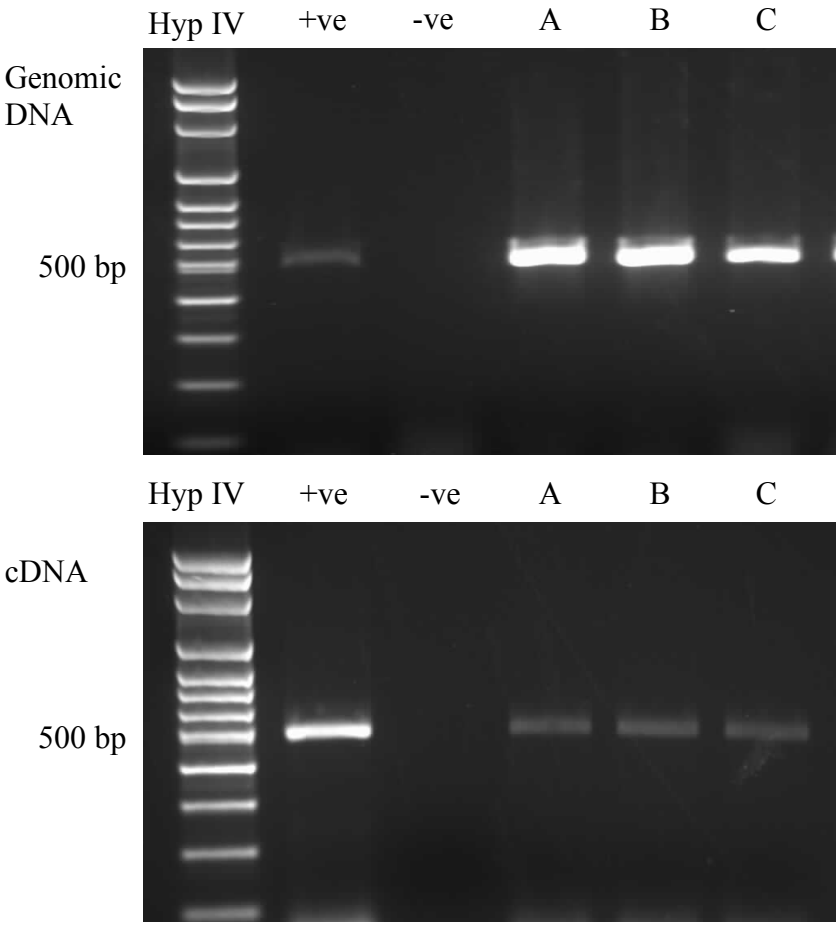


Figure 5-6. The presence of the *ETR1:RFP* construct is confirmed by PCR in genomic DNA and total plant cDNA. The construct was found in three individual positive transformants (A, B and C), with an expected PCR product size of 507 bp. The positive control reaction in lane 1 used the original *pMDC83::ETR1* purified plasmid as a template, the negative control reaction in lane 2 contained *pPLS::PLS:GFP* plant material not transformed with *ETR1:RFP*. The molecular weight marker was HyperLadder™ IV (Bioline), also known as HyperLadder™ 100bp.

5.3.2 Co-immunoprecipitation of PLS and ETR1 proteins

Previous work *in vitro* and *in vivo* (S. Medhi, 2009) suggested that the PLS and ETR1 proteins can interact. Transient expression of tagged *Arabidopsis* PLS and ETR1 proteins in *Nicotiana benthamiana* was employed as an alternative approach to fluorescence localisation studies, with the expressed proteins subsequently used for antibody-based co-immunoprecipitation (Co-IP) experiments.

The *PLS* gene was amplified by PCR without a stop codon, inserted into a Gateway entry vector, and introduced into the pEarleyGate103 expression vector containing a *p35S* promoter and a C-terminal GFP tag (Materials and Methods, 2.7.1 to 2.7.7; Appendix VI). *ETR1* cDNA was introduced into the pEarleyGate301 vector, containing the *p35S* promoter and a C-terminal HA tag sequence (Appendix VI): a general epitope tag with the amino acid sequence YPYDVPDYA.

The constructs were subsequently transformed into *Agrobacterium tumefaciens* (Materials and Methods, 2.7.8). The transformed *A. tumefaciens* cultures were supplied to Dr. Beatriz Orosa Puente, School of Biological and Biomedical Sciences, Durham University, who performed the infiltrations and Co-IP experiments in section 5.3.2, using the gene constructs I generated.

The *PLS:GFP* and *ETR1:HA* constructs, plus a GFP-only control under the expression of the *p35S* promoter, were infiltrated into *Nicotiana benthamiana* leaves (Materials and Methods, 2.9) for transient expression. After three days, leaf tissue was harvested and total protein extracted for use in the pull down assays. The resulting separated protein samples were analysed by SDS-PAGE gel electrophoresis, western blotting and antibody detection of the GFP and HA tags.

The Co-IP experiments revealed that the PLS peptide and ETR1 ethylene receptor protein interact in *N. benthamiana* leaves (Figure 5-7). Both proteins were detected during western blotting after pulling down with either anti-GFP beads (ChromoTek; PLS pulls down ETR1) or anti-HA beads (Miltenyi Biotec; ETR1 pulls down PLS). GFP-only controls did not show binding with ETR1, demonstrating the interaction is exclusively due to the presence of the PLS peptide.

The addition of 0.5 μM copper sulphate ($CuSO_4$) to the protein extract stabilised the PLS-ETR1 interaction. The presence of copper ions resulted in approximately twice the amount of PLS-GFP detected upon pull downs with ETR1-HA (Figure 5-7 A), compared to the

same assay in the presence of 2 mM EDTA, which can chelate metal ions (Ogino and Shimura, 1986). When ETR1-HA was pulled down by the PLS-GFP protein, the addition of copper ions produced up to 4 times the number of binding events (Figure 5-7 B).

Interestingly, the anti-GFP beads bound to two sizes of PLS-GFP protein (Figure 5-7 B),

both of which were larger than a GFP-only control, suggesting that the PLS peptide undergoes a cleavage event. When using ETR1-HA to pull down PLS-GFP, only the larger protein was present, suggesting that ETR1 binds the full length PLS peptide, but that PLS may be cleaved after ETR1 binding.

The sizes of the two PLS-GFP bands correspond to the predicated sizes of PLS-GFP protein sequences if the PLS peptide was cleaved at the previously-predicted cleavage site (Casson et al., 2002) at the arginine residues at amino acid positions 10-12 (refer to Figure 3-1 for PLS sequence). The full length PLS-GFP fusion protein has a predicted molecular weight of 34.4 kDa, whereas a partial PLS-GFP protein, cleaved as above, has a predicted molecular weight of 33.0 kDa. The GFP protein alone has a predicted molecular weight of 28.3 kD. These predicted sizes appear to roughly match the size of the proteins detected in Figure 5-7 B, suggesting that the PLS peptide may indeed be cleaved near amino acid positions 10-12.

To investigate the specificity of PLS binding, synthetic PLS peptide (Cambridge Research Biochemicals, Billingham) was introduced into the infiltrated *N. benthamiana* leaves six hours before the tissue was harvested. The addition of 25 nM synthetic PLS caused a ~40% reduction in PLS-GFP binding to ETR1-HA (Figure 5-8; using anti-HA beads), suggesting that the synthetic PLS peptide is competing for ETR1 binding, and showing the specificity of PLS for ETR1. This experiment is planned to be repeated, investigating whether the 5 nM peptide concentration has any effect on PLS-ETR1 binding, and aiming to ensure that the ETR1-HA protein is detected more clearly during western blotting.

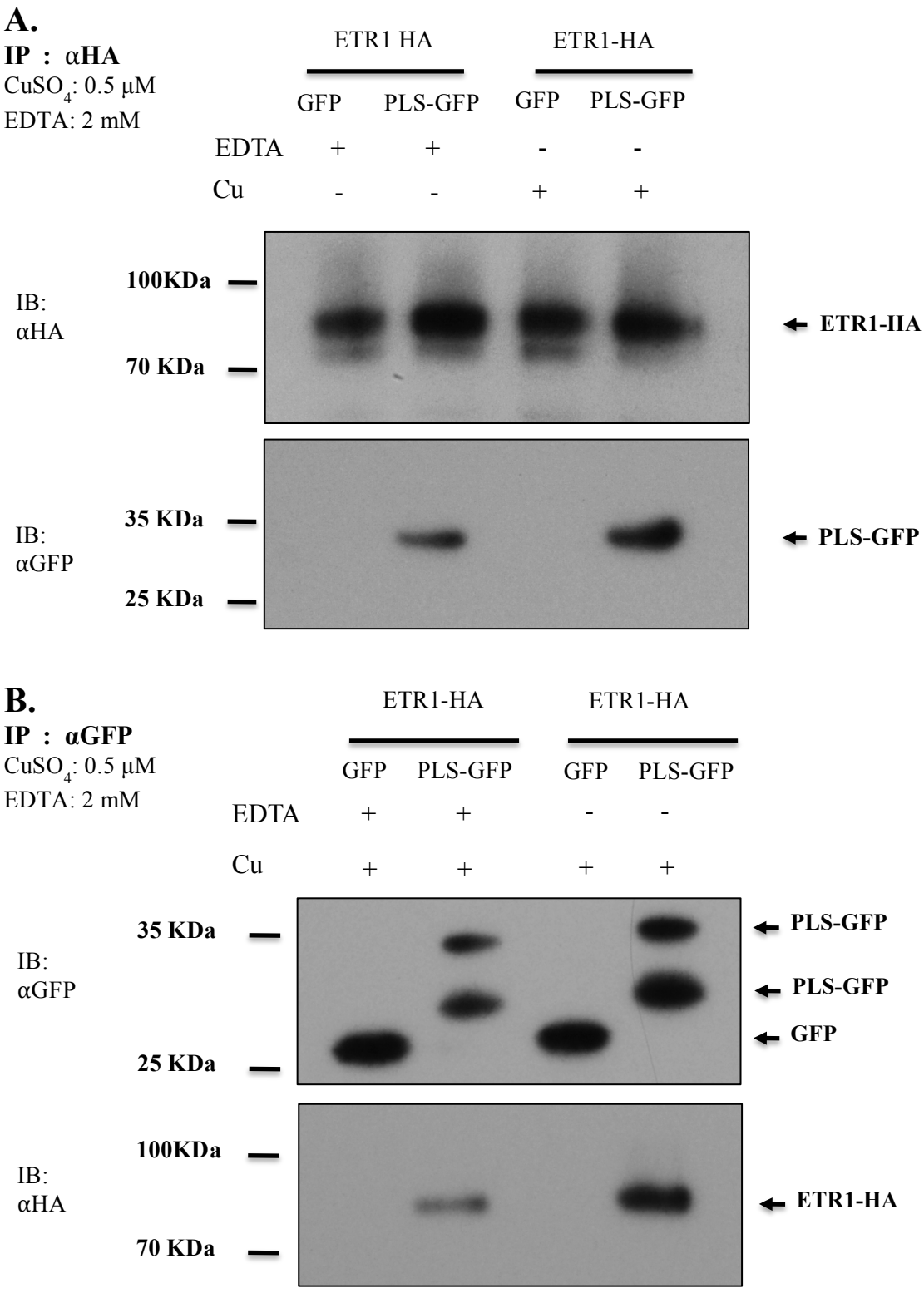


Figure 5-7. PLS and ETR1 interact *in vitro* and each can isolate the other in Co-IP assays. A. Immunoprecipitation of ETR1-HA with anti-HA beads pulls down the larger size of PLS-GFP protein, with double the interaction in the presence of copper. **B.** Anti-GFP beads immunoprecipitate two sizes of PLS-GFP protein, and the co-IP assay pulls down 4 times the amount of ETR1-HA in the presence of copper and absence of EDTA. Protein fusions were transiently expressed in *N. benthamiana* for three days before total protein was extracted as in section 2.9.3.

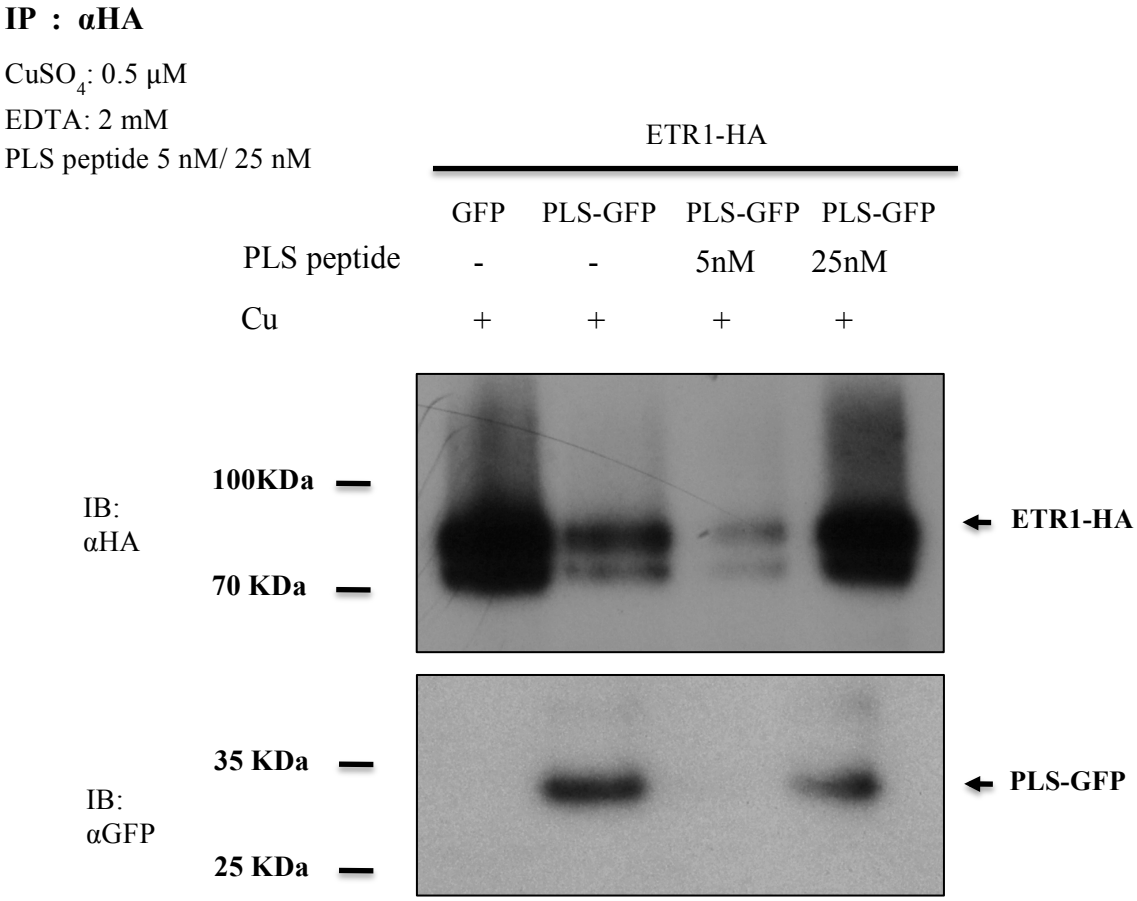


Figure 5-8. Synthetic PLS peptide successfully competes for ETR1 binding with the transiently expressed PLS-GFP protein. The addition of 25 nM synthetic PLS peptide into *N. benthamiana* leaves infiltrated with the *PLS:GFP* and *ETR1:HA* constructs causes a reduction in the amount of PLS-GFP protein pulled down by immunoprecipitation with ETR1-HA, suggesting that the additional peptide is competing for ETR1 binding with the transiently expressed PLS-GFP protein. Protein fusions were transiently expressed in *N. benthamiana* for three days, and the synthetic PLS peptide was introduced to the same leaves 30 minutes before total protein was extracted as in section 2.9.3, in the presence of copper ions to stabilise the PLS-ETR1 interaction.

5.4 PLS binding copper

Experimental work in Chapter 3 revealed that cysteine-containing full length synthetic PLS peptide and PLS(N1) truncated peptide can both increase the short-root *pls* mutant root length (section 3.4.2). Additionally, substitution of the two cysteine residues for serine residues in the full length PLS produced an inactive peptide that could not increase *pls* root length (section 3.4.4).

The cysteine residues, Cys-6 and Cys-17, are therefore presumed to have an important role in PLS peptide function. Proposed mechanisms for PLS function include the peptide interacting directly with ETR1; PLS performing redox reactions with ETR1 using the oxidation state of the sulphur atoms in the cysteine side chain; or possible peptide binding to another ligand, e.g. metal ions, via the cysteine thiol side chains.

The latter idea was investigated by testing whether the role of the PLS peptide involves copper ions. As early as 1965, it was thought that ethylene may bind a metal-containing receptor site (Burg and Burg, 1967). The ETR1 metal ion was shown to be copper by the addition of $CuSO_4$ to ETR1 receptor proteins expressed in yeast membranes, which resulted in increased ethylene-binding activity (Rodriguez et al., 1999). Furthermore, the presence of a copper transporter protein, RAN1, was found to be required for correct ethylene signalling (Hirayama et al., 1999).

Experiments were carried out to determine if the *pls* short-root phenotype can be rescued by the addition or depletion of copper, and whether the PLS peptide is capable of coordinating a copper ion.

5.4.1 Copper ions partly rescue *pls* short root phenotype

C24 wild type and *pls* mutant seedlings were grown for ten days in liquid ½ MS10 growth medium containing increasing concentrations of copper sulphate ($CuSO_4$; Materials and Methods, 2.12.2.2). Primary root length was measured on day ten using ImageJ.

Results of copper feeding experiments showed that the (usually short) *pls* mutant primary root becomes ~7 mm longer than the wild type root at $CuSO_4$ concentrations above 40 μM (Figure 5-9). $CuSO_4$ has an inhibitory effect on root growth in both genotypes after treatment with even the lowest 5 μM concentration, with primary root length decreasing to almost half that of the plants with no $CuSO_4$ treatment, but this effect was shown to be differential at higher concentrations. From 5 μM to 30 μM, both wild type and mutant root

lengths were found to remain between 34 and 44 mm. Mean root length of the C24 plants after treatment with 15 µM $CuSO_4$ was statistically greater than root length after treatment with 10, 25 and 30 µM, but not 5 or 20 µM $CuSO_4$ (ANOVA, $F_{(5,119)} = 7.59$, p = 3.21E-06, followed by Tukey's HSD test). The increasing $CuSO_4$ concentration between 5 and 30 µM produced no difference in the mean length of the *pls* roots (ANOVA, $F_{(5,115)} = 1.36$, p = 0.24).

At 40 and 45 µM, the primary root length of the wild type and mutant is further inhibited by the presence of $CuSO_4$, but the *pls* primary root is 30-50% longer than the wild type, suggesting the mutant responds to copper in a different way. 50 µM $CuSO_4$ was found to be so toxic to both plant lines that seed germination was inhibited.

Student's *t* test analysis revealed that all paired groups had statistically different mean root length values (at $p < 0.05$ threshold), except the 25 µM $CuSO_4$ treatment where p = 0.32. Thus the addition of copper does not simply rescue *pls* root length.

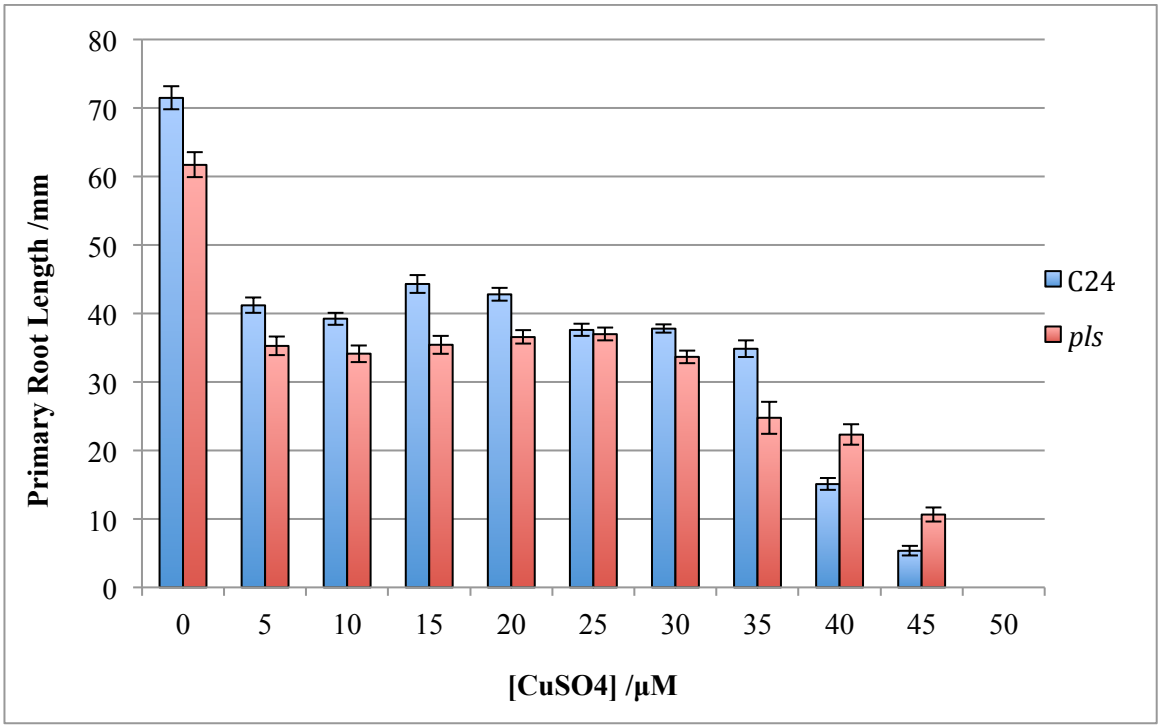


Figure 5-9. The *pls* mutant primary root becomes longer than the wild type upon treatment with copper ions at concentrations above 40 µM. Seedlings were grown for ten days in liquid ½ MS10 media supplemented with 1 mM $CuSO_4$ to create the final µM concentrations above. Root length was measured after 10 days using ImageJ. Error bars show ± 1 standard error.

5.4.2 Copper deficiency causes long *pls* root

$CuSO_4$ concentrations of 40 and 45 µM produced a *pls* mutant primary root which is longer than the wild type (Figure 5-9). A further experiment was carried out to examine the effect of reduced copper on both plant lines.

Wild type C24 and *pls* mutant seedlings were grown for ten days in liquid ½ MS10 media supplemented with the copper chelator bathocuproine disulfonic acid (BCS; (Sancenon et al., 2004) at final concentrations of 0, 10, 50, 100, 250 and 500 µM. The primary root length was measured as before (Materials and Methods, 2.12).

The lower concentrations of BCS in this assay (10 and 50 µM) cause an increase in both wild type and *pls* mutant primary root length (Figure 5-10). Interestingly, although the *pls* primary root conforms to the short-root phenotype in the absence of BCS (Student's *t* test, p = 9.0E-04, the *pls* root length is statistically the same as C24 after BCS treatments of 10 and 50 µM (T-test, p = 0.12 and 0.16 respectively).

After treatment with 100 µM BCS, the *pls* primary root is 50% longer than the C24 root, suggesting again that the *pls* mutant has an altered response to copper deficiency. At 250 and 500 µM BCS, the plants are negatively affected by the lack of copper with both seedlings' primary roots measured at 25-30% of the untreated root length. There is no statistical difference between the root lengths of the two plant lines after either treatment.

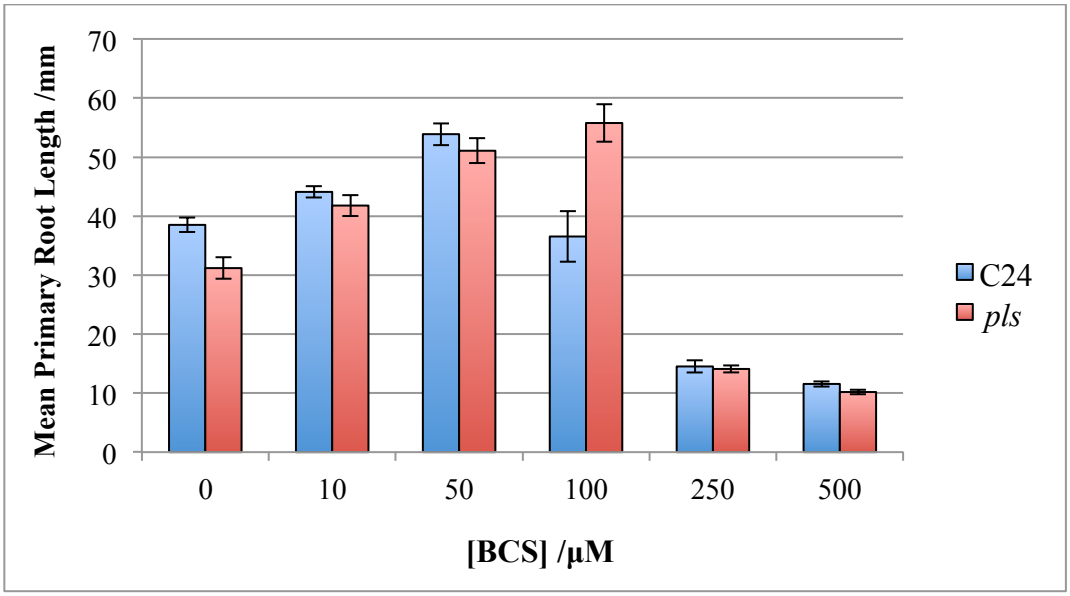


Figure 5-10. Absence of copper reverses the short root phenotype of the *pls* mutant. Seedlings were grown for ten days in liquid ½ MS10 media supplemented with the copper chelator BCS to final concentrations of 0, 10, 50, 100, 250 and 500 µM. Root length was measured after 10 days using ImageJ. Error bars show ± 1 standard error.

5.4.3 POLARIS peptide binds copper ions *in vitro*

The *pls* mutant clearly reacts differently to the addition or depletion of copper compared to the C24 wild type background, highlighting the significance of copper in the role of PLS *in planta*.

Full length synthetic PLS peptide (Cambridge Research Biochemicals) was used in experiments to determine whether PLS can bind a copper ion *in vitro* (Materials and Methods, 2.13). PLS peptide was supplied to Dr. Andrew Foster, School of Biological and Biomedical Sciences, Durham University, who performed the binding experiments in subsection 5.4.3.

Freeze dried PLS peptide was dissolved in 1 ml DMSO creating a final estimated concentration of 395 μM, determined from the absorbance at 280 nm and the PromParam estimated extinction coefficient of 2980 M^{-1} cm^{-1}. The copper ions can only interact with reduced thiol side chains, so the number of reduced thiol groups in the peptide solution was quantified by reaction with Ellman's reagent (DTNB; Materials and Methods, 2.13.1). The reduced thiol group concentration [SH] in the dissolved PLS stock solution was 573 μM. As there are two thiol groups in the peptide, one from each cysteine residue, this suggests the sample was 73% reduced.

The peptide stock was diluted in aqueous buffer to a final PLS concentration of 19.6 μM. The diluted solution was titrated with increasing amounts of reduced Cu^{+} ions and absorbance and fluorescence spectra were recorded after each addition of copper.

The addition of increasing concentrations of Cu^{+} to PLS produces higher intensity UV-vis absorbance at 242 nm, with an inflection observed after the addition of ~20 μM Cu^{+} (Figure 5-11 (A,B)). The fluorescence intensity decreases as the concentration of Cu^{+} is increased, with no further quenching observed by additions above 20 μM (Figure 5-11 (C,D)).

The concentration of the PLS peptide solution used in the binding assays was determined to be 19.6 μM. The inflection in the UV-vis data at ~20 μM and the absence of further quenching in the fluorescence data above 20 μM suggest that PLS binds Cu^{+} ions in a 1:1 stoichiometry.

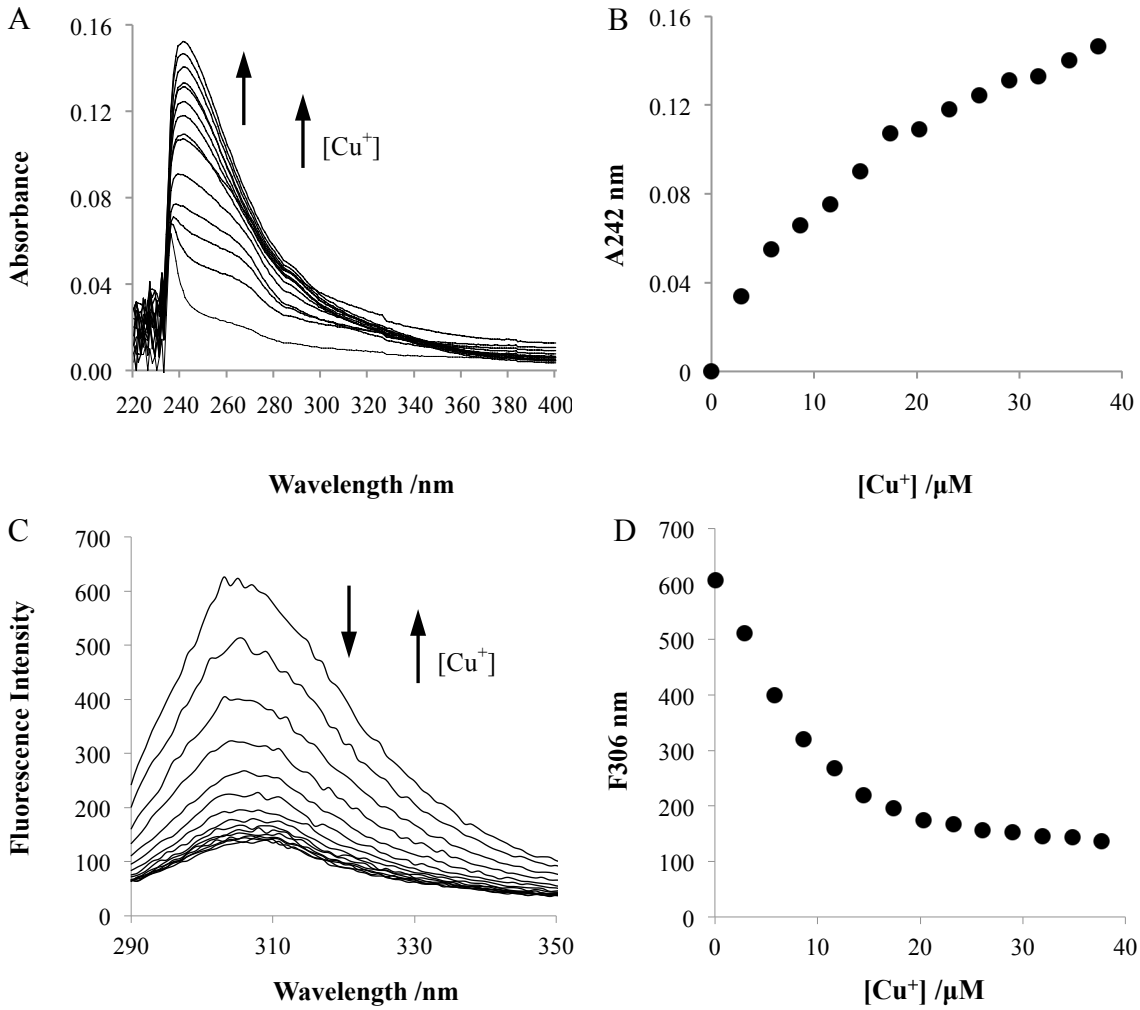


Figure 5-11. Spectral and fluorescence properties of POLARIS upon titration with Cu^+. Cu^+ was added cumulatively to a solution containing PLS peptide and UV-vis (A) and fluorescence (C) spectra were recorded. Arrows illustrate the spectral response to increasing Cu^+ concentration: UV-vis absorbance increases whereas fluorescence intensity decreases. Panels B and D show the intensity of the prominent feature in the UV-vis (242 nm) and fluorescence (306 nm) spectra respectively as [Cu^+] increases**.**

The affinity of the peptide for Cu^+ was tested by the addition of the Cu^+ chelators BCS (bathocuproine disulfonic acid) and BCA (bicinchoninic acid; (Smith et al., 1985). Both chelator molecules bind Cu^+ in a 2:1 (chelator: Cu^+) complex with binding constants of 6.01×10^{-19} M^{-2} and 1.58×10^{17} M^{-2} respectively.

BCS was titrated with Cu^+ in the presence of PLS but no difference was observed between this and the control experiment. This suggested that the PLS peptide was not competing for Cu^+ binding with the BCS chelator, i.e. PLS has a much weaker affinity for Cu^+. Next, PLS peptide was titrated with Cu^+ in the presence of 140 µM BCA (Figure 5-12).

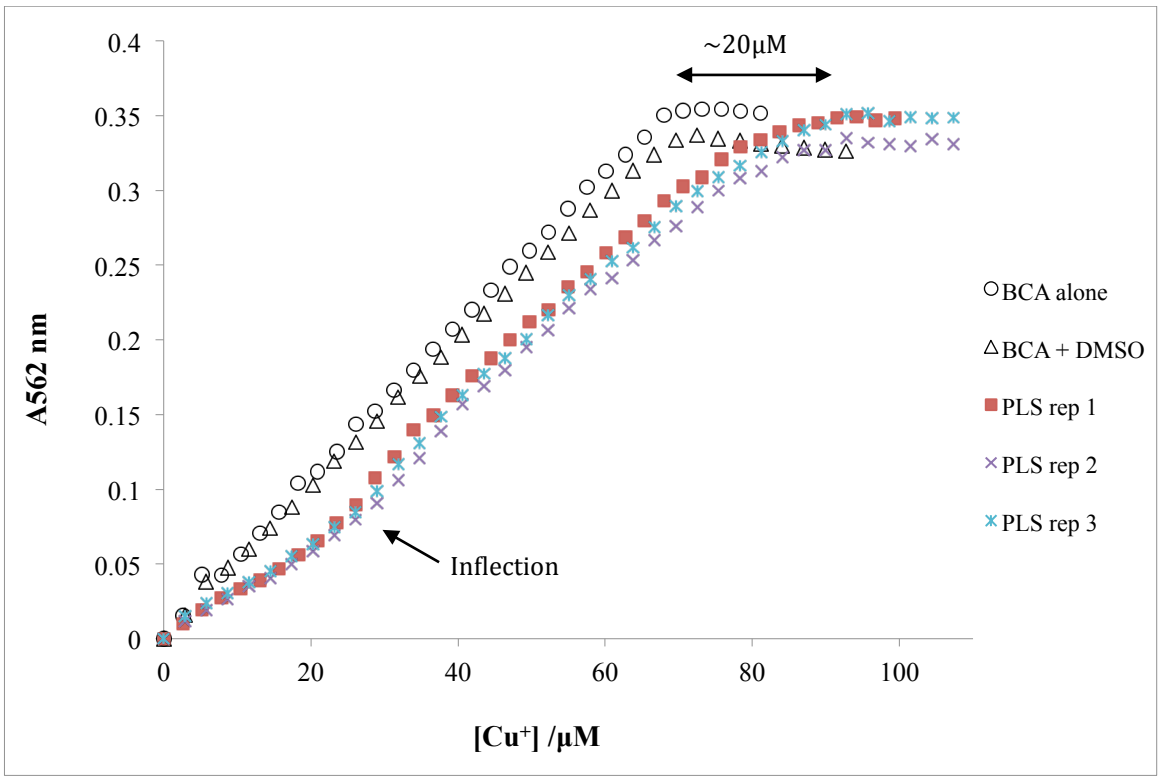


Figure 5-12. Competition between POLARIS and BCA for Cu^+. BCA (140 µM) was titrated with Cu^+ in the absence (open symbols) and presence (coloured symbols) of PLS (19.6 µM). Different symbol shapes represent different experiment replicates. The BCA alone titration was carried out in absence (circles) and presence (triangles) of DMSO (50 µl in 1 ml) to ensure the presence of DMSO doesn't interfere with PLS competitions.

The results are highly reproducible across the three distinct experimental repeats and the addition of DMSO does not interfere with the assay. The curve is shifted to the right in the presence of PLS peptide indicating that Cu^+ is withheld from BCA by PLS. When the PLS peptide is present, the concentration of Cu^+ required to saturate BCA is ~20 µM greater, and the initial gradient is shallower than in the control until a point of inflection (marked, Figure 5-12). These observations demonstrate that PLS is withholding Cu^+ from BCA and therefore possesses a substantial affinity for Cu^+, whilst providing further evidence for the 1:1 stoichiometry.

5.4.4 POLARIS peptide structure and function predictions

Five possible 3D structures of the POLARIS peptide were exhibited in Chapter 3 (3.2.1.1, page 72), generated by the RaptorX server for protein structure predictions (Peng and Xu, 2011b, a; Kaellberg et al., 2012; Ma et al., 2013).

In light of evidence outlined in this chapter that the PLS peptide can bind copper ions, and the *pls* mutant has altered responses to abundant or deficient copper, the five predicted PLS structures from Chapter 3 were analysed for the position of their cysteine residues (Figure 5-13) in order to identify which predicted peptide structures have a more likely pocket for binding copper ions.

The five structures are ranked from best (Figure 5-13(a)) to worst (Figure 5-13(e)) according to Table 3-1 (page 71). The same five predicted structures are displayed in Figure 5-13(f-j), with the position of the two cysteine residues displayed by space-filled molecular models on the linear backbone.

The most likely structures for the peptide are illustrated in panels f, g and i of Figure 5-13, due to the proximity of the cysteine residues to coordinate the copper ion. A disulphide bond length is about 2.05Å in length (Witt, 2008) and Cu^{+}-coordinating residues in the copper-binding protein Cox17 (Cytochrome *c* oxidase 17) in yeast and the Atx1 (Anti-oxidant1) family of copper chaperones bind Cu^{+} with the motifs C*XXX*C and C*XX*C respectively, with the linking amino acids forming a small loop between the Cys residues (Abajian et al., 2004). The two cysteine residues, Cys-6 and Cys-17, in the PLS peptide therefore need to be close enough to bind the copper ion. It is entirely possible however that the flexible loop region in PLS can undergo a conformational change during Cu^{+} binding, thus bringing the cysteine residues closer together.

Another relevant feature of Cox17 and the Atx1 chaperones is the presence of a region of positively-charged amino acids, including conserved arginine residues, which have been proposed to be involved in target recognition and docking via complementary electrostatic charges (Abajian et al., 2004). In all five models produced by the RaptorX server, the three arginine residues in the PLS peptide form an exposed loop between two beta-sheet regions, which could also be involved with target protein interactions.

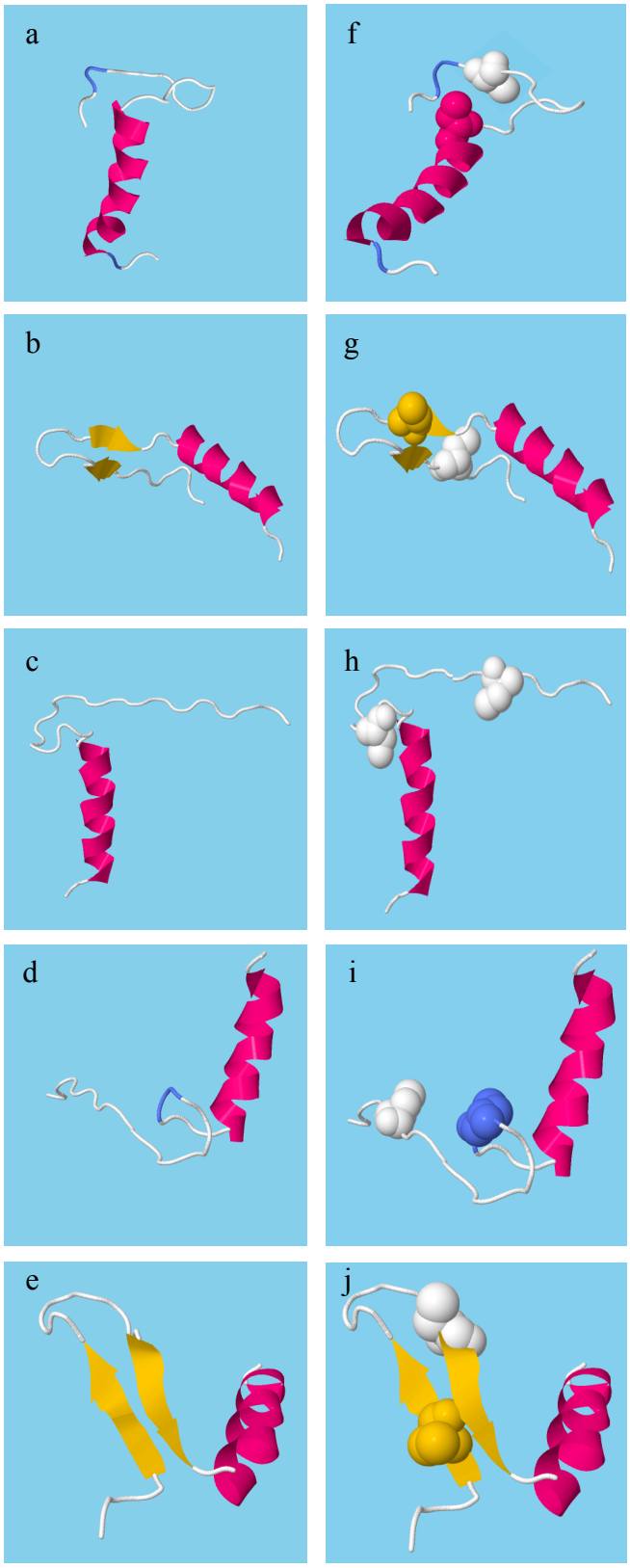


Figure 5-13. Predicted POLARIS peptide 3D structures (a-e) generated by the RaptorX server, ranked from best (a) to worst (e) quality. (f-j) The same PLS peptide 3D structures with cysteine residues highlighted (space-filled) for predicted copper ion binding likelihood.

5.5 Conclusion

This chapter aimed to elucidate the role of the PLS peptide in regulating ethylene signalling.

As observed before, increased ethylene levels resulted in the downregulation of *PLS* transcription in the Col-0 and C24 wild type plant backgrounds, an effect that was unchanged in the *etr1-1* ethylene-resistant mutant suggesting non-functional ETR1 protein does not affect the regulation of the *PLS* gene. Interestingly, the absence of the PLS peptide in the loss-of-function *pls* mutant seems to be causing an upregulation of *PLS* expression, perhaps an attempt to modulate ethylene signalling levels, in both the absence and presence of exogenous ethylene. This therefore provides evidence for a feedback loop to control *PLS* expression. The mutant plant line *eto1,* with a high level ethylene responses, showed no change in *PLS* expression after treatment with additional ethylene.

A proposed role for PLS in modulating the transcription of *ETR1* was rejected after observing that the expression of *ETR1* does not change in either C24 or *pls* upon the addition of ethylene.

The *pls* mutant was found to have altered responses in the presence or absence of copper ions. The mutant plants grew longer roots than the C24 wild type after treatment with high concentrations of $CuSO_4$ (40 and 45 μM). Somewhat unexpectedly therefore, the *pls* mutant also had longer roots than C24 after copper availability was reduced by the copper chelator BCS (100 μM), with the mutant plants growing the same length roots as C24 seedlings at lower concentrations of BCS.

The mechanism by which the POLARIS peptide acts upon the ethylene-signalling pathway has begun to be more fully understood, with the discoveries in this Chapter implying a novel, copper-related role for the peptide. PLS was found to possess a substantial affinity for Cu^+ ions, and appears to bind the ions in a 1:1 stoichiometry, with copper ions strengthening the interaction between PLS and ETR1 proteins. This discovery prompted the re-examination of the previously predicted 3D structures of the PLS peptide (Chapter 3) for the likelihood that their conformations may bind copper. Several of the models have a potential copper binding pocket, with a cysteine residue either side of a flexible turn region which could conceivably change conformation to coordinate copper ions.

Chapter 6 . Discussion

6.1 Introduction

This project aimed to investigate the POLARIS (PLS) peptide in *Arabidopsis thaliana* in order to gain a greater understanding of the role of PLS in ethylene signalling.

The *PLS* gene was discovered using a promoter trapping technique in *Arabidopsis* (Casson et al., 2002). The resulting *pls* mutant seedlings had altered embryo polarity and showed growth characteristics associated with defects in ethylene signalling, including a short, expanded root and hypocotyl, and an increased triple response. The *PLS* gene is necessary for correct plant responses to ethylene and auxin, among other phytohormones, and was previously found to be a point of crosstalk between hormone signalling pathways (Liu et al., 2010).

The *PLS* gene encodes a 36 amino acid peptide which negatively regulates the ethylene signalling pathway and there is evidence that it functions at the level of the ethylene receptors (Chilley et al., 2006; Mehdi, 2009). Although the *pls* mutation was partially complemented by the *PLS* cDNA (Casson et al., 2002), the peptide itself had not been purified, or able to be detected with N-terminal sequence-specific antibodies (P. Chilley; Mehdi, 2009).

This project focussed on the PLS peptide, with more detailed investigation into its structure and function relationships, subcellular localisation and the mechanism by which it regulates plant ethylene signalling. The current chapter will discuss the wider implications of the results presented in chapters 3-5.

6.2 PLS in other plant species

Ethylene is a key plant hormone and plays a role in the regulation of a wide range of plant processes, from developmental roles in tissue patterning and growth, to modulation of external stimuli, response to stresses and organ senescence (Abeles et al., 1992). Given that PLS appears to play a crucial role in ethylene signalling, homologues of *PLS* could be expected to be present in plant species beyond *Arabidopsis thaliana.*

A *PLS* gene, encoding a 22 amino acid peptide with 95% homology to the *Arabidopsis* PLS N-terminus, was identified in *Camelina sativa,* a relative of *Arabidopsis* in the same family, *Brassicaceae.* The *C. sativa* peptide is active in *Arabidopsis* plants, causing an increase in the length of the *pls* mutant primary root, implying PLS is involved in the regulation of root growth in *C. sativa*. PLS-like peptides may therefore have a similar function in other plant species.

A partial *PLS* homologue was also found in the genome of *Brassica rapa* subsp. pekinensis (chinese cabbage), within an open reading frame encoding for a larger, 50-residue protein. The *B. rapa* sequence has several conserved residues with the *Arabidopsis* PLS N-terminus and contains some similar residues within the PLS C-terminus, which might contribute to alpha helical structures.

A wider investigation into PLS homologues and orthologues did not reveal any similar proteins in other plant species beyond the *Brassicaceae* family. This could have been a result of searching for homologous proteins to PLS based on the proteins sharing significant sequence similarity, and thus inferring common ancestry and similar structure. The similarity search tool BLAST, used in Chapter 3, minimises false positives (non-homologues with significant scores; Type I errors) but does not mention false negatives (homologues with non-significant scores; Type II errors). It is also often easier to detect distant homologues when searching in a smaller database, rather than across sequences from a wide range of plant species. The bit-score, used in programmes like NCBI BLAST to infer significant homology between sequences, may have contributed to Type II errors when searching for proteins similar to PLS as the small size of the peptide might have resulted in similar sequences having bit-scores that were too low for the programme to consider them as homologous. In addition, BLAST calculates *local* sequence alignments that identify the most similar region between two proteins resulting in a failure to identify homologous regions found in different sequence contexts in different proteins (Pearson, 2013).

PLS homologues may still be present in other species. Ethylene receptors have been identified in a range of plants, including tomato (Klee and Tieman, 2002), rice (Cao et al., 2003), tobacco (Xie et al., 2002) and melon (Ma et al., 2006), plus a CTR1-like protein has been found in wheat (Bi et al., 2010). If PLS-like proteins act in ethylene signalling pathways beyond the *Brassicaceae* family, perhaps each protein is composed of very

different amino acids except for a few key residues required for an ethylene-regulatory function.

6.3 *PLS* is regulated by ethylene responses

Enhanced ethylene signalling in the *pls* mutant revealed that *PLS* negatively regulates ethylene signalling (Chilley et al., 2006). In this system, the presence of ethylene itself downregulates *PLS* transcription. Therefore, a reduction in PLS reduces its negative regulation on the ethylene signalling pathway, allowing ethylene downstream gene expression and the subsequent plant responses. However, if this system has downregulated *PLS* to the extent that ethylene signalling is stimulated, then a mechanism might exist which allows the plant to return ethylene responses to the original levels. We know that overexpression of *PLS* results in longer roots than the wild type plants (Chilley et al., 2006), so PLS does appear to be able to arrest ethylene responses.

Gene expression studies in this thesis confirmed that the presence of ethylene promotes downregulation of the *PLS* gene in wild type and *etr1-1* seedlings. However, even if the ethylene concentration has since declined, *PLS* expression may stay low which will cause the continuation of ethylene responses. There is therefore a requirement to upregulate *PLS* transcription to mediate signalling and return ethylene responses to their original levels.

One proposed, but ultimately disregarded, mechanism is that modulation of the transcription of the ethylene receptor gene *ETR1* may allow recovery of PLS concentration. The production of active ETR1 receptors would increase inhibition on the ethylene signalling pathway (Chen et al., 2005), thus decreasing ethylene signalling downstream responses and allowing upregulation of the *PLS* gene. However, it is already known that the ethylene receptor gene *ETR1* is not transcriptionally regulated by ethylene (Hua et al., 1998). In this thesis, the expression of *ETR1* was investigated in the context of *PLS* expression and ethylene responses (measured by the expression of the ethylene responsive gene *ERF1*), showing *ETR1* expression remains constant in all plant backgrounds, including the LOF *pls* mutant. This contradicts earlier studies that suggested *ETR1* is upregulated in the *pls* mutant (Mehdi, 2009), and implies that the PLS peptide does not regulate *ETR1* expression.

There appears to be a regulatory feedback mechanism to promote transcription of the *PLS* gene when ethylene signalling responses become high. In the *pls* mutant, the truncated *PLS* transcript (before the gene is interrupted by the T-DNA insertion) is upregulated proportionally to the amount of ethylene signalling, which contradicts the evidence for ethylene downregulating *PLS* expression, and suggests that more *PLS* transcript is produced, conceivably to negatively regulate the ethylene signalling pathway and return ethylene responses to normal levels. There may be a threshold level of ethylene signalling required to activate the positive regulation of *PLS* expression, as upregulated *PLS* was only observed in the *pls* and *eto1* mutants, both of which have increased ethylene-response phenotypes (Guzman and Ecker, 1990; Chilley et al., 2006).

It is accepted that the presence of the hormone ethylene causes a reduction in *PLS* gene expression. However, it has not been previously investigated whether the subsequent ethylene responses can also directly regulate *PLS* expression, in an auxin-independent manner.

The single loss-of-function *etr1-9* mutant harbours a loss-of-function *ETR1* ethylene receptor allele (Qu et al., 2007), producing an *ETR1* transcript which is interrupted by a T-DNA insertion in exon 4 and does not produce an ETR1 protein. The location of the T-DNA insertion would leave the ethylene-binding transmembrane N-terminus intact, but disrupt the C-terminal domain.

The resulting mutant shows ethylene responses similar to the wild type, although it is slightly hypersensitive to ethylene in the light, displaying marginally shorter hypocotyls than the wild type (Qu et al., 2007). In the dark, *etr1-9* has a triple response comparable to that of the wild type, in contrast with the gain-of-function ethylene insensitive *etr1-1* mutant, which shows an elongated hypocotyl and reduced triple response (Appendix IV).

The mild ethylene response phenotype is due to the functional redundancy of the ethylene receptor family, as the creation of plants with ethylene receptor LOF phenotypes requires three or more receptors to have a LOF mutation to avoid the wild type receptors compensating for the mutant proteins (Hua and Meyerowitz, 1998). Although the subfamily I receptors appear to have a dominant role in ethylene signalling, a loss of ETR1 can be mostly offset by the activity of ERS1 (Qu et al., 2007).

When *etr1*-9 is crossed with a *PLS* open reading frame fused to *GFP* DNA under the control of the *PLS* promoter, the expression of the PLS-GFP peptide is significantly

reduced, even in the absence of additional ethylene. If the ethylene signalling pathway functions almost as usual in *etr1-9,* then questions arise as to why *PLS* expression is downregulated to such an extent without the application of ethylene.

The *etr1-9* study suggests that *Arabidopsis* might regulate *PLS* expression directly by feedback from ethylene responses or via ETR1 itself. Although downstream ethylene responses appear normal in *etr1-9,* the pathway may be slightly altered by the absence of functional ETR1 protein. Qu et al. (2007) noted that the *etr-1-9* mutant is slightly hypersensitive to ethylene in the light, revealing that the other four ethylene receptor proteins may not be compensating for the lack of ETR1 as well as the relatively normal *etr1-9* phenotype might suggest. The modulated downstream ethylene responses, whilst perhaps not enough to promote ethylene-mediated root growth inhibition, may be directly affecting *PLS* expression.

It was speculated that the absence of ETR1 itself may be prompting downregulation of *PLS*. The truncated *ETR1* transcript is highly upregulated in *etr1-9,* but no truncated protein could be detected (Qu et al., 2007). As ETR1 is reported to be the dominant receptor, the upregulated *ETR1* transcript may be a feedback mechanism in an attempt to restore normal ethylene detection conditions. If *etr1-9* plants can detect the lack of ETR1, and disturbed ethylene signalling, then the downregulation of *PLS* would be a mechanism by which to relieve any inhibition on ethylene signalling. Furthermore, PLS has only been studied in relation to ETR1 and it is unknown whether it can perform its function on the other ethylene receptor proteins. Another protein which negatively regulates ETR1, RTE1, appears to exclusively associate with ETR1 (Rivarola et al., 2009), which could also be true in the case of PLS. The lack of ETR1 may therefore cause a downregulation of *PLS* because the plant doesn't require its role upon ETR1.

Interestingly, gene expression studies described in this thesis revealed that ethylene appears to downregulate *PLS* expression as normal in the gain-of-function *etr1-1* mutant. This suggests that the reduced capability of *etr1-1* to bind ethylene has no effect on *PLS* transcription and that ethylene binding to the other four receptor proteins is enough to reduce *PLS* expression. It can also be inferred that *PLS* regulation does not require functional ETR1 protein (although the *etr1-9* experiment might suggest that it does at least require the presence of ETR1).

6.4 *PLS* may be upregulated by auxin after ethylene downregulation, altering peptide location in the root

The inhibitory effect of ethylene on root growth requires local auxin biosynthesis in the root tip and auxin responses in the root elongation zone (Swarup et al., 2007; Stepanova et al., 2007). Ethylene also stimulates auxin transport away from the root tip by upregulating the PIN auxin-transport proteins (Ruzicka et al., 2007).

PLS expression is positively regulated by the hormone auxin (Chilley et al., 2006). Work in this thesis showed that ACC treatment causes a change in the location of the PLS peptide in the root tip, with higher ethylene concentration or extended exposure to ethylene causing the region in which PLS is expressed to move further away from the root, resulting in a more proximal localisation. Increased ethylene responses cause the upregulation of auxin reporter genes further away from the root tip, into the elongation zone, as auxin is transported away from the site of biosynthesis (Swarup et al., 2007).

Work in this thesis showed that the expression of the PLS peptide is reduced after treatment with ACC for short periods of time (2 hours), in addition to the downregulation of the *PLS* gene. After longer periods of ACC treatment (24 hours), the level of PLS peptide appeared less reduced. However, rather than the peptide simply being downregulated to a lesser extent, the levels of peptide after extended ACC treatment for 24 hours may actually be recovered slightly, compared to the PLS levels measured following short exposures to ACC. The aforementioned relocation of auxin may therefore start to upregulate PLS in a more proximal position after extended ethylene treatment.

The increased auxin levels in the root, induced by the rise in ethylene responses, may produce a positive feedback loop and upregulate *PLS* expression once the ethylene-mediated responses have occurred (via auxin). The newly synthesised PLS peptide would negatively regulate the ethylene signalling pathway to reduce ethylene responses until greater ethylene levels are detected.

Interestingly, PLS itself is required for ethylene-mediated auxin upregulation in the root tip, demonstrated by the failure of the *pls* mutant to exhibit ACC-mediated auxin synthesis in the root tip (Mehdi, 2009; Ruzicka et al., 2007; Swarup et al., 2007; Stepanova et al., 2007), demonstrating the complex relationship of PLS, ethylene and auxin.

In summary, the presence of ethylene causes downregulation of the *PLS* gene. Increased ethylene responses over a threshold, or after ethylene responsive processes have been

performed, appear to positively regulate *PLS* expression via the upregulation of auxin responsive genes. After long periods of ethylene exposure, PLS is upregulated again in a more proximal position, further from the root tip, corresponding with ethylene-induced auxin transport. In addition, the association of PLS and the ethylene receptor ETR1 appears to be able to regulate *PLS* expression in the case of defective production of ETR1, suggesting there are several elements to the regulation of the PLS peptide itself, which in turn negatively regulates ethylene signalling.

6.5 The POLARIS peptide N-terminus is functional

6.5.1 3D structure of PLS

Structural characteristics of areas of the putative PLS peptide were predicted from the amino acid sequence (Casson et al., 2002). Early studies suggested that the N-terminus of the peptide formed two beta-sheet structures separated by three arginine residues which may form a turn region and a potential site for cleavage. The C-terminus contained a repeated pattern of hydrophobic residues indicating the presence of an alpha-helix.

Structural domain predictions of PLS synthesised by current bioinformatics tools are consistent with these early predictions. Amino acids in the C-terminal 13 amino acids are predominantly hydrophobic and are likely to form an alpha-helix using repeated valine, leucine and phenylalanine residues. Investigations into the N-terminus of PLS confirmed the presence of two beta-sheets and a turn region.

The exact 3D structure of PLS is currently unknown, but bioinformatics tools were recruited to predict the folding of the backbone of secondary structures. The small PLS peptide is unlikely to form any complex tertiary protein structures but five potential 3D structures were produced, showing the relative spatial locations of the key secondary structure domains. Four of the five structures contained a pocket-like structure between the two beta-sheets, either side of a sharp turn formed by the three arginine residues. In all five models, the alpha-helical C-terminus is exposed, corroborating the hypothesis that it may be involved in protein-protein interactions, possibly via a putative ‘leucine zipper’ type motif formed by the periodic pattern of leucine residues (Landschulz et al., 1988; Casson et al., 2002)

Unfortunately, protein structure prediction software tends to base new predictions upon known crystal structures and associated sequences of other proteins. The mostly unique sequence of PLS resulted in low quality scores for the predicted models. Obtaining a crystal structure of the peptide would help to produce a conclusive three-dimensional structure for PLS.

6.5.2 The PLS N-terminus is required for function

The lack of success in isolating the small PLS peptide from plant material promoted the chemical synthesis and use of synthetic PLS peptide. The 36 amino acid backbone is short enough to be synthesised *in vitro* by solid phase peptide synthesis (SPPS), and a wholly synthetic system permitted the creation of truncated PLS domains (Figure 3-8, Chapter 3) to investigate key functional areas of the peptide sequence. *Arabidopsis* seeds were germinated and allowed to grow in hydroponic plant media, supplemented with freeze-dried peptide dissolved in DMSO (Matsuzaki et al., 2010). Fluorescently-tagged peptide molecules were detected within plant root cells, demonstrating uptake of the peptide from liquid growth medium.

Full length synthetic PLS peptide is functional in *Arabidopsis* seedlings, demonstrated by its ability to rescue the primary root length of the short-root *pls* mutant when introduced into seedlings via a hydroponic plant media system. Of the additional four peptide truncations tested, only the 22 amino acid N-terminus (PLS(N1)) demonstrated functional activity, although it could only partially rescue the root length compared to the full length peptide. This suggests key residues for function are located in the N-terminus, but that the whole 36 amino acid peptide is more active or more efficient than the N-terminus alone, suggesting that some key residues required for full function are located in the C-terminus.

It has been proposed that the PLS peptide may undergo proteolytic cleavage within the N-terminus, possibly at the site of the three consecutive arginine residues, and this may have contributed to the failure to detect the peptide using antibodies (which were designed to the N-terminal region; Casson et al., 2002; Mehdi, 2009).

Two truncations were synthesised that comprised the PLS sequence each side of the predicted cleavage site. Neither peptide demonstrated activity in *pls* seedlings, suggesting that the peptide may be regulated by cleavage into non-functional sections, or that the three arginine residues do not form a cleavage site.

Previous work observed that C24/*pls* heterozygous plants showed an intermediate root length between those of the parent lines (Casson et al., 2002; Figure 1-7 B, page 34), which indicated that the role of the PLS peptide may depend on its concentration. Synthetic full length PLS peptide affects primary root length in a dose-dependent way, with higher concentrations of PLS causing an increase in root length.

This strengthens the theory discussed in section 6.3, namely that PLS peptide expression may be regulated by a feedback mechanism resulting in variable regulation of the ethylene signalling pathway dependent on the concentration of PLS.

6.6 PLS acts at the endoplasmic reticulum to modulate ETR1

6.6.1 PLS is localised to the endoplasmic reticulum

The endoplasmic reticulum (ER) houses several components of the ethylene signalling pathway. All five *Arabidopsis* ethylene receptor proteins localise to the ER membrane, anchored by their three N-terminal transmembrane (TM) domains (Chen et al., 2002). Ethylene binding occurs within the hydrophobic TM domains (Rodriguez et al., 1999) in an ethylene/copper binding pocket formed by residues from the TM helices I and II (Wang et al., 2006). The membrane protein RTE1 resides in the ER membrane (Dong et al., 2008) and negatively regulates ethylene signalling, dependent on the ETR1 receptor (Zhou et al., 2007; Rivarola et al., 2009). Furthermore, the CTR1 protein is recruited to the ER membrane by its interaction with the cytoplasmic C-termini of the ethylene receptors (Gao et al., 2003), and the subsequent component in the pathway, EIN2, has been shown to be located in the ER membrane (Bisson et al., 2009).

The PLS peptide was observed to localise to membrane compartments in *Arabidopsis* root tip cells (Mehdi, 2009). Consistent with a role for PLS in the regulation of ethylene signalling, work in this thesis found that the PLS peptide is localised to the endoplasmic reticulum in *Arabidopsis* root cells.

Several of the ethylene signalling components have also been identified at the Golgi apparatus membrane. The ethylene receptors require a copper ion for functional ethylene binding (Rodriguez et al., 2009), which appears to be provided by the Golgi-localised P-type ATPase copper transporter RAN1 (Hirayama et al., 1999). The ETR1 receptor protein

was detected at the Golgi apparatus (Dong et al., 2008) where it is suggested that it receives its copper ion via RAN1 (Dunkley et al., 2006), before being transferred back to the ER to perform its ethylene detection function. However, it has been proposed that ETR1 could be localised differentially to the ER and Golgi organelles, depending on tissue type and developmental stage, to produce variable ethylene signalling responses (Dong et al., 2008). RTE1 has also been found at the Golgi apparatus (Dong et al., 2008) and there is some evidence that CTR1 can be recruited there too (Gao et al., 2003).

Despite the identification of many of the ethylene signalling components at the Golgi apparatus, the PLS peptide does not appear to be localised to the Golgi. This supports the idea that the receptors undergo final processing and receive their copper ion in the Golgi apparatus, via RAN1, and are then transported back to the ER, where PLS can act to negatively regulate ethylene signalling.

6.6.2 PLS interacts with the ethylene receptor ETR1

It has been acknowledged for a decade that the PLS peptide acts at the level of the ethylene receptor, and negatively regulates ethylene signalling (Chilley et al., 2006).

Subsequent work by S. Mehdi (2009) revealed that ETR1 and PLS proteins can interact *in vitro,* using the Yeast 2-Hybrid system (Figure 6-1), and *in vivo,* by bimolecular fluorescence complementation (BiFC) in onion peel cells (Figure 6-2). These assays revealed that the two proteins are able to reside in close enough contact so that the associated reporter molecules, for example the two halves of the split YFP protein in BiFC, are activated. However, it was still unconfirmed whether the two proteins merely associate with one another, or whether stronger PLS/ETR1 binding occurs. A key aspect of this project involved studying the suspected PLS/ETR1 interaction.

Co-immunoprecipitation experiments presented in this thesis showed that full length PLS peptide and the ETR1 receptor protein bind to each other. The PLS peptide appears to be cleaved as two sizes of PLS were detected, but only the larger of the two detected proteins (the full length PLS) was able to bind to ETR1. A cleavage site was proposed previously in PLS (Casson et al., 2002) at the site of the three arginine residues near the N-terminus. The sizes of the two PLS-GFP bands correspond to the predicated sizes of PLS-GFP protein sequences if the PLS peptide was cleaved at this site The full length PLS-GFP fusion protein has a predicted molecular weight of 34.4 kDa, whereas a partial PLS-GFP protein, cleaved as above, has a predicted molecular weight of 33.0 kDa. The GFP protein alone

has a predicted molecular weight of 28.3 kD. These predicted sizes appear to roughly match the size of the proteins detected in Figure 5-7 B, suggesting that the PLS peptide may indeed be cleaved at the three arginine resdiues. However, truncated versions of the PLS peptide (PLS(C2) and PLS(N2)) which mimic cleavage at this site were not functional when introduced into *Arabidopsis* plants (Figure 3-9). The peptide may therefore be cleaved at a different site, or the cleavage occurs after PLS has performed its regulatory function upon ethylene signalling to modulate root growth, revealing a mechanism by which the function of the PLS peptide itself may be regulated. The PLS peptide has been detected bound to the metallopeptidase Aminopeptidase M1 (APM1) (Angus Murphy and Wendy Peer, personal communication), a peptide isomerase enzyme which might be involved in processing of PLS.

There is evidence that other proteins in the ethylene signalling pathway are subjected to hormone-induced cleavage. The endoplasmic reticulum membrane protein EIN2, situated downstream of ETR1 and CTR1 in the ethylene signalling pathway, also undergoes a cleavage event in the presence of ethylene, producing a free C-terminal domain which is translocated to the nucleus, with the help of a nuclear localisation signal, where it acts to stabilise EIN3 and activate ethylene responses (Wen et al., 2012).

It is likely that the PLS peptide uses its alpha helical C-terminus to interact with a similarly hydrophobic site in the ETR1 protein, possibly within the hydrophobic N-terminus in the ER membrane. The repeating positively-charged lysine residues in the C-terminal helix could be involved in protein-protein interactions with complementary electrostatic charges, a feature conserved in *Arabidopsis* copper chaperone proteins (Abajian et al., 2004). Cleavage of the peptide could conceivably disrupt the PLS/ETR1 interaction and cause the removal of PLS.

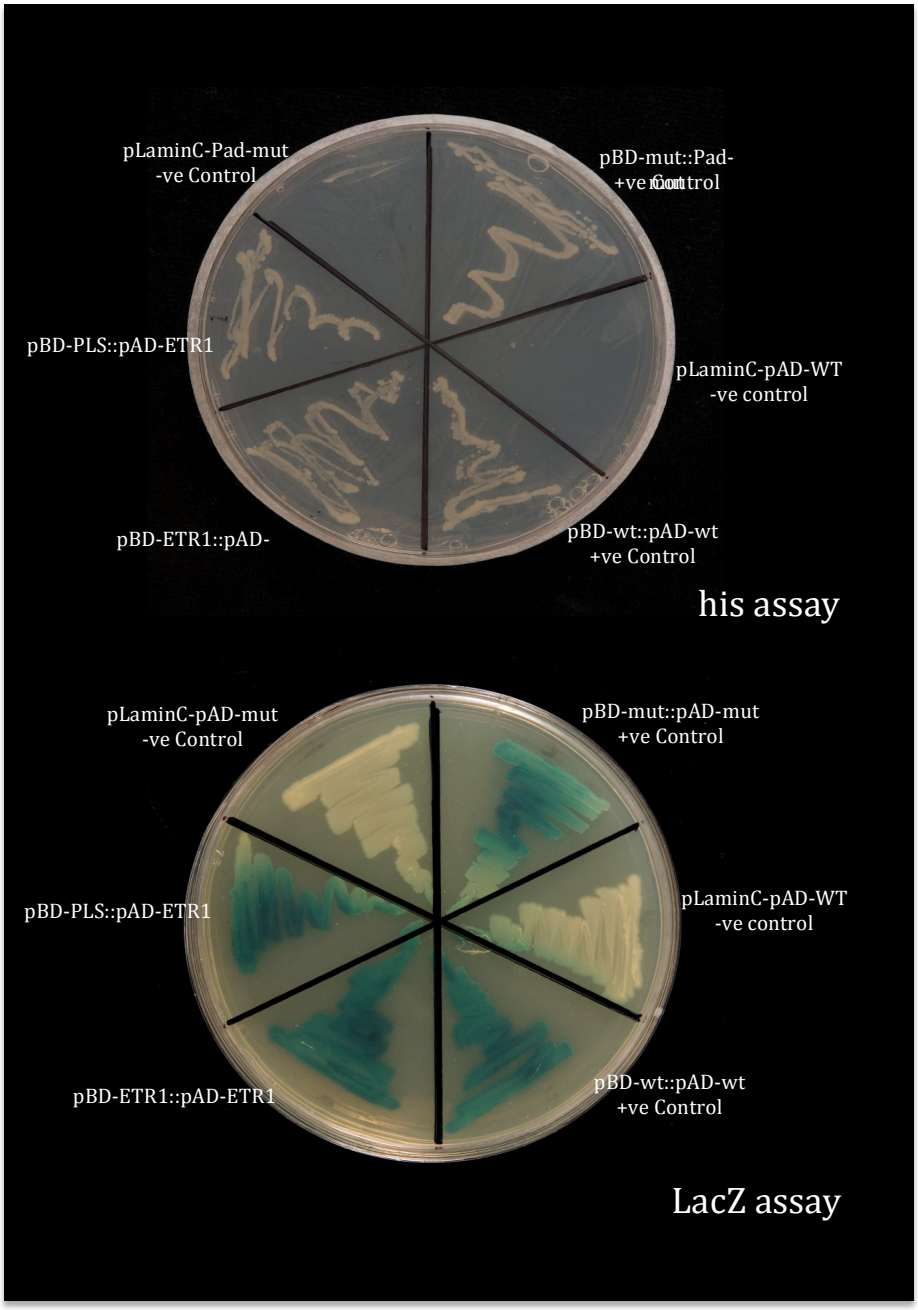


Figure 6-1. PLS interacts with ETR1 in yeast 2-hybrid assays (Mehdi, 2009). Gal4 two hybrid vector systems were used to detect an interaction between PLS (the 'bait') and ETR1 (the 'target') proteins, with the help of two reporter genes, β-galactosidase (*lacZ*) and the histidine synthesis gene *HIS3*. The two proteins are tagged with two separate domains of a transcriptional activator. The interaction between PLS and ETR1 reconstitutes the activator which causes expression of the reporter genes. Top: media lacks the amino acid histidine. Colonies can only grow due to the specific interaction between the bait and target proteins, resulting in expression of the *HIS3* gene, to compensate for the lack of histidine in the media. Bottom: media contains X-GAL, showing expression of the *lacZ* gene (blue colonies) due to the interaction between bait and target proteins.

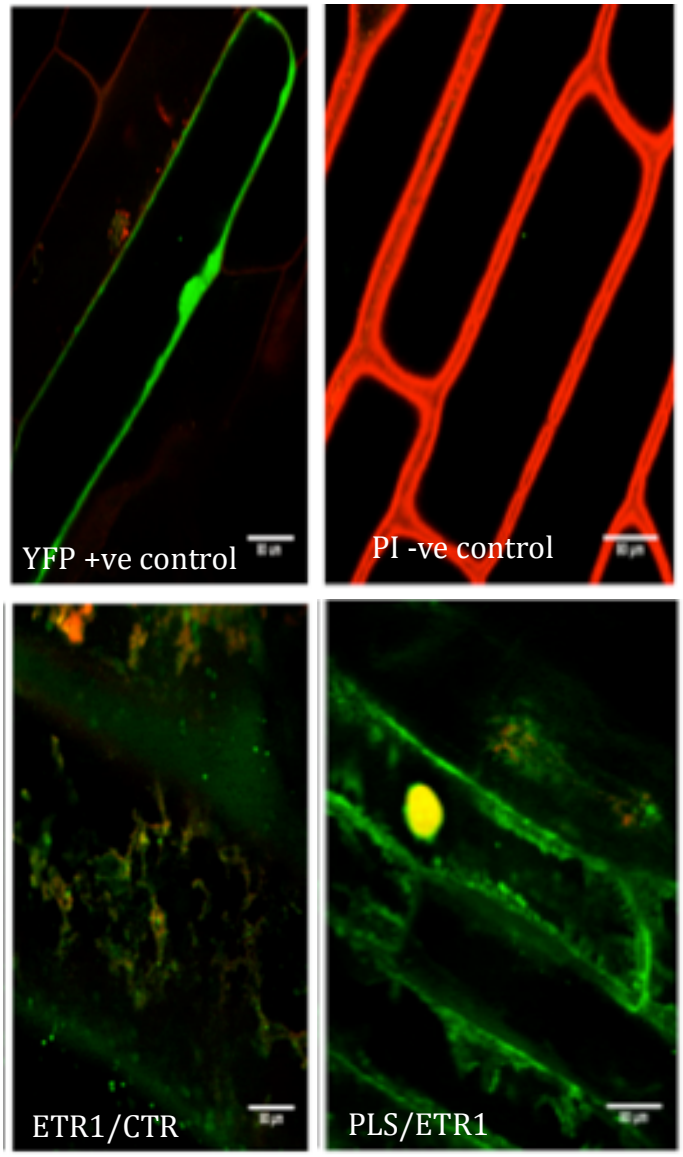


Figure 6-2. Bimolecular Fluorescence Complementation (BiFC) illustrated an interaction between PLS and ETR1 in onion peel cells (Mehdi, 2009). PLS and ETR1 were each tagged with one half of a YFP fluorescent protein. If the two proteins interact, the YFP domains become close enough to produce fluorescence (coloured green). The intact YFP positive control localises to cell membranes, the ETR1-CTR1 interaction is localised to membrane structures, predicted to be the endoplasmic reticulum (ER), and the PLS-ETR1 interaction also appeared to localise to membranous structures, with some localisation to the cell periphery.

6.7 The role of PLS involves copper ions

Copper (Cu) is a redox active transition metal and exists in two oxidation states in the cell environment, Cu^{2+} and Cu^{+}. Its redox potential makes copper biologically useful and it can therefore be used in electron transfer reactions (Pilon et al., 2006). Copper has a structural role in a number of metalloproteins and acts as a cofactor in proteins involved with electron transport in the chloroplasts (e.g. Cu/Zn-superoxide dismutase (Cu/ZnSOD)) and mitochondria (e.g. cytochrome *c* oxidase), among other oxidases. Copper is also required for cell wall metabolism, oxidative phosphorylation, and signalling to the cellular transcription and protein trafficking machinery (Yruela, 2009). Proteins which bind copper for transmembrane transport or delivery tend to limit copper ion binding to a single oxidation state (Wu et al., 2010).

Copper ions have been identified in having a crucial role in ethylene binding, with each ethylene receptor homodimer requiring one copper ion for correct function (Rodriguez et al., 1999; McDaniel and Binder, 2012). Furthermore, the Golgi apparatus-localised copper transporter RAN1 is required for ethylene binding activity (Woeste and Kieber, 2000; Dunkley et al., 2006), and is predicted to be involved with ethylene receptor biogenesis (Binder et al., 2010). The mechanism by which the copper ion cofactor is delivered from RAN1 to the ethylene receptor proteins is unknown. RAN1 has not been found in the ER membrane (Binder et al., 2010), but there is evidence of ethylene receptor proteins in the Golgi apparatus membrane (Dong et al., 2008), suggesting that the receptors may receive their copper ion in the Golgi apparatus before being trafficked back to the ER to perform their ethylene binding function. Alternatively, the copper delivery into the membrane compartments may not be tightly controlled, demonstrated by the rescue of the *ran1* mutant by flooding with excess copper ions (Woeste and Kieber, 2000).

Genetic analysis of mutations in the RAN1 copper transporter and the ethylene receptor ETR1 reveal that copper ions have more than one role in ethylene signalling. A lack of copper delivery in the strong *ran1-3* and *ran1-4* null alleles causes constitutive ethylene responses (Woeste and Kieber, 2000), producing a triple response phenotype similar to the *pls* mutant (Casson et al., 2002). These *ran1* mutants fail to bind ethylene due to the lack of copper available for the ethylene binding site (Binder et al., 2010). It has been shown that the decrease in ethylene binding in the *ran1-3* and *ran1-4* mutants is not due to reduced levels of the ETR1 protein (Binder et al., 2010), which corresponds to the stable levels of *ETR1* transcript in the *pls* mutant shown in this thesis, and regulation of the

ethylene receptor ETR1 is generally regarded to be independent from *ETR1* transcription or degradation (Hua et al., 1998). The weaker *ran1-1* and *ran1-2* alleles only show the triple response phenotype if they are grown in the presence of copper chelators (Binder et al., 2010). Therefore, defective RAN1 results in no copper delivery to wild type receptors, which causes constitutive downstream ethylene responses and a triple response phenotype.

However, the *ran1-3* and *ran1-4* mutants with constitutive ethylene responses are distinct from the ethylene insensitive *etr1-1* mutant. *etr1-1* harbours a mutation in a key cysteine residue, Cys-65, within the ETR1 ethylene binding domain which results in the *etr1-1* mutant receptor protein failing to bind copper and lacking saturable ethylene binding activity (Rodriguez et al., 1999). As they cannot coordinate copper, the *etr1-1* receptors maintain their inhibition on the ethylene signalling pathway (no ethylene responses) due to reduced ethylene binding, therefore displaying longer roots and a reduced triple response phenotype (Bleecker et al., 1988); the opposite phenotype to that of the strong *ran1* mutants.

Therefore, both the *etr1-1* mutant receptor and the strong *ran1* mutant alleles have reduced ethylene binding capability but the *etr1-1* mutation is distinct from the ethylene hypersensitive wild type receptors simply lacking copper due to defective delivery (in *ran1-3* and *ran1-4).* The *etr1-1* mutation is maintained in its 'active' state and constitutively inhibits downstream ethylene responses, whilst the *ran1* alleles cause constant ethylene signalling as ETR1 remains in the 'inactive' state, allowing ethylene responses.

Why then does the loss of ethylene binding affect the two receptor alleles in opposite ways? A possible answer is that copper has multiple roles in the regulation of ETR1 and ethylene signalling: it is crucial for binding ethylene molecules but it is also needed for the process of signal transduction by the receptor protein to regulate downstream ethylene responses.

In *etr1-1*, copper is available in the plant, but the mutated receptor protein cannot coordinate copper properly to facilitate ethylene binding, and therefore cannot become 'inactivated' by ethylene to promote downstream ethylene responses. Without copper in *ran1-3* and *ran1-4,* the receptor proteins appear to stay in their 'inactive'/ethylene-bound state, even when ethylene cannot bind, leading to strong ethylene responses. This suggests that, firstly, copper is required for ethylene binding to 'inactivate' the receptors, and then

subsequently, copper needs to be present for the receptors to be 'reset' back to their 'active' state, in which they activate CTR1 and block downstream responses.

The *pls* mutant has a similar phenotype to the strong *ran1* alleles, with constitutive ethylene responses (Chilley at al., 2006). The lack of the PLS peptide appears to affect the ETR1 protein in the same way as *ran1* mutant copper deficiency. Even with copper ion delivery occurring via RAN1, *pls* mutant plants have a constantly active ethylene signalling pathway.

This observation places PLS in two possible roles. If the constitutive ethylene responses in *pls* are due to a failure to 'reset' the ethylene receptor, as apparent in *ran1-3* and *ran1-4*, then PLS mediates receptor signal transduction. Alternatively, constitutive ethylene responses in *pls* could also be a result of continuous ethylene binding to the receptors because the PLS peptide is not present to negatively regulate receptor function at the ethylene binding domain.

Removing copper ions from both wild type C24 and *pls* mutant seedlings causes an apparent decrease in ethylene signalling, illustrated in this thesis by increased root length in both plant lines during copper depletion. This inhibition of ethylene signalling could be a result of a decrease in ethylene binding, due to reduced copper ion availability for the ethylene binding domain site; or alternatively the reduction in copper causes more receptor conversion from 'inactive' to the 'active' state, thus repressing ethylene responses. However, without copper in *ran1*, the receptors have a signal transduction defect, residing in the 'inactive' state and promoting ethylene signalling. In contrast, the reduction in ethylene signalling in depleted copper *pls* plants is likely attributed to a the failure to bind ethylene in reduced copper conditions, hence the seedlings show the same increased root length phenotype as *etr1-1*. The upstream ethylene binding effect appears to be dominant over any copper-mediated signal transduction effect, and so ethylene responses are limited due to a failure to bind ethylene.

At very low levels of copper, the *pls* mutant has longer roots than the wild type. In the absence of both PLS and copper combined, plants unsurprisingly continue to show reduced ethylene signalling as ethylene cannot bind without copper present. However, at the same depleted copper concentration, the wild type plants also exhibit a shorter root than *pls*. This may be due to a *ran1* mutant-type phenotype, where the absence of copper causes enhanced ethylene signalling, even in the presence of the PLS peptide. Possibly, at very

low copper levels, the limiting factor on ethylene signalling regulation switches from the ability to bind ethylene, to the ability to transduce the ethylene signal.

With excess copper, the plants seem to exhibit a toxic stress response. Seedlings dramatically decrease their biomass and root growth upon treatment with excess copper (Wang et al., 2015). Flooding the plant cells with copper can result in protein disruption as ions bind to proteins, and copper has the capacity to initiate oxidative damage, interfere with cellular processes such as photosynthesis and plasma membrane permeability, and causes severe inhibition of shoot and root growth (Yruela et al., 2009), reportedly by blocking the division of root meristem cells (Peto et al., 2011).

Having noted this, there is a small response of *pls* in the presence of high concentrations of excess copper, producing *pls* seedlings with longer roots than the wild type, suggesting reduced ethylene signalling (although many other growth and stress-related plant processes may be also affected by excess copper, resulting in the differential root growth). Addition of copper ions to the *ran1-3* mutant was able to partially suppress its strong constitutive ethylene responses (Woeste and Kieber, 2000). If the longer roots in *pls* can be attributed to reduced ethylene responses, this might suggest that adding extra copper when PLS is absent may bypass a need for both PLS and copper to negatively regulate ethylene receptor signal transduction, implying that the PLS peptide may usually be required to 'present' the copper ion for the regulation of receptor signalling.

It has been recently reported that a salicylic acid receptor in *Arabidopsis* also requires copper ions (Wu et al. 2012). The hormone salicylic acid (SA) is essential for plant immune responses and induces broad-system systemic acquired resistance (SAR) to pathogens through global transcriptional reprogramming (Fu et al., 2012). SAR in *Arabidopsis* requires the NPR1 SA receptor protein to activate SA-dependent defence genes. NPR1 binds SA via a copper ion, dependent on the presence of two crucial cysteine residues, abolishing an interaction between the N- and C-terminal domains of NPR1 and allowing the C-terminal 'transactivation' domain to regulate SAR gene transcription (Wu et al., 2012). The copper-dependent binding of SA to NPR1 has clear similarities to the coordination of ethylene with the copper ion in the ethylene binding pocket of the ethylene receptor ETR1 (Rodriguez et al., 1999), suggesting that copper may play a critical function in other hormone-receptor interactions and their signal transduction pathways.

6.8 PLS coordinates copper ions

Copper ions tend to associate with soft ligands; Cu^{2+} is often bound by nitrogen in histidine side chains whereas Cu^{+} preferentially binds to sulphur atoms in the side chains of methionine and cysteine residues (Crabtree, 1994). In biological systems, 35% of the copper-coordinating ligands are cysteine residues with many proteins losing the capacity to bind copper ions if the Cys residues are removed (Zheng et al., 2008; Wu et al., 2010). In the ethylene signalling pathway, a number of cysteine and histidine residues were identified to be crucial for copper coordination in the ETR1 ethylene binding domain (Rodriguez et al., 1999).

Like copper ions, the sulphur-containing thiol side chains of the amino acid cysteine can exist in oxidised and reduced states, and they have the capacity to form internal disulphide bridges for the folding and internal stability of proteins (Sevier and Kaiser, 2002), interact with other proteins, and coordinate metal ions (Crabtree, 1994).

The PLS peptide contains two cysteine residues, Cys-6 and Cys-17, which were originally predicted to form an internal disulphide bond to aid peptide folding, with a possible role in protein-protein interactions (Casson et al., 2002). Removal of both cysteine residues in the full length PLS peptide rendered the peptide inactive, demonstrated by its failure to rescue the short root phenotype of the *pls* mutant. The inactivity of PLS could still be a result of the disruption of any of the thiol side chain interactions mentioned above, including binding to the ETR1 protein via intermolecular disulphide bonds.

The presence of multiple potential metal ligands in the small PLS polypeptide led to investigations into the metal-binding capacity of PLS. As ETR1 requires a copper ion, studies concentrated on copper-specific binding. Protein interaction studies in this thesis revealed that the presence of a copper ion stabilises binding of PLS to ETR1, suggesting that PLS may be able bind copper ions.

Further investigation demonstrated that synthetic full length PLS peptide is capable of coordinating Cu^{+} ions in a 1:1 stoichiometry, conceivably by the thiol side chains from the Cys-6 and Cys-17, although the N-terminal methionine residue could also play a role. PLS may therefore be important for the integration of the copper ion into the ethylene/copper site of the receptor proteins, or for the copper-dependent receptor conformational changes. It is unconfirmed in which oxidation state the copper ion resides when acting as a cofactor in ETR1. Cu^{+} ions are transported into the cell by COPT (COPPER TRANSPORTER

PROTEIN) membrane transporters (Sancenon et al., 2003) and are delivered to RAN1 by copper chaperones (Himelblau et al., 1998), suggesting that Cu^{+} ions are more likely to act as the cofactor. However, some copper binding proteins can bind both Cu^{+} and Cu^{2+} (Loftin et al., 2005), and the capacity of PLS to bind Cu^{2+} ions is still to be tested.

6.9 PLS does not appear to deliver the copper ion to ETR1

It is apparent that PLS does not regulate the activity of ETR1 by delivering the copper cofactor into the ethylene binding domain. Although the mechanism of copper delivery from RAN1 to the ethylene receptor proteins is unknown, it is speculated that copper ions may be delivered by a copper chaperone mechanism, or simply diffuse into the active site of ETR1 (Binder et al., 2010).

It is known that the four members of the COPT transporter protein family reside in plant cell membranes and transport Cu^{+} ions into the cytosol (Sancenon et al., 2003). *Arabidopsis* COPT1 is highly expressed in root tips, and plants lacking functional COPT1 show a root elongation phenotype (Sancenon et al., 2004), similar to that observed in C24 and *pls* with forced copper deficiency. Copper is cytotoxic as it catalyses reactions which lead to increased oxidative stress (Yruela, 2009) and so delivery needs to be carefully controlled, with *Arabidopsis* copper chaperone proteins ATX1 (Anti-oxidant1) and CCH (Copper Chaperone(Himelblau et al., 1998) transporting copper from the influx transporters to organelle membrane transporters, such as RAN1 in the Golgi apparatus (Andres-Colas et al., 2006).

The mechanism on the other side of RAN1, by which ETR1 receives its copper ion, is unknown. Considering the tight control of copper delivery to avoid toxic effects, it seems plausible that RAN1 recruits other copper chaperones. However, it may be that once the copper ions enter the Golgi apparatus lumen, such tight control is no longer required and available copper ions are taken up by proteins which contain copper-coordination sites. Flooding *Arabidopsis* seedlings with copper appears to produce effects on root growth, which may support the latter mechanism. It was observed that the separate additions of excess copper ions and functional RAN1 protein could restore ETR1 function in yeast cells lacking the yeast copper transporter Ccc2, a homologue of RAN1. The fact that Ccc2 can deliver copper ions to the foreign ETR1 protein, which has no similarity to any native

yeast proteins, suggests that copper delivery to proteins in the secretory compartments is not strictly regulated (Binder et al., 2010) and may not require copper chaperone proteins.

Three additional copper chaperones were identified in *Arabidopsis* from their yeast homologues. Sco1p (synthesis of cytochrome *c* oxidase), Cox11p and Cox17p (cytochrome *c* oxidase-11 and -17) bind copper via thiol side chains and deliver copper ions for the formation of cytochrome *c* oxidase in the mitrochondrial membrane (Carr and Winge, 2003). Cox17 is a 69 residue, cysteine-rich chaperone and coordinates one copper ion, which it passes onto Sco1p and Cox11p by docking via a number of conserved, positively charged amino acids (Abajian et al., 2004).

ATX1, CCH, HCC1 (*Arabidopsis* homologue of Sco1p), Cox11p and Cox17p have all been shown to bind copper. However, none of these proteins share any nucleotide or amino acid residue sequence similarity with PLS, and PLS does not possess the conserved C*XX*C or C*XXX*C cysteine-containing copper binding motifs (Abajian et al., 2004). PLS seems unlikely therefore to act as a chaperone to mediate copper delivery into the ethylene binding domain.

Studies on the *ran1* copper transporter mutants provide further evidence that PLS does not have a role in delivering copper ions to the ETR1 N-terminus to facilitate ethylene binding. The weak *ran1-1* and *ran1-2* mutants are partially rescued by the addition of silver ions, suggesting RAN1 can also transport silver into the vicinity of the ethylene receptors (Binder et al., 2010). Silver can replace copper in the receptor ethylene binding pocket, but prevents the transmission of the signal onto downstream proteins (Binder, 2008). However, silver can still block ethylene responses in the *pls* mutant and rescue the ethylene response phenotype (Chilley et al., 2006), showing that PLS is not required in the delivery of silver into the ethylene receptor and suggesting that PLS might not be required for the delivery of metals ions into this domain. PLS may be a copper-specific chaperone, but it seems unlikely from the evidence discussed.

Furthermore, it is unknown how the copper ions are transferred from RAN1 transport through the Golgi membrane to the ethylene receptor binding sites. PLS is not localised to the Golgi apparatus, only the ER, so it is improbable that PLS acts as the missing link for copper delivery.

6.10 A model for PLS action

The work in this thesis, combined with previous studies on the *PLS* gene and PLS peptide, has given rise to a model for the function of the PLS peptide in negatively regulating ethylene signalling by modulating the availability of copper ions in the ethylene binding domain of ETR1, or by acting in tandem with copper to regulate the signalling state of the ethylene receptor ETR1.

In the absence of ethylene, the receptors rest in their 'active' state in which they negatively regulate the ethylene signalling pathway and repress the induction of ethylene responses (Chen et al., 2005). Ethylene binds to the receptor proteins, which subsequently induces their 'inactivation', thus relieving their inhibition on the pathway, promoting downstream ethylene responses (Hua and Meyerowitz, 1998; Bleecker, 1999). The proposed model of ethylene binding involves three states of receptor signalling (Figure 6-3).

In state 1, the receptor is actively inhibiting the signalling pathway and no ethylene is bound. Upon ethylene binding, the receptor enters the unstable intermediate state 2, in which the ethylene signalling pathway is still inhibited and receptor-CTR1 interactions are maintained. While ethylene is bound, the unstable receptor state 2 is in equilibrium with the more stable ethylene-bound state 3, in which the receptor is rendered inactive thus allowing downstream signalling and ethylene transcriptional responses to occur (Wang et al., 2006; Binder, 2008; Binder et al., 2010).

PLS negatively regulates the ethylene signalling pathway (Chilley et al., 2006). The PLS N-terminus containing Cys-6 and Cys-17 is required for peptide function, the peptide can bind Cu^{+} ions, and PLS interacts with the ethylene receptor ETR1 at the endoplasmic reticulum membrane, an interaction which is stabilised by copper. Although the PLS peptide had no copper-binding motif similarities with known *Arabidopsis* copper chaperone proteins, 3D structural predictions of the peptide revealed an N-terminal pocket-like structure with a Cys residue on either side, with a potential flexible loop region to accommodate copper ions. Two sizes of the PLS peptide have been detected, suggesting the peptide is cleaved, but only the larger peptide can bind ETR1, suggesting cleavage occurs after PLS has performed its role.

The evidence points towards PLS binding the copper ion in the transmembrane ethylene binding domain (EBD) in the ETR1 receptor protein (Figure 6-4). A copper ion is required for ethylene binding, so in the absence of ethylene, PLS may act to remove the copper ion

via coordination by its cysteine residues. The PLS-Cu complex could be routinely removed from ETR1 and degraded, accounting for the two sizes of PLS detected during protein studies, thus preventing ethylene binding and preserving ETR1 in signalling state 1, which prevents downstream ethylene responses.

PLS may not bind strongly to the ETR1 protein when a copper ion is absent. If copper ion delivery into the EBD is not tightly controlled, then the arrival of a copper ion may strengthen the interaction of PLS and ETR1, illustrated by the co-immunoprecipitation experiments, before PLS-Cu is removed.

The *PLS* gene and the resulting peptide concentration are downregulated by the presence of ethylene and ethylene responses. The absence of PLS preserves the copper ion in the EBD, supporting ethylene binding and promoting signal transduction from the ethylene receptor, which has entered signalling state 3 (Figure 6-5). This provides an explanation for the constitutive ethylene response phenotype observed in the *pls* loss-of-function mutant (Chilley et al., 2006). The transcriptional regulation of *PLS* reinforces the idea that the peptide is degraded; reducing the amount of *PLS* transcript would not have any effect on the PLS-mediated regulation of ethylene signalling unless the peptide itself was being removed from the system.

It must be noted that there is some evidence that PLS does not completely block ethylene signalling. Plants overexpressing the *PLS* gene have longer roots than the wild type plants, but root length can still be reduced by ACC treatments, suggesting the plants can still respond to ethylene (Chilley et al., 2006). It is feasible that the PLS peptide only acts upon ETR1, and so the other four ethylene receptors continue to respond to ACC as normal. Alternatively, PLS may not block ETR1 function completely, or ACC may have a second function that does not work via the receptors, possibly via auxin.

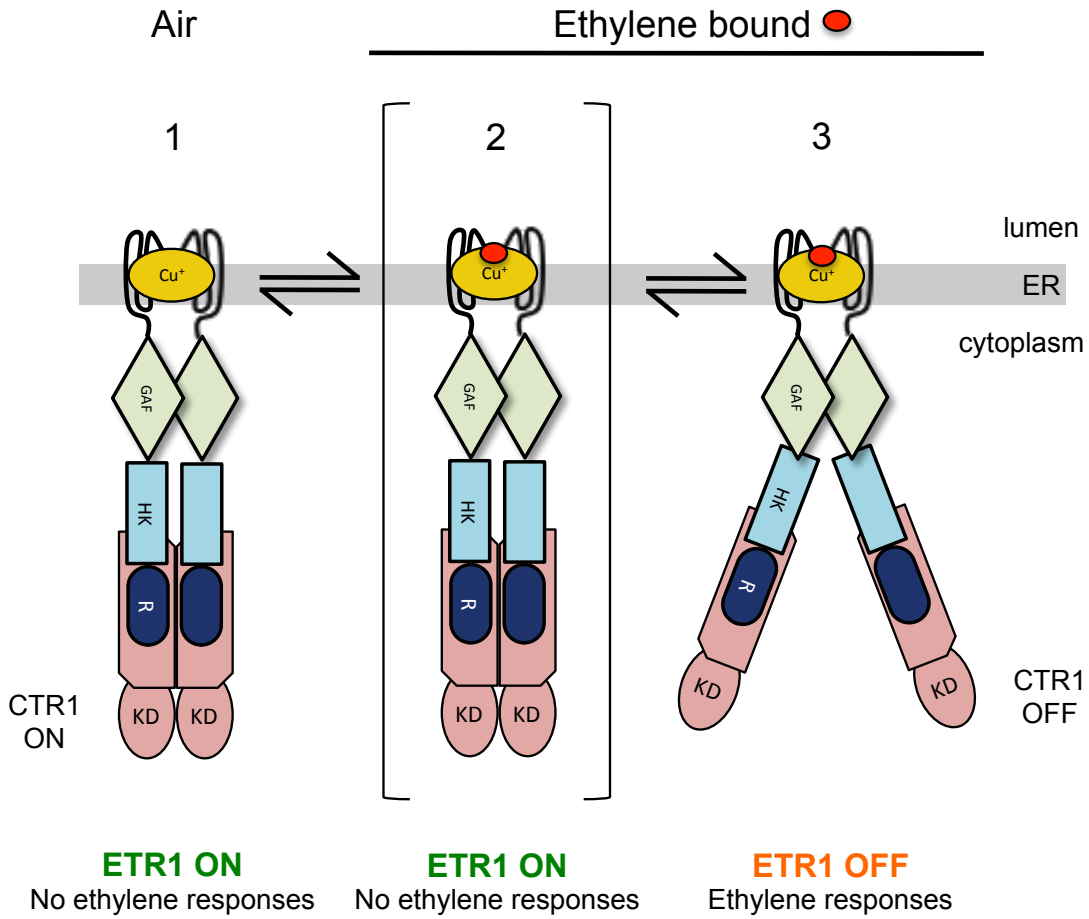


Figure 6-3. The three-state model for receptor signalling. The ethylene receptor ETR1 is shown as a homodimer anchored in the endoplasmic reticulum membrane by three transmembrane domains which constitute the ethylene and copper binding domain. The remainder of the receptor protein extends into the cytoplasm. In air, the ETR1 receptor is 'active/on' (state 1) and it interacts with the CTR1 protein via its cytoplasmic C-terminus, acting to inhibit ethylene responses. When ethylene binds, the receptor enters an unstable intermediate state (2) in which the receptor is still active and inhibits ethylene responses. State 2 is in equilibrium with state 3, in which ethylene binding causes inactivation of both ETR1 and CTR1 and allows the downstream ethylene signalling pathway to continue. Adapted from Wang et al., 2006; Resnick et al., 2008; Lacey and Binder, 2014.

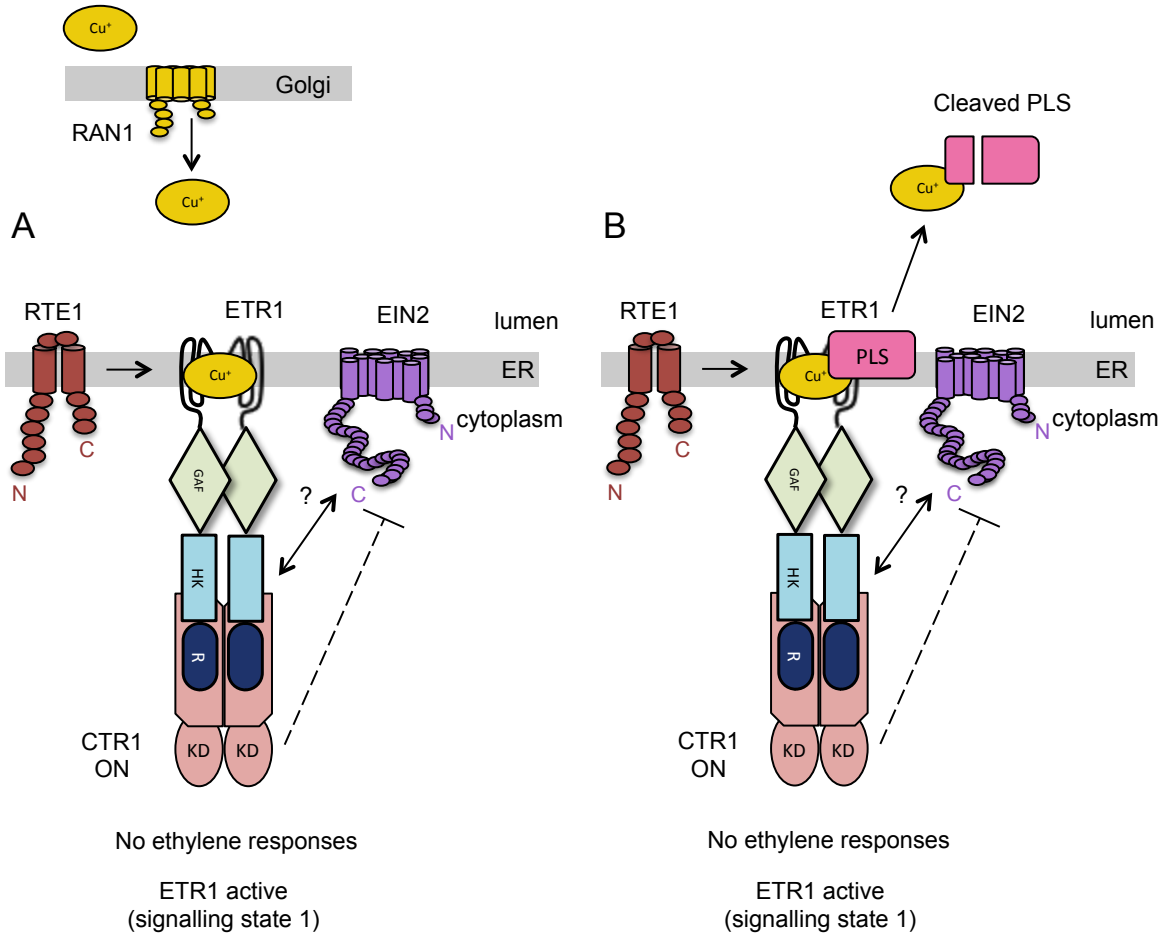


Figure 6-4. A model for the role of POLARIS in the ethylene binding domain whilst in the absence of ethylene. A. When ethylene is absent, the endoplasmic reticulum-located ethylene receptor ETR1 is active and maintains its inhibition on the ethylene signalling pathway by interacting with CTR1, which in turn inhibits the function of EIN2. In order to bind ethylene, ETR1 requires a copper ion in the ethylene binding site, which is transported over the Golgi apparatus membrane by RAN1. B. In this model, PLS negatively regulates ethylene responses by interacting with both ETR1 and the copper ion, and removing the copper ion from the ethylene binding site. This produces ETR1 receptors which cannot bind ethylene and maintain their inhibition on the signalling pathway. The PLS-Cu complex is degraded to release PLS from ETR1.

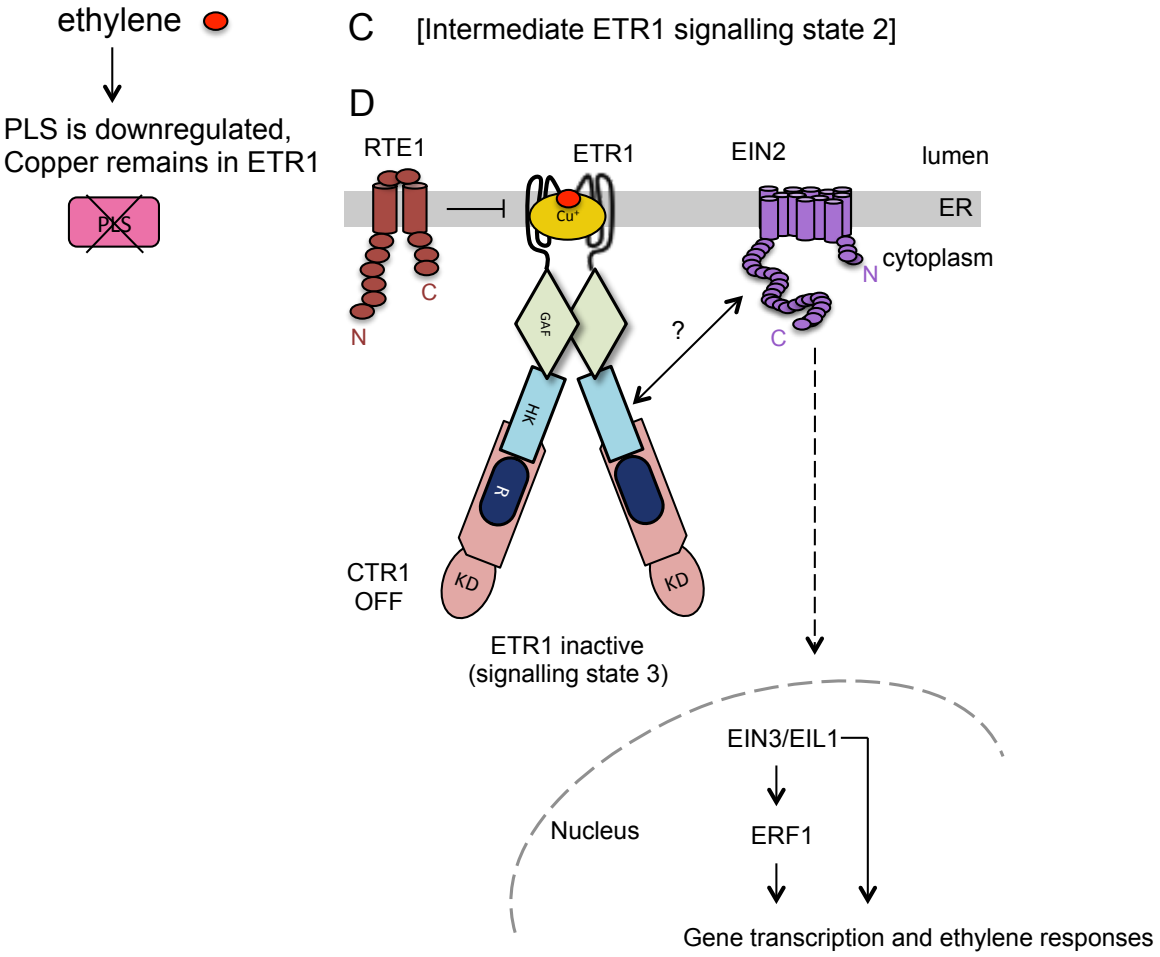


Figure 6-5. A model for the role of POLARIS in the ethylene binding domain in the presence of ethylene. The presence of the hormone ethylene reduces expression of PLS. Subsequent to the events outlined in Figure 6-2, the downregulation of PLS allows the copper ion to remain in the ethylene binding domain. The ethylene receptor binds ethylene and transitions to the inactive state 3 (D; refer to Figure 6-1), via the intermediate signalling state (C). The inactive receptor protein induces CTR1 to become inactive, relieving the inhibition of CTR1 on EIN2, and causing downstream ethylene-mediated transcriptional responses.

As demonstrated by the contrasting phenotypes exhibited by the *etr1-1* and *ran1-3* mutations, copper is also required to regulate receptor signal transduction, possibly by protein conformational changes. It has not been ruled out that PLS also has a role in the regulation of the signal transduction (Figure 6-6). The *pls* mutant has constitutive ethylene responses like the copper-deficient *ran1-3,* and the latter appears to fail to reset the receptors from state 3 to state 1 (Chilley et al., 2006; Binder et al., 2010). However, the activity of the RTE1 protein should be considered in the context of receptor signal transduction regulation.

RTE1 acts upon the ethylene receptor ETR1 in the endoplasmic reticulum membrane (Dong et al., 2008). It is proposed that RTE1, as a specific positive regulator of ETR1 but a negative regulator of ethylene signalling (like PLS), may promote ETR1 transition into state 1. Alternatively, it may inhibit ETR1 transition into state 3 (Resnick et al., 2008). It was thought that RTE1 may act as a copper chaperone, helping to deliver copper ions transported by RAN1, but genetic analysis revealed that RTE1 and RAN1 act in different pathways: *rte1* can suppress the receptor conformational change mutant *etr1-2,* but *ran1* cannot (Wang et al., 2006; Resnick et al., 2008). This suggests that RTE1 does not require copper from RAN1 for its function.

If RTE1 is a negative regulator of ethylene responses, which acts upon ETR1, it perhaps suggests that PLS does not also negatively regulate ethylene responses by mediating the receptor signalling state. It seems unlikely that both PLS and RTE1 act in the same way upon ETR1, corroborating the proposal that PLS regulates ETR1 activity at the ethylene binding level. Even so, the ethylene receptors have to detect ethylene over a wide range of concentrations and mediate differential signal outputs, depending on growth conditions and the plant tissue involved (Grefen et al., 2008), leading to the idea that the receptor family forms different higher order complexes (Chen et al., 2010).

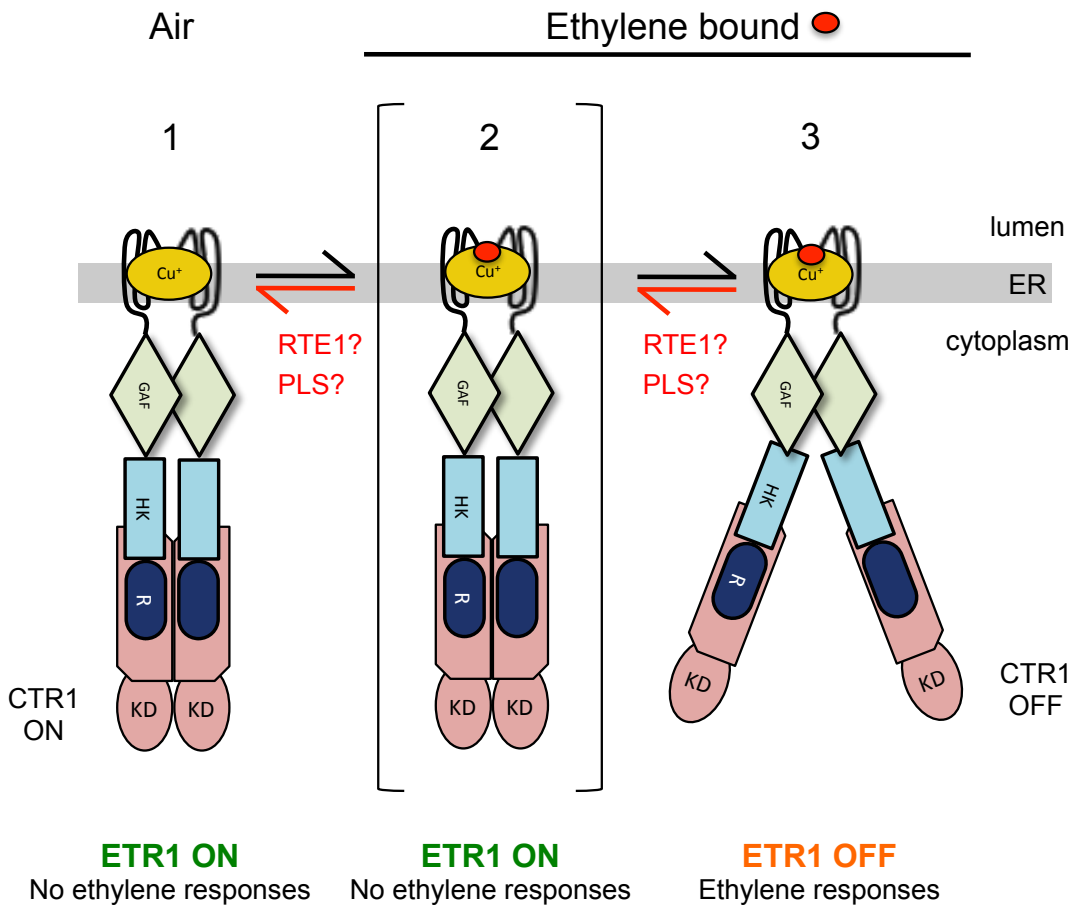


Figure 6-6. A model for the role of POLARIS in mediating receptor signal transduction. PLS and a copper cofactor act upon the inactive ETR1 protein (state 3) to promote the transition back into the active state (1), thus negatively regulating ethylene responses. In the presence of ethylene, PLS is downregulated which promotes receptor transition to state 3, thus allowing the continuation of the ethylene signalling pathway. The membrane protein RTE1 is proposed to have a similar role in the negative regulation of receptor signal transduction.

The low expression of *PLS* and perhaps a higher expression of *RTE1* could conceivably aid the regulation of ethylene signalling over various ethylene concentrations, providing sensitive control over receptor regulation. *RTE1* transcription is under the control of a feedback mechanism, with ethylene treatment causing *RTE1* transcript accumulation within 2.5 hours (Zhou et al., 2007). The recovery of *PLS* transcript levels occurs over a longer time frame, suggesting that regulation by PLS could follow the more immediate regulation of ethylene signalling by RTE1. RTE1 also appears to have a greater role in the

aerial regions of the plant (Zhou et al., 2007) compared to PLS, which appears to function predominantly in the *Arabidopsis* root (Casson et al., 2002).

Interestingly, the gene locus of *RTE1* on chromosome 2 has some similarity with that of the *PLS* gene on chromosome 4. Transcription of the *RTE1* gene produces mRNA transcripts of two lengths with the longer fragment coding for RTE1 (Zhou et al., 2007), similar to the two transcript lengths detected from the *PLS* gene locus (Casson et al., 2002). Perhaps there is a conserved regulatory function of both genes.

It must not be forgotten that PLS is a point of crosstalk between a number of plant hormones so the role of PLS is more complicated than solely negatively regulating the ethylene signalling pathway. PLS is required for ethylene-mediated auxin accumulation and synthesis in the root (Mehdi, 2009) with an increase in the rate of auxin biosynthesis predicted to be associated with a PLS-induced decrease in the concentration of the hormone cytokinin (Liu et al., 2010). The dependence of auxin concentration on ethylene signalling can be flexible, with increasing ethylene responses able to promote both increases and decreases in auxin concentration (Liu et al., 2010). In addition, it was proposed in this thesis that the ethylene-mediated auxin accumulation in the root elongation zone may upregulate the *PLS* gene in order to reinitiate its negative regulation on the ethylene signalling pathway.

PLS therefore has a complex part to play in the regulation of root growth, although the work in this thesis has gone some way to elucidate the role of the peptide in ethylene signalling.

6.11 Future perspectives

Although the work in this thesis has identified some key structural and functional characteristics of the PLS peptide, there is a need for further investigation into the mechanism of how PLS mediates ethylene signalling.

The discovery that PLS can bind copper ions and thus regulate ETR1 function is a big step forward in our understanding of the regulation of the ethylene signalling pathway, but the mode of PLS action has not yet been explicitly determined. The strong *ran1-3* allele has reduced ethylene binding capacity (Binder et al., 2010) and it would be useful to know whether the *pls* mutant shows a similar ethylene-binding defect. The creation of the double

mutants *pls/ran1-3, pls/rte1* and *pls/etr1-2* should reveal whether PLS has a role in the transduction of the ethylene receptor signal. Aside from the mutant phenotypes, further experiments using these double mutants, involving the addition or depletion of copper ions, may help to elucidate the relationship between PLS and copper with respect to ETR1. It would also be interesting to observe the response of the PLS overexpressing lines and C24/*pls* heterozygous plants to modulated copper concentrations. When performing copper feeding or copper chelation experiments, ethylene signalling could be quantified by qPCR (detecting a downstream gene like *ERF1)* to detect whether changes in morphology are due to altered ethylene signalling or simply stress responses.

Binder et al (2010) used a yeast (*S. cerevisiae*) cell system with a non-functional copper transporter RAN1 analogue to show that ETR1 recovers its ethylene binding capability in the presence of additional copper ions. The PLS peptide could be introduced into such a system for easy manipulation of the copper environment, to study the effects of PLS on ETR1 ethylene binding activity.

These experiments would provide information that could be fed into a mathematical model to explore the relationship between PLS, copper and ETR1, as performed previously with auxin, ethylene and cytokinin (Liu et al., 2010).

The cysteine residues were identified to be crucial for PLS function. The capability of the full length PLS peptide to bind Cu^{2+} ions needs to be investigated, and further characterisation of the synthetic PLS peptide mutant containing Cys to Ser substitutions will reveal whether the two cysteine residues do indeed coordinate the copper ion. More detail may be gleaned from further copper chelator competition assays, using the chelator at lower concentrations to emphasise the competition posed by PLS. Mathematical modelling could be used to describe the competition data and extract a Cu^{+} affinity for the PLS peptide, which would provide information on the copper binding reaction kinetics.

New amino acid substitutions in synthetic peptides will help to establish whether the three arginine residues are important for peptide docking to ETR1, and which residues are required for PLS cleavage. As the PLS peptide from *C. sativa* shows activity in *Arabidopsis* plants, it would be interesting to treat *Arabidopsis* seedlings with the full length or smaller domains of the 50-residue *B. rapa* PLS homologue identified in this thesis. The creation of a crystal structure for the PLS peptide would clarify the 3D structure, and if possible, a crystal structure for PLS bound to ETR1 would likely resolve

the mechanism of PLS action upon ETR1. This information may also allow for modelling of the PLS/ETR1 binding site and interaction.

Further immunoprecipitation experiments could provide information about the activity of the PLS N-terminus. Root length assays showed that the synthetic N-terminus containing two cysteine residues had some activity in *Arabidopsis* seedlings. A competition assay using this N-terminal peptide, analogous to the pull down experiment using full length synthetic PLS, could reveal whether this domain can compete with PLS-GFP for binding to ETR1. The creation of ETR1 proteins containing mutations in residues already considered important for receptor function and signal transmission (Wang et al., 2006) could be used for further immunoprecipitation experiments alongside the PLS-GFP protein. Careful experimental design may identify which ETR1 residues are required for PLS binding. Considering that work in this thesis implied that PLS may function alongside the other four members of the ethylene receptor family (when ETR1 is absent in the *etr1-9* mutant), pull down experiments should be performed with ERS1, EIN4, ETR2 and ERS2 proteins to investigate their relationships with the PLS peptide.

Additional techniques need to be employed to fully understand the localisation of both ETR1 and PLS. Fluorescence microscopy experiments were planned to investigate whether the subcellular localisation of the PLS peptide changes upon ethylene treatment. Unfortunately, because endogenous *PLS* expression is so low and ethylene downregulates *PLS* expression further, any fluorescent signal was undetectable. Secondly, fluorescently-tagged ETR1 protein also showed no detectable signal. This construct was under the control of the constitutive *p35S* promoter, so the lack of signal may have been due to transgene silencing. The addition of the silencing inhibitor gene p19 from the tomato bushy stunt virus (TBSV) may help to overcome this problem if the constructs were transiently expressed in *N. benthamiana* (as demonstrated in Grefen et al., 2008). Even when using this system, the group found that the *p35S*-powered tagged ETR1 protein was only present at wild type levels in the plant, revealing that *ETR1* RNA production is tightly controlled. Furthermore, the addition of large fluorescent tags at the C-terminus of the ETR1 protein may have impaired its interaction with CTR1 and the defective protein may have been destroyed.

If an effective fluorescently-tagged ethylene receptor construct could be made, modern fluorescent microscopy techniques, such as light sheet microscopy for large living samples (Ovecka et al., 2015), could record the interaction between ETR1 and PLS in real time

over hours or even days during growth of the *Arabidopsis* root. The interaction between ETR1 and PLS has now been observed in onion cells and *N. benthamiana* leaves, but the interaction is yet to be investigated within *Arabidopsis* itself.

As ethylene is such an important hormone in plant growth and development, it was surprising to discover *PLS* homologues in only a few relatives of *Arabidopsis.* It is possible that other plant species contain peptides or proteins which share only a few key residues with PLS, perhaps for copper binding, but which perform a similar regulatory role upon ethylene signalling. More comprehensive homologue searches or mutant screens could be undertaken and looking for key copper-binding ligands rather than concentrating on sequence alone may reveal PLS-like proteins.

6.12 Concluding remarks

The work in this thesis has established a novel mechanism by which the POLARIS peptide could regulate ethylene signalling, and subsequently root growth, in the model plant *Arabidopsis thaliana.*

It has been identified that the N-terminus of PLS is required for peptide function and that PLS resides at the endoplasmic reticulum in root cells, confirming previous localisation work. It has been confirmed that PLS does bind with the ethylene receptor ETR1, an interaction which is stabilised by the presence of copper ions, and that the peptide itself is capable of binding copper ions *in vitro.* These findings influenced the production of a new model, in which the PLS peptide negatively regulates ethylene receptor function by mediating the availability of copper ions to the receptor proteins, although there is scope for future work to investigate this interaction further.

Homologues of the PLS peptide are yet to be identified in plant species unrelated to *Arabidopsis thaliana.* However, the importance of PLS in ethylene signalling, as well as the function of the peptide as a point of crosstalk between other plant hormones, suggest that PLS-like proteins may be conserved across plant species to regulate root growth; a vital organ for plant survival.

Appendix I: Preliminary data for root length assay experiments

DMSO Assays

To encourage uptake of the synthetic PLS peptides by the root, cell membrane permeability was increased by including dimethyl sulfoxide (DMSO) in the plant media (Yu and Quinn, 1994). DMSO is also an excellent solvent in which to dissolve the PLS peptides. Preliminary assays with the growth media containing only DMSO were carried out to investigate whether DMSO affects root growth, and more importantly, maintains the mutant short-root length phenotype.

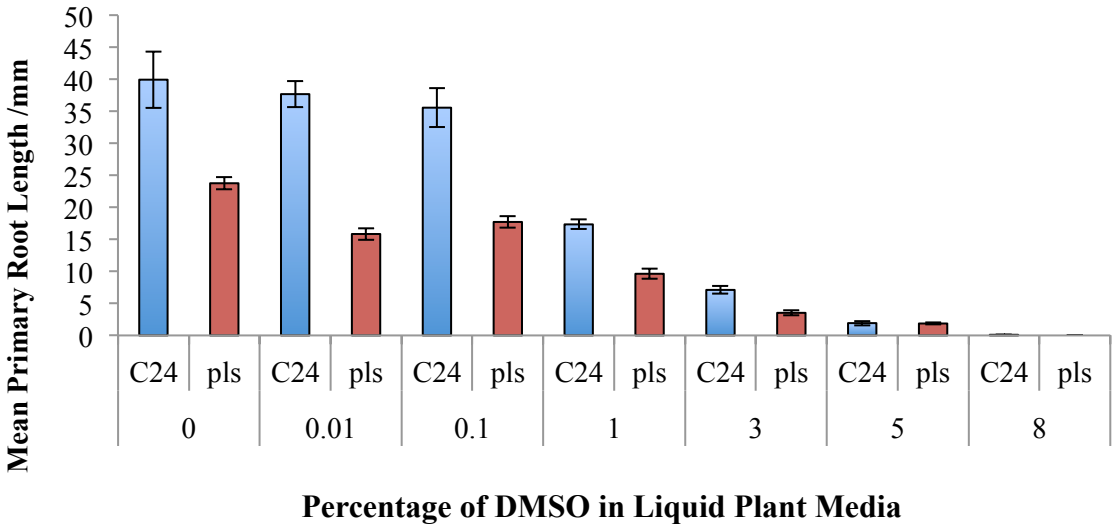


Figure I-1. C24 wildtype and *pls* mutant primary roots maintain their difference in length after treatment with </= 3% DMSO.

C24 wildtype and the *pls* mutant were grown for ten days in media containing a percentage of DMSO. A significant difference in primary root length continues to be observed up to and including 3% DMSO ($p = 8.286E^{-5}$). At 5% DMSO and above, the difference in root length is no longer significant (ANOVA, $p = 0.871$).

DMSO has an inhibitory effect on primary root length, but concentrations up to and including 3% DMSO by volume still show the *pls* mutant short-root phenotype, and can therefore be used in root length rescue assays. Further experiments were undertaken to show how the membrane permeability increases under treatment with DMSO. C24 and *pls* mutant seedlings were grown in media containing DMSO until seven d.a.g, root tips were removed, stained with a 1x solution of acridine orange/ethidium bromide and imaged.

Membrane damage as a result of DMSO treatment is observed by the increased access of ethidium bromide (light blue colour) into the root cells.

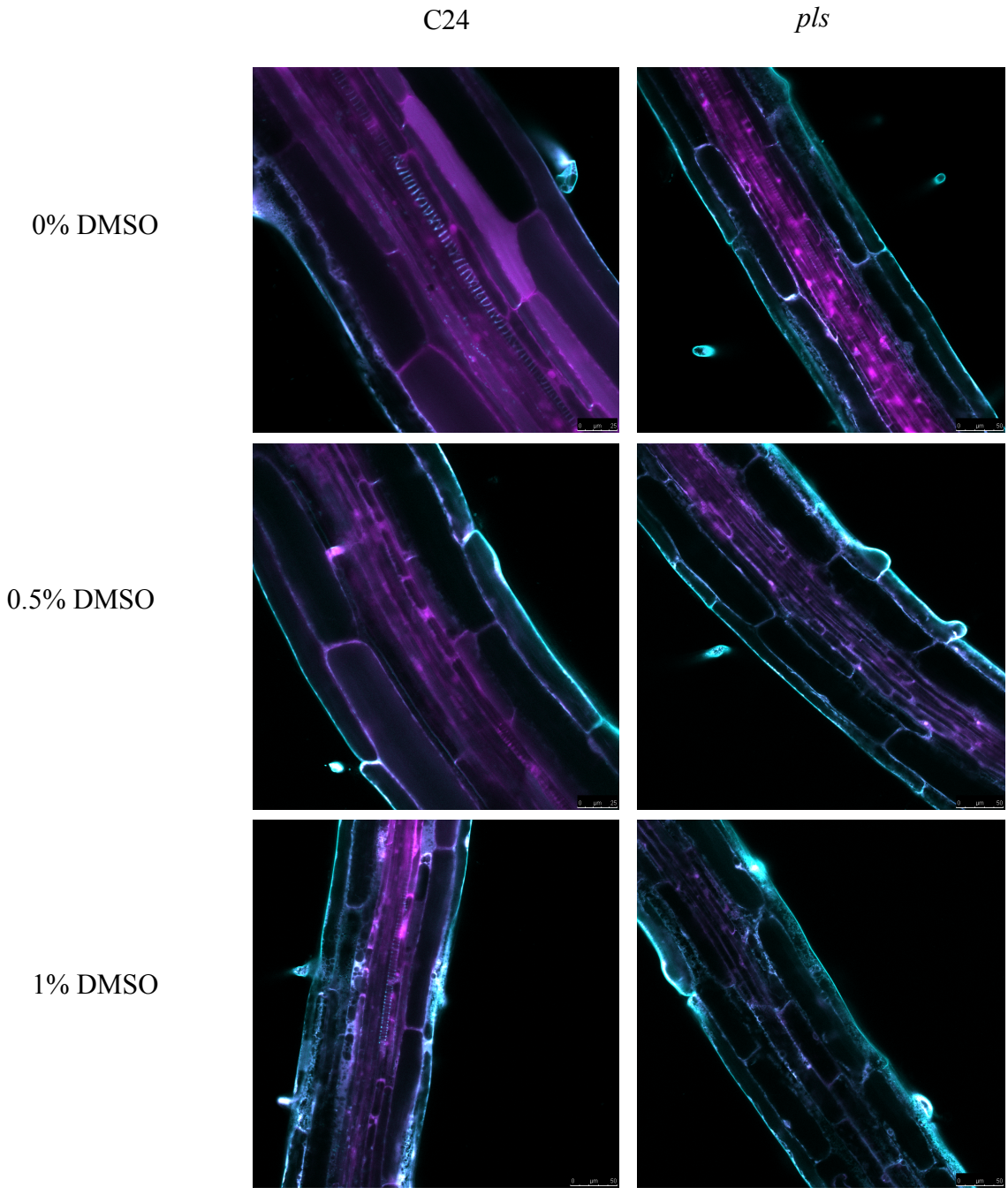


Figure I-2. DMSO treatment increases cell membrane permeability. Seedlings were treated with 0, 0.5 or 1% DMSO and stained with AO/EB. Healthy cell membranes are stained with acridine orange (purple), damaged membranes with ethidium bromide (light blue; Materials and Methods, 2.10.2.3.

Evidence to show liquid growth method does not interfere with growth

The easiest method for applying the synthetic peptide to plant roots is to supplement the plant growth media with peptide, with liquid 1/2MS10 media providing the best system for easy peptide uptake. Preliminary experiments were undertaken to check that the *pls* short-root length phenotype, compared to that of C24, can still be observed in such conditions.

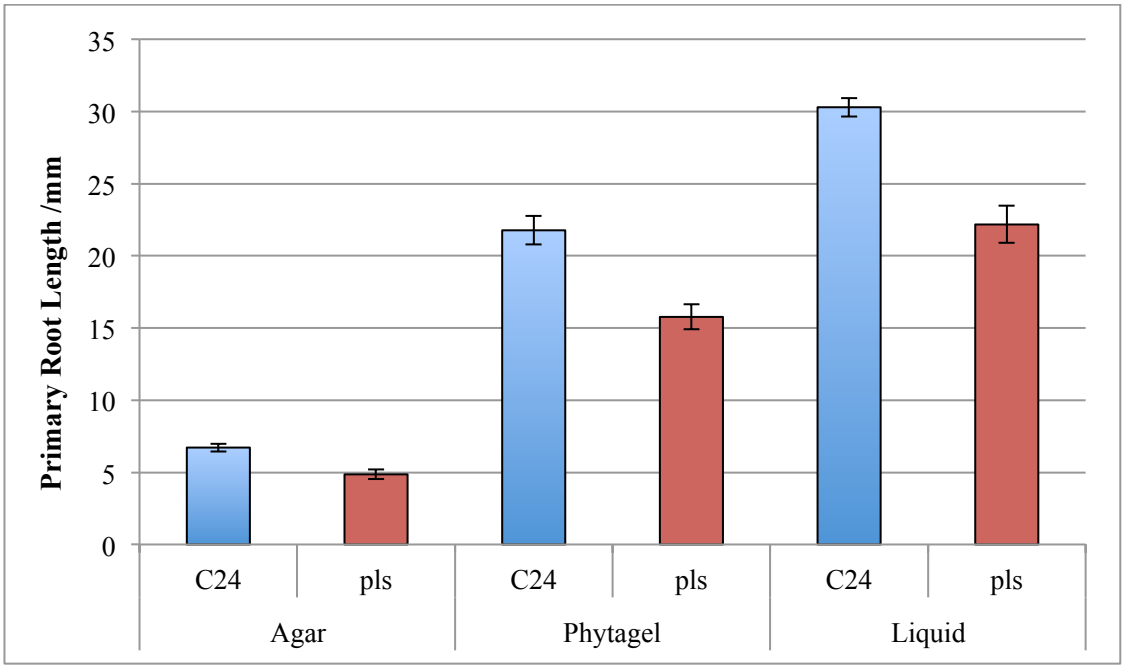


Figure I-3. Primary root length of C24 wildtype and *pls* mutant on plant media containing a range of gelling agents. Roots measured when seedlings were 10 days old. Error bars are ± 1 standard error.

The difference in root length is maintained in all three media types. Roots encountering the reduced resistance of the phytagel media, and vastly reduced resistance in the liquid media, are able to grow longer than those grown on agar media, but still show the required root lengths for use in root length rescue assays.

As a result of the preliminary experiments, it was decided that liquid media containing 0.01% DMSO would be the best conditions for root length rescue assays. Neither C24 wildtype nor *pls* mutant seedlings showed much change in their root lengths with 0.01% DMSO present in the media, so it is not damaging the plant cell membranes to the extent that it's significantly affecting growth. It should however help the plant with uptake of large peptide molecules. Liquid media continues to show the difference in root length between the wildtype and *pls* mutant, and provides an environment where synthetic PLS peptide can diffuse easily throughout the media for uptake by plant roots.

Appendix II: Solid Phase Peptide Synthesis

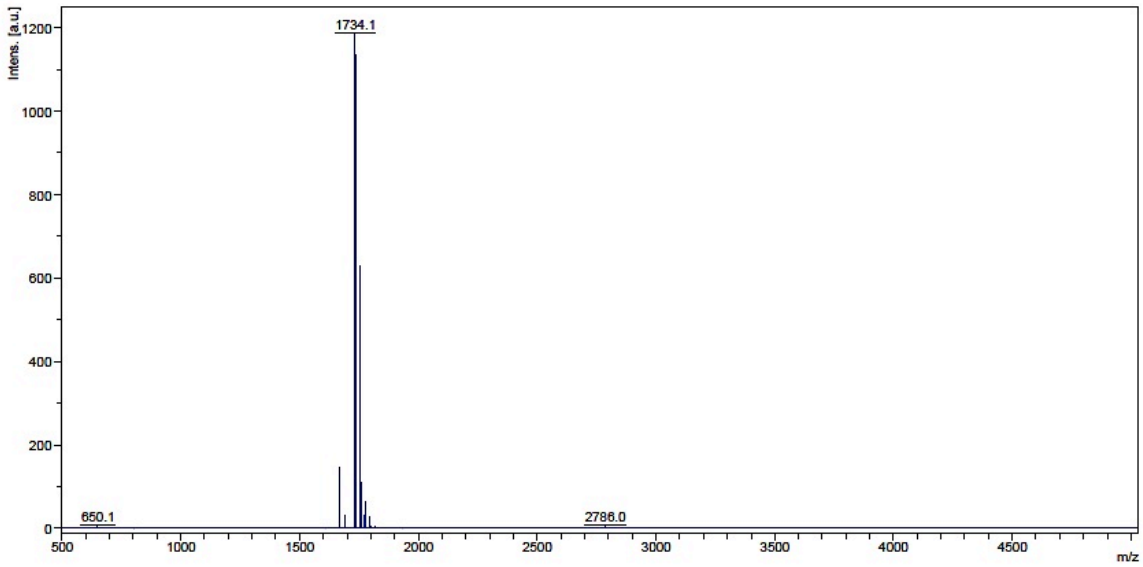
Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry

Peptides synthesised under the conditions in (Materials and Methods, x) were subjected to MALDI-TOF MS analysis to check the expected mass was present, purified by high performance liquid chromatography (HPLC), and HPLC fractions with high signals were submitted for MALDI-TOF MS again to confirm the mass of the peptide. Post-HPLC MALDI-TOF MS spectra are presented, with the corresponding freeze-dried peptide from these fractions used for *Arabidopsis thaliana* root length assays.

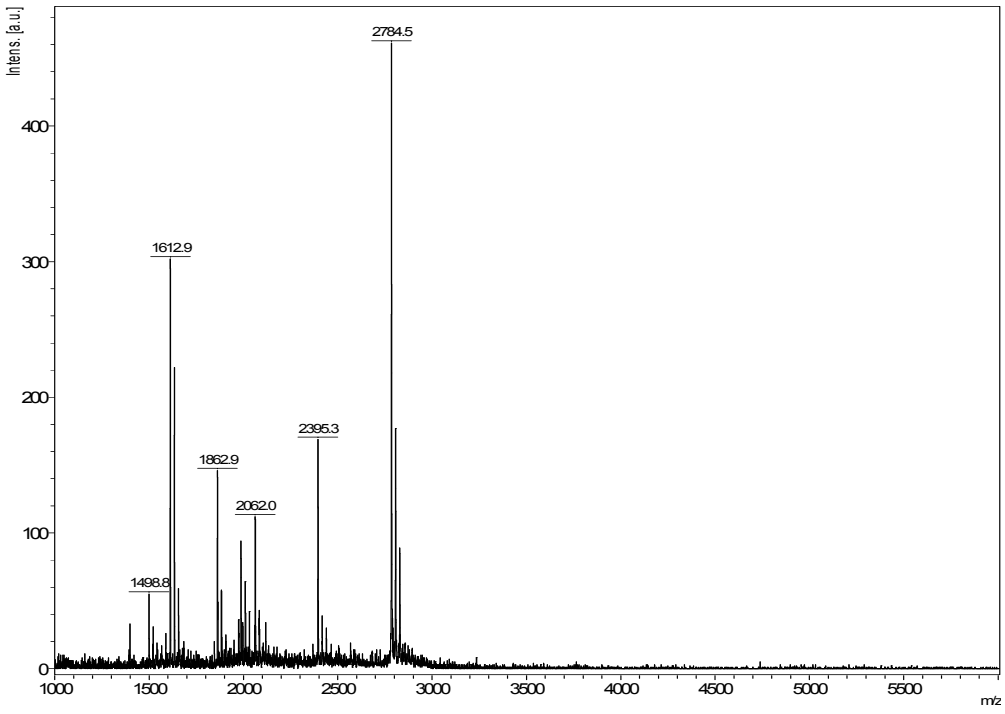
Full Length	MKPRLCFNFRRRSISPCYISISYLLVAKLFKLFKIH
C1	YLLVAKLFKLFKIH
C2	SISPCYISISYLLVAKLFKLFKIH
N1	MKPRLCFNFRRRSISPCYISIS
N2	MKPRLCFNF
PLS(FL)*C6S,C17S*	MKPRLSFNFRRRSISPSYISISYLLVAKLFKLFKIH

Figure II-1. Amino acid sequences of POLARIS and peptide truncations.

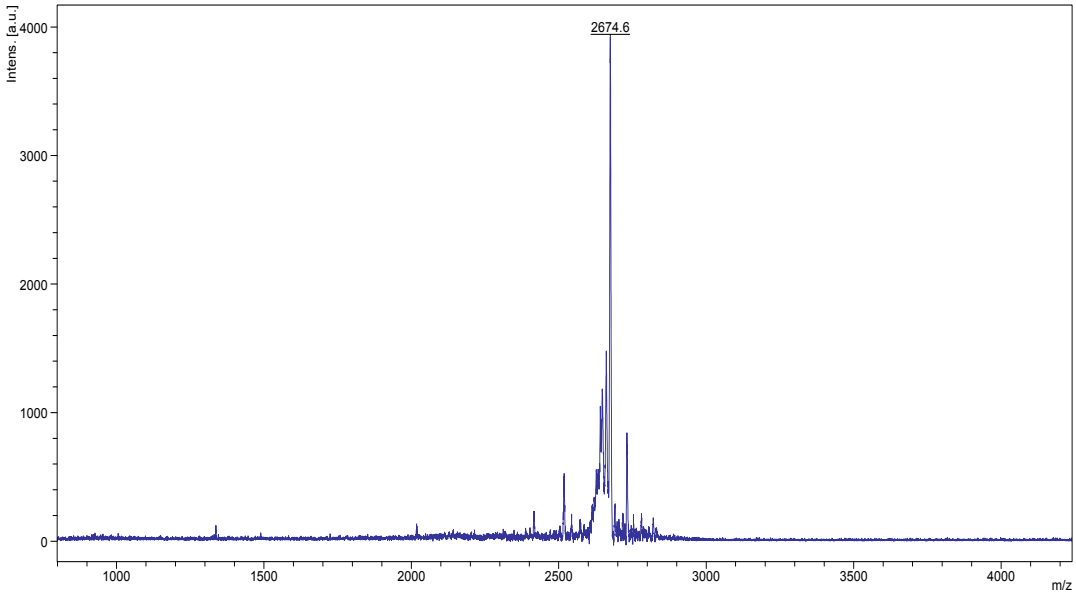
NB due to the difficulty of purifying the full length PLS peptide, synthesis was outsourced to Cambridge Research Biochemicals, Billingham.



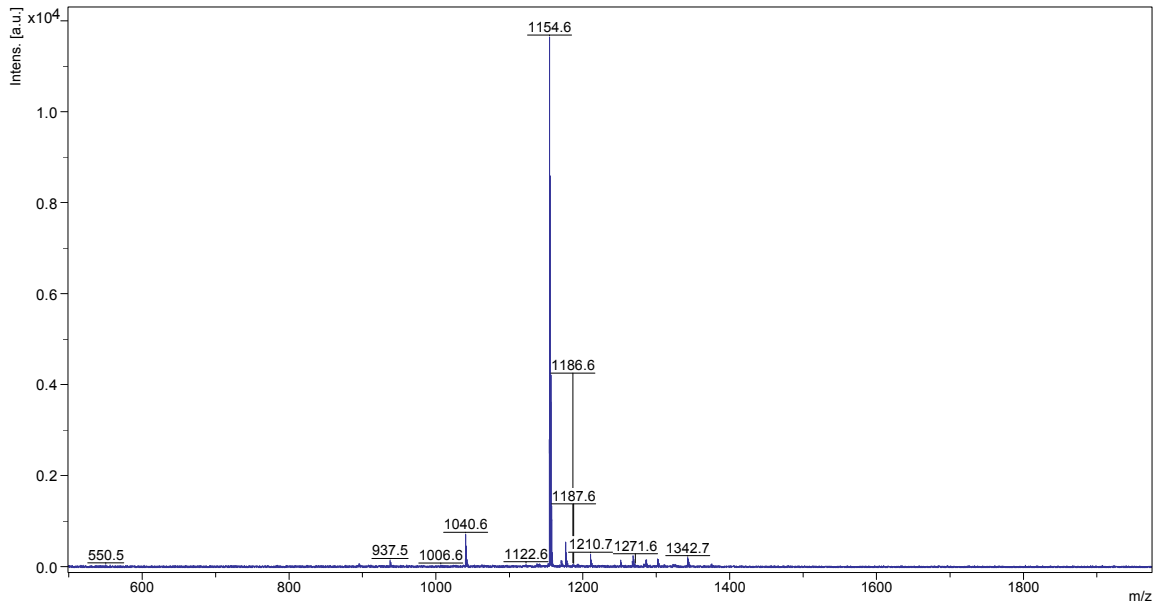
PLS(C1). Expected mass 1732.09. Mass found 1734.1.



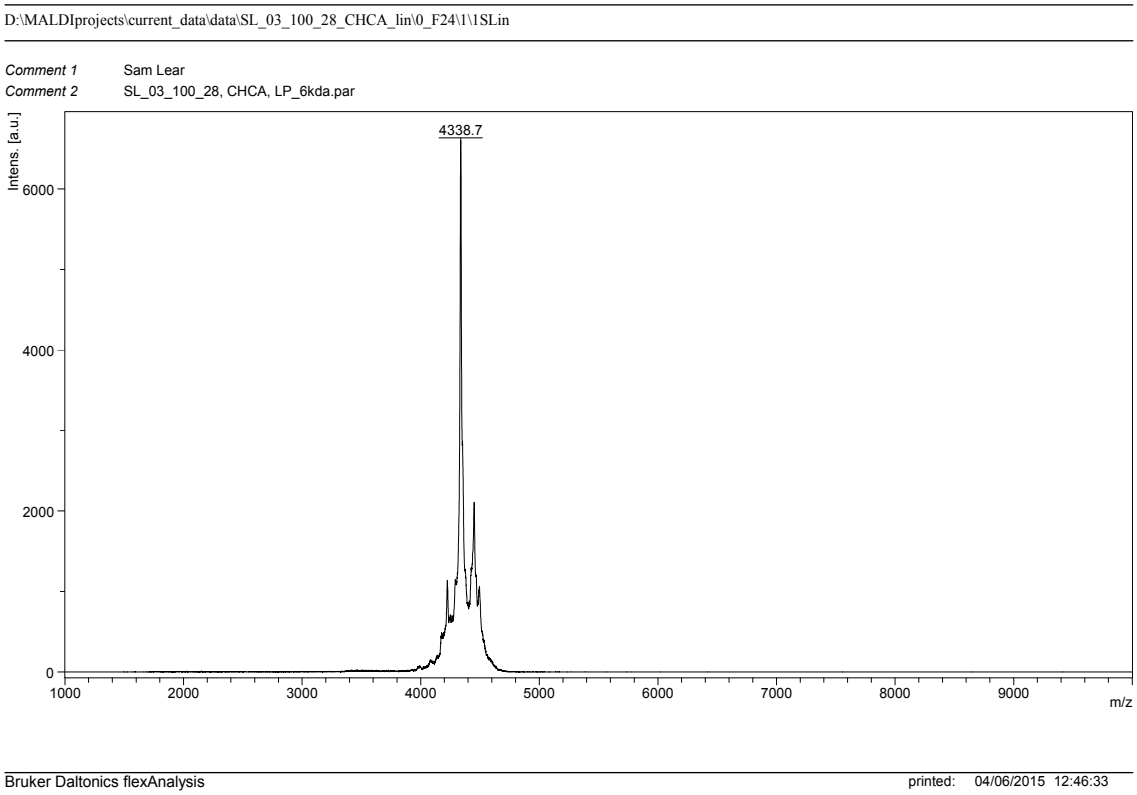
PLS(C2). Expected mass 2782.59. Found mass 2784.5.



PLS(N1). Expected mass 2673.39. Mass found 2674.6.



PLS(N2). Expected mass: 1154.58. Mass found: 1154.6.



PLS(FL)*C6S,C17S*. Expected mass: 4339.5 Mass found: 4338.7.

Appendix III: Expression level comparison between the *POLARIS* and *p35S* promoters powering *GFP* transcript

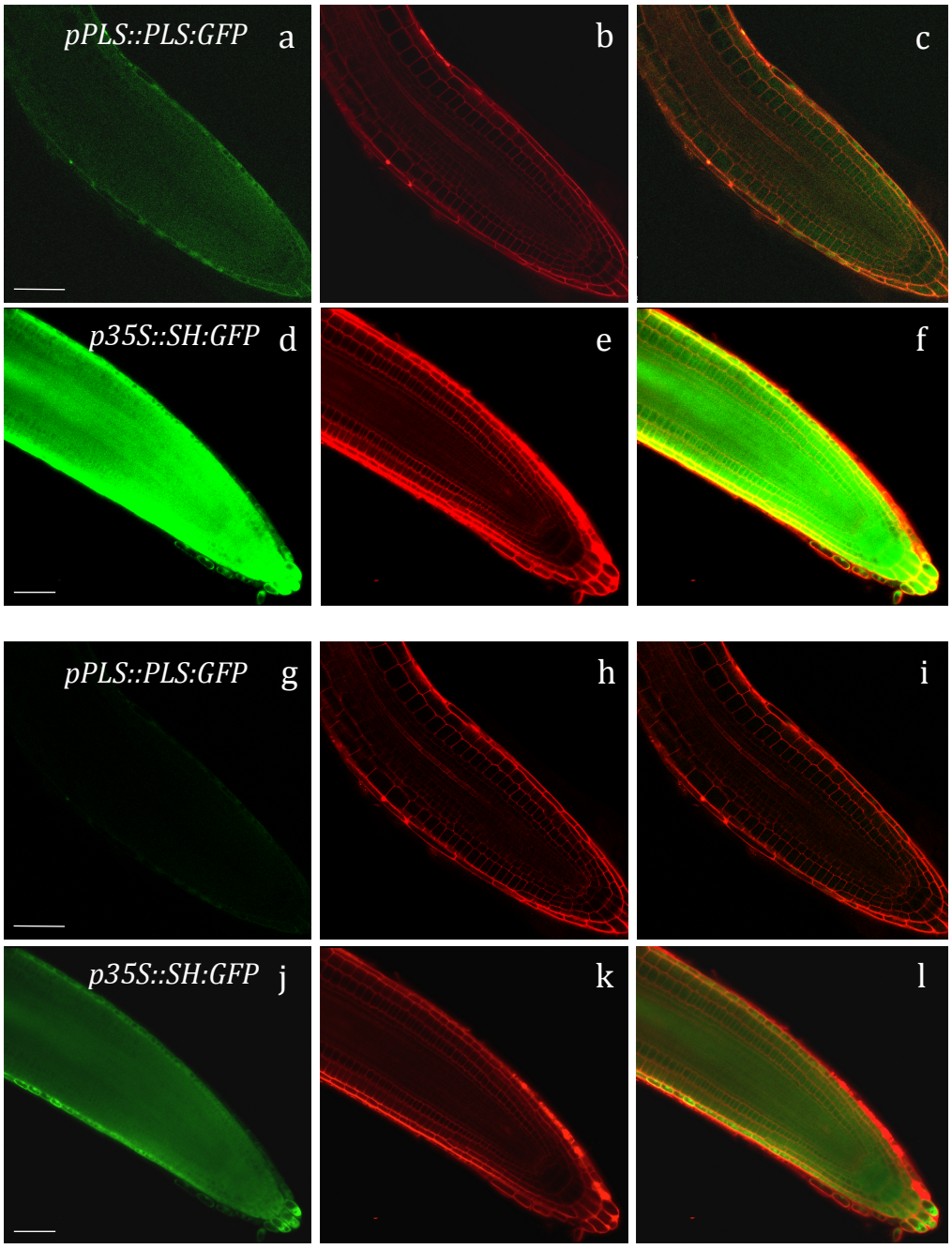


Figure III-1. *pPLS::PLS:GFP* (a-c & g-i) and *p35S::GFP* (d-f & j-l) in root tips. Laser settings were maintained at 40% power (488 nm at 20 mW) 1013 V gain, for all images. Images a-f are displayed using 44/255 brightness settings, g-l are displayed using 14/255.

Appendix IV: *etr1-9* ACC phenotype studies

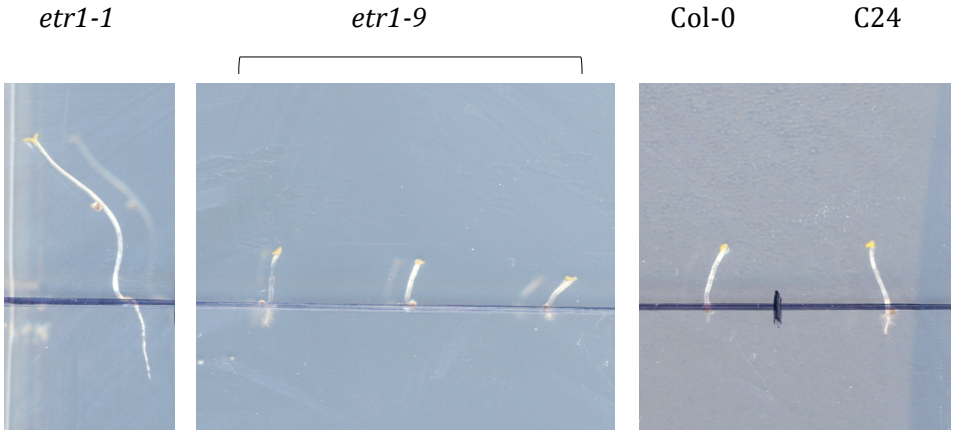


Figure IV-1. Dark-grown phenotypes of *etr1-1, etr1-9,* and wild type plants Col-0 and C24 on agar plates supplemented with 10µM ACC (ethylene precursor). Seeds were sterilised and germination on the ACC-treated agar plates. Seedlings were grown in the dark for 5 days at 21°C long days. *etr1-9* seedlings do not show the ethylene-insensitive phenotype of the gain-of-function *etr1-1* mutant, and are slightly ethylene hypersensitive compared to the wild type.

Appendix V: Primer Lists

ETR1 primers

Name	Sequence	Length (bp)	Tm	GC (%)	Product Size	Start/end
For whole **W**	ATGGAAGTCTGCAATTGTA	19	45.95	36.84	2217	1
ETR1 Rev whole **W**	TTACATGCCCTCGTACA	17	46.29	47.06		2218
ETR1_for **1**	GAATTCATGGAAGTCTGCAATTGT	24	56	38	2230	-6 (1)
ETR1_rev **1**	GTCGACTTACATGCCCTCGTACA	23	60	52		+6 (2218)
AM ETR1_for **2**	TCGAGAACCGTGGCGCTTGTG	21	51.98	61.90	678	238
AM ETR1_rev **2**	TACAGCCACCTGATCAGCGACGA	23	59.75	56.52		916
SM ETR1 Mid_for **3**	CTGGGAAATATATGCTAGGGGA	22	50.52	45.45	736	717
SM ETR1 Mid_rev **3**	GCGGTTACGGAGATACTACCTT	22	59.38	50.00		1452
SM etr1 _for (new) **4**	TGCTTTGATGGTTTTGATGCTTCC	24	54.89	41.67	205	820
SM etr1_rev (new) **4**	GATTGCTGTTTCTGCTTCTCGTCT	24	55.76	45.83		1024
AM etr1_for6 **5**	CCATCACACTAAATCTTGCACC	22	57.64	45.45		1320
SM etr1_for (2) **6**	GTCGGGCATACCGAAAGTTCC	21	55.59	57.14	372	1776
SM etr1_rev (2) **6**	ACGGGTTTGAGCAACACACCG	21	58.14	57.14		2147
AM etr1_rev **7**	TCTATACGGAAACACGGCTG	20	55	50		157
ETR1 qPCR For **8**	ACTCAGGAAGAAACCGGAAG	20	57.16	50.00	132	413
ETR1 qPCR Rev **8**	CAATGCACACTCCTCCAAAG	20	57.00	50.00		544
ETR1 For **9**	TCTCATGGAGCAGAATGTTGC	21				
ETR1 qPCR For **10**	CTCTGGAGCAGGAATAAATCCTC	23	58.36	47.83	141	1706
ETR1 qPCR Rev **10**	CCCTCCATCAGATTCACAAACC	22	58.98	50.00		1825

Primer pairs 1-4 and individual primers 5 & 7 were used for sequencing throughout the ETR1 gene when it was inserted into a vector. They can also be used interchangeably or in combinations with primers from other pairs, depending on the desired product size or specific Tm values required. Primer pairs 8 & 10 are designed for use in qPCR.

PLS primers

Name	Sequence	Length (bp)	Tm	GC (%)	Product Size	Start/end
PLS F1	TCCACGTAGCTGCAGAGAGA	20	60.32	55.00	151	3037
PLS R1	CATGGAGAAATGGACCTTCG	20	56.21	50.00		3187
PLS F2	AAGAGAAGAGCACGTGAGGC	20	60.04	55.00	121	3057
PLS R2	TGGACCTTCGCCTGAAATTA	20	56.84	45.00		3177
PLS F3	CAGAGAGAAAGAGAAGAGCACG	22	58.50	50.00	130	3049
PLS R3	TAATTTCAGGCGAAGGTCCAT	21	57.36	42.86		3178
pls_for whole	ATGAAACCCAGACTTTGTT	19	52.14	36.84	110	3138
pls_rev whole	CAATGGATTTTAAAAAGTT	19	44.61	21.05		3247
qPCR PLS loc F (JR)	AGACTTGTTGTGGTGATGTT	20	53.2	40	96	3093
qPCR PLS loc R (JR)	ACATGGAGAAATGGACCTTC	20	55.3	45		3189

PLS primer pairs 1, 2 & 3 are located throughout the PLS gene. Different combinations of these primers may be useful with respect to their Tm values, or for sequencing the PLS locus in a vector.

Colony PCR primers

Name	Sequence	Length (bp)	Tm	GC (%)	Product Size	Start/ end
SM etr1_for **4**	TGCTTTGATGGTTTTGATGCTTCC	24	54.89	41.67	633	820
SM ETR1 **3**	GCGGTTACGGAGATACTACCTT	22	59.38	50.00		1452

Quantitative PCR primers

Name	Sequence	Length (bp)	Tm	GC (%)	Size	Start/ end
ETR1 qPCR For **8**	ACTCAGGAAGAAACCGGAAG	20	57.16	50.00	132	413
ETR1 qPCR Rev **8**	CAATGCACACTCCTCCAAAG	20	57.00	50.00		544
PLS F3	CAGAGAGAAAGAGAAGAGCACG	22	58.50	50.00	130	3049
PLS R3	TAATTTCAGGCGAAGGTCCAT	21	57.36	42.86		3178
PP2C HK For	AGC AGG GTG AGG ATT TGG TG	20	59.40	55.00	136	
PP2C HK Rev	ATT CAC CTG GCA AAT CCG GT	20	57.30	50.00		
qPCR For (pre-tDNA)	GCAGTGTCTCACTGAAACATG	21	57.47	47.62	132	
qPCR post-tDNA rev	CAATGGATTTTAAAAAGTTTAAA CAATTTTGC	32	58.35	21.88		

qPCR primers above were used in the qPCR experiments contained in this thesis. Other primer pairs with qPCR in their names are also suitable for use in qPCR studies.

Genotyping and sequencing primers

Name	Sequence	Length (bp)	Tm	GC (%)	Start	End
Etr1-9 For (genotyping)	GCGGTTGTTAAGAAATTACCCATCACACT	29				
Etr1-9 Rev WT	ATCCAAATGTTACCCTCCATCAGATTCAC	29				
Etr1-9 Rev tDNA LB	CATTTTATAATAACGCTGCGGACATCTAC	29				
GUS 4247	TGAACAACGAACTGAACTGG	20	56.23	45.00	613	400
GUS 3636	AGCATCTCTTCAGCGTAAGG	20	57.41	50.00		1013*
pMDC107 GFP F	CTTCTTCAAGAGCGCCATGC	20	59.90	55.00	562	
pMDC107 GFP R	AGACCGGCAACAGGATTCAA	20	59.60	50.00		
pEG301 bb For	TCACGTCTTGCGCACTG	17	57.93	58.82	1891 in empty	1287
pEG301 bb Rev	AGGCGTCTCGCATATCTC	18	56.31	55.56		3177
GFP end Rev (Cterm cloning)	TTAGTGGTGGTGGTGGTGG	19	59.16	57.89		
pMDC107 NEW F	GGAAAACTACCTGTTCCATGG	21	56.51	47.62	467	
pMDC107 NEW R	GCAGATTGTGTGGACAGG	18	55.68	55.56		
HA tag (N) For	TACCCATACGATGTTCCAGAT	21	55.94	42.86		
HA tag (C) Rev	TAATCTGGAACATCGTATGGG	21	54.90	42.86		
RFP For	ccaagctgaaggtgaccaag	20	58.76	55	507	
RFP Rev	gttccacgatggtgtagtcc	20	57.99	55		
pMDC83 For	gctcccttatacacagccag	20	57.75	55.00	932 in empty 83	
pMDC83 NOS Rev	caagaccggcaacagg	16	54.77	62.50		
pFGCSTGolgi RFP R	GAGCCGTACATGAACTGAGG	20	58.07	55.00		

Appendix VI: DNA, protein and plasmid sequences

POLARIS genomic DNA locus and open reading frame

The *POLARIS* gene promoter, starting 1.5kb before the start of the *PLS* open reading frame, and the 108-nucleotide *PLS* gene (highlighted and capitals), located on chromosome 4.

ctttagcccgtgcgggaagttttgtacgtgagaaagaagcctcgagaatgaataaaactacgataagattgat
tcaacttcagatttccaaccccataactaggtatatatacatatatataaattgattaatgattatgtattta
cgtacacatcgcaatcctccacttatatgccttacagaaaaatatctaaatgcatggaccgtcttgcacttga
cccatatttacatatttagagcaattttatatgtacataatcagatacatgacgacgtttaaacgtgaattat
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gagcgaagacagtccacgtagctgcagagagaaagagaagagcacgtgaggcacacgttccttgtgtaagact
tgttgtggtgatgttggcgcagtgtctcactgaaacatgaATGAAACCCAGACTTTGTTTTAATTTCAGGCGA
AGGTCCATTTCTCCATGTTATATATCAATCTCTTATTTATTAGTAGCAAAATTGTTTAAACTTTTTAAAATCC
ATTGAtcaccctatcattttcaatatctacatacaatcttatgtctcgataaaggtttatctttatcttatta
tgcaatacatatccctcccatttctatattgcaaattatgacatcaaaaaaccattcttttgattctacttgg
gccaataacaaaatcaatagtaatggaaaaaataacgtagatggatataaatatagtccaacggt

Transcription start site 1 in pink

Transcription start site 2 in grey

T-DNA insertion site in blue

POLARIS amino acid sequence

MKPRLCFNFRRRSISPCYISISYLLVAKLFKLFKIH*

***ETR1* cDNA**

Complete cDNA sequence of the *Arabidopsis thaliana* ethylene receptor *ETR1*, used with or without final stop codon for N- or C-terminal DNA cloning fusions.

atggaagtctgcaattgtattgaaccgcaatggccagcggatgaattgttaatgaaataccaatacatctccg
atttcttcattgcgattgcgtatttttcgattcctcttgagttgatttactttgtgaagaaatcagccgtgtt
tccgtatagatgggtacttgttcagtttggtgcttttatcgttctttgtggagcaactcatcttattaactta
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ETR1 amino acid sequence

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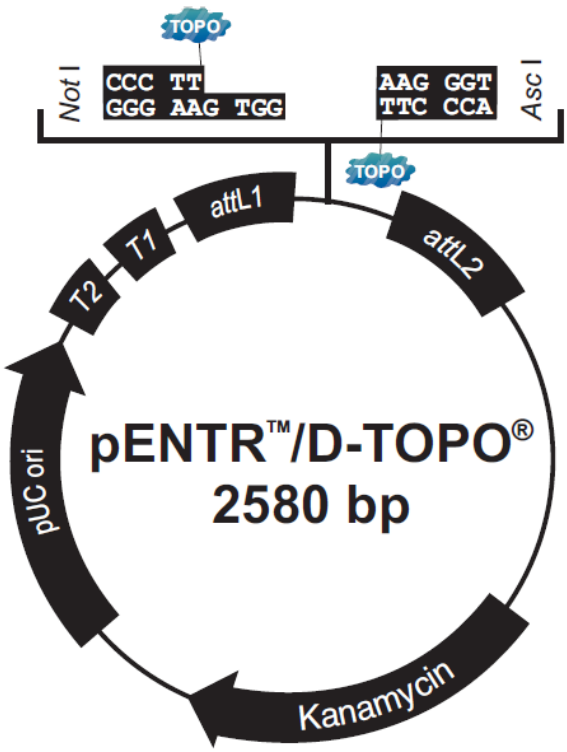
Plasmid Construction

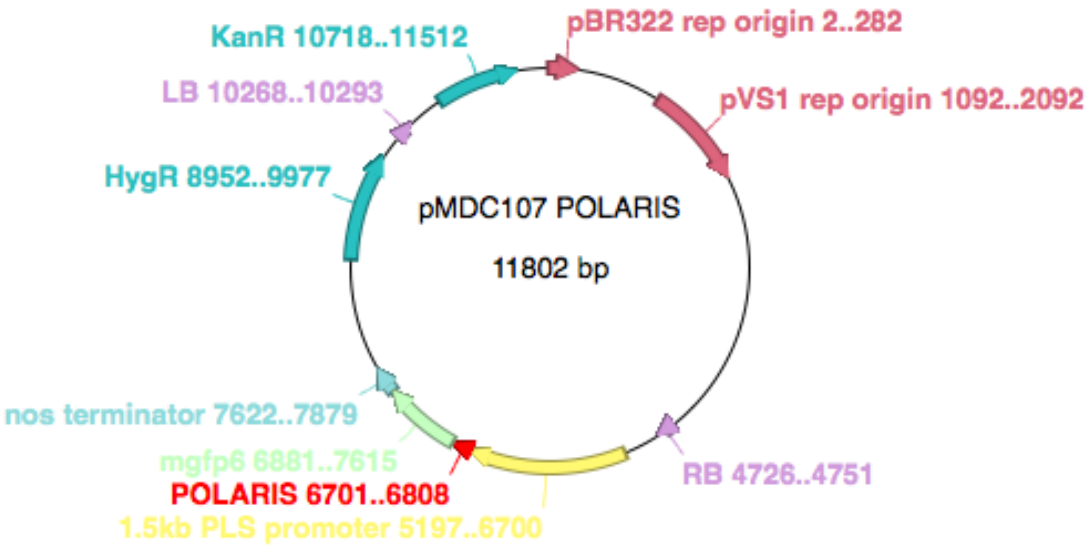
pENTR™/D-TOPO

The pENTR™/D-TOPO® vector (Life Technologies) was used as an entry vector into Gateway cloning.



pENTR™/D-TOPO® vector map, showing key features and the insertion site with overhang.

pMDC107::pPOLARIS:POLARIS



Graphical map of the pMDC107 vector and *POLARIS* promoter:*POLARIS* gene DNA.

pMDC107 and *POLARIS promoter:POLARIS* construct DNA sequence.

POLARIS promoter in yellow, *POLARIS* open reading frame represented in red and capital letters.

Location of construct features in the DNA sequence below correspond to plasmid above with colours roughly matching.

Start: nucleotide position 1

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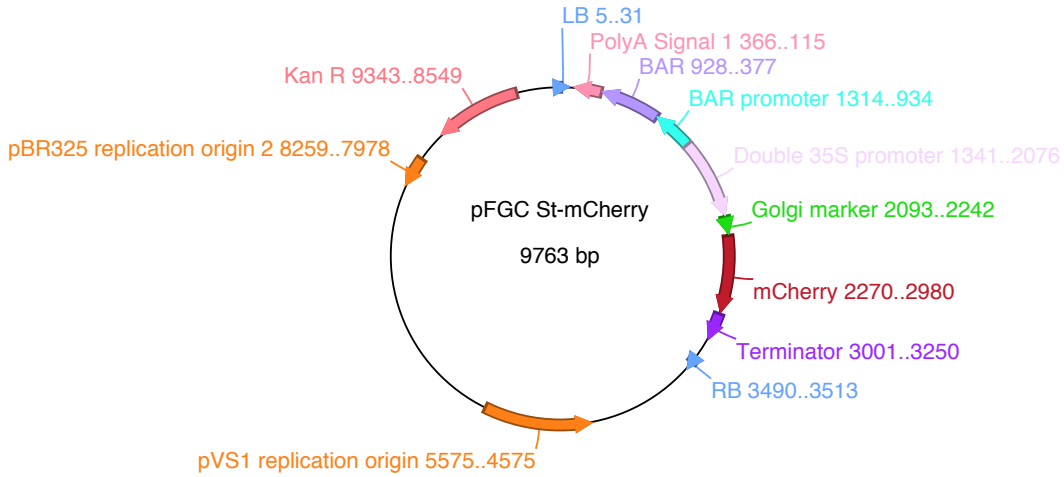
End: nucleotide position 11802

pFGC::ST:mCherry

Golgi marker cytoplasmic tail + TM domain of soybean



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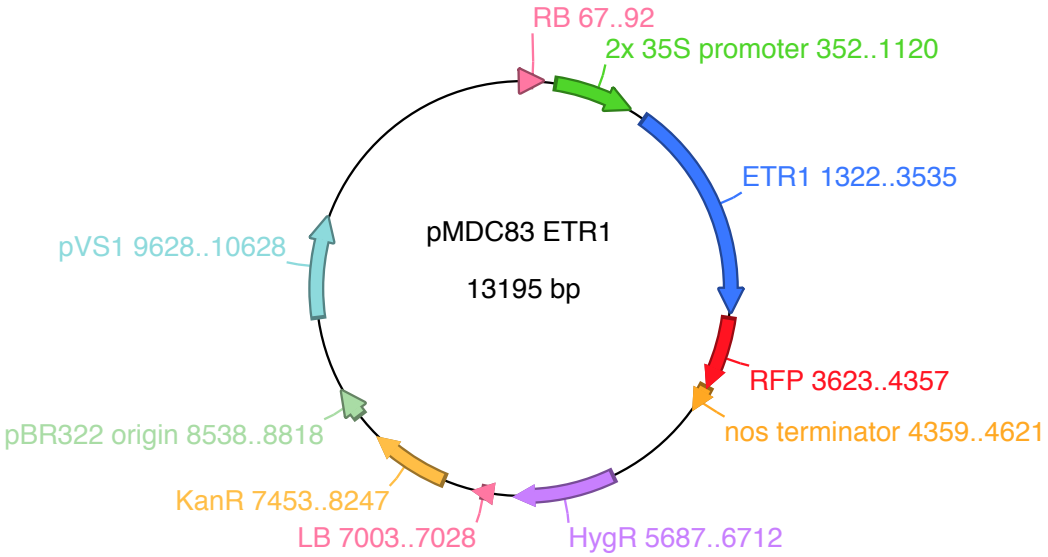

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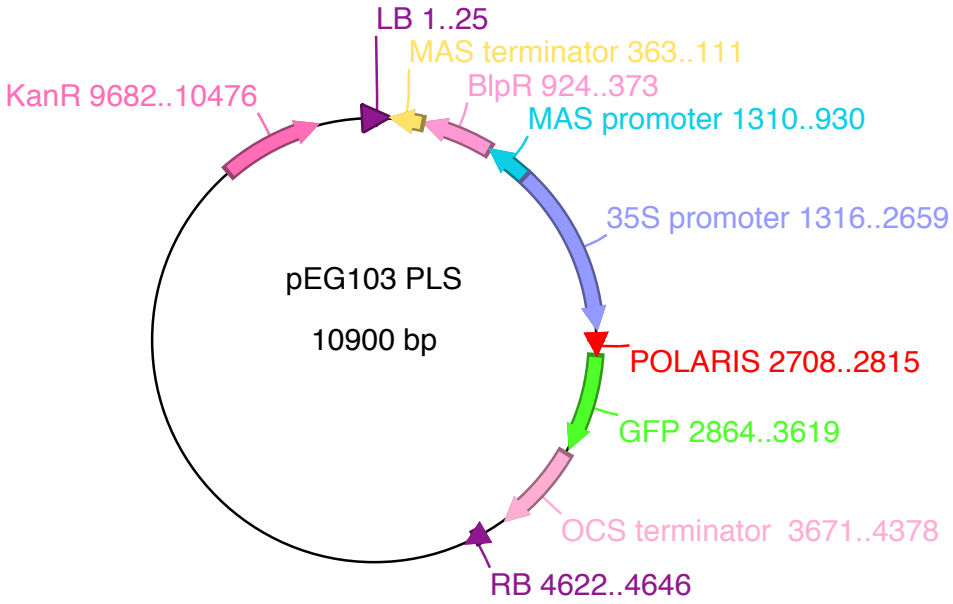

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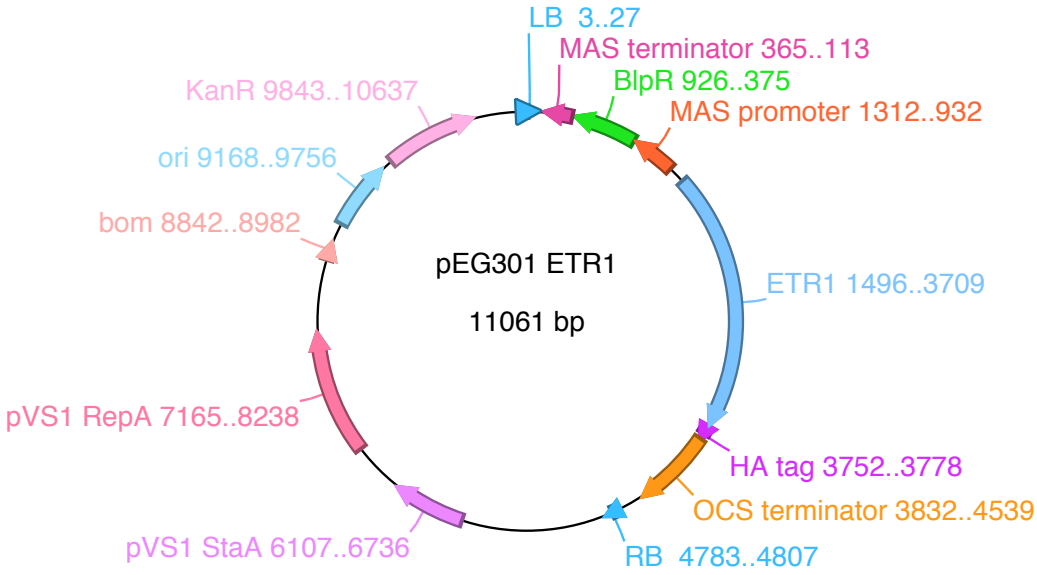

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