Protein- Hormone Interactions Patterning the Gynoecium

By Joyita Deb

University of East Anglia

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ABSTRACT

The gynoecium is among the most intricately patterned organs of the plant, comprising different tissue sub-structures, all with the purpose of facilitating propagation to the next generation. It is therefore representative of the complexity involved in the initiation and establishment of organ patterning and presents a unique model to study the mechanisms coordinating development. As with all other organs, the interplay between genetic and hormonal factors specifies carpel development. However, although much is known about the genetic components involved in carpel development, our understanding of hormonal regulation and the cross-talk between these two pathways is limited. Thus, the aim of this thesis has been to address this issue by obtaining an integrated view of the genetic and hormonal regulatory mechanisms which act to coordinate gynoecium development. It has done so using broadly two approaches: first, by characterising the transcription factor interactions which pattern the carpel, and second, by elucidating the cross-talk between these interactions and the plant hormone auxin. Further, it has also studied the role of auxin in carpel morphogenesis.

Observations from this research have uncovered a novel auxin co-receptor complex formed by the transcription factors IND and ETT. The co-receptor binds the IAA molecule directly and exhibits specificity for IAA over the synthetic analogues NAA and 2,4-D. This co-receptor functions to coordinate the development of the style and stigmatic tissues of the carpel, possibly via the regulation of *PID* kinase. Further, this work has also identified novel roles in protein-protein dimerisation for the domains involved in this interaction. Analyses also indicate that this novel auxin signalling pathway may also be conserved in the Brassicaceae through the *ETT* orthologues in this family. Finally, this project has analysed how ETT's role as an auxin receptor could be translated into precise spatio-temporal regulation of its target genes to specify the boundaries necessary for gynoecium patterning.

Together, the results from this work have posed new questions as to the signalling mechanisms through which auxin coordinates its varied and numerous functions in plants.

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LIST OF ABBREVIATIONS

-AD……..	GAL 4 activation domain
AFB…….	Auxin-Binding F-Box
ARF…….	AUXIN RESPONSE FACTOR
-BD……..	GAL4 DNA-binding domain
bHLH……..	basic helix-loop-helix
CFP……..	Cyan Fluorescent Protein
ChIP……	Chromatin Immunoprecipitation
Col-0……	Columbia-0
2,4-D……	2,4 Dichlorophenoxy Acetic Acid
DBD…….	DNA Binding Domain
DEX…….	Dexamethasone
DMSO……..	Dimethyl sulfoxide
DNA…….	Deoxyribonucleic acid
dNTP……...	Deoxyribonucleotide triphosphate
ds……..	Double stranded
EDTA……..	Ethylenediaminetetraacetic acid
ETT…….	ETTIN
ESdomain……	ETT-Specific domain
FRET-FLIM……...	Fluorescence Resonance Energy Transfer and Fluorescence-lifetime imaging microscopy
FUL……	FRUITFULL
GFP…….	Green Fluorescent Protein
GUS…….	β-Glucuronidase
HEC…….	HECATE
IAA……..	Indole-3-acetic acid
iaaL……..	Indoleacetic acid-lysine synthetase
iaaM…….	Indoleacetic acid monooxygenase
IBA……..	Indolebutyric acid
IND……..	INDEHISCENT
ISdomain……	IND-Specific domain
LiAc…….	Lithium acetate
LWHA…….	Leucine Tryptophan Histidine Adenine
MR……...	Middle Region
NAA…….	α-naphthalene acetic acid
NPA…….	1-N-Naphthylphthalamic acid
OD……	Optical density
ONPG……..	o-nitrophenyl ß-D-galactopyranoside
PCR……	Polymerase chain reaction
PEG…….	Polyethylene glycol
PID……...	PINOID Kinase
PIN1/3…….	PIN-FORMED1/3
RNA…….	Ribonucleic acid
RNAi……	RNA interference
RT……	Room Temperature
SD……	Synthetic Drop-out
SEM…….	Scanning Electron Microscopy
SPR……..	Surface Plasmon Reasonance
SPT……..	SPATULA
ss……..	Single stranded
STY……..	STYLISH
TE buffer…….	Tris EDTA buffer
TIR1……	TRANSPORT INHIBITOR RESPONSE 1
WT……...	Wild Type
YFP……..	Yellow Fluorescent Protein
YPD…….	Yeast extract-peptone-dextrose

CHAPTER 1

CHAPTER 1- General Introduction

The angiosperms constitute the largest and most diverse group among land plants. Having arisen in the Late Jurassic period approximately 160 million years ago (MYA), this group of plants has diversified to include almost 300,000 extant species (Scutt et al., 2006). Several factors have contributed to the evolutionary success of this group, but among them the feature that has perhaps played the most major role is the evolution of the gynoecium or the carpel. The gynoecium is the female reproductive organ of the plant and it is the distinguishing feature of the angiosperm group as it forms a protective organ enclosing the ovules- the future seeds of the plant. Besides this, it has also evolved a variety of structures which aid pollination, fertilization and seed dissemination, all of which have contributed to the diversification and adaptive success of the angiosperms.

The carpel is thought to have evolved by the fusion of leaves, more specifically from the sporophylls of gymnosperms which are leaf-like structures bearing the reproductive organs (Scutt et al., 2006). This hypothesis is supported by observations involving homeotic transformations of the floral organs into leaves by misexpression of floral identity genes. Loss of function of the ABC function genes (Coen and Meyerowitz, 1991) and E- function *SEPALLATA (SEP)* genes results in the conversion of the floral organs into leaves (Pelaz et al., 2000); conversely, ectopic expression of the ABC function genes with the *SEP* genes results in the conversion of leaves into different floral organs (Honma and Goto, 2001) . Also consistent with this hypothesis is the high degree of conservation seen between the genetic networks patterning the leaves and the carpel (Balanza et al., 2006). Architecturally however, these two organs bear little resemblance as the carpel has evolved into a far more complex organ with tissue sub-structures each with a distinct function.

Carpel development has been characterized extensively in *Arabidopsis thaliana* and the genes involved in both leaf and carpel development have been studied in-depth. This study has also used the *Arabidopsis* gynoecium as the model to investigate carpel development and hence forth all references to the gynoecium will be based on this model system.

1.1 The structure of the mature gynoecium

The gynoecium is a complex structure comprised of several different tissues. These tissue domains are described along three axes of symmetry: apical-basal, medio-lateral and adaxial-abaxial (Fig. 1).

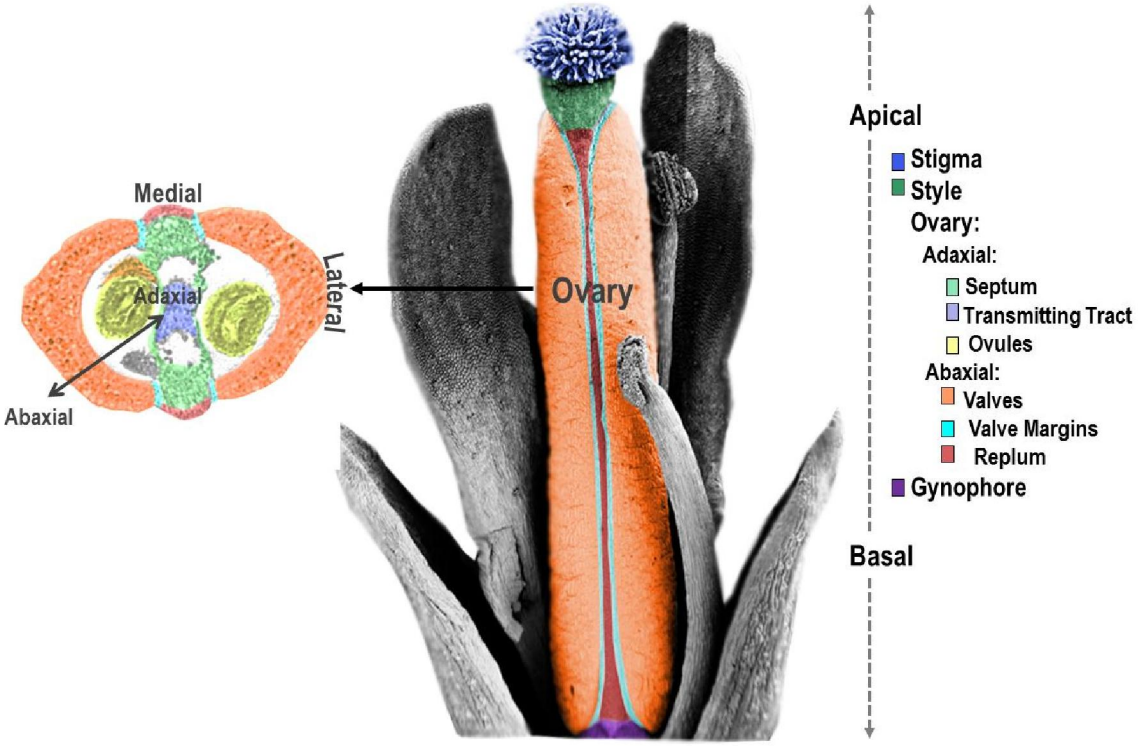

Figure 1 Gynoecium patterning along different axes of polarity. The *Arabidopsis* gynoecium has three axes of polarity: apical-basal, adaxial- abaxial, medio-lateral. Colours in the figure legend correspond to false colouring.

The apical-basal axis consists of, sequentially, the stigma, style, ovary and the gynophore. The stigma consists of elongated papilla cells which help in pollen capture and pollen germination. This is followed by the style which is a cylindrical mass of tissue with the transmitting tract in the centre. The ovary forms the main body of the carpel and houses the ovules. Finally the gynophore, which is found at the base of the ovary, is a stalk-like structure which attaches the carpel to the plant.

The adaxial-abaxial axis (Fig. 1) forms the internal-external axis of the carpel. Internally, the carpel is divided into two halves by the septum. The transmitting tract is located at the centre of the septum as a continuation from the style and stigma. Like the stigma, the transmitting tract secretes a polysaccharide-rich extra-cellular matrix which aids pollen tube growth towards the ovules. The ovules are found within each locule of the carpel, and

each is attached to the placenta by a stalk-like funiculus. Externally, the walls of the ovary are called the valves which are separated by the replum. On either side of the replum is found a narrow constricted zone of elongated cells known as the valve margins. At maturity the fruit dehisces along this zone which is a few cell files wide. Prior to dehiscence, the cells of the valve margins differentiate into two layers: the separation layer and the lignified layer. The separation layer consists of small cells which secrete hydrolytic enzymes such as polygalactouronase which aid cell separation. On the other hand the lignified layer consists of larger cells which become lignified just before dehiscence and this provides the necessary tension for the shatter-like mechanism of seed dispersal which is a characteristic of *Arabidopsis*.

Finally, along the medio-lateral axis (Fig. 1) can be found the valves and the valve margins, which are considered lateral tissues, whereas the medial part consists of the replum externally and the septum and the transmitting tract tissue internally.

1.2 Stages of Gynoecium Development

The gynoecium in *Arabidopsis* is derived from two congenitally fused carpels, which emerges as a single primordium in the centre of the flower. Floral development has been divided into stages which serve as landmarks detailing key developmental events (Smyth et al., 1990). Carpel development begins from stage 6 - when it emerges as a protuberance - to stage 13 at the time of anthesis when it begins to develop into a fruit post-fertilization (Fig. 2).

Stages 6-8 involve the elongation growth of the carpel to form a hollow tube-like structure without any tissue differentiation. Stage 8 marks the first stages of vascular tissue development as the medial vascular bundles begin to develop. In the later phases of this stage, the inner walls of this hollow tube fuse. By stage 9 medial tissue fusion is complete and the septum is formed.

Stage 9 is characterized by the initiation cell identity which consequently establishes the overall patterning of the gynoecium (Fig. 2). At this stage cells at the top of the gynoecium begin to differentiate and acquire a more rounded shape indicating the development of the stigmatic papillae which is complete by stage 11-12. Style development occurs below the apex concurrently and this structure becomes morphologically distinct from the main body of the carpel. Internally, septum fusion is complete and the ovule primordia begin to appear.

Stages 10-11 involve further elongation and expansive growth coupled with differentiation and demarcation of the various tissue regions of the gynoecium. By stage 12 the mature gynoecium takes shape and at this stage the valve margins and the replum - both of which are necessary for fruit development - also become morphologically distinct. By the end of stage 12 all the tissues necessary for pollination and fertilization are fully developed, the ovules are mature and the carpel is ready for anthesis (Fig. 2).

Stage 13 is marked by anthesis - the opening of the flower. *Arabidopsis* self-pollinates and the pollen grains land on the now fertile stigma and germinate (Fig. 2). Once they germinate, pollen tube growth occurs between the cells of the transmitting tract in the extracellular matrix towards the ovules to fertilize them. It is at this point, following fertilization, that stage 14 begins and the gynoecium develops into a fruit.

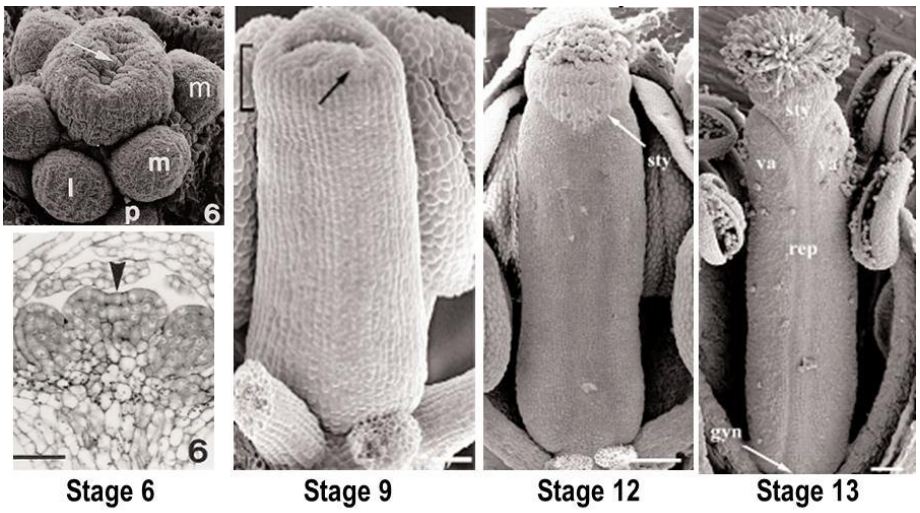

Figure 2 Major early stages of development of the WT carpel. Stage 6 marks the emergence of the carpel primordia with an invagination in the centre (indicated by arrows). At stage 9 the style becomes conspicuous and stigmatic tissue develops during late 9-10. At stage 12 the carpel is fully mature and the tissue domains are developed. At stage 13 anthesis occurs and the carpel is pollinated. (*Roeder and Yanofsky, 2006)*

1.3 Genetic Regulation of Gynoecium Development

In the last few decades, the major genetic players involved in developing and patterning the gynoecium have been revealed primarily through reverse genetic approaches. This has revealed complex and coordinated transcription factor pathways which function within each tissue domain of the gynoecium. The interactions patterning the carpel are thus best described along the different axes of polarity and the genetic pathways functioning along these planes will be discussed in the following sections.

1.3.1 The adaxial-abaxial patterning factors

When viewed along the adaxial-abaxial axis, the internal tissues comprising the septum, the transmitting tract and the placentae, and the external tissues -the style, stigma and the replum are together known as the marginal tissues. This is because they are derived following the fusion of the medial ridge of the carpel which occurs around stage 9. The genes which have identified as playing a major role in defining these tissues early on in development include *LEUNIG* (*LUG*), *ANTINTEGUMENTA* (*ANT*), *SEUSS* (*SEU*) and *FILAMENTOUS FLOWER* (*FIL*). These genes function together in developing the marginal tissues with a major role played by *LUG* which is a transcriptional co-repressor. Double mutant phenotypes of *ant lug*, *fil ant* and *lug seu* are very similar as they exhibit severe losses of replum, style, septum and placental tissues. Further, LUG and SEU can also interact indicating that these proteins probably function cooperatively and share common targets (Balanza et al., 2006, Østergaard, 2010).

Functioning downstream of these genes are members of the *STYLISH (STY)*/*SHORT INTERNODE (SHI)* gene family, which encode zinc-finger transcriptional activators (Kuusk et al., 2006) (Fig. 3). Single mutants in these genes show minor defects in both stigmatic tissue and style development which is increased in a dose-dependent manner with increasing mutant combinations suggesting partial redundancy in their function. These genes function to regulate auxin biosynthesis in the style via the *YUCCA4 (YUC4)* auxin biosynthesis gene (Sohlberg et al., 2006).

Also important for marginal tissue development are a group of basic Helix-Loop-Helix (bHLH) transcription factors which have been identified as playing a role in establishing stigmatic tissue and style identity. These include SPATULA (SPT) (Groszmann et al., 2010, Heisler et al., 2001) and the IND/HEC sub-family which comprises the bHLH

proteins INDEHISCENT (IND) and HECATE (HEC) 1, 2 and 3 (Gremski et al., 2007, Heim et al., 2003). As these proteins are the subject of this study, their roles will be discussed in detail (Fig. 3).

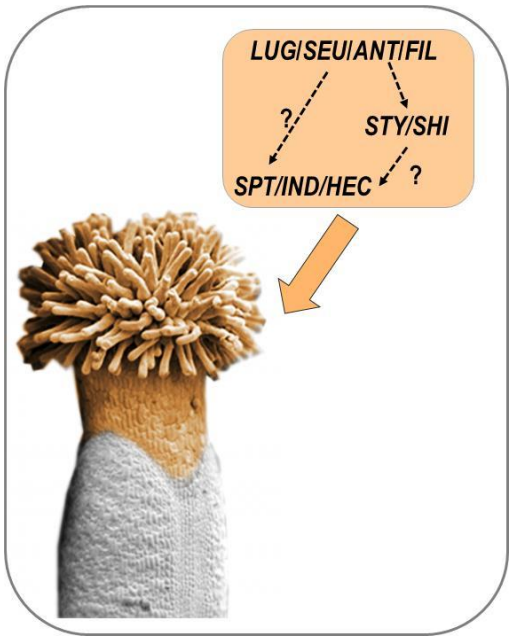

Figure 3 Transcription factor pathways patterning the apical tissues.
LUG/SEU/ANT/FIL function upstream of both the *STYLISH* genes and the bHLH group of proteins. It is not clear whether SPT and the IND/HEC family of proteins function downstream of the STY/SHI proteins or in a parallel pathway.

SPT expression is first seen when the gynoecium is specified from inflorescence meristem and as the gynoecium develops, its expression is restricted adaxially in the region of the developing septum. Later, during stage 9 when the other marginal tissues are specified, *SPT* expression can also be detected in the style and stigmatic tissues. In accordance with its expression pattern, *spt* mutants show conspicuous defects in marginal tissue development which include a bifurcated style and a reduction in stigmatic tissue, transmitting tract and septum development (Heisler et al., 2001).

Most of the phenotypic defects seen in the *spt* mutant are also shared by *ind* and the *hec* mutants but they are less extreme (Gremski et al., 2007, Girin et al., 2011). Single mutants show reduction in stigmatic tissue development and transmitting tract development which results in reduced pollen tube germination on the stigma and fewer pollen tubes reaching the ovules. Phenotypic analyses of these mutants suggest that there is substantial functional redundancy between these genes in marginal tissue development. Gradual loss of each gene in higher mutant combinations of *hec1 hec3* HEC2-RNAi (Gremski et al., 2007) or *ind-2 spt-12* (Girin et al., 2011) result in the complete loss of stigmatic tissue, style, transmitting tract and septum and as a result these mutants are sterile. Although these

proteins have overlapping roles in marginal tissue development, IND has acquired a specialized role in fruit dehiscence (Liljegren et al., 2004). It is required for valve margin development and *ind* mutant fruits completely lack a valve margin and do not dehisce. While IND is required to specify the lignified layer of the valve margin, specification of the separation layer requires both IND and SPT function (Girin et al., 2011).

The specific role of these proteins as marginal tissue identity factors can be seen when the proteins are ectopically expressed. When either *IND* or *HEC1*, *2* or *3* genes are constitutively expressed under the 35S Cauliflower Mosaic Virus (CaMV) promoter, the overexpression of these genes results in carpelloid features such as stigmatic tissue developing ectopically on the floral organs. *35S::SPT* expression does not exhibit any phenotype and it is only when the IND protein is misexpressed in the presence of SPT that this aberrant tissue development is seen. This suggests the involvement of heterodimeric complex formation among these two proteins and interaction between SPT and IND proteins has been shown through both yeast two-hybrid and *in planta* interaction assays (Girin et al., 2011). Additionally, SPT can also interact with all three HEC proteins however they do not heterodimerise amongst themselves. The interaction between IND and SPT occurs via their bHLH domains and this is likely also to be the case with SPT and the HECs although it is yet to be proven (Girin et al., 2011, Gremski et al., 2007).

IND and the HEC proteins share considerable homology in their amino acid sequences and among them HEC1 and HEC2 are the most closely related showing 61% amino acid identity and 100% homology in their bHLH domains. These proteins have been classified as a separate sub-group among the bHLH proteins in plants. This is due to substitutions of key amino acid residues in bHLH domains of the IND/HEC proteins which are otherwise highly conserved within bHLH proteins (Heim et al., 2003, Toledo-Ortiz, 2003). bHLH proteins interact with their DNA target sequence via amino acids at positions 5, 9 and 13 of the bHLH domain (Shimizu et al., 1997). While most bHLH proteins have the amino acid residues His (H), Glu (E) and Arg (R) at positions 5, 9 and 13 respectively, the IND/HEC sub-family proteins possess instead the amino acid residues Gln (Q), Ala (A) and R respectively at those positions. The ‘E’ at position 9 in this domain is necessary for binding the E-box (5’- CANNTG-3’) which is a palindromic DNA-binding motif recognized by bHLH proteins (Toledo-Ortiz, 2003). IND and the HEC proteins do not possess this residue and predictably IND has been shown to bind to a variant of the E-box with the non-

palindromic sequence 5'-CACGCG-3'. The mechanism of binding is yet to be understood (Girin et al., 2011) and it is not known whether the HEC proteins can bind this motif.

1.3.2 The apical-basal patterning factors

The apical-basal axis of the carpel is distinctive as it exhibits a precise zonation of tissue domains and relies on the precise scaling of these domains. Among the factors which affect both the above parameters is *ETTIN* (*ETT*). ETT is an Auxin Response Factor (ARF) and is also known as ARF3. *ett* mutants have pleiotropic effects on the number and patterning of different organs such as the sepals, petals, stamens and leaves (Sessions et al., 1997, Pekker et al., 2005). In the gynoecium, *ett* mutants show patterning defects along the medio-lateral as well as adaxial-abaxial plane in addition to the apical-basal domain and it could be considered as a master regulator of gynoecium patterning (Fig. 4; Sessions and Zambryski, 1995).

Defects in *ett* gynoecia can be seen very early from stage 7-8 as the carpel assumes a trumpet-like shape instead of a cylindrical one and often there is precocious development of stigmatic tissue at stage 8. At the later stages *ett* mutants show reduction in ovary size and a corresponding increase in basal gynophore length and apical stigmatic and stylar tissue domains suggesting defects in establishing apical-basal boundaries (Fig. 4,A) (Sessions and Zambryski, 1995, Sessions, 1997, Sessions et al., 1997). The top of the carpel is sometimes split or open such that in intermediate and strong alleles the ovules are often exposed. Defects along the adaxial-abaxial boundaries are also seen as the transmitting tract develops externally in the mutants and pollen grains are often seen germinating on the abaxial (outer) surface of *ett* mutants. The phenotypic severity of the above defects increases with increasing allelic strength, such that in the strong *ett-1* mutant, ovary development is negligible (Sessions et al., 1997). Ovule positioning and development is affected and the ovules have defects in integument development (Kelley et al., 2012).

Within the gynoecium, *ETT* expression is directly regulated by GIANT KILLER (GIK), a chromatin modifier, which negatively regulates this gene by binding directly to its promoter (Ng et al., 2009) and the STV1 protein which regulates *ETT* transcription via upstream Open Reading Frames (uORFs) found within the *ETT* gene (Nishimura et al., 2005). Correct spatial expression of *ETT* is maintained post-translationally by TAS3 trans-acting siRNA, which has been shown to be necessary for the apical-basal patterning of

gynoecium and adaxial-abaxial patterning of leaves, which ETT also coordinates (Fahlgren et al., 2006).

The downstream targets of ETT represent a point of intersection of adaxial-abaxial and apical-basal patterning pathways. ETT regulates *SPT* expression and restricts the expression of *SPT* within the marginal tissue domains. In the *ett* mutant *SPT* expression is extended ectopically into the abaxial-lateral surface of the carpel. This is in agreement with the phenotype of the *ett-3 spt-2* double mutant, which shows a rescue of the apical-basal zonation as the extended apical domain and overgrowth of stigmatic tissue seen in the *ett* mutant is partially recovered in the *ett-3 spt-2* double mutant (Heisler et al., 2001). Expression of the *HEC* genes is also regulated by ETT and like *SPT*, the expression of the *HEC* genes is also ectopically extended abaxially in the *ett* mutant (Gremski et al., 2007).

Besides ETT, coordination of apical-basal patterning is also regulated by *PINOID (PID)*. *PID* belongs to the AGC group of serine-threonine kinases and is an auxin inducible gene (Benjamins et al., 2001). It is involved directly in the auxin signalling pathway as it regulates auxin transport by modulating the polarity of PIN-formed (PIN) proteins which are polar auxin efflux transporters (Friml et al., 2004). In the stronger *pid* alleles there is no differentiation of the tissues of the gynoecium and it appears only as a cylindrical structure topped by stigmatic tissue indicating that *pid* affects gynoecium development from a very early stage (Fig. 4B). Weak *pid* alleles show mispatterning of the ovary and an overgrowth of stigmatic tissue although, unlike the *ett* mutant, the boundary positions of these domains are shifted only slightly and the adaxial-abaxial patterning is not affected.

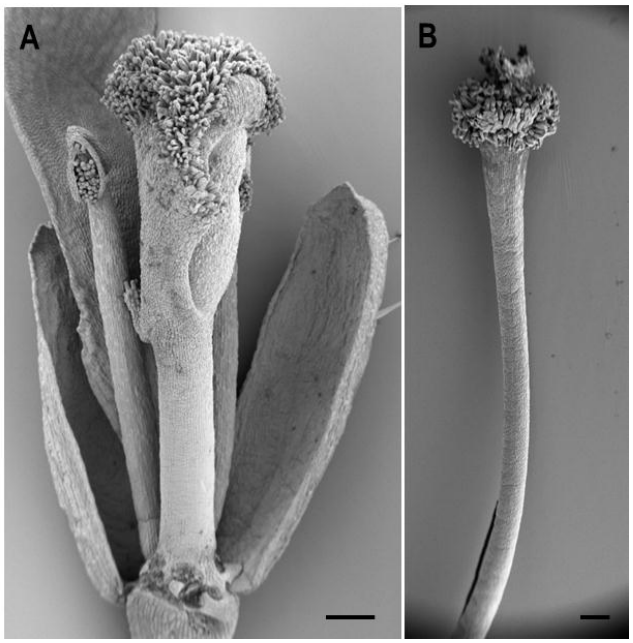

Figure 4 Phenotype of apical- basal patterning mutants.(A) *ett-3* mutant- intermediate allele. **(B)** *pid-9* mutant- strong allele. Scale Bars represent 200µm.

As illustrated above, correct apical-basal patterning relies on the proper development of each domain of the carpel. If we consider the valves of the carpel, a distinct set of transcription factors are involved in developing this tissue region. Among the factors which are crucial to pattern the valves is *FRUITFULL (FUL)*. *FUL* encodes a MADS Box transcription factor and it is expressed in the valves. The fruits of the *ful* mutant are small and stunted as cell expansion, occurring post-fertilisation, is affected in these mutants (Gu et al., 1998). Although the fruit mutant phenotype is most conspicuous, the defects in valve development are evident early on in the carpel itself. While defects in cell expansion are clearly observed, the cells of the inner epidermis of the carpel possess supernumerary cells which are much smaller than WT suggesting that FUL has tissue specific effects in its ability to control cellular differentiation and growth.

FUL expression is controlled upstream by the transcription factors *FILAMENTOUS FLOWER (FIL)*, *YABBY3 (YAB3)* and *JAGGED (JAG)*. All three of the factors are required for the development of lateral organs and within in the carpel they function redundantly to regulate *FUL* expression in the valves (Dinneny et al., 2005). While *FIL* and *YAB3* are involved in establishing lateral organ polarity, *JAG* is specifically required for lateral organ growth. All three genes are expressed very early on during carpel development and although they do not function as carpel identity factors, they are hypothesized to function in concert with the identity factors to define the precise domains of the carpel. Definition of the lateral domains is also achieved by *ASYMMETRIC LEAVES* 1(*AS1*) which restricts the expression of the replum factors *REPLUMLESS (RPL)* and *BREVIPEDICELLUS (BP)* and prevents their ectopic expression into the valves. Conversely, it was recently shown that both *BP* and *RPL* also restrict the expression of the all the above lateral domain factors within the valves to ensure precise replum development and definition (Gonzalez-Reig et al., 2012).

Also required for correct apical-basal patterning is *CRABS CLAW (CRC)*, a YABBY transcription factor. *crc* mutant fruits are shorter and wider than WT fruits and also have a split style (Bowman and Smyth, 1999). Overexpression of *CRC* under a 35S promoter results in the carpel developing into a solid cylinder topped with stigmatic tissue (Eshed et al., 1999). This phenotype is reminiscent of auxin mutants and is worth investigating in the future.

As seen from the above examples, correct gynoecium patterning is not only dependent on the coordinated activities of different transcription factors but it is also coupled with the regulation of hormone dynamics, particularly the hormone auxin. Aspects of auxin biology will now be elucidated in the following section.

1.4 Hormonal Regulation of Gynoecium Development

1.4.1 Auxin: Signalling and Transport

The plant hormone auxin has numerous and varied functions in plant development which includes organ patterning. The active and most abundant auxin found in plants is Indole 3-Acetic Acid or IAA and it is this compound that will be referred to as 'auxin' throughout the thesis. Precise organ patterning and growth by this hormone is dependent on two factors - auxin signalling and subsequently polar auxin transport.

Auxin signalling involves the initial perception of the auxin molecule by the receptors - Transport Inhibitor Response1 (TIR1) and/or Auxin Binding F-BOX (AFB) proteins. TIR1 was the first auxin receptor to be characterized (Kepinski and Leyser, 2005, Dharmasiri et al., 2005) following which the 5 AFB proteins in *Arabidopsis* have also been shown to function as auxin receptors (Calderon Villalobos et al., 2012). The TIR1/AFB proteins are F-Box proteins which are components of a larger protein complex called the SCF (Skp1-Cul1-Fbox) complex. This complex is a class of E3 ubiquitin ligases which mark target proteins for degradation by ubiquitinating them following which they are degraded by the 26S proteasome (Mockaitis and Estelle, 2008). The structure of the TIR1/AFB proteins is characterized by the presence of 18 leucine rich repeats (LRRs) within which is found the auxin binding pocket. The auxin binding pocket of TIR1 is formed between a long loop projecting out from the second TIR1 LRR and the inner concave surface of the carboxy-terminal half of the TIR1 LRR domain (Tan et al., 2007). The binding pocket is largely hydrophobic in nature and contacts made with auxin are primarily with the carboxyl end of the molecule. The carboxyl group undergoes hydrogen bonding and forms a salt bridge with a conserved basic residue in the floor of the auxin binding pocket. The contribution of the indole-ring to the interaction is minimal and mainly involves spatial accommodation of the molecule in the binding pocket (Calderon-Villalobos et al., 2010). For this reason, the TIR1/AFB proteins show a degree of promiscuity in the ligands they can bind. Several

auxin analogues can also bind to TIR1/AFB which include the commonly used synthetic analogues α-Naphthalene Acetic Acid (NAA) and 2,4-Dicholorophenoxy Acetic Acid (2,4-D). These molecules are similar to IAA in that they possess a carboxylic acid end but they differ in their aromatic ring structures as a result of which the affinity of these compounds to the TIR1 receptor differs slightly.

The second step in auxin signalling involves the relay of the auxin signal to modulate gene expression. The control of the expression of target genes is achieved by a transcription factor complex comprising the AUX/IAA and Auxin Response Factor (ARF) proteins. Critical to the auxin signal mediation is the interaction of TIR1/AFB auxin receptors with the AUX/IAA proteins which occurs in the presence of auxin. The AUX/IAA proteins function as transcriptional repressors and 29 such proteins are encoded by the *Arabidopsis* genome. AUX/IAA proteins possess a modular structure comprising four domains. The N-terminal domain Domain I (DI) is required for transcriptional repression, while Domain II (DII) is the primary domain required for the interaction with the TIR1 receptor. The C-terminal domain has two domains- Domain III and IV- and is a dimerisation domain through which AUX/IAA dimerise amongst themselves and with ARF proteins (Tiwari et al., 2001, Guilfoyle and Hagen, 2007) .

Domain I of AUX/IAA proteins possesses an Ethylene response factor Associated Repression (EAR) motif which is responsible for recruiting co-repressors of transcriptional regulation. One of the major partners is TOPLESS (TPL) and TOPLESS RELATED (TPR) proteins. These proteins can recruit histone deacetylases which keep chromatin in a closed state favouring transcriptional repression (Szemenyei et al., 2008, Krogan et al., 2012).

The DII domain of AUX/IAA proteins contains a highly conserved Gly-Trp-Pro-Pro-Val (GWPPV) degron motif which is necessary for the interaction with the $SCF^{TIR1/AFB}$ complex. However, recent studies have shown that upon auxin binding, considerable variation exists in the affinities of the different AUX/IAAs to complex with the TIR1/AFB proteins thus adding an additional layer of specificity in the auxin signalling pathway. This difference in affinities has been attributed to amino acid residues lying outside the core degron motif (Calderon Villalobos et al., 2012).

DIII/IV of the AUX/IAA proteins share homology with the C-terminal domain of ARF proteins and facilitates heteromeric AUX/IAA-ARF protein interactions as well as homo- and hetero- dimer formation between the AUX/IAA proteins themselves. Recent crystal

structure and bioinformatics studies on this domain have revealed that this region has a Phox and Bemp1 type I/II (PB1) like structure which is associated with higher order complex formation. The PB1 type I/II domain has an acidic face and a basic face and the interactions proceed directionally between oppositely charged faces. *In planta* analyses also support the idea that AUX/IAA proteins function preferentially as multimers. Overexpression of dominant-negative IAA proteins, with mutations compromising their oligomerization capacity, result in their inability to repress plant growth (Nanao et al., 2014, Korasick et al., 2014).

As mentioned above, the direct downstream targets of AUX/IAA repression are the ARF proteins. 23 ARF proteins exist in *Arabidopsis* with diverse functions and expression patterns (Guilfoyle and Hagen, 2007). Of particular relevance to gynoecium development are ARF3 (ETT), ARF4, ARF5 (also known as MONOPTEROS (MP)) and ARF8 (Pekker et al., 2005, Østergaard, 2010, Vivian-Smith et al., 2001). Like AUX/IAA proteins, ARFs also have a modular structure and are characterized by the presence of 3 conserved domains. The most highly conserved among them is the B3-type DNA Binding Domain (DBD) which is found in all ARFs. Recently, this domain was found to have three distinct regions which include the DBD, a Dimerisation Domain (DD) and an Ancillary Domain (AD) both of which enable dimerisation among ARFs. The structure of the DBD is such that it is surrounded both N and C-terminally by the DD and is thus embedded within it, in a manner much like what is described as a 'taco-like' shape (Boer et al., 2014). The target cis-element of ARFs is called the Auxin Response Element (AuxRE) which has the sequence 5' TGTCTC 3' (Ulmasov et al., 1997). Recently however, a higher affinity AuxRE has been discovered with the DNA sequence 5'-TGTCGG-3'. Additionally it has been found that the nucleotide spacing between two adjacent AuxRE elements isan important determinant of the DNA binding specificity of ARF-ARF dimers (Boer et al., 2014).

C-terminal to the DNA binding region is located the Middle Region (MR). The amino acid sequence in this region classifies ARFs as either transcriptional repressors or activators. ARFs with MRs rich in Glu, Ser and Leu amino acids are considered activators, while ARFs with MRs rich in Ser, Leu and sometimes Pro and Gly are classified as repressors. Transient expression in protoplasts and amino acid sequences indicate that most ARFs appear to be repressors (Guilfoyle and Hagen, 2007, Tiwari et al., 2003).

Domain III/IV as mentioned previously serves as the dimerisation domain with AUX/IAA proteins. The structure of this domain, like the AUX/IAA domain is a PB1 type I/II like domain and thus the mechanism of dimerisation is also conserved among these two families of proteins as determined from crystal structures. All ARFs, except ARF3 and ARF13, possess this domain (Guilfoyle and Hagen, 2007, Korasick et al., 2014).

Thus, auxin signalling proceeds in the following manner (Fig. 5):

In low auxin environments, AUX/IAA proteins along with the co-repressor TPL form a repressive complex binding to the ARFs. Based on new crystallographic evidence, it is likely that the AUX/IAAs repress ARFs in the form of multimeric heterotypic or homotypic complexes. ARF proteins on the other hand remain bound to DNA as either hetero- or homo-dimers and interaction between these two protein families occurs via their C-terminal DIII/IV regions.

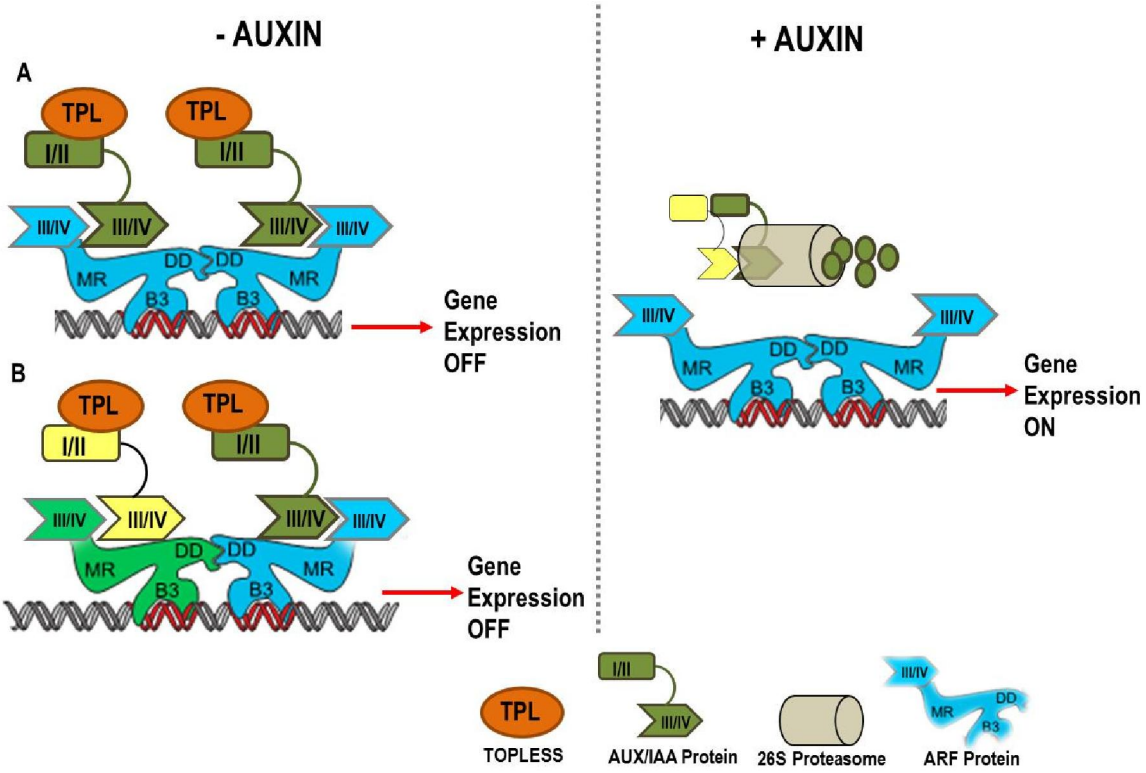

Figure 5 Auxin signalling pathway. Under low auxin conditions AUX/IAA proteins repress ARF proteins either as homodimers **(A)** or as heterodimers **(B).** This is aided by TPL a transcriptional repressor which keeps chromatin in a closed conformation. Once auxin reaches a threshold level, it binds to the SCF $^{TIR/AFB}$ receptor which ubiquinates AUX/IAA proteins which are then degraded by the 26S proteasome. The ARF proteins are then free to regulate the transcription of their target genes. *Adapted from Boer et al.,2014 and Korasick et al.,2014.*

Once auxin levels increase, IAA binds to the F-box proteins in the $SCF^{TIR1/AFB}$ complex, which then recruit and target AUX/IAA proteins. Auxin binding to the co-receptor complex between TIR1/AFB and AUX/IAA proteins allows the SCF complex to ubiquitinate the AUX/IAA proteins which are then degraded by the 26S proteasome. Degradation of the AUX/IAA proteins releases the ARFs from repression and they are then active to regulate their target genes.

Although TIR1/AFB proteins are the primary receptor molecules involved in auxin signalling, two other proteins have also been identified as capable of binding auxin. These however, do not function via the canonical auxin signalling pathway i.e through the association with the AUX/IAA proteins. The first receptor SKP2A, belongs to the F-Box protein family like the TIR1/AFB proteins and has also shown to bind IAA and its analogues 2,4-D and NAA. Although the crystal structure of the protein has not been obtained, site-directed mutagenesis experiments suggest that the mechanism of auxin-binding might be analogous to that seen in the TIR1/AFB receptors. SKP2A is a regulator of cell division and upon auxin binding, the protein is degraded by an unknown mechanism. Analyses in seedling roots indicate that the primary function of the regulation of SKP2A by auxin is to create a balance between cell division and cell growth activities to ensure proper root growth (Jurado et al., 2010).

The other receptor identified is Auxin Binding Protein1 (ABP1). ABP1 has been investigated since the 1970's however its role in auxin signalling is only now being understood. Experiments in maize in the 1970's provided the first indirect evidence for the existence of ABP1, as membranes from maize coleoptiles were reported to have auxin binding affinity (Sauer and Kleine-Vehn, 2011). Later in 1980's, ABP1 was identified as the protein conferring this property (Sauer and Kleine-Vehn, 2011) though conclusive evidence of this was obtained following the crystallization of the protein by Woo et al. in 2002.

Structurally ABP1 is similar to the germin/seed storage 7S protein family and possess a characteristic β-jellyroll barrel structure formed by two antiparallel β sheets. The auxin binding site is highly hydrophobic lying deep within the β barrel and it precludes the bound auxin almost entirely from surrounding solvents. Like in the TIR1/AFB binding site, auxin binds via its carboxylic end but unlike the TIR1 binding pocket, it requires the presence of

a zinc molecule to anchor it to the binding site. Much like in TIR1/AFB proteins, the binding of IAA does not cause any conformational change to the protein (Woo et al., 2002).

ABP1 is located both in the ER and the plasma membrane. The presence of the KDEL ER-retention motif coupled with immunolabelling experiments have shown that a large fraction of the protein is present in the ER although the functional significance of this is still unknown, while a small fraction of the protein is secreted in the plasma membrane (~ 22% in *Arabidopsis*) (Jones and Herman, 1993, Xu et al., 2014).

Among the major functions ABP1 include the regulation the cell expansion, control of PIN accumulation at the plasma membrane by clathrin-dependent endocytosis and the development leaf pavement cells by cytoskeletal reorganisation.

ABP1's role in cell expansion was observed very early during the studies on this protein both in maize and *Arabidopsis*. Recently, it has been shown that ABP1 regulates cell expansion through the modulation of the expression of cell wall related genes which include expansins, glycosyl hydrolases, xyloglucan endo/transglycosidase hydrolases among others. The changes initiated do not affect the composition of the cell walls but involve changes in the chemical structure of the cell wall components primarily the xyloglucan polysaccharides (Paque et al., 2014).

Auxin and ABP1 work antagonistically in clathrin-dependent endocytosis of PIN proteins. While auxin prevents PIN protein internalization to promote PIN accumulation at the plasma membrane thereby regulating its own efflux; ABP1 on the hand appears to promote endocytosis and internalization of PINs. Auxin binding interferes with its function as mutations in the auxin-binding pocket of ABP1 leads to the internalization of PINs even in the presence of auxin (Robert et al., 2010).

Finally, auxin promotes the association of ABP1 with members of the transmembrane kinase (TMK) family which then activate ROP2 and ROP6 (Rho-like guanosine triphosphatases). Subsequently ROP2 and ROP6 induce actin and microtubular rearrangements, respectively, in these cells thereby regulating leaf epidermis patterning and expansion (Xu et al., 2010).

1.4.2 Auxin transport

Patterning of biological structures such as organs of multicellular organisms involves the precise localization of tissues composed of different cell types. Auxin plays an important role in organ patterning, which is brought about through carefully controlled spatial localization coupled with the generation of appropriate concentration gradients. Both of these are achieved by the activity of auxin-specific carriers which actively transport auxin across cell files.

The major transporters of auxin efflux from a cell belong to the PIN-formed (PIN) family of transmembrane proteins. There are 8 annotated PIN proteins encoded in the *Arabidopsis* genome out of which most are localized to the plasma membrane while a few are localized to the endoplasmic reticulum. Plasma membrane PINs display polarity in their localization and are apically, basally or laterally localized in the cell (Grunewald and Friml, 2010, Viaene et al., 2013). This polarity is context dependent and PIN protein specific - the same PIN protein may have different polarities in different organs and within the organs themselves, different PINs have different orientations. For example, PIN1 is basally localized in the tissues of the root tip while in the apical tissues such as the gynoecia, PIN1 is apically localized (Benkova et al., 2003). PIN2 on the other hand is apically localized in the root cap and root epidermal cells (Grunewald and Friml, 2010). The orientation of the PINs on the plasma membrane is coordinated by a phosphorylation-dependent mechanism. PINs are the targets of phosphorylation of the three Ser/Thr Kinases PID, WAG1 and WAG2 (Friml et al., 2004, Dhonukshe et al., 2010). These three kinases are able to phosphorylate the Ser residues of an evolutionary conserved TPRXS(S/N) motif in PIN proteins and the level of phosphorylation determines the shift in polarity. For instance, overexpression of these kinases results in a basal-apical shift in the polarity of PIN1 and PIN2 in roots (Dhonukshe et al., 2010). In most cases there is feedback between auxin and the localization of PINs and these kinases serve as the molecular switches which determine the orientation of auxin flux.

Other auxin efflux carriers include members of the ABCB/PGP family of auxin transporters. Unlike the PIN proteins, these transporters have a non-polar distribution around a cell and therefore their main function appears to be to facilitate auxin efflux from cells which would then be transported in a polar manner by the PIN proteins (Geisler et al., 2005, Grunewald and Friml, 2010)

Although auxin influx into the cell occurs primarily by simple diffusion in the form of the IAAH molecule, auxin is also transported against the concentration gradient by the AUX/LAX family of auxin influx carriers. The AUX/LAX transporters belong to a small family of transmembrane proteins whose members are highly conserved, which include AUX1 and LIKE-AUX1 (LAX) genes, LAX1, LAX2, and LAX3. The most well characterized member of this family is AUX1 which has roles spanning several aspects of root development including root gravitropic responses and lateral root development; additionally, all the members of the AUX/LAX family function redundantly in phyllotactic patterning (Swarup and Peret, 2012) .

More recently a novel class of transporters has been discovered called the PIN-likes (PILS). These were discovered by *in silico* methods and comprise 7 members which are localized to the ER. The PILs appear to regulate the internal accumulation of IAA and are thus speculated to have a role in regulating auxin homeostasis in specific cellular compartments (Barbez et al., 2012).

1.4.3 The role of Auxin in gynoecium patterning

Auxin functions as a classic morphogen in patterning the gynoecium coordinating both organ growth and tissue identity. This is evident, for example, from the phenotype of the *pid* mutant where not only carpel growth is affected but the distinct tissue domains are also missing. *pid-9* mutants have very strong apical-basal defects as tissue zonation is non-existent and the gynoecium assumes the form of a hollow tube-like structure (Fig. 4,B). Weak *pid-8* alleles also show defects as the valve length often differs in the mutants, the style is shorter and the stigmatic tissue also appears overgrown.

The observed phenotypes are indicative of a role of auxin early during carpel primordium development, however, auxin distribution imaging using a *DR5*:*GFP* reporter construct shows that the functions of this hormone continue into the later stages as well. From stage 10 onwards an auxin maximum is seen as a ring around the apex of the carpel which continues into stage 12 (Girin et al., 2011, Benkova et al., 2003). Prior to this apical ring formation, auxin is concentrated at two lateral foci which are seen from stages 7 to early 9 (this study). The precise function of this auxin maximum is not clear however it appears to be necessary for style development. Mutants in *SPT* show a split-style phenotype and *DR5*:*GFP* expression in these mutants remains localized to two lateral foci never forming an auxin maximum ring (Girin et al., 2011). Complementation of this split-phenotype is

only possible by inducing high levels of auxin within this region, for instance by treating gynoecia with polar auxin transport inhibitors such as 1-N-Naphthylphthalamic acid (NPA) rescues the bifurcated style in the *spt* mutant and this is suggested to be because auxin accumulates in the apical tissues (Nemhauser et al., 2000). A similar effect is seen when overexpressing the *STY1* gene. STY1 has been shown to regulate the auxin biosynthetic gene *YUC4* and overexpression of *STY1* results in elevated auxin levels (Sohlberg et al., 2006). Expression of *STY1* under the 35S promoter is able to rescue the *spt* style phenotype effectively mimicking NPA treatment (Staldal et al., 2008). These are of course artificially created situations and the endogenous mechanism to concentrate auxin in these tissues would involve a combination of auxin biosynthesis and directional transport, both of which have been shown to occur.

Several auxin biosynthesis genes including *YUC4*, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* (*TAA1*) and *TRYPTOPHAN AMINOTRANSFERASE RELATED1* (*TAR1*) are expressed in the apical tissues of the gynoecium and although single mutants do not show any phenotype, double mutant combinations have severe apical-basal defects suggesting that they function from the early stages (Cheng et al., 2006, Stepanova et al., 2008). A second mechanism to ensure proper auxin distribution involves correct auxin transport. The PIN1,3,7 transporters are all localized in an apolar manner within the apical tissues (Moubayidin and Ostergaard, 2014) ensuring auxin is correctly distributed within the stylar tissues. This involves regulation of *PID* and *WAG2* by the IND-SPT heterodimer which can bind to the promoters of the both *PID* and *WAG2* genes. The complex upregulates *WAG2* expression in the stylar tissues while it down-regulates *PID* thus preventing apical-basal localization of PINs and establishing an apolar orientation (Girin et al., 2011).

Although the tissue specific roles of auxin are being deciphered, the mechanism(s) by which coordination of overall growth with correct domain scaling is achieved by auxin is still unclear. The dependence of tissue zonation on the correct levels of auxin in the carpel is apparent as when auxin signalling mutants *axr1*, *tir1* and *ett* mutants and the auxin biosynthetic mutant *yuc4* are treated with NPA, they all show a hypersensitive response to the treatment - tissue differentiation in the carpel body is no longer seen as the valves are no longer present and the carpel is just topped by stigmatic tissue (Fig. 6) (Staldal et al., 2008).

Treating WT gynoecia with NPA significantly alters the ratio of the length of the domains in the apical-basal axis. At high concentrations of NPA (100µM) the ovary becomes extremely reduced while the style, stigmatic tissue and gynophore become conspicuously longer (Nemhauser et al., 2000). This is however not accompanied by any changes in cell identity suggesting that it is the distribution and the levels of auxin which determine the tissue boundaries (Fig. 6).

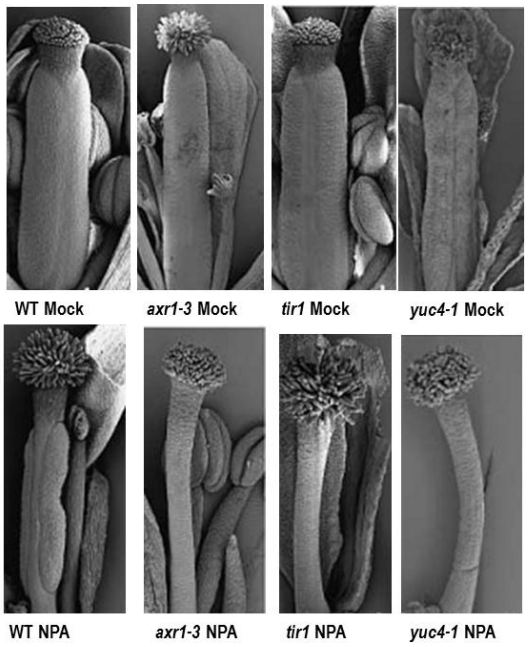

Figure 6 NPA treatment of auxin mutants. Gynoecia of different auxin mutants when treated with 100µM NPA. *Adapted from Staldal et al.,2008.*

Based on these observations Nemhauser et al. (2000) proposed the existence of a gradient in the carpel, with high auxin levels at the top, which is basipetally transported to the gynophore. The readout of levels of auxin in different parts of the carpel would determine the nature of the tissue, for instance, high auxin levels correspond to apical tissue differentiation, medium level would define the ovary and low levels mark the gynophore. The threshold levels of auxin are determined by two hypothetical boundaries which are established during the early stages of development. The first boundary lies between the style and the ovary while the second lies between the ovary and the gynophore. Changes in gradient of auxin or in establishing the boundaries would affect overall development and thereby the position of the different tissue domains.

Although this hypothesis is very attractive and was considered valid for a long time, the development of novel auxin reporter constructs, have now shown that the auxin gradient is unlikely to exist. Both *DR5:GFP* and DII-VENUS (Brunoud et al., 2012) auxin signalling

reporters show an auxin maximum at the top of the gynoecium but no gradient appears to exist in the main body of the carpel (Laila Moubyidin unpublished results, this study).

Based on the known auxin distribution patterns, (Hawkins and Liu, 2014) have recently proposed a new model of gynoecium patterning called the 'Early-action Model'. According to this model, gynoecium development relies on the correct establishment of the adaxial-abaxial (AD/AB) boundary early at the time of the emergence of the carpel primordium. The boundary is found at the apex of the primordium and is defined by the sites of auxin biosynthesis. The definition or sharpness of the boundary relies on correct auxin transport flows from the sites of synthesis. Like in the leaf, the AD/AB boundary functions both as a patterning determinant and also drives the overall growth of the organ. Lack or irregular development of the boundary such as in *pid*, *yucca* or *ett* mutants results in irregular or no tissue differentiation analogous to a blade-less leaf. This new model provides an alternative perspective on carpel development and could be tested by expression analysis of the AD/AB factors patterning the gynoecium, along with cell ablation of the auxin biosynthesis foci.

In conclusion, the role of auxin as a major morphogenetic regulator of the carpel is evident from the phenotype of mutants with defects in various aspects of auxin dynamics. To ensure correct patterning however, a necessary requirement would be to incorporate feedback between auxin and the downstream targets of auxin signalling to ensure correct spatio-temporal regulation of target genes. Currently, genetic networks patterning the gynoecium have been well characterized and concurrently, the role of auxin has also been studied, but what is lacking is an understanding of how these two components of patterning intersect. Thus, what is now required is an integrated view of the transcriptional and hormonal regulatory mechanisms that serve to carry out the role of auxin in gynoecium morphogenesis.

1.4.4 Role of Cytokinin in Gynoecium Patterning

As illustrated in the previous sections, the roles of auxin in carpel development have been studied extensively in the last few decades. Relatively little, however, is known about the function of cytokinin in patterning the gynoecium and novel roles for this hormone are just emerging. Cytokinin distribution can be visualized using the *TCS*:*GF*P reporter construct, and within the developing carpel the expression of this reporter is localized to the marginal tissues (Muller and Sheen, 2008). Mutants in cytokinin signalling however do not show

any observable phenotype but modifying cytokinin levels either exogenously or endogenously affects both marginal tissue development and the apical-basal patterning of the carpel (Fig. 7) (Zuniga-Mayo et al., 2014).

The most conspicuous effect is seen with the application of the synthetic cytokinin benzylaminopurine (BAP) which induces over-proliferation of marginal tissue causing the development of outgrowths on the gynoecium. Ectopic expression of the cytokinin biosynthesis gene *IPT7* under fruit specific promoters also has a similar effect, as the size of the replum increases (Marsch-Martinez et al., 2012). Increase in cytokinin levels has been shown to promote the meristematic activity of reproductive meristems including the placental tissues which results in the development of more ovules and consequently increased seed set per silique (Bartrina et al., 2011). Thus, the effect of cytokinin at the carpel marginal tissue can be explained as a promotion of the meristematic identity of the medial tissues which possess a quasi-meristem like state (Girin et al., 2009) .

Affecting the levels of cytokinin also disrupts the apical-basal patterning of the gynoecium mirroring the phenotypes observed when gynoecia are treated with NPA (Fig. 7). The cross-talk between the auxin and cytokinin is clear as BAP treatment leads to ectopic and apolar localization of PINs in the valves of carpels while NPA treatment appears to increase the levels of cytokinin in the marginal meristem as visualized from the *TCS*:GFP reporter (Zuniga-Mayo et al., 2014). Auxin and cytokinin appear to function in a positive feedback loop as mutants in auxin signalling, transport and biosynthesis are hypersensitive to BAP treatment and show enhanced apical-basal defects; conversely mutants in cytokinin signalling are hypersensitive to NPA treatment (Zuniga-Mayo et al., 2014) .

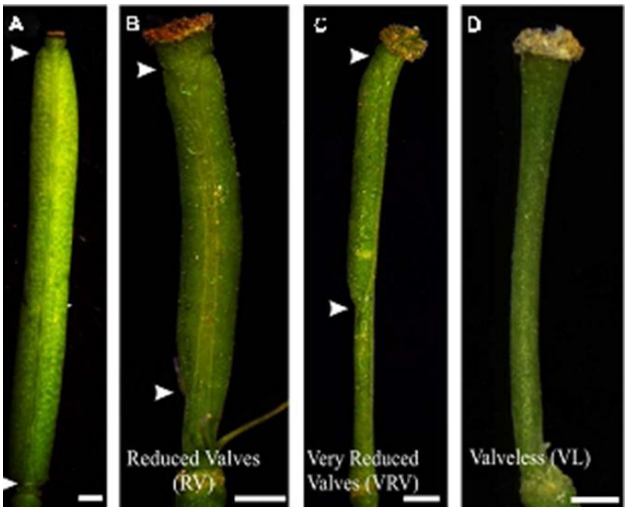

Figure 7 BAP treatment of gynoecia. Phenotypic variability of gynoecia treated with 100μM BAP. **(A)** Mock treated gynoecium, **(B)**, **(C)**, **(D)** categories of valve phenotypes (arrows indicate end of valves). Scale bars (A) 1mm, (B,C) 400μm, (D) 200μm. *Adapted from (Zuniga-Mayo et al., 2014)*

It is unclear whether cytokinin affects auxin transport directly or whether this is mediated at the level of biosynthesis. These data present a new a line of investigation where there appears to be a synergistic role for both hormones in establishing the axes of polarity of the carpel. Analysis of the distribution of both hormones during the early stages of carpel primordium development would perhaps give more clarity as to the morphogenetic roles of each hormone in organ patterning.

1.5 Project Aims

This project aims to further our understanding of the regulatory mechanisms at play during the development of the gynoecium. It has attempted to bridge the gap between genetic regulation and hormonal regulation by detailed analysis of regulatory pathways where there is cross-talk between the two. It has approached the problem of gynoecium patterning in two ways: first, by focusing on the protein-protein interactions within the transcription factor networks functioning in the gynoecium, and second, by using reporter lines to monitor spatio-temporal auxin dynamics in the gynoecium. Finally, the conservation of these protein-hormone interactions in other *Brassica* species has been explored to determine their significance through evolution.

CHAPTER 2

CHAPTER 2- Materials and Methods

2.1 Introduction

The methods described in this chapter have been in regular use during the duration of this project. Methods used for specific experiments have been described in the relevant chapters.

2.2 General Methods

2.2.1 Edwards Quick DNA Extraction

Two young leaves of the plants to be genotyped were collected in Eppendorf tubes. 400 µl of DNA extraction buffer (200mM Tris HCl pH7.5, 250mM NaCl, 25mM EDTA, 0.5% SDS) was added to the tissue which was ground using a pestle. This mixture was subsequently centrifuged at 13,000rpm for 5-7 minutes and 300µl of the supernatant was transferred to a fresh Eppendorf tube. The supernatant was mixed with 300µl isopropanol and left at room temperature for 2 minutes. Following centrifugation at 13,000rpm for 5 minutes, the supernatant was discarded and the pellet was resuspended in 70% ethanol. After a second round of centrifugation at 13,000rpm for 5 minutes, the pellet was air dried and dissolved in 80µl H2O. The extracted DNA samples were stored at -20ºC (modified from Edwards et al, 1991).

2.2.2 General PCR Protocols

PCR reactions were prepared to a final volume 20µl and contained 1X PCR Buffer, 0.2mM dNTPs each, 1µM each of the Forward and Reverse Primer, 1-2µl of Taq Polymerase and 50-100ng of gDNA. PCR reactions were carried out in GSTORM Thermocyclers and products were analyzed using agarose gel electrophoresis. The PCR programmes used are listed below:

PCR programme used for Phusion Taq Polymerase (NEB) and Standard Taq Polymerase (NEB)	
1	110^{o}C : Heated Lid
2	98^{o}C for 30sec
3	98^{o}C for 10sec
4	55^{o}C (Or as per primer requirements) for 30sec
5	72^{o}C as per amplicon size
6	Repeat steps 3-5 for 35 cycles
7	72^{o}C for 5min

For colony PCR the programme is modified and a 5min boiling step at 95^{o}C is added prior to the start of the PCR to lyse the bacterial cells.

2.2.3 Sequencing Reactions

Sequencing reactions (10µl) contained 1µl Big Dye v3.1, 1.5µl 5xSequencing reaction buffer, 0.32µM of the specific primer and 1µl DNA (approx. 100-200 ng/µl) or PCR products (2-5ng/µl per 100bp). Sequencing reactions were carried out in a GSTORM PCR Thermal cycler with the following cycle:

PCR programme used for Sequencing Reactions	
1	110^{o}C : Heated Lid
2	96oC for 1min
3	96°C for 10sec
4	50°C for 5sec
5	60°C for 4min
6	Repeat steps 3-5 for 25 cycles

2.2.4 Cloning Methods

Cloning of most constructs used in this project utilized standard Restriction Enzyme-based cloning as described in Sambrook and Russell, Third Edition, 2001. Gateway cloning was carried out according to the manufacturer's instructions.

(Invitrogen, https://tools.lifetechnologies.com/content/sfs/manuals/gatewayman.pdf). Ligation Independent Cloning was used to clone fluorescent protein reporter constructs as described in (De Rybel et al., 2011).

2.2.5 E.coli Heat-shock Transformation

DH5α or Top10 *E. coli* competent cells were used for transformation. Competent cells were thawed and kept on ice for 5-10min. 1-10µl of the DNA ligation or plasmid to be transformed was gently mixed with 100µl of competent cells and left on ice for another 20min. Following this, competent cells were heat-shocked for 90sec at 42ºC and chilled on ice for 1-2min. Finally, 500µl of LB was added to the cells and incubated at 37ºC shaking at 200rpm for 1hr. Transformed cells were plated on selective media and incubated at 37ºC overnight until colonies appeared.

2.2.6 Electroporation-based transformation

Transformation of *Agrobacterium tumefaciens* was carried out using AGL1 electro-competent cells (25µg/ml carbenicillin, 25µg/ml rifampicin) were used for transformation. 50-200ng of the DNA to be transformed was added to 40µl of electro-competent cells and mixed gently. This mix was placed in a prechilled electroporation cuvette and subjected to a pulse length of 8-12 ms in Biorad GenePulser (settings: 2.50kV, 25µFD and 400 Ohms). 1ml of LB was immediately added to the cuvette following the pulse and, subsequently, cells were incubated at 28°C with shaking at 250rpm for 2-3hrs. Transformed cells were plated on the appropriate selective media (which included the AGL1 specific antibiotics and the plasmid specific antibiotics) and incubated at 28°C for 2-3 days until colonies appeared.

For electroporation of *E. coli*, the same methodology was used with the following modifications: Genepulser settings for *E. coli* were the same except a lower resistance setting of 200 Ohms was used. Following the electric pulse 500µl SOC medium was added

to the cells which were then incubated for 1hr at 37^{o}C shaking. Cells were then plated in the appropriate selective media and incubated overnight.

2.2.7 Arabidopsis floral dip transformation

A single *A. tumefaciens* colony carrying the plasmid to be transformed was grown in 5 ml of LB medium with the appropriate antibiotics (which included 25µg/ml rifampicin and the plasmid specific antibiotics) at 28°C with shaking at 250rpm for 24hrs. This preculture was used to inoculate 500ml of LB with the vector specific antibiotic(s) and the new culture was incubated at 28°C with shaking at 250rpm overnight until a saturated OD_{600} was reached (OD_{600}>1.5). The next morning the OD_{600} of the overnight culture was recorded and the culture was centrifuged at 5000rpm for 20 mins at room temperature. The pellet was resuspended in 5% sucrose to an OD_{600} approximately of 0.8 taking the saturation OD_{600} as reference. Just before the floral dipping, Silwet L77 was added to 0.05%. Ten pots containing five Arabidopsis plants each were dipped per plasmid to be transformed. Plants were dipped for approximately 2mins with the occasional shaking. After the dipping, plants were bagged overnight to maintain the humidity and the next day they were transferred to containment.

2.2.8 Yeast Methods

2.2.8.1 Yeast Transformation

Stains Y187 and AH109 were used to transform the necessary construct. For consistency during the project, all constructs in plasmid pGAD424 (which possess the GAL4 AD domain) was transformed into the yeast strain Y187 and constructs in plasmid pGBT9 (which possess the GAL4 BD domain) were transformed into the yeast strain AH109. Yeast strains were vortexed in 2ml YPDA (with 2% Glucose added) and this was then used to inoculate larger 20-30ml cultures of YPDA in 50ml Falcon tubes. Cultures were then kept shaking at 180rpm overnight at 28^{o}C. The next day the cultures were centrifuged at 4000rpm for 10min to pellet the cells. The pelleted cells were resuspended in 10ml 1x LiTE solution [0.1M Lithium Acetate and 1x TE Buffer (0.01M Tris HCl and 1mM EDTA) pH 7.5] and centrifuged for 10min at 4000rpm. The pelleted cells were resuspended in 1.5ml 1x LiTE Buffer, transferred to an Eppendorf tube and centrifuged at 6000rpm for 5min. The supernatant was discarded and the pelleted cells were resuspended in 1.2ml 1x LiTE Buffer. For the transformation approximately 500- 1µg of plasmid DNA was pipetted

into an Eppendorf tube and if a strain was cotransformed then the concentrations of both plasmids in the mixture were kept approximately the same. The following components were added to the plasmids in the following order: 8µl Salmon or Herring sperm DNA, 50µl of the appropriate yeast strain, 10µl DMSO and 300µl of PEG/LiTE solution [50% Polyethylene Glycol (3000 or 4000) and 1x LiTE solution]. The yeast cells were then mixed gently either by pipetting or by inverting the Eppendorf tube gently and subsequently transferred to a 28^{o}C incubator for 30min with gentle agitation. The cells were then heatshocked at 42^{o}C for 30min. Cells were then centrifuged for 5min at 6000rpm and the pellet was resuspended in 100µl sterile water taking care to remove all of the PEG/LiTE solution. 100µl of the cell pellet was then plated on selective SD agar media (Formedium) and incubated at 28^{o}C for 3-4 days.

Transformations for yeast one-hybrid were performed as above with a few modifications. The yeast strain YM4271 was used for these experiments the plasmid vector used to clone the cis-element was linearized by the appropriate restriction enzyme as per manufacturer instructions (Clontech, MATCHMAKER One-Hybrid System, User Manual- PT1031-1).

2.2.8.2 Yeast Mating

Each yeast strain was resuspended in 2ml YPDA medium and vortexed for 1 min. 500µl of AH109 and 500µl of Y187 was mixed together in a universal and kept at 28^{o}C overnight. The next day 2µl of the overnight culture was diluted in 15µl of sterile water and this was plated on selective SD-LT plates. Plates were kept at 28^{o}C for 3-4 days.

2.2.8.3 Yeast Interaction Plate Setup

All interaction tests were setup on SD agar selective plates. 2 large yeast clones to be tested were taken a diluted in 30µl of water and 5µl of this solution was then plated on the selective plate. The process was repeated for 2 technical repeats and 2 biological repeats.

CHAPTER 3

CHAPTER 3- Characterization of Protein-Protein Interactions among Gynoecium Patterning Factors

3.1 Introduction

In the last decades, mutants defective in carpel development have helped elucidate the genetic pathways which function to pattern the gynoecium. These genetic analyses have revealed the roles of different transcription factors which function to develop the different domains of the gynoecium (Balanza et al., 2006). These proteins belong to divergent transcription factor families, but it is evident that transcription factors with roles in leaf development have been co-opted to assume similar and additional roles in the carpel, while other carpel-specific factors have also emerged to define novel carpel-specific domains (Ostergaard, 2009). Genetic interactions have of course shed some light on our understanding of gynoecium development, however, as in leaf and floral development (Causier et al., 2010), combinatorial protein-protein interactions are an important element in organ development. This is an aspect that requires further investigation in the study of gynoecium development.

The prominent role of transcription factor regulatory networks in carpel development is seen, for instance, among apical-basal patterning factors. SPT and the IND/HEC subfamily of bHLH proteins (Toledo-Ortiz, 2003) are all required for marginal tissue development which includes the apical tissues: the stigma and the style, and the medial tissues: the septum and the transmitting tract (See Chapter 1, Fig1). Overexpression of SPT using a *35S::SPT* construct does not exhibit any phenotype indicating that SPT requires other interacting partners to carry out its carpel-specific functions (Girin et al., 2011). Indeed, overexpression of both IND and SPT results in ectopic carpelloid tissue development indicating the significance of this heteromeric complex to form the apical tissues. Both proteins also interact in yeast two-hybrid assays and SPT also interacts with the HEC proteins which share the same functions as the above two proteins (Gremski et al., 2007) suggesting that these proteins are likely to function *in planta* by forming large oligomeric complexes.

Establishing apical-basal patterning is also a function of ETT/ARF3 which can be considered a master regulator of gynoecium patterning (Sessions et al., 1997, Sessions and

Zambryski, 1995). ETT is an Auxin Response Factor and has been shown to function as a transcriptional repressor. It has a unique structure among ARFs as it does not possess the C-terminal dimerisation domains which facilitate ARF-ARF and ARF-Aux/IAA protein interactions (Guilfoyle and Hagen, 2007).

ETT is able to homodimerise (Boer et al., 2014) and the proteins that are known to interact with it include KAN4 (Pekker et al., 2005) which is required for ovule development and SEUSS where the interaction is most likely important for developing whorls 1-3 of the flower (Pfluger and Zambryski, 2004).

Both ETT and SPT functions converge for correct apical-basal patterning. ETT has been shown to restrict SPT expression to the marginal tissue domains as in the *ett-3* mutant, *SPT* expression extends ectopically into the abaxial domain and the phenotype of the *ett-3 spt-2* double mutant suggests that at least some of the patterning defects seen in the *ett* mutant are a consequence of *SPT* misexpression (Groszmann et al., 2010). However, as discussed previously, both SPT and IND proteins are necessary for marginal tissue development, therefore, the phenotype of the *ett* mutant also suggests an involvement of IND. Thus this project has analysed the role of protein complexes in the gynoecium patterning- specifically the roles of IND, ETT and SPT in apical-basal patterning.

3.2 Materials and Methods

3.2.1 Expression and analysis of proteins

The following protocol was used for expression of recombinant proteins in *Escherichia coli*. Unless otherwise specified all plasmids were transformed into *E. coli* strain BL21 (DE3). Constructs were not codon optimized therefore the helper plasmid pRI952 was co-transformed to overcome codon bias. 10ml of bacterial cultures were incubated overnight at 37^{o}C and 2ml of these were used to inoculate 50ml flasks of Luria-Bertani media containing the appropriate antibiotic selection. Flasks were kept at 37^{o}C shaking for 3hrs. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside added to a final concentration of 1mM. Following induction flasks were kept for different time periods to assess the best conditions for protein expression and protein folding. 10ml aliquots were collected at different time points to monitor growth and the corresponding OD_{600} was taken.

To extract the proteins cell pellets were resuspended in 1ml of Buffer A [50mM Tris pH8.0, 50mM dextrose, 1mM EDTA], centrifuged for 3min and resuspended in 100µl Buffer A. 100µl 2x lysozyme (8mg/ml lysozyme prepared in Buffer A) was added and samples were incubated at RT for 15min. Samples were kept on ice and sonicated with 10sec pulses (avoiding sample frothing) until cells were lysed. Samples were then centrifuged for 5min and the supernatant was transferred to a new centrifuge tube. To determine whether the proteins were precipitating into insoluble inclusion bodies, the pellet stored after sonication was resuspended in Buffer A containing 8M Urea and boiled at 80^{o}C for 2 min. All samples were analysed on a denaturing SDS page gel.

SDS gel electrophoresis was carried out on Expedeon precast gels and sample preparation, running buffer and staining post-electrophoresis was conducted according to the manufacturer's protocol [http://shop.expedeon.com/products/15-Protein-Electrophoresis/].

3.2.2 In vitro translation using Wheat Germ Protein Expression System

For *in vitro* translation IND, SPT, ETT proteins were cloned into the plasmid vector pSPUTK using the *NcoI* restriction site. *In vitro* translation of proteins was carried out using the Promega TNT SP6 Wheat Germ Protein Expression System [https://www.promega.co.uk/resources/protocols/technical-manuals/0/tnt-sp6-highyield-wheat-germ-protein-expression-system-protocol/]. All the components supplied by the manufacturer were used according to the manufacturer's instructions, the following components were added to a final reaction volume of 50µl: 1ug target DNA, RNAsin (from Amersham) to a final concentration of 40units, 1µl non-labelled amino acid mixture (Catalogue No: L4461). Translation was carried out at 30^{o}C for 2hrs. Samples were stored at -20^{o}C until further use.

3.2.3 Western Blotting

Western blot was carried out on His-tagged IND protein produced by *in vitro* translation (See above). Following protein production, samples were run on precast 12% SDS gels. Samples were transferred to a nitrocellulose sheet by wet blotting for 1hr at 90V in 1x Transfer Buffer [100ml 10x Transfer Buffer (480mM Tris, 390mM Glycine, 0.375% SDS) + 200ml Methanol + 700ml H_2O to 1L final volume]. The membranes were then stained with Ponceau Red for 10-15min to detect protein transfer. Membranes were blocked overnight at 4^{o}C with Blocking Solution [4% Milk powder (Marvel) + 1x PBS]. Blots were then washed for 5 min (shaking) in Wash Solution [1X PBS + 0.1% Tween]. Blots were incubated in 1:1000 dilution of AbCam Anti-His antibody (HIS.H8) in Blocking Solution for 2h at RT. They were then washed 3X for 10min each in wash solution and incubated in 1:20,000 dilution of HRP-conjugated Anti-mouse antibody (ThermoScientific) for 2h at RT. Blots were then washed 4x for 10min each in wash buffer and then incubated for 10-15min in Thermoscientific SuperSignal West Pico Chemiluminiscent Substrate for detection.

3.2.4 Surface Plasmon Resonance Assay

Surface Plasmon Resonance (SPR) experiments were carried out in collaboration with the Lawson Lab in the Biological Chemistry Department at the John Innes Centre. SPR measurements were conducted at 25^{o}C with a BiacoreT200 system (GE Healthcare). All experiments described herein were performed using ReDCaT (Stevenson et al., 2013) with a single Sensor Chip SA (GE Healthcare), which has four flow cells each containing SA

pre-immobilized to a carboxymethylated dextran matrix. For the experiments, flow cells 1 and 2 were used as the reference (FC_{ref}) and test flow cells (FC_{test}), respectively. All samples were prepared and SPR experiments were performed in HBS-EP+ Buffer [150mM NaCl, 3mM EDTA, 0.05% (v/v) surfactant P20, 10mM, HEPES (pH 7.4); GE Healthcare]. For Experiment 5 (See Table 2) which has been discussed in detail, HBS P+ Buffer [Same composition as HBS-EP+ without 30mM EDTA] was used. For Experiment 5, IAA was added to the HBS P+ running buffer at a final concentration of 100µM and protein samples were prepared in the same. DNA samples were purchased from Sigma-Aldrich as desalted ss oligomers at 100mM concentration in water. The test DNA samples were prepared by annealing together the oligomers using a slight excess (1.2:1) of the oligomer with the adapter to minimize the likelihood of the free type (without adapter) would compete with annealed test DNA for the immobilized linker. Complementary oligomers were annealed by heating to 95^0C for 10 min, before cooling to 20^0C. The various DNA oligomers were diluted to their working concentrations using HBS-EP+ buffer.

The SPR assay protocol used for this experiment is as follows: test DNA was injected for 60sec over Flow Cell 2 at 10µl min^{-1} followed by a Buffer Cycle to stabilize the baseline for 120sec. The samples were injected for 60sec over Flow Cell 1 and 2 at 30µl min^{-1} followed by a Stabilization Cycle for 60 sec. The protein was removed with 0.5M NaCl over Flow Cell 1 and 2 for 60sec at 30µl min^{-1} which was followed by a Stabilization Cycle for 60 sec. The oligomers hybridized to the chip was removed using a combination of 1M NaCl and 50mM NaOH for 60 sec at 10µl min^{-1} over Flow Cell 1 and 2 which was followed by a Stabilization Cycle for 120sec.

To interpret the results from SPR experiments the theoretical maximum response, $R_{max,}$ for the proteins tested (the "analyte") binding to the test DNA (the "ligand") was calculated using the formula:

Theoretical R_{max} = (mol. mass analyte/mol. mass ligand) x (response for ligand capture) x (stoichiometry).

However, when the ligand is DNA, it has been suggested that the result needs to be multiplied by a factor of 0.78 because the response associated with nucleic acid binding to the surface is not the same as that for a protein of equivalent mass.

Therefore, for IND protein the R_{max} calculated was:

Theoretical R_{max} = (mol. mass of IND dimer/mol. mass test DNA) x DNA captured x 1x 0.78

The oligos used for the experiments are listed below. The adapter sequence is highlighted in green.

Primer Set	Primer Sequence	Description	Copies of cis-element in oligo
1	SPT SPR1: 5' AGCTGGCGCGTGACAGAC 3' SPT SPR2: 5'GTCTGTCACGCGCCAGCTCCTACCCTACG TCCTCCTGC 3'	E-box in *SPT* promoter	1
2	PID SPR1: 5' CTCTCTCACGCGTTGAAA 3' PID SPR2: 5' TTTCAACGCGTGAGAGAGCCTACCCTACGT CCTCCTGC 3'	E-Box in *PID* promoter	1
3	EboxY1H SPR1: 5'AATTCTCTCACGCGTTGTCTCACGCGTTGT CTCACGCGTTGC 3' EboxY1H SPR2: 5' GCAACGCGTGAGACAACGCGTGAGAC-AACGCGTGAGAGAATTCCTACCCTACGTCC	Oligo used for yeast one-hybrid assays (Girin et al.,2011)	3

	TCCTGC 3’		
4	PIDnative SPR1: 5’TTTTCTGTTTACCTC**TCTCACGCGTTG**AAAAGTGCAATCAAT 3’ PIDnative SPR2: 5’ATTGATTGCACTTTTCAACGCGTGAGAGAGGTAAACAGAAAACCTACCCTACGTCCTCCTGC 3’	Longer segment of *PID* promoter (36bp in length)	1

Table 1 DNA oligos used for SPR experiments

3.2.5 Yeast Methods

The protocols used for the yeast two-hybrid and three hybrid interactions have been listed in Chapter 2- Material and Methods. For the yeast three-hybrid assays, constructs indicated by (-BD) were developed by Sara Fuentes in the lab by modifying the pGBT9 yeast vector to express the protein of interest without the GAL4 BD

3.3 Results

3.3.1 IND and ETT proteins interact in yeast two-hybrid assays

In order to uncover novel protein-protein interactions functioning in the gynoecium, a candidate gene approach was adopted and the yeast two-hybrid screening system was used to identify possible interactions among these proteins. Considering the importance of IND, SPT and ETT in apical-basal patterning (Girin et al., 2011, Sessions et al., 1997), yeast two and three-hybrid assays were performed to test whether these proteins dimerise and/or trimerise. Complex formation among proteins is often sensitive to temperature, therefore the yeast matings among these proteins were setup on SD –LWHA plates and kept at 28^{o}C- the standard temperature optimal for yeast growth- and also at room temperature (RT). A novel interaction between IND and ETT was detected and this interaction was weaker than the IND-SPT interaction as yeast growth was observed after 4 days of incubation and the interaction was also stronger at RT than at 28^{o}C (data not shown) (Fig. 8).

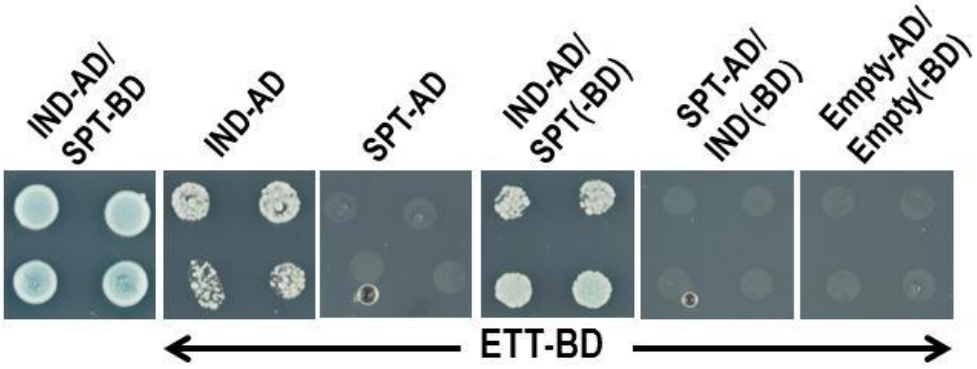

Figure 8 Interaction between IND and ETT. Yeast two-hybrid and three-hybrid interactions between IND, ETT and SPT showing a novel interaction between IND and ETT. Yeast colonies were plated on SD –LWHA plates and kept at RT. Figure shows 2 biological repeats and 2 technical repeats. IND and SPT mating was used as a positive control. (-BD) are constructs expressing the protein of interest without a GAL4 AD or BD domain.

SPT and ETT do not appear to interact in this assay as no colony growth was detected. In the three-hybrid test with these proteins, colonies are only seen in an interaction with IND-AD, ETT-BD and SPT (- BD) (a construct expressing proteins without the GAL4 domains). Conversely, a three-hybrid assay with SPT-AD, IND (-BD) and ETT-BD did not show any yeast growth (Fig. 8). The most likely reason for this is that in the first case, since IND-AD and ETT-BD can form a complex, reporter gene expression occurs. In the second case, as

SPT-AD and ETT-BD cannot interact and IND is expressed without a yeast domain in this interaction, the GAL4 domains are not in proximity of each other and no reporter gene expression occurs.

IND belongs to a specialized clade of bHLH proteins which includes the three HECATE proteins- HEC1, 2 and 3 (Toledo-Ortiz, 2003). The HEC proteins are expressed in the same regions in the carpel as IND and are partially redundant in their roles for marginal tissue development (Gremski et al., 2007). Among them HEC3 shares 62.5% similarity with IND and is its closest homologue. A two-hybrid interaction test between HEC3 and ETT showed that these proteins do not interact, therefore, among the proteins tested which are specifically required for marginal tissue development only IND interacts with ETT (Fig. 9).

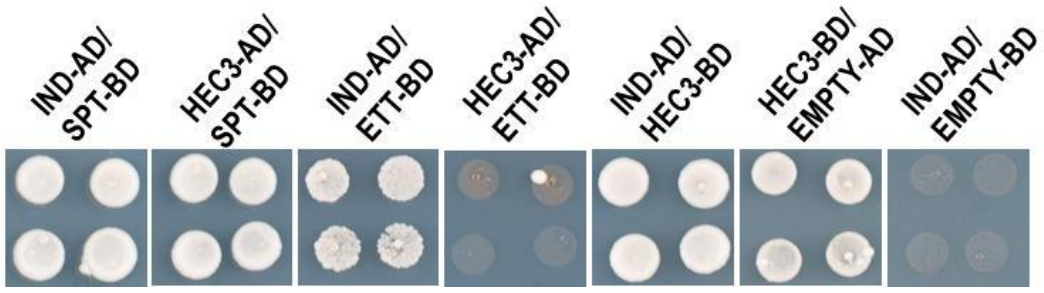

Figure 9 HEC3 and ETT do not interact. Yeast two-hybrid interactions between HEC3 and ETT show no colony growth. The positive control HEC3-AD and SPT-BD shows an interaction. The HEC3-BD /Empty-BD shows yeast colony growth as HEC3-BD is autoactive in yeast. The yeast colonies represent 2 technical repeats and 2 biological repeats. Yeast colonies were plated on SD – LWHA plates and kept at RT.

To determine which domains of IND are required for the IND-ETT interaction, truncated versions of the IND protein were tested (Fig. 10,A). IND has a modular structure and is characterized by the bHLH domain at the C-terminal end. It also possesses a HEC domain which it shares with the HECATE proteins and an N-terminal region -56 amino acids in length- which is unique to this protein and will be referred to as the IND-IS (IND-specific) domain from this point for convenience. The yeast experiment was setup as before at both temperatures. No interaction between the full-length ETT-BD/AD and INDΔ2-AD/BD, INDΔ3-AD/BD was observed, however, a weak interaction was observed with INDΔ1-AD and ETT-BD (Fig. 10,B)

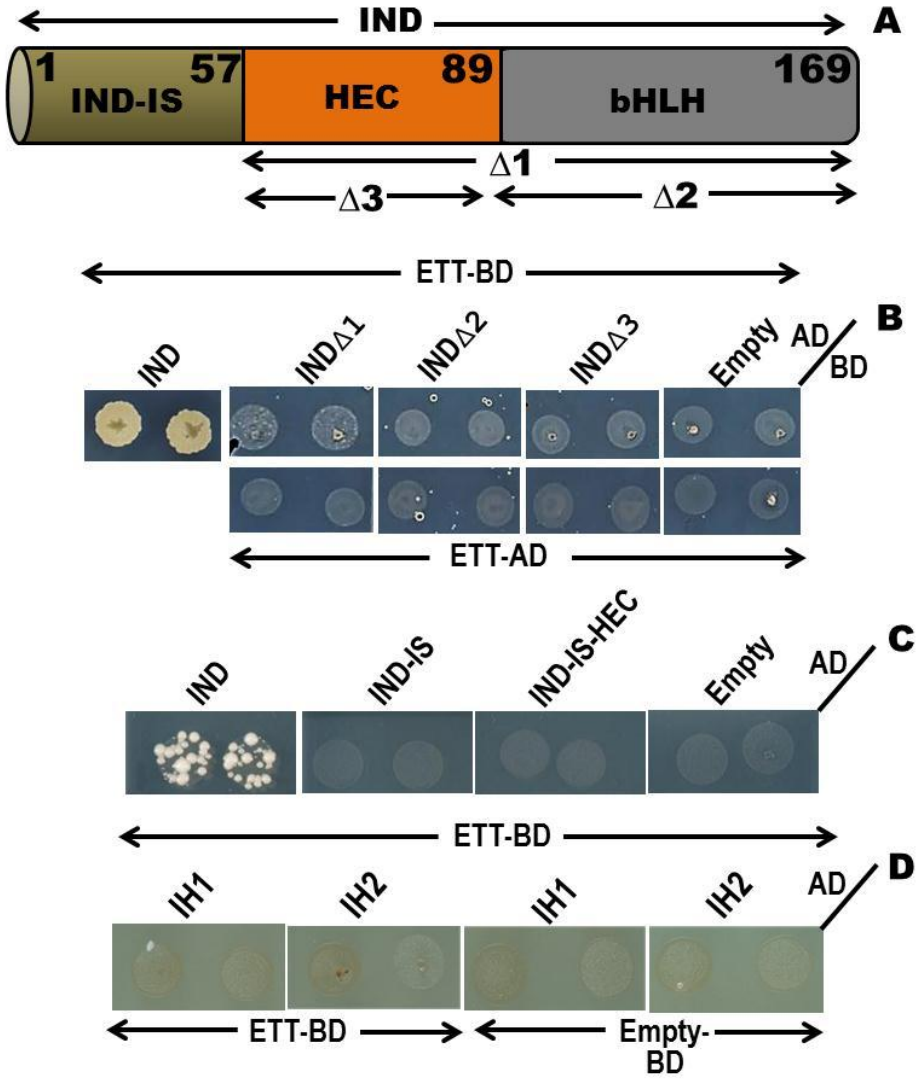

Figure 10 The three domains of the IND protein are required to interact with ETT.
(A) Schematic representation of the IND protein structure. **(B)** Yeast two-hybrid assay showing strong interaction between the full-length IND protein and ETT and a weaker interaction between IND△1 and ETT.The IND-BD construct is auto-active *(Girin et al., 2011)* and was therefore excluded from this assay. **(C)** The IND-IS and IND-IS/HEC truncated proteins do not interact with ETT. **(D)** Chimeric proteins with domains from IND and HEC3 do not interact. IH1: IS domain+ HEC3- (HEC+bHLH), IH2: IND-(IS domain+Hec domain)+HEC3 bHLH domain. Yeast colonies were plated on SD –LWHA plates and kept at RT

A suggesting a weak interaction between ETT and the HEC-bHLH domains. Since a large proportion of the interaction appears to be facilitated by the IND-IS domain the IND-IS domain and the IS domain with the HEC domain were then cloned individually to identify the region which is sufficient for the interaction with ETT. However, no yeast colony growth was observed with ETT for either of the constructs (Fig. 10,C)suggesting that either the entire IND protein is required for the interaction with ETT or that the cloned isolated domains do not adopt the right conformation for the interaction to occur.

To determine if it was the latter case, domains from HEC3 were cloned fused to the IND domains thus forming chimeric IND-HEC3 proteins for expression in yeast, as IND and HEC3 share 84.5% identity in their bHLH domains and 78.6% identity in their HEC domains (EMBOSS Water- Local Alignment). The first construct IND:HEC3-1(IH1) consisted of the IS domain with the HEC and bHLH domain from HEC3, and the second construct- IND: HEC3-2(IH2) - comprised the IND- IS and HEC domain with the HEC3 bHLH domain. The assays revealed that neither of the constructs was able to interact with the full-length ETT. This suggests all three domains of IND are required for this interaction although a large proportion of the interaction occurs via the IND-IS domain (Fig. 10,D).

Interactions among bHLH proteins and ARF transcription factors have been reported, for instance, the bHLH protein BIGPETALp has been shown to interact with ARF8. This interaction proceeds via the C-terminal end of both proteins and a motif –GRSLD-was found necessary for this interaction (Varaud et al., 2011). This motif was found in domain III of ARF8 which is lacking in ETTIN- an ARF with a non-canonical structure (Guilfoyle and Hagen, 2007) and no such motif was found in the protein. Truncated versions of the ETT protein were therefore constructed to determine the domains of the protein which interact with IND and these were cloned in-frame with the GAL4 yeast domains (Fig. 11,A).

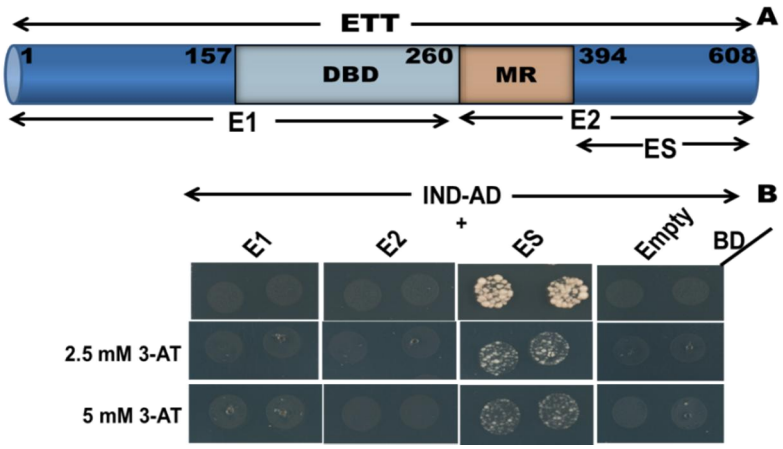

Figure 11 The ES domain is necessary and sufficient for the interaction with IND. **(A)**Schematic representation of the different domains of the ETT protein.**(B)** Truncations of the ETT protein showing an interaction between the ES domain and IND in the presence of yeast growth inhibitor 3-AT. Yeast colonies were plated on SD –LWHA plates and kept at RT.

Domain 1 (E1) comprised the N-terminal half of ETT including the B3 DNA-binding domain, domain 2 (E2) comprised the C-terminal region after the DNA binding domain

and domain 3 (E3) comprised a region specific to ETT and henceforth will be referred to as the ETT-specific or ES domain (Appendix 1, Figure 1). A strong interaction between IND-AD and the ES domain was observed with addition of up to 5mM 3-AT. IND did not interact with domains E1 and E2 (Fig. 11,B).

3.3.2 Indole Acetic Acid can affect the IND-ETT interaction

Studies indicate that transcriptional regulation of gynoecium development is coupled with regulation by the hormone auxin and several nodes exist where both these pathways converge (Staldal et al., 2008, Sundberg and Ostergaard, 2009). In the case of IND, a direct link with auxin has been established as IND has been shown to regulate PIN polarity in the valve margin of the fruit and does so by directly regulating the AGC Kinases PID and WAG2 (Sorefan et al., 2009). Furthermore, ChIP experiments in the lab revealed a curious observation wherein IND-binding and consequently the pull-down of the IND *cis*-element in the *PID* promoter could only be facilitated in the presence of IAA (Personal communication Lars Ostergaard and Pauline Stephenson). This suggests that either IAA influences the IND DNA-binding activity (e.g. through modulation of protein structure), or that IND or its binding site is blocked by an auxin-sensitive molecule such as an Aux/IAA protein.

ETT has been suggested to participate in auxin-dependent pathways involved in polarity establishment in the ovules and leaves (Kelley et al., 2012; Pekker et al., 2005). Within the gynoecium, it has been implicated in interpreting auxin gradients as the phenotype of *ett* mutants closely resemble WT gynoecia treated with the polar auxin inhibitor NPA (Nemhauser et al., 2000). The mechanism(s) by which ETT might be able to translate auxin levels into developmental outputs is unclear, however the lack of dimerisation domains DIII and DIV suggest that ETT might function through an alternative auxin signalling mechanism possibly not involving the canonical TIR1/AFB auxin signalling pathway. This project therefore tested whether IND and ETT proteins could directly bind auxin using the established yeast two-hybrid system.

Yeast two-hybrid interactions were conducted as previously but this time only at RT as repeated experiments showed that this temperature was most conducive for the IND-ETT interaction. IAA was added to the SD –LWHA media at concentrations of 10, 50, 100 and 200μM IAA and the results were observed after 4-6 days of growth. Remarkably, the IND-ETT interaction (as well as the weak INDΔ1-ETT interaction) was disrupted from 50μM

IAA onwards, while the addition of IAA did not affect the IND-SPT interaction thus clearly demonstrating specificity for the IND-ETT complex (Fig. 12, A).

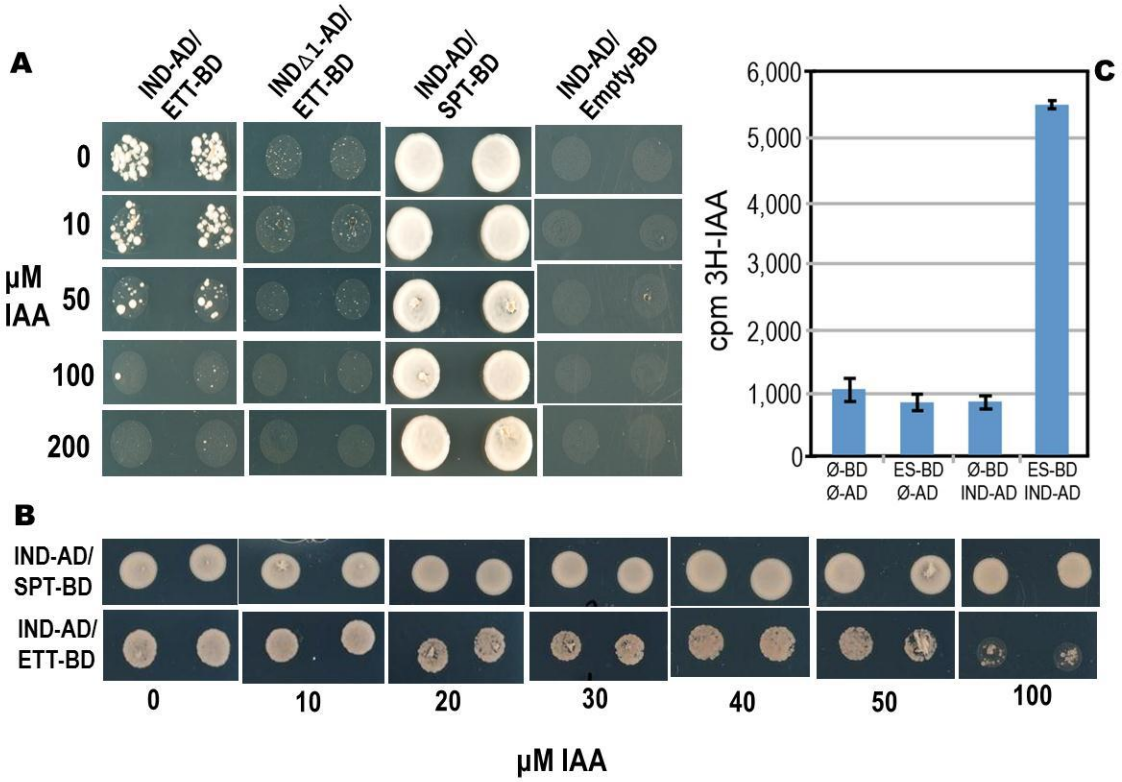

Figure 12 IND and ETT can bind IAA (A) IAA sensitive interaction between IND and ETT. IND△1-ETT interaction is also sensitive to IAA. **(B)** IAA sensitivity in yeast two-hybrid assays is seen from 50µM IAA onwards. Yeast colonies were plated on SD –LWHA plates and kept at RT for both of the above experiments. **(C)** Radioactive IAA retention assay in yeast showing that both IND and ETT are required to bind IAA. Figure shows 2 technical repeats.

Based on these results one could conclude that either one of the proteins is able to bind IAA or the proteins bind IAA together as a complex. The IAA-sensitivity of IND without the IS domain suggests that if the IND protein is able to bind IAA, the IAA binding pocket must be present within the HEC and bHLH domains of the protein. The IAA-sensitivity of this interaction suggests two possible scenarios- either IAA-binding to the protein(s) causes a disruption of the interaction, or that binding results in a conformational change of the protein(s) structure which prevents the yeast GAL4 domains from interacting, thus preventing reporter gene expression. Addition of IAA in a concentration series resulted in a clear effect from 50µM IAA onwards (Fig. 12,B).

In order to determine whether this complex can directly bind auxin and if so which of the protein partners binds IAA, a radioactive IAA-retention assay in yeast was performed by our collaborators in the Friml Lab at the IST in Vienna. The experiments revealed that IAA was bound only when both proteins were present together. Neither of the proteins was able to bind IAA alone as matings with the empty plasmids with either IND-AD or with the ES domain had levels comparable to the control empty plasmid matings (Fig. 12,C). Thus, this suggests that the lack of colony growth in the IND-ETT interaction, when IAA is added to the medium, is possibly caused due to a conformational change in one or both proteins when they bind the IAA molecule.

Conventional IAA signalling is dependent on the F-Box protein TIR1 binding IAA. As an F-box protein, TIR1 is part of the SCF (Skp1-Cul1-Fbox) E3 ligase complex which binds Aux/IAA proteins in the presence of IAA and ubiquinates them, thus targeting them for degradation by the 26S proteasome (Tan et al., 2007). To exclude the possibility that the IAA-sensitive effect is due to an analogous ubiquitination-dependent mechanism in yeast targeting either IND or ETT for degradation, either directly or via a third mediating protein, the proteasome inhibitor MG132 was added to the yeast medium and the assay was repeated. The results show that even in the presence of a proteasome inhibitor, IAA is still able to affect the interaction between IND and ETT, further supporting that IAA is able to directly affect the interaction (Fig. 13).

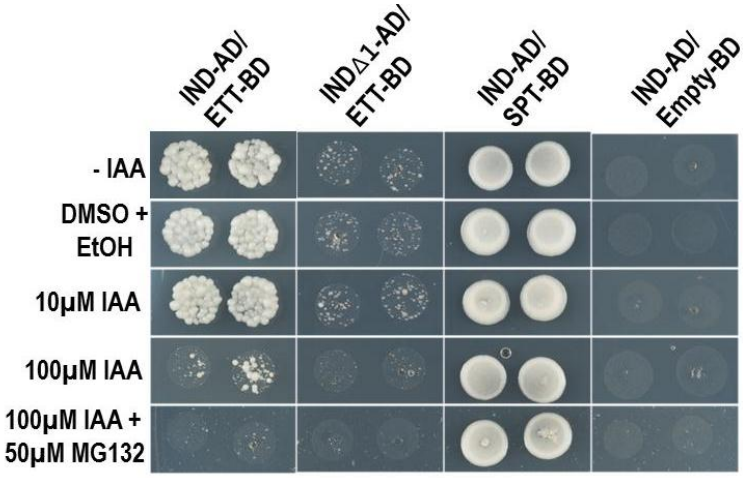

Figure 13 Addition of MG132 does not affect IAA binding to the IND-ETT complex. DMSO and EtOH were used as solvent controls. Yeast colonies were plated on SD –LWHA plates and kept at RT.

The IAA binding pocket of TIR1 is promiscuous and can accommodate the auxin analogues NAA and 2,4-D, albeit with lower affinities than IAA due to their different ring structures. Auxin acts as molecular glue which helps enhance the interacting surface between Aux/IAA proteins and TIR1 (Calderon Villalobos et al., 2012, Calderon-Villalobos et al., 2010). In order to determine whether this interaction also occurs using a similar mechanism, both NAA and 2,4-D were added to the yeast medium to observe their effect on the IND-ETT complex. Surprisingly, neither NAA nor 2,4-D affected the interaction between IND and ETT indicating a novel IAA-specific binding mechanism operating in this receptor complex. The interaction was seen even up to 200µM NAA and 2,4-D at which concentrations adding IAA completely inhibits the interaction (Fig. 12, 14)

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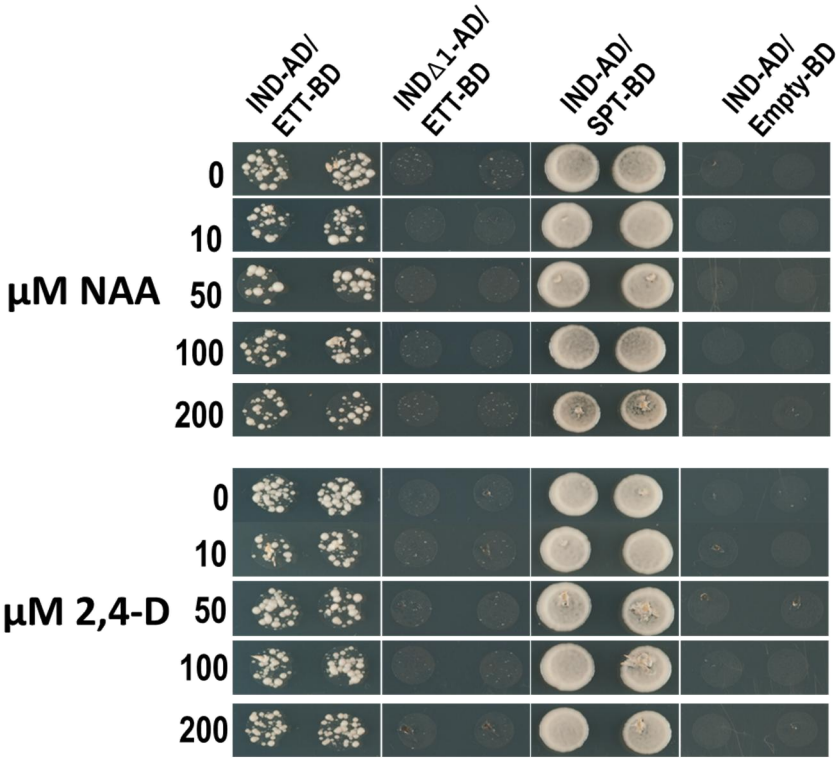


Figure 14 Auxin analogues NAA and 2,4-D do not affect the IND-ETT interaction. Yeast colonies were plated on SD –LWHA plates and kept at RT.

The lack of specificity seen in the TIR1 auxin binding mechanism is because the interaction proceeds through the carboxyl group of these compounds, and therefore, the presence of the different ring structures does not compromise binding. The IAA-specificity of this interaction could be in part due to the Indole ring of the IAA molecule. To test this hypothesis Indole 3-Butyric Acid (IBA)- a naturally occurring auxin precursor- was used. This compound has a very similar structure to IAA except that it possesses a longer carbon

chain and different plant organs demonstrate differential specificity and responses to this molecule (Strader and Bartel, 2011).

IBA was not able to disrupt the interaction between IND and ETT (Fig. 15). On closer inspection however it appeared to affect the INDΔ1-ETT interaction as at higher concentrations this interaction was inhibited (Fig. 16) suggesting that the longer carbon chain also plays a role in causing the conformational change of the protein complex. Thus the above results indicate that unlike the mechanism of IAA-binding to TIR1, this interaction is possibly influenced by the entire IAA molecule binding to the complex where the indole-ring confers IAA specificity.

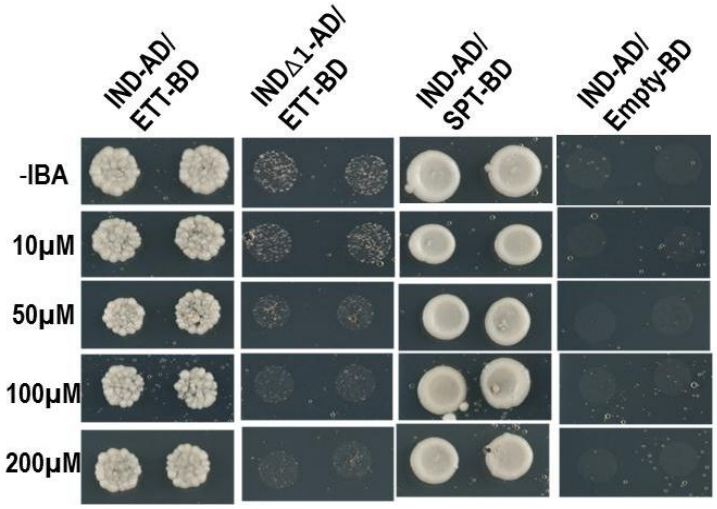


Figure 15 Effect of IBA on the IND-ETT interaction. The interaction between full-length proteins is not affected by the addition of IBA. IBA only affects the interaction between IND△1 and ETT. Yeast colonies were plated on SD –LWHA plates and kept at RT.

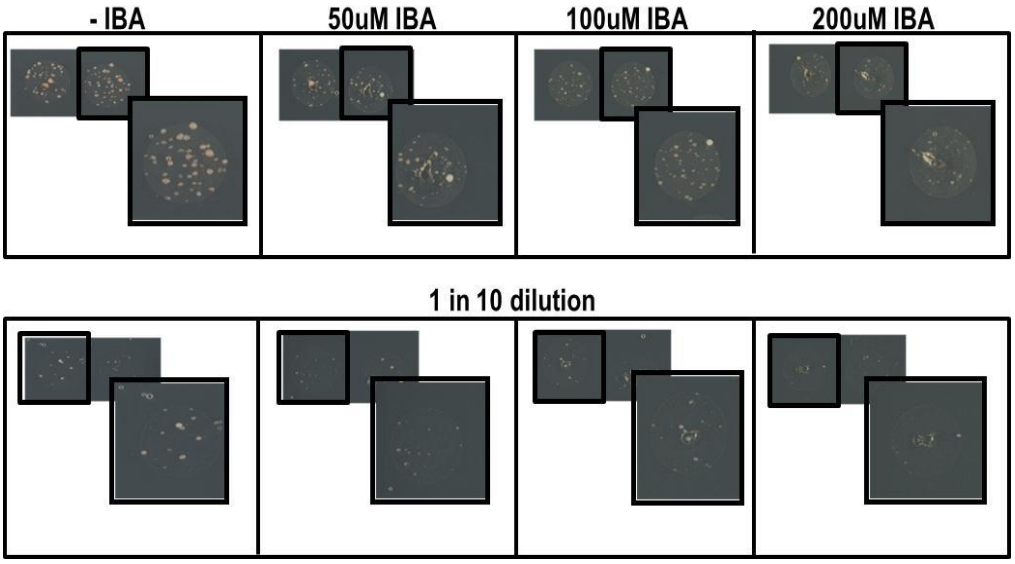


Figure 16 IBA only affects the interaction between IND△1 and ETT. Close-up view of the IND△1-ETT interaction shows gradual loss of colony growth from 50µM IAA onwards. Yeast colonies were plated on SD –LWHA plates and kept at RT.

3.3.3 Partially successful in vitro analysis of the IND-ETT interaction

The results from yeast two-hybrid experiments indicated that IND and ETT proteins are able to bind IAA. The yeast two-hybrid system, however, is a qualitative assay and it does not provide any details of the mechanism of the interaction. Furthermore, as this assay relies on the heterologous expression of the proteins of interest, it does not exclude the possibility of endogenous yeast proteins from potentially influencing the assay. It was therefore necessary to recapitulate the IND-ETT interaction in an *in vitro* system to assess the effect of IAA on this interaction and also to provide insight into the mechanism of this interaction.

For this purpose the Surface Plasmon Resonance (SPR) system was chosen and the experimental design was based on the ReDCaT assay (Stevenson et al., 2013) developed in the Lawson Group at the John Innes Centre. Briefly, this technique uses a single-stranded (ss) DNA linker which is covalently linked to the sensor chip. The dsDNA sequence of interest is synthesized with an adapter (which is complementary to the single-stranded DNA linker) and this is captured on the chip by hybridizing to the linker. For the purpose of this experiment, the DNA-oligo used was the non-canonical E-Box which is the *cis*-element bound by IND on the *PID* promoter (Girin et al., 2011). The assay involved using heterologously produced IND and ETT proteins to test (i) whether IND can bind to the E-box in complex with ETT, or (ii) whether ETT sequesters IND and prevents it from binding the non-canonical E-box, and (iii) how IAA influences this interaction.

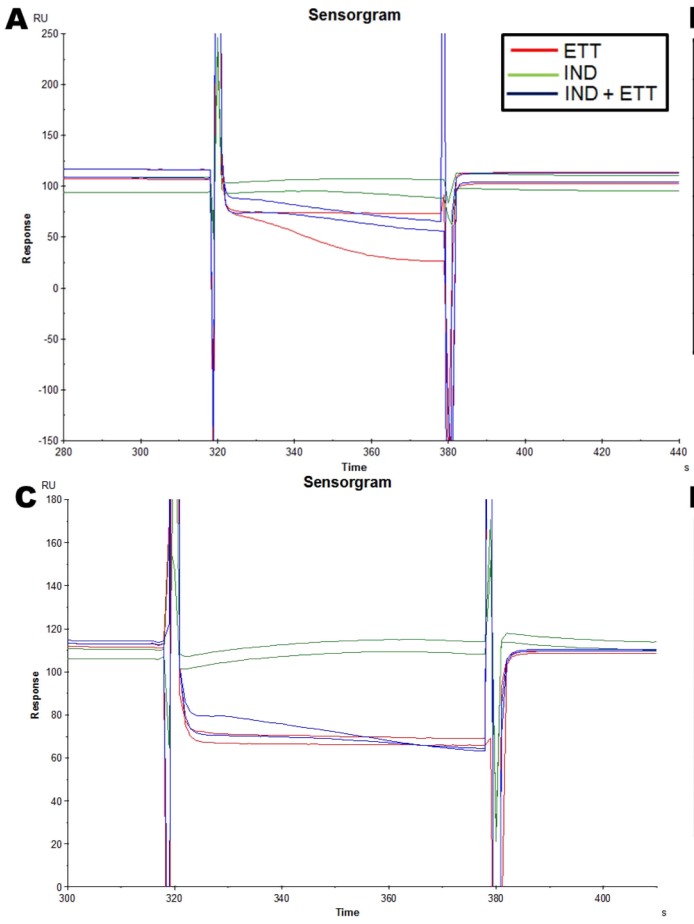
During the course of the project different systems and strategies were attempted to produce IND and ETT proteins using different heterologous systems, however, expression of both proteins in their native conformation proved to be challenging. In addition, two different versions of the DNA- oligos were used to aid protein binding and these have been described in the Material and Methods Section (3.2.4) The strategies used and SPR results from these attempts have been listed in Table-2 (See Appendix I for further results). A last attempt at SPR was made using refolded proteins and this yielded some promising results.

S.No.	Expression System	Protein	Oligo Used	SPR Results
1	*E. coli*	IND (Produced by Chromus Pvt. Ltd.)	1,2	No binding detected
2	*E. coli*	IND (Produced by Chromus Pvt. Ltd.)	3,4	No binding detected
3	*E. coli*	Co-expression of IND and ETT. IND protein with N-terminal 6xHis-Tag	NA	NA
4	TNT SP6 Wheat Germ Protein Expression System	IND: N-terminal 6xHis-tag + 4xAlanine Linker; SPT, ETT.	1,2,3,4	No binding detected
5	*E. coli*	IND, ETT Refolded proteins.	3,4	Weak binding of IND to both oligos. IND/ETT dimer did not bind.

Table 2: Table listing the proteins and assays attempted for SPR experiments. For details on the oligos used check Materials and Methods of this Chapter.

Results from Experiment 5 (Table 2) indicate that the IND protein is able to bind both versions of the oligos used (Fig. 17, A,C) which suggests that a single copy of the cis-element is sufficient for binding. As a segment of the native PID promoter was used it is possible that some residues flanking the element are needed to stabilize the protein-DNA interaction. The strength of the response was much lower than the calculated R_{max} in both cases which suggests weak binding of the protein to the cis-elements which may be because a large fraction of the protein is not folded in the right conformation. When IND and ETT were added as a complex, IND was not able to bind the E-box suggesting that there is a competition between DNA-binding and protein-protein complex formation (Fig. 17,A,B).

Addition of 100μM auxin does not alter the results considerably (Fig. 17, C,D). IND appears to bind DNA stronger in the presence of IAA, however, auxin also appears to slightly enhance the binding response from the control suggesting that the addition of IAA changes the buffer conditions which is aiding protein stability and/or the DNA binding property. Significantly, addition of IAA does not seem to disrupt the interaction between IND and ETT as IND does not bind the E-box in the presence of ETT and IAA (Fig. 17, C,D). This corroborates the results from the radioactive yeast retention assay which suggests that the receptor complex binds auxin together.



B

Cycle	Response 2-1	Sample	Oligo
1	3.5	IND	3
2	-4.4	ETT	3
3	-4.0	IND/ETT	3
4	3.3	IND	4
5	-3.1	ETT	4
6	-3.6	IND/ETT	4
7	432.6	control	control

D

Cycle	Response 2-1	Sample	Oligo
1	6.6	IND	3
2	-3.4	ETT	3
3	-4.0	IND/ETT	3
4	5.9	IND	4
5	-2.9	ETT	4
6	-3.3	IND/ETT	
7	458.6	control	control

Figure 17 SPR experiment using IND and ETT refolded proteins in the presence and absence of IAA. (A) Sensogram of IND binding behaviour to immobilized oligos without IAA **(B)** Response readings for the same **(C)** IND binding behaviour to immobilized oligos in the presence of IAA **(D)** Response readings for the same. Readings from Flow cell 2 are normalized to Flowcell 1 to take buffer effects into account. For description of oligo numbers in Tables B,D refer to *Material and Methods Section (3.2.4)*.

3.4 Discussion

3.4.1 The IND-ETT Interaction

This project has attempted to uncover novel interactions among transcription factors patterning the gynoecium, in particular the interactions that are required to pattern the domains in the apical-basal axis of the carpel- the stigma, style, ovary and the gynophore. Using a targeted yeast two-hybrid approach a novel interaction between IND and ETT was discovered.. There is strong evidence that all three domains of the IND protein interact with ETT as constructs with the individual domains and chimeric constructs with HEC3 protein domains did not interact with ETT in yeast. This interaction appears to be stabilised by the IND-IS domain as the interaction is substantially weakened when IND constructs without the IND-IS domain (INDΔ1, Fig. 10,B) were tested for their interaction with IND. This perhaps also indicates that IND is the only protein among the HEC family of proteins as HEC1,2 and 3 do not possess the IS domain. Hence future work will involve verifying this result in a plant system such as BiFC or FRET-FLIM. Ultimately, crystallography studies would provide the most conclusive description of the interaction.

Truncations of the ETT protein showed conclusively that the ETT-specific ES domain is necessary for heterodimerisation with IND. ETT is capable of binding DNA (Cheng et al., 2012) and homodimerising via its DBD (Boer et al., 2014). The discovery of the ES dimerisation domain suggests that this ARF could potentially form higher order protein complexeswhile still bound to DNA. The mechanism of heterodimerisation via this domain remains unclear. Several interacting partners of ETT which interact with the ES domain have been identified in a yeast two-hybrid screen conducted in the lab, but these belong to diverse transcription factor families and do not possess domains similar to the IS domain of IND. Therefore, it is possible that some motif in the secondary structure of these proteins exists which enables their interaction with the ES domain.3.4.2 Mechanism of Gene Regulation by IND, SPT and ETT

IND and SPT cooperatively regulate a number of genes and the proteins dimerise through their bHLH domains (Girin et al., 2011). However, a ternary complex among the three appears unlikely as all three domains of IND are required to interact with ETT and IND, SPT and ETT did not interact neither in the two- or three-hybrid assay. Therefore, if these three proteins were to regulate common targets two scenarios could occur. In the first case, there might be a competition between SPT and ETT to bind with the IND molecule and

consequently gene expression would depend on the stoichiometry of the molecules prevalent at the time. The SPR suggests that complexation with ETT prevents IND from binding DNA, thus IND could interact with SPT only if there is an excess of SPT during the time of regulation or, as will be discussed later, when IND is relieved from interacting with ETT. This competitive mechanism could not be verified through the interaction tests conducted here, as two-hybrid and three-hybrid IND-AD/ETT-BD interactions did not show any marked differences in yeast colony growth. This system, however, is not adequate to detect subtle effects and a method that could be used is the pBridge Vector system in yeast which is optimized to detect competition among molecules in three-hybrid interactions (Clontech). In addition, the SPR system can also be used by immobilizing one of the proteins to the sensor chip and then assessing the binding of either of the other two proteins. The second situation that could occur is that these three proteins may form a complex together but a bridging protein might be necessary to facilitate oligomerisation. This would not occur in yeast based systems as an additional plant-specific protein would be needed.

3.4.3 The IND-ETT IAA Receptor-Complex

The major finding from this project is the discovery that the IND-ETT complex may function as an IAA co-receptor. Radiolabelled IAA retention assays in yeast indicate that both proteins participate in binding auxin. This possibly results in a conformational change in the complex upon IAA-binding, which in the yeast two-hybrid assays may cause the GAL4 domains to be separated resulting in no colony growth. The IAA-sensitivity seen in the interaction could have arisen through a proteasome-dependent degradation mechanism, similar to the one seen in the AUX/IAA-TIR1 pathway, however the addition of MG132 did not have any effect suggesting that neither of the proteins are being degraded in this process and additionally no degradation-sensitive yeast protein- which might have influenced the interaction -is involved. However, there is evidence that the uptake of MG132 in yeast cells is very low (Lee and Goldberg, 1996), therefore it is necessary to repeat this experiment *in planta*, perhaps using a protoplast transient expression assay to verify this result and effectively replicate *in vivo* conditions.

A question still remains as to which of the proteins is capable of binding the auxin molecule. In the case of TIR1, the majority of the binding occurs in the TIR1 binding pocket and this is aided by the Domain II degron motif of Aux/IAA proteins (Tan et al.,

2007). In the case described here, it seems likely that the ES domain forms the binding pocket as several IAA-sensitive interactions using ETT as bait have been found in the yeast two-hybrid screen conducted in the lab (Sara Simonini, personal communication). In this scenario, IND would therefore be the accessory protein in the interaction and perhaps like the TIR1-Aux/IAA interaction complete the binding pocket. The HEC and bHLH domains of IND most likely also contribute to the contact with the binding pocket as the weak interaction between IND without the IS-domain and ETT is IAA-sensitive.

A unique aspect of this putative receptor-complex is that it appears to specifically bind IAA and not the synthetic analogues 2,4-D and NAA. The TIR1/AFB family of proteins exhibit different sensitivities to these three compounds (Calederon Villalobos et al., 2012; Lee et al., 2013), however, the ETT-IND co-receptor is the first evidence of a complex that exhibits IAA selectivity. This suggested that the majority of the electrostatic contacts made in the auxin-binding pocket are with the indole-half of the IAA molecule. However, when IBA was used in the yeast assayit only affected the weaker interaction between INDΔ1 (IND minus the IS domain) and ETT, which suggests that the carbon chain could also potentially have a role in facilitating auxin binding to the binding pocket. It is possible the IS domain provides a selective mechanism for the molecules entering the binding site. The domain could restrict access to only those molecules adopting the correct spatial conformation or which can make the electrostatic contacts necessary to access the binding pocket. In the case of the INDΔ1-ETTcomplex , the binding site may be more accessible, thus allowing the IBA molecule to bind the pocket and influence the interaction.

The structural considerations are of course only speculations that need to be verified by biochemical and structural studies. Future work will for example involve a repetition of the SPR experiment with NAA, 2,4-D and IBA along with crystallographic studies.

CHAPTER 4

CHAPTER 4- Understanding the Role of the IND-ETT Auxin Co-receptor in the Hormonal Regulation of Gynoecium Patterning

4.1 Introduction

The gynoecium is structurally the most complex organ of the plant. This is primarily as a consequence of its functional complexity as it coordinates all the three processes of pollination, fertilization and seed dissemination. It has subsequently evolved to develop various tissue sub-structures in the body of the organ which facilitates all three functions. These structures are best described along the different axes of symmetry. Among the major axes, the apical-basal axis is defined by the presence of the stigma, style, ovary and the gynophore, respectively (Fig. 18). The adaxial -abaxial axis comprises the medial tissues – the septum and transmitting tract, and the ovules adaxially, while the abaxial region comprises the lateral tissues- valves and the valve margin, and the replum.

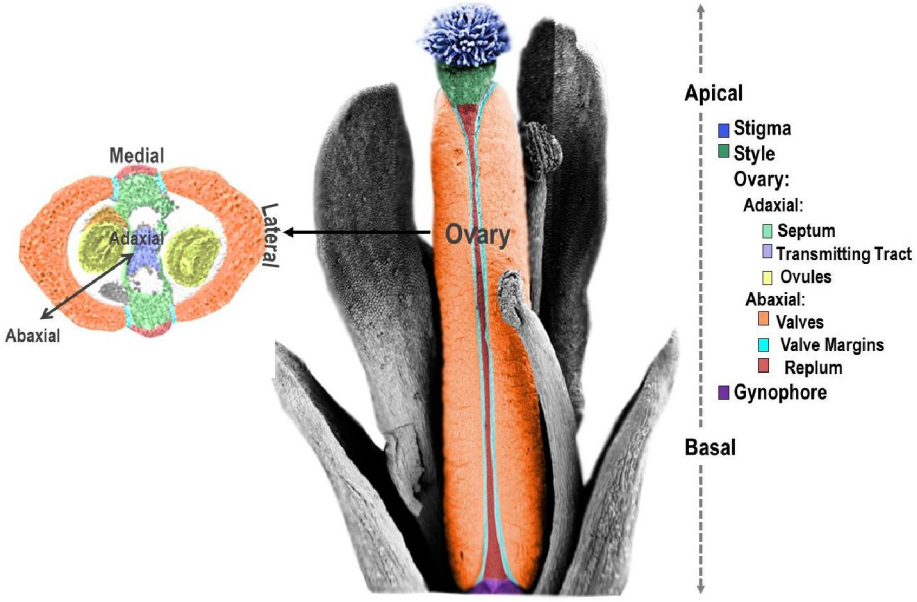


Figure 18 The axes of symmetry of the *Arabidopsis* gynoecium

The study of the morphogenesis of the carpel has proven to be challenging. In the initial stages, carpel morphogenesis involves initial cellular expansion and division to grow into the recognizable cylindrical structure from a ball of primordium cells (Stages 6-7). This is then followed by sequential establishment of tissue identity while division and expansion

also occur concurrently. These tissue domains have well defined boundaries which are scaled precisely to the overall organ.

This precise morphogenesis of the carpel is the output of both genetic and hormonal regulation. Among the hormones which are necessary morphogens for carpel development are auxin and cytokinin. For the purposes of this study the role of auxin will be considered. Auxin clearly functions in the very early stages of carpel development as mutants in auxin signalling and biosynthesis develop carpels which lack tissue patterning and develop as hollow tubular structures, topped with stigmatic tissue (Cheng et al., 2006)(Chapter 1, Fig. 4, B). Also necessary is the maintenance of the right levels of auxin in the gynoecium. Perturbing auxin transport thorough the application of polar auxin transport inhibitors such as NPA, results in the shift of the boundaries which determine the apical-basal tissue zonation (Figure 19). Based on these observations the prevailing model for auxin-regulated gynoecium development was proposed by Nemhauser et al. (2000) who hypothesized the presence of an auxin gradient in the carpel acting as the morphogenetic determinant of this tissue zonation. The gradient is initiated at the apical end of the carpel where the concentration of auxin is highest which then reaches a minimum at the basal end. The gradient defines two hypothetical boundaries at the top of the ovary and at its base before the gynophore which provide positional information to the developing tissue thus coordinating tissue differentiation.

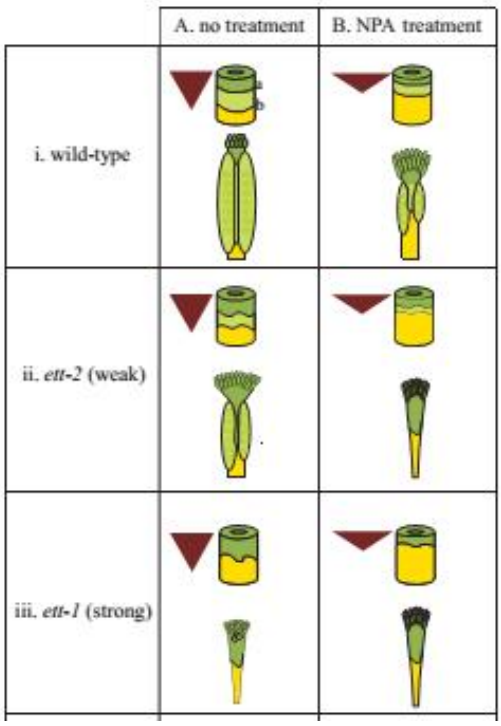


Figure 19 Gradient Model of Gynoecium Patterning. Triangles indicate relative concentration of auxin, as well as the direction of auxin flux. Cylinders represent gynoecium primordia. Horizontal lines, a and b, within the primordium represent emerging regional boundaries dependent upon defined auxin concentrations. Wavy horizontal lines in the primordia of *ett* mutants represent destabilization of these boundaries, as a result of reduced ETT function. *Adapted from Nemhauser et al., 2000*.

This hypothesis was conceived when tools to visualize auxin distribution were unavailable. Since the development of auxin reporter constructs such as *DR5::GFP* and the DII Venus, it is now considered unlikely that the hypothesized gradient in the carpel exists. Thus the mechanism of carpel development by auxin still remains unknown.

The genetic regulation of carpel development involves the activities of multiple transcription factors many of which have been co-opted from leaves. The activities of these genes can be loosely grouped as identity determinants, regulators of cell division and expansion and boundary genes which ensure correct patterning. Among them, ETTIN/ARF3 (ETT) is a master regulator of carpel development as it functions to coordinate all of the above three aspects. In strong e*tt* mutant alleles carpel growth is severely stunted while in weaker alleles in addition to growth defects, patterning is also significantly altered. The boundaries of the tissue domains are shifted as the apical and basal boundaries are extended while ovary development is compromised. In the hierarchy of genetic regulation, ETT appears to function upstream of many of the carpel genes in order to specify boundaries correctly. ETT also presents a point of convergence of the genetic and hormonal control of carpel development. ETT is an Auxin Response Factor (ARF) and mutant phenotypes phenocopy the distortions in patterning seen when gynoecia are treated with NPA. The strongest mutant alleles exhibit the characteristic phenotypes of auxin deficient mutants (Fig. 19) and this is evident by the application of NPA on these mutants. Treating the weak *ett-2* alleles and intermediate *ett-3* alleles with NPA causes the gynoecia to assume the phenotype of the strongest *ett-1* mutant allele, while treating *ett-1* carpels has no effect (Nemhauser et al., 2000) (Fig. 19). This suggests that in the strongest alleles either the levels of auxin are already at the lowest possible levels or that auxin signalling is strongly affected such that these mutants are insensitive to the treatment. Additionally, the dominance of the apical and basal domains, and the reduction in the ovary is also conspicuous in all three mutant alleles which is a trend also seen when WT gynoecia are treated with higher concentrations of NPA. The difference between NPA treated gynoecia and *ett* mutants, is that in the mutants, tissue identity is also affected as the marginal tissues- stigmatic and transmitting tract tissues- develop ectopically along the abaxial regions

It is unclear whether the regulation of tissue patterning by ETT is via both auxin-dependent and auxin-independent signalling mechanisms. .Thus , *ett* mutants could be considered

useful tools to dissect the mechanisms coordinating carpel development and could be utilized to answer some of the major questions which remain unsolved.

This chapter addresses the second aim of this study which is to understand the mechanisms behind the cross-talk of genetic and hormonal pathways. The discovery of the novel auxin-sensing mechanism between IND and ETT in this study has provided some insight into how this might occur. Here, the relevance and function of this auxin co-receptor complex in the context of gynoecium patterning has been analysed. Further, the regulation of auxin distribution has also been analysed using the *ett-3* mutant as a model for dissecting hormonal regulation of carpel development.

4.2 Material and Methods

4.2.1 Scanning Electron Microscopy

4.2.1.1 Tissue fixation

All specimens were collected and fixed in FAA solution [50% Ethanol, 3.7% Formaldehyde, 5% Acetic Acid] and vacuum infiltrated in 20ml scintillation vials. Vacuum was applied until all samples began to sink to the bottom of the vials after which samples were incubated in the FAA solution at RT for 4hrs with gentle agitation. The FAA solution was then removed and replaced with 50% ethanol and this was incubated at RT for 30min. This process was repeated by sequentially increasing the concentration of ethanol from 50% to 60%, 70%, 80%, 90% and 95% and incubating the samples for 30min at RT at each step. Samples were then stored at 4^{o}C until further use.

On the day of the Critical Point Drying, samples were incubated in 100% ethanol twice each for 30min at RT and this was then followed by 30min incubation in 100% dry ethanol. Samples were then subjected to Critical Point Drying using the Leica EM CDP300. The programme used was as follows: 12 cycles of fast flow, fast exchange and mixing. Temperature at 15^{o}C and Pressure 50bar.

The dried samples were then dissected and fixed on metal stubs and sputter coated for 75 seconds with a 20nm coating of gold using the AGAR Sputter Coating machine following which the samples were analysed and imaged using the Zeiss Supra55 FE-SEM.

4.2.2 DEX Treatment

All treated plants were grown at 22^{o}C at long days throughout the period of the treatments. The inflorescences of plants were dipped in 100µM Dexamethasone + 0.015% Silwet-55 solution until fully coated with the solution. Samples collected 4 days post-application were treated with DEX twice during the 4-day period. Samples collected 7-days post-application were treated thrice during the 7-day period.

4.2.3 GUS treatment

Two different protocols were used to GUS-stain the carpel specimens shown here which differ in the vacuum infiltration times and the incubation times at 37^{o}C. The treatment is indicated in legends to figures. All images were taken using the Leica DM6000 Microscope.

4.2.3.1 Treatment 1:

Samples were incubated in Acetone for 1hr at -20^{o}C. Following this, they were washed twice in 0.1M Sodium Phosphate Buffer (0.1M NaH_2PO_4 + $Na_2HPO_{4)}$ for 5 min each rocking. Samples were then incubated for 1hr, rocking in 1mM Potassium Ferricyanide ($K_3[Fe(CN)_6]$ and Potassium Ferrocyanide ($K_4[Fe(CN)_6$) solution at RT. They were then vacuum infiltrated for 3 min in X-Gluc Buffer Solution [1mg/ml X-Gluc, 0.1M Sodium Phosphate Buffer, 1mM $K_3[Fe(CN)_6$ + $K_4[Fe(CN)_6$ Solution, 1mM EDTA and 0.001% Triton] and incubated overnight at 37^{o}C.

4.2.3.2 Treatment 2:

Samples were incubated in Acetone for 1hr at -20^{o}C. Following this, they were washed twice in 0.1M Sodium Phosphate Buffer (0.1M NaH_2PO_4 + $Na_2HPO_{4)}$ for 5 min each rocking. Samples were then incubated for 1hr, rocking in 1mM Potassium Ferricyanide ($K_3[Fe(CN)_6]$ and Potassium Ferrocyanide ($K_4[Fe(CN)_6$) solution at RT. They were then vacuum infiltrated for 7 min in X-Gluc Buffer Solution [1mg/ml X-Gluc, 0.1M Sodium Phosphate Buffer, 1mM $K_3[Fe(CN)_6$ + $K_4[Fe(CN)_6$ Solution, 1mM EDTA and 0.001% Triton] and incubated for 6 hrs. at 37^{o}C.

4.2.4 Methods for FRET-FLIM

4.2.4.1 Cloning of constructs

All genes were cloned using Ligation Independent Cloning into vectors kindly provided by the Weijers Laboratory in Wageningen. Cloning was carried out according to the published protocol (De Rybel et al., 2011).

4.2.4.2 Arabidopsis Mesophyll Protoplast Isolation

The leaves from 4-5 week old plants were used and mesophyll cells were isolated from the lower epidermis of leaves using magic tape to pull away the cells. The tape with the tissues attached was placed in a petri-dish with 20ml Enzyme Solution [20mM MES pH5.7, 20mM KCl, 0.4M Mannitol, 1% Cellulase R10 (Yakult), 0.25% macroenzyme (Yakult)- incubate at 55^{o}C for 10min- add 10mM $CaCl_2$, 0.1% BSA]. The tissues were incubated in the solution for 2.5 hrs at 40rpm rocking at RT. Protoplasts were then filtered through a 70µm mesh into 50ml Flacon tubes and centrifuged at 1000g for 5min. Protoplasts were then washed with 10ml cold W5 Solution [2mM MES pH 5.7, 154mM NaCl, 125mM

$CaCl_2$, 5mM KCl] and spun for 3min. Samples were then resuspended in 5ml W5 Solution and a haemocytometer was used to count the number of protoplasts isolated. For optimum transformation the concentration of cells obtained should be close to 2-5 x 10^5 cells/ml. Cells were kept on ice for 30min and then centrifuged for 3min at 900g. The isolated protoplasts were then resuspended in MMg Solution [4 mM MES, 0.4M Mannitol, 15mM $MgCl_2$] to a final concentration of 2-5 x 10^5 cells/ml.

For the transformation with plasmids, 10-20µg plasmids were suspended in 20µl water in a 1.5ml Eppendorf. To this was gently pipetted, 100µl of competent protoplasts with a cut-off blue tip. To this was added 120µl PEG/Ca Solution [40% PEG 4000, 0.2M Mannitol, 100mM $CaCl_2$, H_2O to 10ml (Use freshly made PEG)] along the side of the Eppendorf tube. The reaction was mixed gently by inversion ensuring proper mixing. The mixture was incubated for 10min. To this was added 600µl W5 solution very gently and the tube was mixed by inversion following which 600µl W5 was added additionally and this mixture was mixed gently. Samples were spun at 100g for 1min and all of the PEG/W5 Solution was removed by pipetting. Samples were then resuspended in 250µl W5 Solution and pipetted (using a cut off blue tip) into 25 well plates coated with filter sterilized 1% BSA solution to prevent the protoplasts from sticking to the sides of the petri-dish. Samples were left overnight in a culture room covered by a blue-roll.

4.2.4.3 FRET-FLIM

FRET-FLIM was carried out using LaVision TriM Scope II multi-photon microscope at the University of East Anglia with help from Grant Calder and Paul Thomas (NRP BioImaging). The data was analysed using the Inspector Pro software. Statistical analysis was carried out using GenStat Edition 15.

4.2.5 Construction of Fluorescent Reporters

The *pIND::IND:YFP* reporter was constructed by amplifying 2.7Kb of the native IND promoter in frame with the 510bp gene from genomic DNA. PCR primers with adapters for Ligation Independent Cloning were used to amplify the product (See Appendix 2-Table 1). The amplicon was cloned into the destination vector pPLV16 which comes with eYFP. The resulting construct was a translational fusion of *pIND::IND* with YFP at the C-terminal end of the protein. Refer to De Rybel et al., 2011 for details of Ligation Independent Cloning and the plasmid used.

The *pETT::ETT:GFP* reporter was constructed using 3.7Kb of the native *ETT* promoter fused to the CDS of the *ETT* gene. The plasmid template used for amplifying the gene was kindly provided by Charlie Scutt of the Universite de Lyon and details of the construct can be found in Finet et al., 2010. The amplified product was cloned between the AgeI and SacI sites (See Appendix 2- Table 1 for primer details) of the plasmid pEGAD which comes with a single copy of eGFP. The final construct consisted of a translational fusion of the native 3.7Kb promoter of *ETT* fused to the 1827bp CDS of the *ETT* gene with eGFP at the C-terminal end of the protein.

4.2.6 Confocal Microscopy

All confocal microscopy and images were taken using Leica SP5II Confocal Microscope. Images were analysed using ImageJ software.

4.3 Results

4.3.1 Expression Analyses of IND and ETT Genes

IND is a carpel-specific protein and *pIND::IND:GUS* reporter analysis shows that the gene is expressed from stages 9-10 of carpel development. Initially, the gene has a wider expression domain extending into the medial replum region and the developing style. By stage 11-12, the expression is restricted to the style and the valve margins (Girin et al., 2011, Figure 23 A,B). *In situ* analyses have shown that *ETT* is expressed from the initiation of the carpel primordium up to stage 11 when the expression is detected on the abaxial surface and within the ovules (Sessions et al., 1997, Sara Simonini personal communication). However, a sequential analysis of *ETT* expression through gynoecium development has not been thoroughly conducted. Thus, for the purpose of this study fluorescence reporter constructs for both genes were developed. The constructs were translational fusions of the ETT open reading frame with GFP under the control of a 3.7 Kb segment of its promoter, and a *pIND::IND:YFP* reporter was constructed using 2.7Kb of the *IND* promoter plus the single-exon open reading frame of 510 bp fused to the YFP coding region.

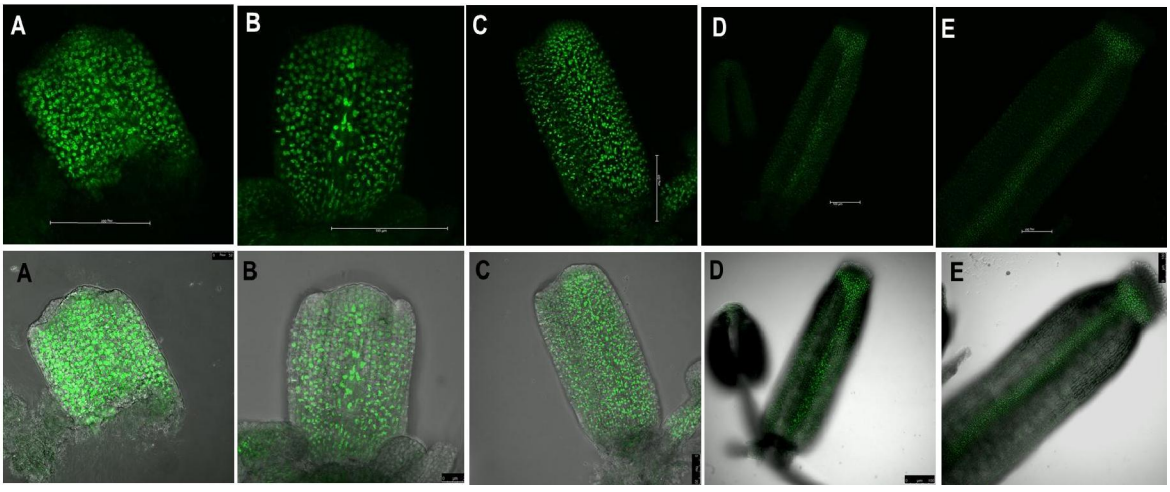


Figure 20: *pETT::ETT:GFP* expression in the WT gynoecium. A,B Early and mid- stage 8 respectively. **C,** Stage 9 gynoecium. **D,E** Stage 12 and 13 gynoecia respectively. Top panel shows GFP channel, lower panel overlay with bright field. Scale Bars=100μm

ETT expression was detected from stages 7-13 of carpel development and it is expressed along the entire abaxial surface of the carpel (Fig.20). Expression in the style- which is visible from stage late stage 10 onwards- was also clearly visible. It also appears to be expressed in the medial and lateral vascular bundles from stages 9-10, and this is more

evident around stage 12 when the expression can be clearly detected in the medial vascular bundles and additionally within the vascular strands of funiculi at stage 13 (Fig. 20, Appendix Fig. 1,2). *ETT* expression in the ovules, which has been detected in *in situ* experiments, was not detected with this reporter, which may be due to the presence of thick integuments making it difficult to detect the fluorescence signal from within the ovules.

Analysis of the *pIND::IND:YFP* reporter clearly reveals that the IND is a nuclear-localized protein. This was indeed expected based on data from Girin *et al.* (2011), but has not been shown directly before. *pIND::IND:YFP* expression was clearly detected within the valve margins from stage 12 onwards (Fig. 21), however the stylar expression that has been detected using the GUS reporter system, could not be detected here.

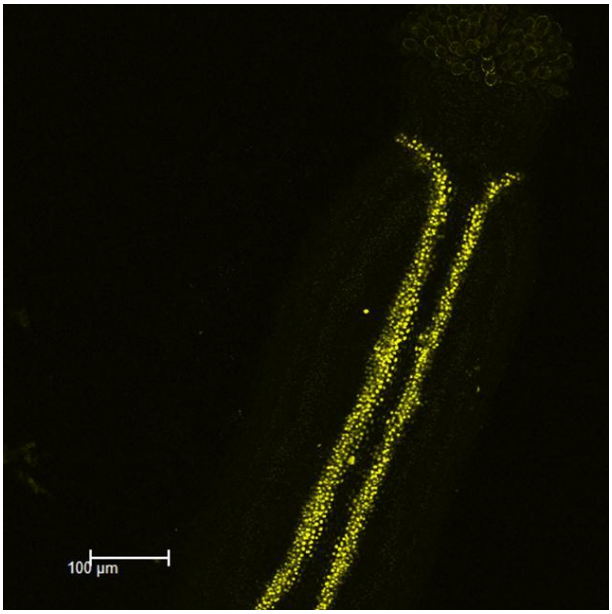


Figure 21: *pIND::IND:YFP* expression in the carpel. Stage 12 carpel showing IND:YFP expression in the valve margin.

Different lines transformed with this construct were tested to rule out the possibility that the expression of the construct was partly suppressed by its insertion position in the genome, however no stylar expression was detected in any the lines analysed. This suggests that the expression of the gene is possibly weak within these regions and therefore cannot be detected by confocal microscopy. As the GUS reporter system is sensitive to incubation times, weaker expression is better detected using this assay. It was not possible to develop an enhanced reporter construct for the IND protein during the time-frame of this project, thus future work would involve optimizing this construct possibly using a larger fragment of the promoter or by a translational fusion of IND with two YFP molecules.

Taking together the results of the *IND:GUS* construct and the *ETT:GFP* expression pattern, the expression of both proteins shows an overlap from stage 9 onwards in the stylar region of the carpel. In the later stages, there also appears to be an overlap in the valve margin region although this would be best confirmed by co-expressing the constructs in the same plant which is currently under development. As the significance of IND activity in the valve margin is primarily in the later stages of fruit development, ETT expression would need to be analysed in the fruit from stage 16 onwards for this overlap to be functionally relevant.

To confirm whether both proteins interact *in planta*, FRET-FLIM technology was used by co-expressing both proteins in *Arabidopsis* mesophyll protoplasts. Using ETT:CFP as the donor and IND:YFP as the acceptor, a clear interaction between both proteins was detected as the life-time of the CFP molecule showed a clear decrease in the presence of the IND:YFP molecule (Fig. 22,A).

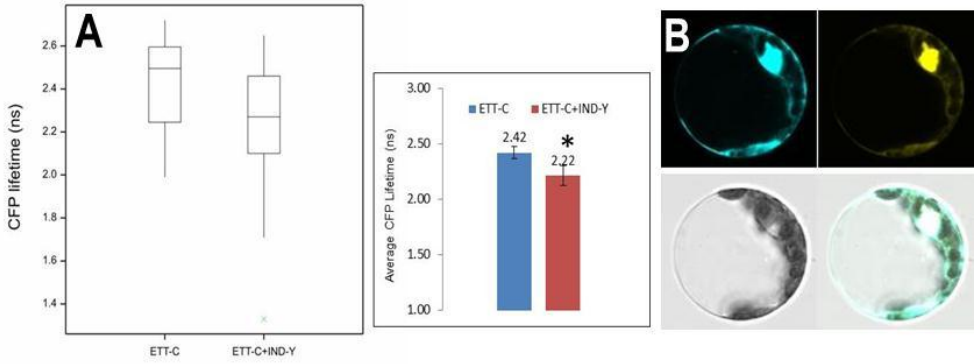


Figure 22 :FRET-FLIM analysis of IND-ETT interaction in mesophyll protoplasts. A, FRET-FLIM measurements of CFP-tagged ETT (ETT-C) and interaction between ETT-CFP and YFP-tagged IND (IND-Y). Histogram shows mean values of FLIM decay rate(ns) of CFP indicating an interaction between IND and ETT (p-value for Student's t-test = 0.056). n=16. **B,** Nuclei expressing both constructs.

This result was further confirmed in an independent FRET-FLIM experiment in collaboration with Alejandro Freire-Rios in Dolf Weijers laboratory (Univ Wageningen). Thus, to conclude, the above results show that ETT and IND can interact in plant cells suggesting that these proteins function in a complex during carpel development.

4.3.2 Ectopic IND and SPT expression results in the patterning defects of the ett-3 mutant

The expression pattern of *ETT* in the carpel is consistent with its role as a master regulator of carpel development. ETT functions not only to ensure correct tissue patterning but also

appears to coordinate the development of the different domains of the gynoecium. In the context of its interaction with IND, ETT's function would be two-fold: first, to ensure correct spatial expression of *IND*, and second, to regulate its activity so that style development occurs synchronously with the development of other tissue domains. A clue to the former of the two functions comes from ETT's regulation of *SPT*. *SPT* expression extends ectopically into the abaxial domain in the *ett* mutant and this, in part, causes the abaxialization of the transmitting tract tissue and over growth of stigmatic tissue, both of which are rescued in the *spt-2 ett-3* double mutant (Heisler et al., 2001). However, as both IND and SPT proteins are necessary for the development of these marginal tissues, it indicates that *IND* could also be expressed ectopically in the *ett* mutant. Thus, to test this hypothesis, the *pIND::IND:GUS* construct was crossed into the *ett-3* mutant. The results clearly show that *IND* expression extends ectopically into the valve region in the *ett-3* background (Fig. 23 C,D; Fig. 28,B). At stages 11-12, *pIND::IND:GUS* expression is seen wherever ectopic stigmatic tissue is found on *ett-3* gynoecia giving credence to the hypothesis that this aberrant tissue growth could, in part, be due to ectopic IND expression (Fig. 23,D). This observation is corroborated by the phenotype of the *ind-2 ett-3* double mutant (Fig. 25) which shows a partial rescue of the *ett-3* phenotype.

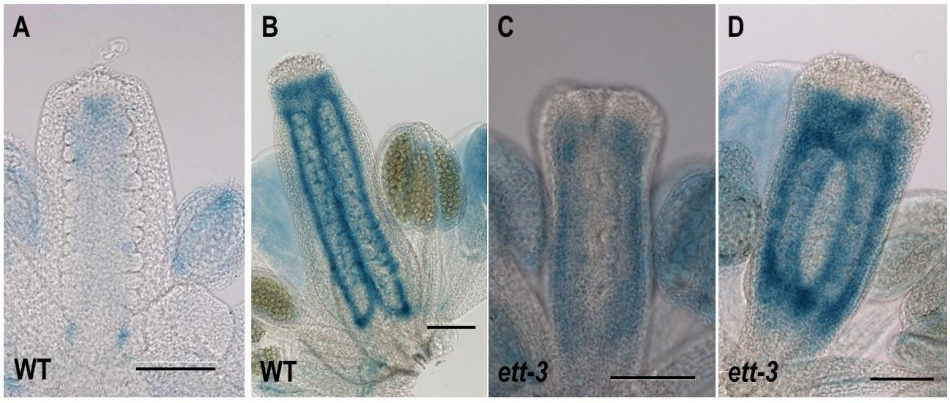


Figure 23 pIND::IND:GUS expression in WT (A,B) and ett-3 (C,D) gynoecia. A,B: IND:GUS expression in Stage 9 and late stage 11 gynoecia, respectively. **C,D** IND:GUS expression in Early Stage 9 and Stage 11 gynoecia, respectively. Samples were treated using GUS Treatment 1 *(See Materials and Methods 4.2.3.1)*. Scale Bars=100µm.

The apical-basal patterning defects are reduced, ovary development is significantly rescued and the stigmatic tissue develops in the correct domains towards the apical end (Fig. 24, 25).

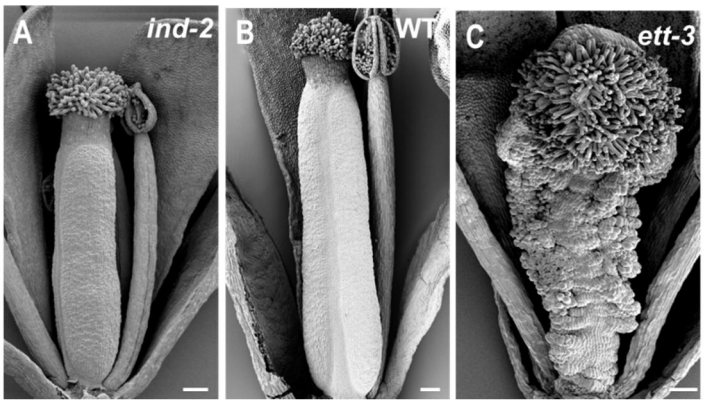


Figure 24 *ind-2* gynoecium at stage 13, **B**, WT gynoecium at stage 13, **C,** *ett-3* gynoecium at stage 13.
Scale Bars=100μm

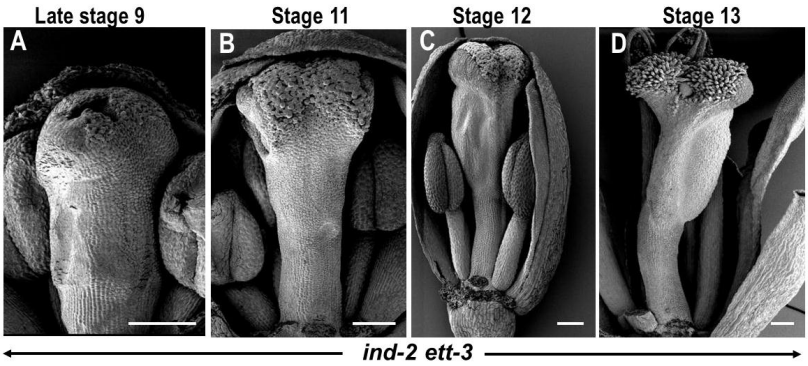


Figure 25 *ind-2 ett-3* carpel phenotype at different stages of development. Scale Bars=100μm

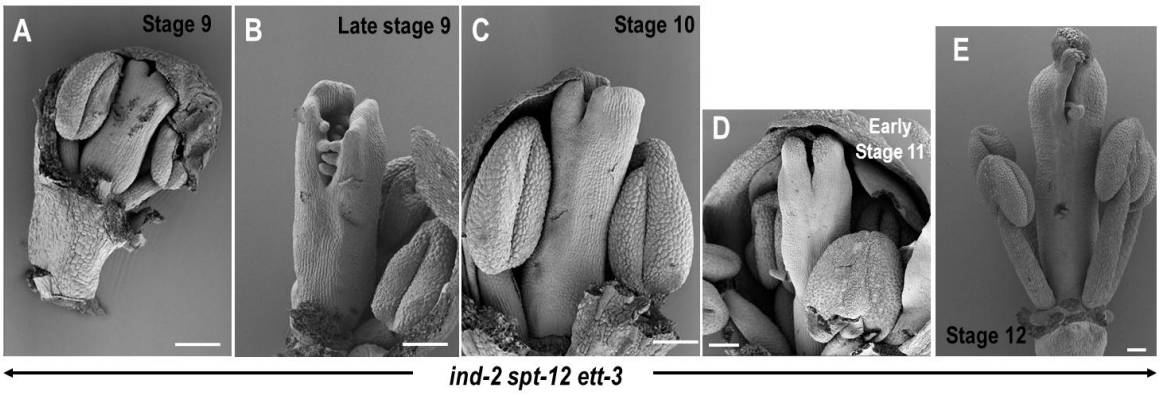


Figure 26 ***ind-2 spt-12 ett-3*** **carpel phenotype at different stages of development.** Scale Bars=100μm.

However, zonation defects are still visible as the domains are not scaled correctly and the gynoecium does not elongate to WT levels. In the *ind-2 spt-12 ett-3* triple mutant there is further rescue of the phenotype and the apical-basal patterning is almost entirely recovered. However, in the style region the triple mutant shows the characteristic split-style of the *ind-2 spt-12* double mutant (Fig. 24, 26) (Girin et al., 2011) showing that *IND* and *SPT* function epistatically over *ETT*.

Unlike the *ind-2 spt-12* double mutant however, the triple mutant shows some development of stigmatic tissue at the apex of the style. This might be as a result of the expression of the *HEC* genes which are also repressed by ETT in the valves (Gremski et al., 2000). A comparison of the fruits of the *ett-3*, *ind-2 ett-3* and *ind-2 spt-12 ett-3* mutants shows that fruit elongation in the triple mutant is severely compromised (Fig. 27, A,D,E,F). In this mutant, fruits are normally split at the top and are reduced which is due to the additive effects of the *spt-12* mutant (Girin et al., 2011) and the defects from the *ett* mutant. The conspicuous split would result in fertility defects and ETT probably also regulates the auxin-induced elongation responses post-fertilization, both of which contribute to the reduced fruit development seen in the *ind-2 spt-12 ett-3* mutant. The *ind-2 ett-3* mutant shows a significant rescue of fruit length although a few patterning defects still remain (Fig. 27, B,C). The rescue of the fruit phenotype is possibly because style and transmitting tract development in the double mutant are not as severely affected as in the triple mutant.

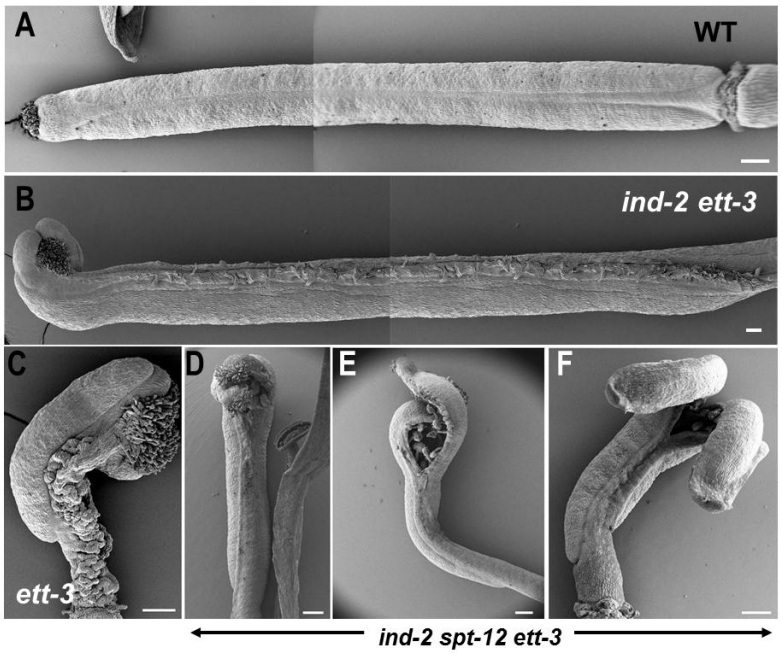


Figure 27 A, WT fruit, **B**, *ind-2 ett-3* fruit**, C,** *ett-3* fruit**, D-F** Phenotypes of the *ind-2 spt-12 ett-3* triple mutant. Scale Bars=200μm.

The above results therefore indicate that the extension of *IND* and *SPT* expression ectopically within the main body of the carpel results in the apical-basal patterning defects seen in the *ett-3* mutant. This also results in the abaxial development and overgrowth of the marginal tissues (the transmitting tract and stigmatic tissues) which normally develop adaxially.

4.3.3 Gynoecium patterning and the role of auxin

As seen in the previous section, a characteristic phenotype of the *ett* mutant is the distortion of apical-basal patterning. Sessions and Zambryski,1995, ascribe this patterning defect to irregular apical-basal boundary positioning in the mutant. They propose the presence of two hypothetical concentric boundaries: an apical boundary present below the style and a basal boundary which is located below the ovary. The boundaries are established early during stage 6 of gynoecium development and therefore provide positional information to the developing tissues, hence the apical boundary would specify the style while the lower boundary would specify the valve tissues. This theory suggests the existence of a pre-pattern in the gynoecium and therefore gene expression would proceed according to these positional cues from the start. Another mechanism by which correct patterning could occur is by ensuring correct spatio-temporal regulation of gene expression. Considering the genes under study for instance, ectopic expression of *IND* coupled with its precocious expression would result in the premature establishment of cell identity in the wrong tissue domains. Consequently, the identity of the cells in these tissues is 'fixed' early-on and they would not divide and differentiate synchronously with their neighbouring cells. This would then affect the overall patterning and growth of the carpel as is seen in the case of *ett* mutants. Consistent with this hypothesis is the observation that *ett* mutants have often reported to develop stigmatic tissue precociously at stage 8 (Sessions and Zambryski,1995, this study)

In order to determine if IND is expressed prematurely in the *ett* mutant, *IND:GUS* expression was monitored prior to stage 9 in the carpel. The expression of this construct was observed in *ett-3* carpels at a slightly earlier time point in stage 9 than the WT (Fig. 28 E,C,G). The gene was expressed ectopically at this stage along the abaxial surface beyond the apical domain. The levels of expression of the construct also appear to be stronger than in the WT, as the GUS signal strength is stronger in the *ett-3* mutant than in WT in two independent experiments using different incubation times (See Materials and Methods, Section 4.2.3).

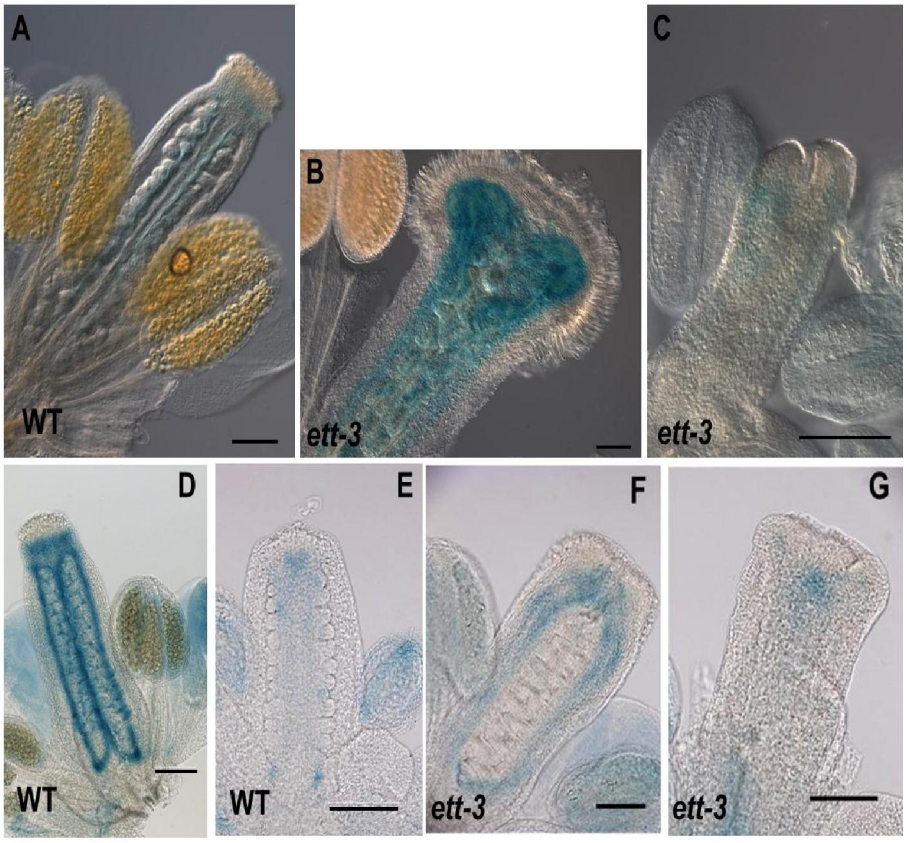


Figure 28 *pIND::IND:GUS* expression in WT and *ett-3* carpels. IND:GUS expression in WT (A, D, E) At stages 12 (A,D) and stage 9 (E). IND:GUS expression in *ett-3* (B, C,F,G) at stages 12 (B), 11(F) and early 9 (C,G). Scale Bars=100μm

These data therefore suggest that spatio-temporal regulation of gene expression could be a significant determinant of the overall patterning of the gynoecium.

The Gradient Model proposed by Nemhauser et al.,2000, also suggests that the patterning defects seen in *ett* mutants are due to the destabilisation of the apical-basal boundaries, such that the apical boundary which is normally restricted by ETT is extended, presumably due to the pooling of auxin in this region. This shifting of the apical boundary coupled with the reduction of auxin levels in the medial domain, results in the loss of the ovary in the *ett* mutant. In order to determine if this is the case, *DR5:GFP* distribution was analysed in the *ett-3* mutant. Remarkably, instead of an increase in the levels of auxin signaling and distribution which was expected in the apical domain, DR5 levels were seen to be lower in the *ett-3* mutant (Fig. 29, 30). In WT, auxin is concentrated at two foci at the lateral apical ends up to early stage 9. By stage 10, auxin then forms a distinct stylar ring which is seen up to stage 12, after which the ring becomes weak (Fig. 29).

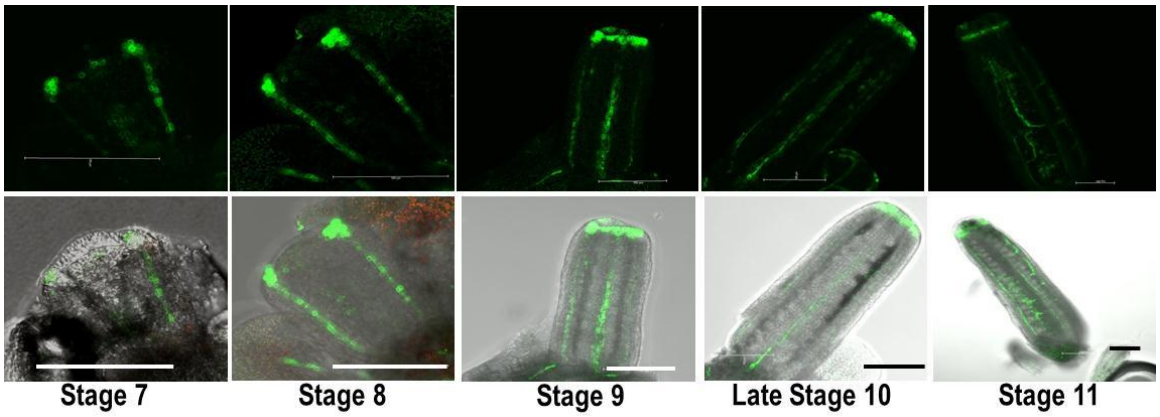


Figure 29 *DR5:GFP* expression in WT. Scale Bars=100μm

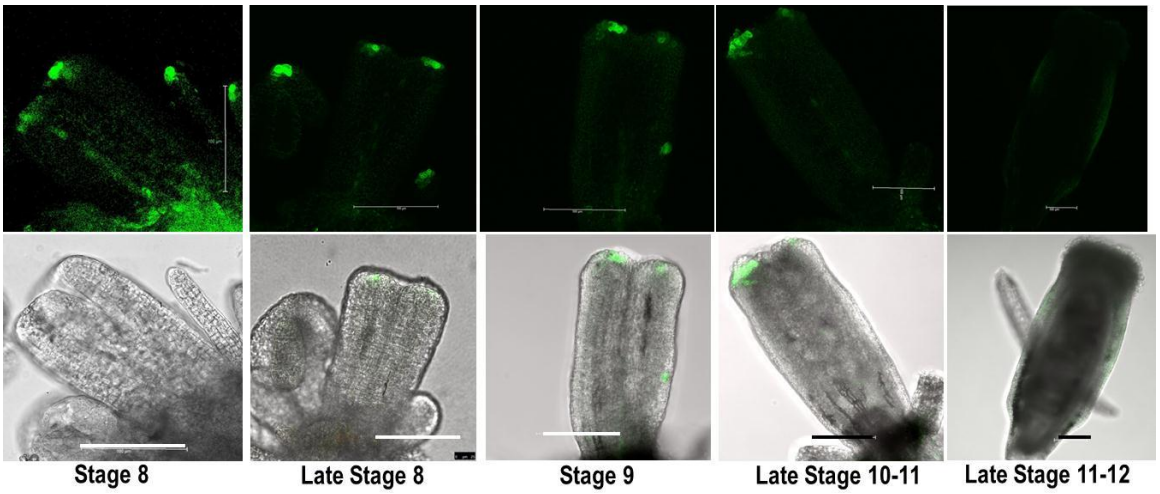


Figure 30 *DR5:GFP* expression in *ett-3*. Scale Bars=100μm

In the *ett* mutant however, the auxin ring is never formed and it remains localized to the foci. WT carpels also show very clear *DR5:GFP* signals in the medial and lateral vascular bundles and these are also very low in the *ett-3* mutant. At this point it is difficult to conclude whether this is due to lower auxin levels or as a consequence of defective vascular tissue development which is seen in *ett-3* mutants. Thus, unlike the expected accumulation of auxin in the apical domain and the extension of the apical auxin boundary which was expected in the *ett* mutant, what is seen is a reduction in overall auxin distribution. The formation of the apical auxin ring is hypothesized to occur via the regulation of PIN proteins (PIN1 and PIN3 primarily) by IND and SPT. These proteins co-regulate WAG2 and PID kinases which coordinate PIN polarity. IND and SPT repress *PID* expression during the early stages of carpel development and this has been shown to allow

apolar localization of PINs which would transport auxin laterally from the foci and accumulate auxin in this region (Moubaiyidin and Ostergaard, 2014).

Like the *ett* mutant, the *ind-2 spt-12* double mutant also lacks the apical ring and possesses a split-style, presumably due to defective PIN localization. Thus, to determine whether PIN localization patterns were also disrupted in the *ett* mutant, *PIN1:GFP* and *PIN3:GFP* reporter constructs were crossed into the *ett-3* background. *PIN1:GFP* localisation in the *ett-3* mutant was similar to the WT at the very early stages of carpel development (Fig. 31,32).

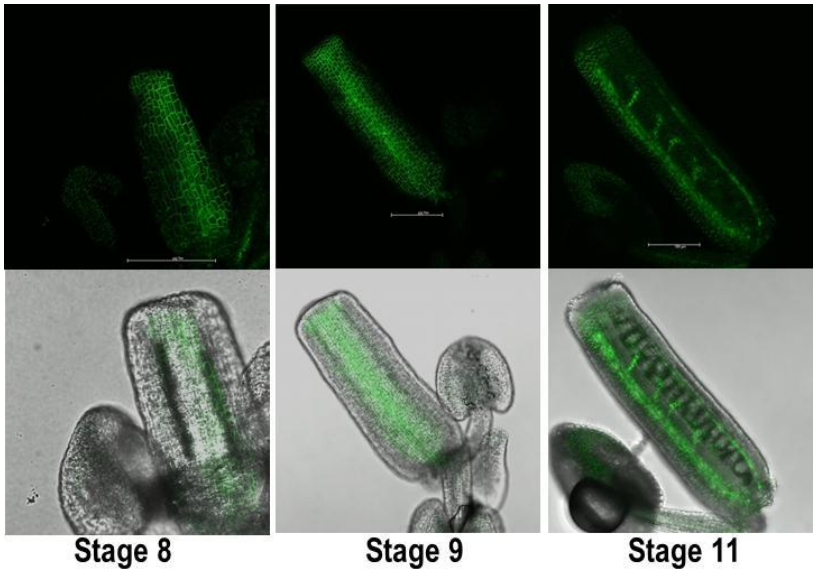


Figure 32 *PIN1:GFP* distribution in WT gynoecia. Scale bars= 100 μm

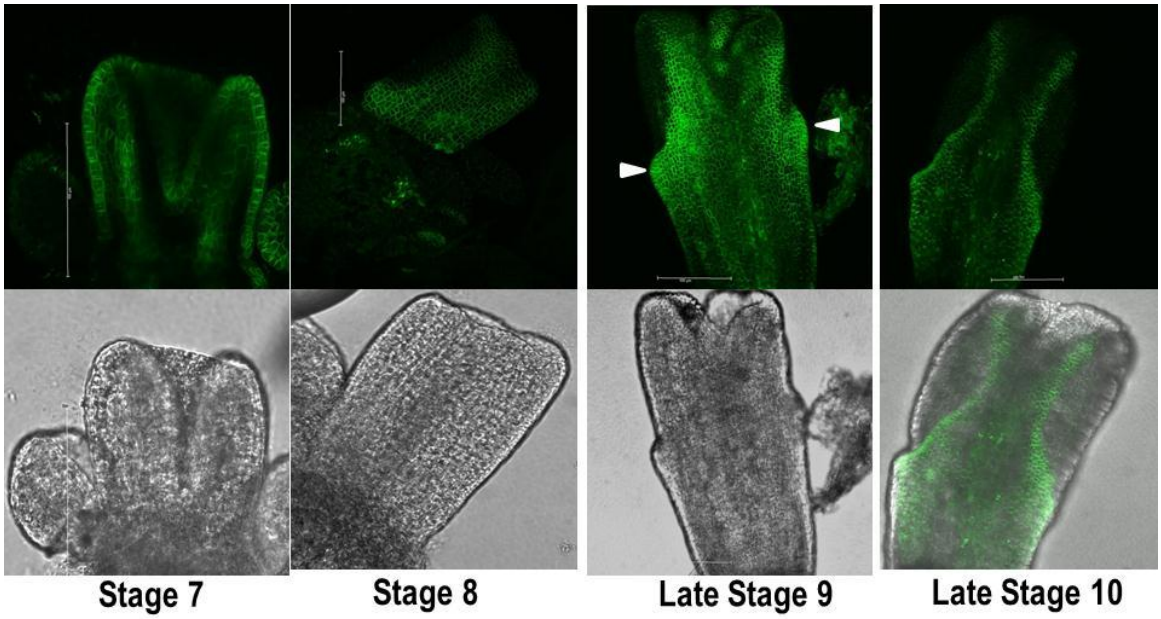


Figure 31 PIN1:GFP localization in *ett-3* carpels. Scale Bars=100μm

The orientation of PIN1 shows an apical trend in its localization in the carpel tissues, which is probably to transport auxin apically to facilitate carpel elongation. However, in the later stages of development, PIN1 appears to localize towards the abnormal outgrowths which are often seen in the *ett-3* mutant, and in these regions, PIN1 localization is largely apolar (Fig. 32). At the apical end, the distinctive apolar localization of PIN1 which is seen in the WT does not occur in the mutant. Thus, PIN1 expression patterns suggest that mislocalized PIN1 in the apical region is likely one of the reasons for the lack of the auxin ring.. It is clear, however, that the development of lateral outgrowths in the *ett* mutant would involve pooling of auxin at these sites which is suggested by PIN1 localization patterns. This would affect auxin flux through the gynoecium and consequently also affect overall carpel growth.

The localization of *PIN3:GFP* was also analysed in the *ett-3* mutant. In WT, PIN3 is localized at the apical end throughout gynoecium development (Fig. 33). Interestingly in the *ett-3* mutant, it appears that the *PIN3* gene is ectopically expressed in the main body of the gynoecium around stages 7-8 (Fig. 34). This observation indicates that mislocalized PIN3 may contribute to the apical-basal pattering defects seen later in the carpel. Further, it might also be involved in the precocious establishment of stigmatic tissue identity in these cells as in the later stages, *PIN3:GFP* expression is coincident with ectopic stigmatic tissue (Fig. 34).. As IND regulates PIN polarity, the ectopic *PIN3* expression could be a consequence of precocious and ectopic IND activity. .

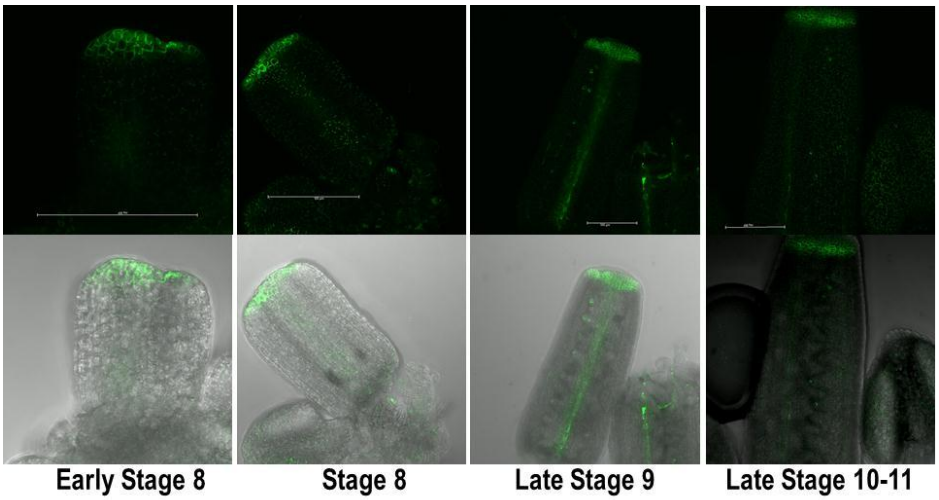


Figure 33 PIN3:GFP localization in WT carpels. Scale Bars=100μm

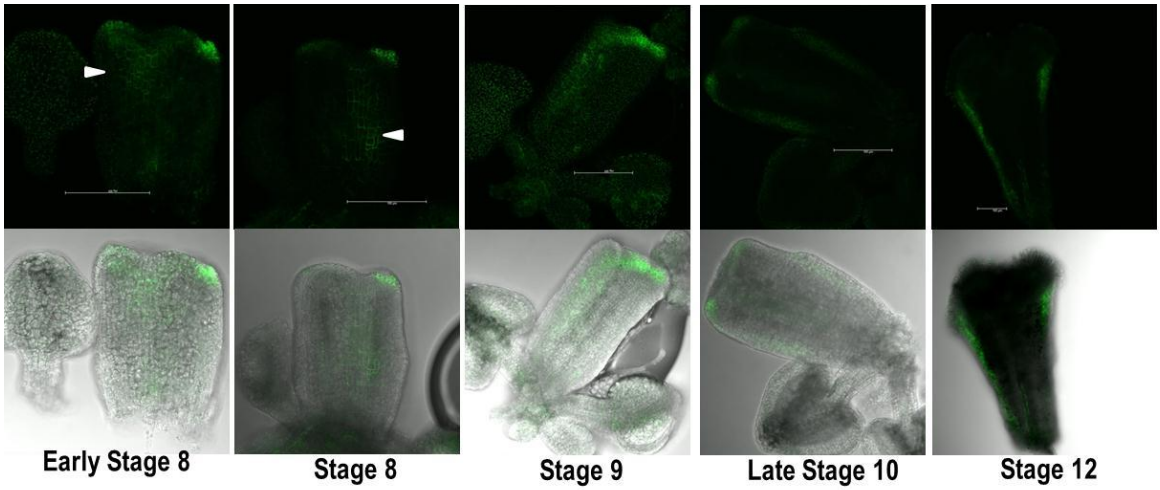


Figure 34 PIN3:GFP localization in *ett-3* carpels. Scales Bars=100μm

IND regulates PIN polarity in the style by repressing *PID*. It is possible that ectopic *IND* expression in the *ett-3* carpel also represses *PID* in these regions and this results in the patterning defects seen in the mutant. Based on this hypothesis, a DEX-inducible construct expressing *PID* under the control of the IND promoter was transformed into the *ett-2* mutant to check whether the construct could rescue the patterning defects seen in the mutant. It was not possible to obtain transformants in the *ett-3* background owing to the poor fertility and lack of seeds from many of the plants. SEM analysis on *ett-2* plants expressing this construct showed that expressing *PID* within the ectopic IND-expression domain did not rescue the apical-basal patterning defects (Fig. 35 E-G). On the contrary, the patterning defects were exacerbated as the carpels of these plants were shorter than the WT and lacked apical-basal pattering. The valves in these plants either did not develop or were highly reduced and in some cases the style did not develop either although all the carpels had stigmatic tissue at the apex. The phenotype of these mutants is similar to many auxin deficient mutants. Lower levels of auxin in the carpels of these plants could be caused either if PID destabilizes the PIN proteins in the regions it is expressed or if the PINs are localized in a manner which disrupts auxin distribution such that it is concentrated in some regions and lower in the neighbouring areas. Either of the above mechanisms would result in a disruption in auxin flux through the carpel which would consequently affect genetic regulation and growth activities necessary for carpel development.

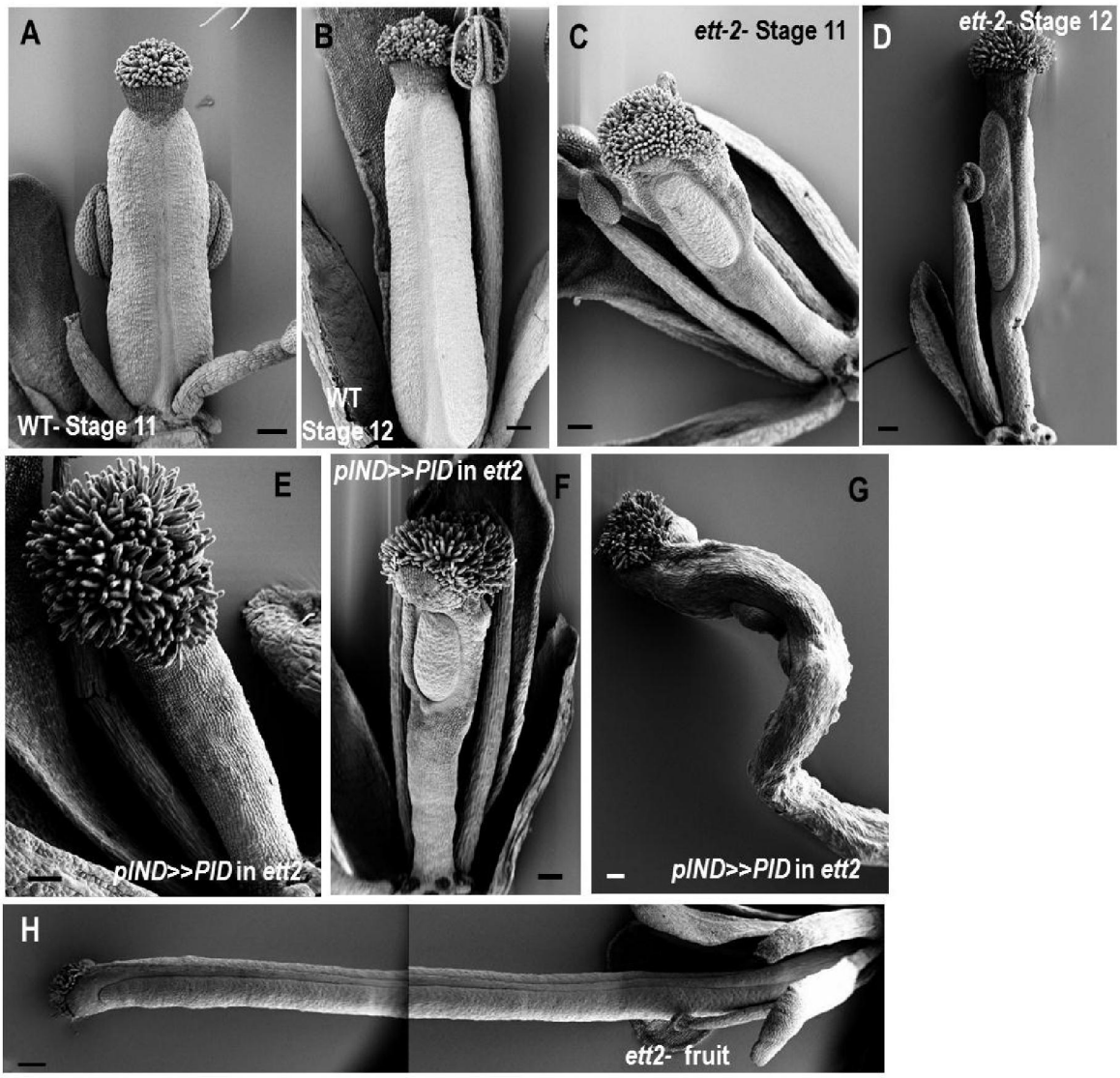


Figure 35 *pIND>>PID* increases the patterning defects of the *ett-2* mutant. A,B WT carpels at stage 11 and 12 respectively**.** C,D *ett-2* mutant at stages 11, 12 and the fruit phenotype (H). E,*F pIND>>PID* in *the ett-2* background at stages 11 and 12 respectively and the fruit phenotype (G). All plants were treated with 100µM DEX twice during a 4 day period. As controls, WT and *ett-2* were transformed with a DEX-inducible *pIND>>mCHERRY* construct. Scale Bars= 100 µm (A-G) 200µm (H)

4.3.3 Analysis of the role of INDas a potential auxin receptor in carpeldevelopment

During the course of this study it was found that IND and ETT function as a novel co-receptor for auxin. Auxin binding to this co-receptor appears to cause a conformational change which in yeast two-hybrid assays results in the lack of reporter gene expression. To uncover the role of this receptor-complex in plants, Sara Simonini, a post-doctoral researcher in the lab, utilized the error-prone PCR technique to identify a point mutation in the IND protein, which makes the protein insensitive to IAA in yeast assays.

This auxin-insensitive version of IND (INDmut) was shown to be fully functional in plants as it was able to compliment the *ind-2* mutant conferring the dehiscence phenotype. The carpels of these plants show defective style development such that the styles are conspicuously reduced and there is a proliferation of stigmatic tissue (Fig. 36). This suggests that auxin acts to modulate the activity of IND to ensure that there is coordination between style and stigmatic tissue development i.e. it restrains IND activity to ensure that stigmatic tissue only develops after the style is sufficiently developed and elongated. The mutants also showed reduced seed set and pollen tube growth; therefore, the defective style development also affects fertilization and seed set.

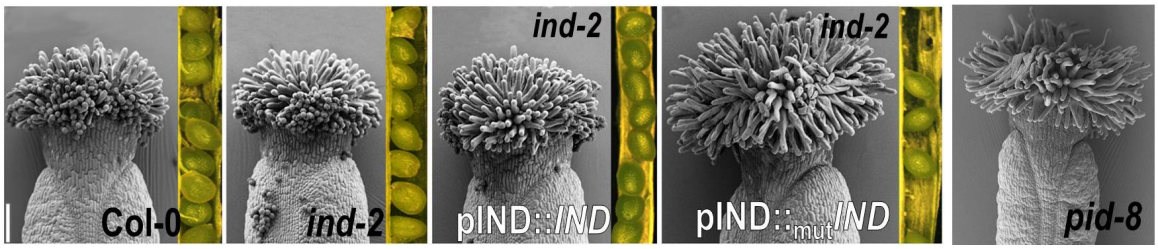


Figure 36 Phenotype of plants with the IAA-insensitive *pIND::INDmut* construct. Both *pIND::IND* and *pIND::INDmut* construct were transformed into *ind-2* plants. *pIND::INDmut* plants resemble the *pid-8* mutant. *Acknowlegment to Sara Simonini for SEM and seed images.*

In the WT, the activity of the INDmut construct can be replicated if the levels of auxin in the style are below a threshold concentration. Thus, in order to determine if low auxin levels in the style can replicate the INDmut phenotype, the auxin degrading iaaL enzyme was expressed under the IND promoter in WT. Conversely, iaaM the bacterial auxin biosynthetic enzyme was also expressed to check whether this would have the opposite effect of delaying stigmatic tissue development and form a longer style. Both constructs were DEX inducible and expressed under the IND promoter. Plants were treated with 100µM DEX twice during a 4 day treatment period. Neither of the two constructs appeared to have any effect on the plants as SEM analysis showed that the treated plants did not have any distinguishable phenotype (Fig. 37). To rule out the possibility that the DEX treatment was insufficient, the transgenic plants were grown for 7 days and treated 3 times with 100µM DEX. These plants also didn't exhibit any distinct phenotype (data not shown). It is likely that this experiment did not yield any results due to technical reasons and would have to be optimized as other constructs in the lab using this system of induction and under the control of the *IND* promoter, have also shown reduced promoter activity (Xinran Li, personal communication).

The experiment will therefore be repeated and additionally, lines with these two constructs in a *DR5:GFP* background have also been developed in the lab and these will be used to monitor auxin distribution when either *iaaM* or *iaaL* is expressed in the style.

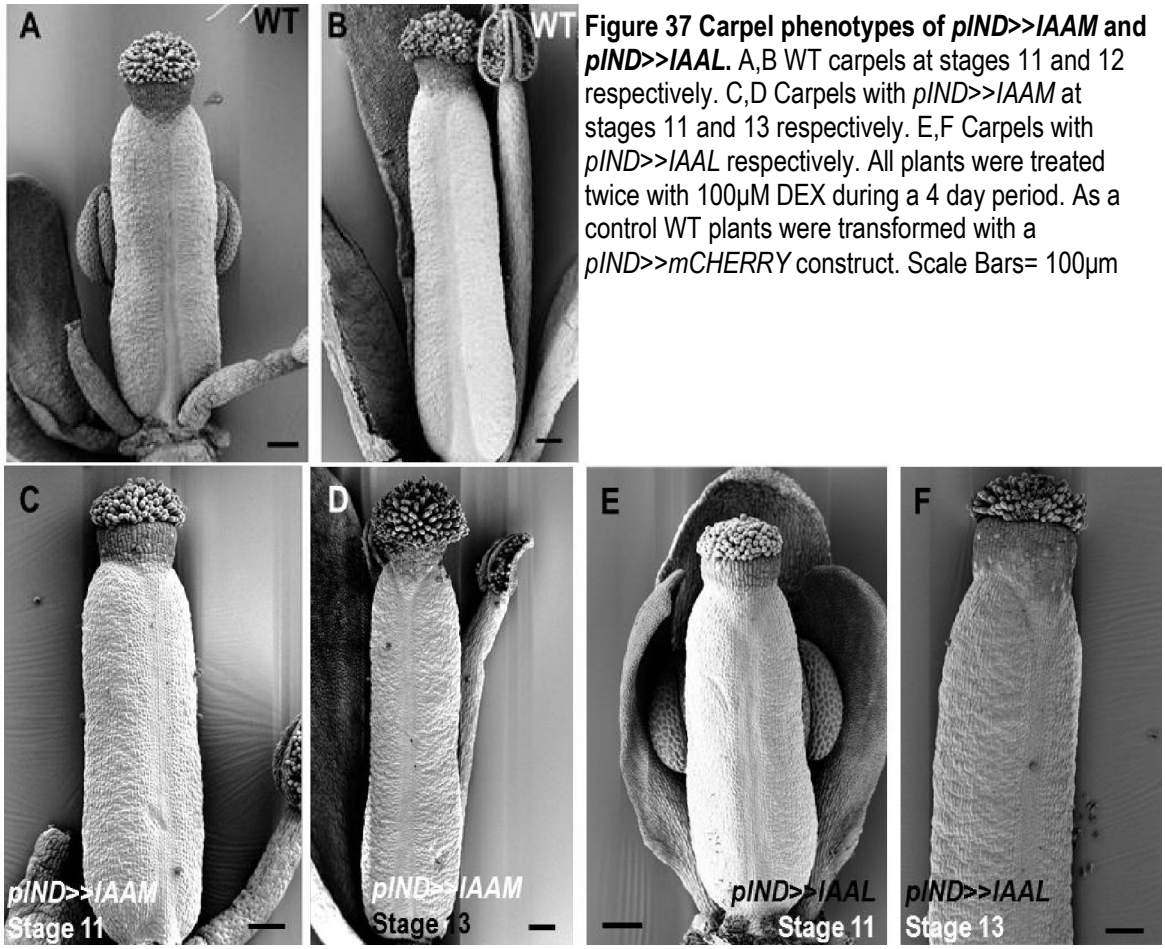


Figure 37 Carpel phenotypes of *pIND>>IAAM* and *pIND>>IAAL*. A,B WT carpels at stages 11 and 12 respectively. C,D Carpels with *pIND>>IAAM* at stages 11 and 13 respectively. E,F Carpels with *pIND>>IAAL* respectively. All plants were treated twice with 100µM DEX during a 4 day period. As a control WT plants were transformed with a *pIND>>mCHERRY* construct. Scale Bars= 100µm

4.4 Discussion

One of the major aspects of gynoecium patterning which we are yet to understand is how gene expression regulation and hormonal regulation are coupled during gynoecium development. There is clearly feedback between the two (See Section 4.1), however, correct morphogenesis entails a mechanism to interpret auxin levels and second, a mechanism to ensure organ patterning is synchronized. The Gradient Model of gynoecium development was based on the existence of an auxin gradient in the carpel which is then interpreted by the ETT molecule to establish boundaries and coordinate patterning. As a master regulator of carpel patterning and with mutants exhibiting auxin-affected phenotypes, the proposition that ETT is able to interpret auxin levels is credible. However, since no auxin gradient has been found in the carpel, the mechanism of carpel morphogenesis is still open to interpretation. Another mechanism, by which ETT could translate auxin levels into different tissue specific outputs, would be if it was expressed as a gradient in the carpel and tissue identity specification would depend on ETT protein dosage (Galinha et al., 2007). A *pETT::ETT:GFP* construct was thus developed in this study to visualize ETT distribution in the gynoecium. Confocal analysis however did not reveal any gradient of *ETT* expression.

The findings from this study suggest that regulation of gene targets by ETT could be via auxin-dependent and auxin independent mechanisms. The former case would rely on its putative function as an auxin co-receptor to provide a direct read-out of auxin levels with different protein partners.

4.4.1 The role of ETT as a putative auxin receptor

The IND-ETT interaction and FRET-FLIM assays together support a model in which these proteins function as a co-receptor complex in the style. This was further substantiated from the phenotype of plants expressing the mutated IAA-insensitive version of IND (INDmut). In these plants, the style appeared less developed while stigmatic tissue showed excessive development which is a similar phenotype to that of the weak *pid-8* allele. This reveals an additional layer of regulation for style development to occur correctly and in coordination with stigmatic tissue proliferation. It was hypothesized that the auxin maxima formation in this region relied on the apolar distribution of PINs - mainly PIN1 and PIN3 - and this mechanism was under regulation by the IND-SPT heterodimer. This protein complex represses *PID* expression in the style (Girin et al., 2011) thus supressing the apical-basal

orientation of PINs which PID would otherwise promote. These results indicate that constitutive repression of *PID* by IND and SPT does not allow the style to develop completely and auxin probably functions as a switch to relieve *PID* repression by IND. Indeed, very weak expression of a *PID:GUS* construct is observed from stage 11 onwards in the style (Girin et al., 2011). Thus, one could hypothesize that in the early stages when the concentration of auxin is low in the style, the IND-ETT complex represses *PID* facilitating maxima formation. Around stage 10-11 when the auxin concentration appears to accumulate and stigmatic tissue differentiation is observed, the IND-ETT complex could bind auxin which would change the conformation of this co-receptor, allowing *PID* expression and subsequent style elongation. This conformational switch is also perhaps necessary to regulate IND activity so that it does not result in over-proliferation of stigmatic tissue. *PID* expression would probably result in the apical localisation of PINs in the plasma membranes of stylar cells. There are indications of an apical trend in PIN1 localisation in style cells from stage 11, however, this requires further observation. No such switch is visible in the orientation of PIN3 around stage 10 and its localization at the later stages will have to be observed. Future work would involve determining whether *PID* is a target of ETT and also confirming the mechanism of regulation by both IND and ETT. At present, a ChIP assay will be carried out by Pauline Stephenson in the lab to verify if *PID* is a target of ETT. During this project, a *pid-9 ett-3* double mutant was generated, however, owing to problems with the genotyping PCR, the double mutant could not be identified in the time frame of this project. Attempts were also made to establish a yeast two-one hybrid assay involving IND and ETT and the cis-element from the *PID* promoter that IND has shown to bind to elucidate the mechanism of regulation by these proteins on the *PID* promoter, however, a high degree of auto-activity was observed and even after several attempts at optimisation, the levels of auto-activity could not be reduced and the assay could not be completed.

This auxin co-receptor presents a novel mechanism by which ETT could translate the same auxin input into differential gene expression outputs. Through a yeast-library screen, Sara Simonini in the lab has also found auxin-sensitive interactions between ETT and other carpel-specific and non-specific proteins. Among the ones which are expressed in the carpel include *BREVIPEDICELLUS* (*BP*) which is expressed in the replum and functions with *REPLUMLESS (RPL)*, a homoedomain protein which is required for replum development. As no common motif has been found among ETT's interacting partners (data

not shown), it is possible that these specific interactions possess different thresholds of sensitivity to auxin, much like the different affinities of the AUX-IAA proteins to the TIR1/AFB proteins (Calderon Villalobos et al., 2012). Thus, this mechanism negates the necessity for a gradient of a protein or a hormone occurring in the carpel, as different sensitivities to auxin ofthe different protein complexes with ETT would ensure differential gene expression outputs.

4.4.2 Transcriptional Regulation of Gynoecium Patterning by ETT

The findings from this study also indicate a possible alternative mechanism of patterning than previously proposed. Sessions et al., (1997) and Nemhauser et al., (2000), ascribe the patterning defects of the *ett* mutant to misplaced boundary positions. However, two observations appear to contradict this hypothesis. The first assumption made was that the extension of the apical boundary in *ett* mutants is a consequence of high levels of pooled auxin in this region. In this study, *DR5:GFP* expression in the *ett-3* mutant showed the contrary i.e the distribution of auxin in the style is disrupted and the overall levels appear lower. Further, the DR5 signal is concentrated at the foci and does not extend with the apical tissues. This brings to front the second contradictory finding which is the stochastic nature of patterning in the *ett-3* mutant. Although a trend showing misplaced apical-basal boundaries is apparent in this mutant, the development of these ectopic tissues does not follow a consistent pattern and tissues often appear in different patches. Further, the inversion of the adaxial- abaxial boundary also cannot be explained with this theory. The nature of the *ett-3* phenotype suggests that rather than positional information from boundaries, spatio-temporal gene expression regulation by ETT is perhaps more significant to coordinate patterning in the carpel. The *ett-3* phenotype is partially rescued in the *ind-2 ett-3* mutant and a further rescue is seen in the *ind-2 spt-12 ett-3* triple mutant suggesting ectopic expression of these proteins is in part one of the contributors of aberrant patterning in the *ett-3* mutant and this was confirmed using a *pIND*:: *IND:GUS* translational fusion construct which showed ectopic expression in the ovary and often extending to the base of the *ett-3* mutant. Ectopic *IND* expression was also seen at stage 8-early 9 in the *ett-3* mutant. This early specification of tissue identity would therefore perturb the overall patterning of the gynoecium as is seen in the *ett-3* mutant. Further, as IND also regulates PIN protein polarity, ectopic *IND* expression could also affect auxin flux through the gynoecium. Although *PIN1:GFP* localisation did not show any prominent changes at the early stages, the localisation of the protein was perturbed in the later stages (stage 10

onwards, Fig. 32) although it is difficult to conclude whether this is a consequence of the patterning defects or could be the cause of the observed carpel defects. PIN3:GFP did show some ectopic expression in the early stages, which was clearly evident in the later stages. Thus, in the future, *ett-3* carpels at stage 8- early 9 will have to be analysed to determine whether this ectopic *PIN3:GFP* localization is seen in more samples. It will also be necessary to confirm whether ETT directly regulates *IND* by binding to its promoter by ChIP. Further, in order to test whether precocious *IND* expression can perturb overall gynoecium patterning, a DEX inducible *IND* construct under the *ETT* promoter could be developed and expressed in the *35S:SPT* background. Induction of this construct in the gynoecium should be able to phenocopy the *ett* mutants.

In conclusion, the findings from this study suggest that temporal regulation coupled with precise spatial regulation of genes provide the required cues to ensure synchronized pattern establishment. Whether the spatial regulation of genes is coordinated by a pre-pattern in the gynoecium is difficult to conclude at this point, however it is also possible that sequential temporal regulation following the interpretation of auxin levels by the proposed auxin-sensing property of ETT, might be sufficient to initiate the chain of patterning events. At this point, as the auxin-sensing domain of ETT has been identified, transforming plants without this domain would help decouple the auxin-dependent and auxin-independent regulatory functions of ETT which would be a useful tool in developing a better understanding of the regulation of carpel patterning.

CHAPTER 5

CHAPTER 5- Analysis of the Evolutionary Conservation of the IND-ETT Auxin Co-receptor Complex in the Brassicaceae

5.1 Introduction

The central role played by auxin is clear from its many roles in plant development; these range from the time of the inception of the plant in the emerging embryo, to the development of organs in the adult. Although extensively studied and well characterized in *Arabidopsis thaliana (A. thaliana)*, the existence of similar auxin-regulated processes in other plant species was evident from the time of the discovery of auxin itself. These emerged from studies on phototropism, initially by Charles Darwin in canary grass and subsequently from the discovery of the IAA molecule by Went in *Avena* coleoptiles (Abel and Theologis, 2010) .Auxin regulates key growth and developmental processes in evolutionarily divergent plants; in the moss *Physcometrilla patens (P. patens)*, for instance, auxin is both necessary and sufficient for the transition from the chloronema stage to the caulonema stage via the regulation of two bHLH proteins (Jang and Dolan, 2011). Among higher plants changes in auxin distribution patterns is thought to be a possible mechanism underlying the diversity in leaf morphologies (Kuchen et al., 2012). But within the plant families themselves, differential auxin outputs also appear to contribute to diversity in morphologies, for instance in the Brassicaceae, the change from a simple leaf as seen *A. thaliana* to a dissected leaf like that of *Cardamine hirsute*, is thought to have occurred due to the formation of local auxin foci in the rachis of developing leaves (Barkoulas et al., 2008). In recent times, phylogenetic studies have identified orthologues of 'auxin'genes' in the earliest land plants. These genes encode components of auxin biosynthesis, transport and signalling, however, whether the function of many of these genes has been conserved through evolution is yet to be fully determined (Finet and Jaillais, 2012).

Considering the evolution of auxin signalling pathways, the presence of orthologues of Aux/IAA proteins, ARFs and TIR1/AFB proteins in the genomes of *P. patens* and *Selaginella moellendorffii*, suggest that the canonical auxin signalling pathway is highly conserved among the land plants. This view is further supported by the observations such as the ability of moss AUX/IAA proteins to interact with *A. thaliana* TIR1 homologues (Finet and Jaillais, 2012). The present day ARF protein sub-families also appear to have ancient origins dating back to the gymnosperms and these were clearly established in the

ancient ANA grade angiosperms (*Amborella trichopoda, Cabomba aquatica*, and *Illicium parviflorum*). Whole-genome duplication events which are hypothesized to have lead to the evolution of the seed plants seen today probably lead to the diversification of the ARF protein family, and this, followed by their neo-functionalization and sub-functionalization, has led to the range of functions exhibited by the ARF proteins (Finet et al., 2013). In addition to duplication events, evidence of several alternatively spliced forms of ARF proteins have also been found in primitive angiosperms and this is hypothesized to have resulted in the evolution of ARF proteins without protein dimerisation domains (Finet et al., 2010, Finet et al., 2013) suggesting that changes in protein-protein interaction abilities may have also lead to the diversity in auxin signalling outputs seen today.

This is evident in the case of ARF4 and ARF3/ETTIN. These genes can be traced to the ANA grade angiosperms as both genes are expressed within the carpel tissues of *Amborella trichopoda* (*A. trichopoda)* and *Cabomba aquatica* (*C. aquatica*) (Finet et al., 2010). AtETT and AtARF4 share a high degree of homology, however, ETT does not possess the dimerisation domains III and IV, while ARF4 has a canonical ARF structure and possess these domains. There is evidence that this non-canonical domain structure of ETT may have arisen from alternatively spliced transcripts and truncated versions of ARF4 lacking domains III and IV which exist in *A. trichopoda* and *C. aquatica*, respectively. The truncation of ETT appears to have been necessary for the acquisition of its carpel-specific functions, as versions of AtETT with domain III and IV added are unable to fully complement the carpel phenotype of the *ett-1* mutant and conversely, *ett-1* mutants with truncated versions of ARF4 have defective carpels (Finet et al., 2010). Thus, the diversification of ARF structure, probably lead to the acquisition of novel functions by these proteins (Finet et al., 2013).

This project has revealed the existence of a novel auxin co-receptor complex between IND and ETT proteins in *A. thaliana*. Although *IND* plays a role in regulating auxin distribution in the carpel, this gene appears to be specific to the Brassicaceae family and is highly conserved among its members. The function of IND has been studied in *Brassica rapa (B. rapa)* and *Brassica oleraceae (B .oleraceae)*. Both diploid species only have one copy of the *IND* gene. The function of the protein is highly conserved in both species as mutational analysis and RNAi techniques have both demonstrated that a lack of IND function leads to the development of indehiscent fruits without valve margin development. The expression pattern of the gene is also conserved and is restricted to the valve margins in *B. rapa* (Girin

et al., 2010). Although the functions of the *Brassica IND* gene in the fruit have been well characterized, whether it regulates gynoecium and valve margin development through the manipulation of auxin distribution in these species, is still an open question.

This chapter looks into the conservation of the IND-ETT co-receptor complex in the Brassicaceae family. In addition to *A . thaliana*, three members of this family have been chosen to assess this which are- *Capsella rubella* (*C. rubella*), *B. rapa* and *B. oleraceae*. Furthermore, another aim of the following experiments is to use the diversity in the gene sequences of these closely related members to potentially identify key residues which might be essential for auxin binding. The yeast two-hybrid approach has been adopted to answer both of the above questions regarding the evolutionary significance of this novel auxin-sensing mechanism.

5.2 Material and Methods

5.2.1 Cloning methods

The IND genes from *B. rapa* and *B. oleraceae* are intron-less and were therefore amplified directly from genomic DNA. Leaf tissue from *B. rapa* var. R-o-18 and *B. oleraceae* var. AG DH1012 was used as a template to PCR amplify *BraA.IND.a* and *BolC.IND.a* genes (Ostergaard and King, 2008), using the Phusion PCR protocol specified in Chapter 2, section 2.2.2. *B. rapa* has two *ETT* genes. *BraA.ETT.a* and *BraA.ETT.b* and both were amplified from cDNA synthesized from RNA isolated from *B. rapa* var. R-o-18.

Both *IND* and *ETT* genes from *C. rubella* (*CrIND* and *CrETT*, respectively) were cloned using Gateway Cloning (Invitrogen) into yeast vectors from Clontech with Gateway sites. *CrETT* was cloned into pGADT7 and pGBKT9 (Clontech). *CrIND* was cloned into a modified version of pGADT7g vector for a C-terminal fusion of the AD with the protein (Stellberger et al., 2010).

5.2.1.1 cDNA Synthesis

A total of 1µg of RNA from stage 14 *C. rubella* fruit was used for first-strand synthesis with 1µl oligo dT (18) primers in a 11µl reaction volume. This was then incubated at 65^{o}C for 15 min. To this reaction was then added 1µl of MMLV-RT (Invitrogen), 1µl of 10mM dNTP mix, 2µl 0.1mM DTT, 4µl 2.5mM $MgCl_2$, 2µl 10x RT Buffer for a total reaction volume of 20µl.The reaction was incubated at 50^{o} for 50min following which 80µl RNAse-free water was added to the reaction mixture for 100µl of total cDNA.

5.2.1.2 Cloning of genes from *B. rapa* and *B. oleraceae*

The *BraA.IND.a* and *BolC.IND.a* genes and *BraA.ETT.a* and *BraA.ETT.b* genes were cloned into the yeast vectors pGAD424 and pGBT9 using the EcoRI and PstI sites in the multiple cloning site of both vectors.

5.2.1.3 TOPO Cloning and Gateway Cloning Methods

Genes were subcloned into the TOPO vector PCR8 (Invitrogen) using TA cloning. The PCR products were gel extracted and purified. About 100-300ng of the gel extracted product was used in the A-tailing PCR reaction to which was added 1µl 1mM dATP, 0.2µl

Standard Taq Polymerase (NEB), 1µl 10X Reaction Buffer and sterile water to a total reaction volume of 10µl. This was then incubated at 65°C for 30min.

For TOPO cloning, 4µl of the above PCR reaction was used to which was added 0.25µl of PCR8 vector, 1µl of Salt Solution and 0.75µl water. This was incubated for 10min at RT followed by 15min on ice. The reaction was then transformed into TOP10 *E. coli* cells (Invitrogen) using the heat-shock method (See Chapter 2, section 2.2.5)

The subcloned products were then cloned into the appropriate destination vectors by an LR reaction according to the manufacturer's instructions

(Invitrogen https://tools.lifetechnologies.com/content/sfs/manuals/gatewayman.pdf).

5.2.2 Bioinformatic Analyses

Sequences for *B. rapa IND* and *ETT* genes were obtained from the Brassica Database (http://brassicadb.org) while the sequence of *BolA.IND.a* was obtained from the NCBI database. *CrIND* and *CrETT* sequences were obtained from the Phytozome database (www.phytozome.net). Multiple sequence alignment was carried out using the software T-Coffee (http://www.ebi.ac.uk/Tools/msa/tcoffee/). Translation of gene sequences was carried out using the Expasy Translate Tool (http://web.expasy.org/translate/) while the amino acid content of the proteins was analysed using Expasy ProParam (http://web.expasy.org/protparam/).

5.2.3 Yeast Methods

All yeast transformation procedures were according to the protocol described in Chapter 2, section 2.2.8. *CrETT* and *CrIND* genes were co-transformed into the yeast vector AH109. For all other combinations yeast matings were carried out to identify possible interactions.

5.3 Results

5.3.1 CrIND and CrETT are able to interact in yeast two-hybrid assays

The origins of the canonical auxin signalling pathway can be traced to the evolution of the first land plants as functional components have been identified in *P. patens* (Finet and Jaillais, 2012). Further, parallel auxin signalling pathways also appear to have been conserved in plants as orthologues of the ABP1 protein can be traced back to the first land plants (Rensing et al., 2008). There remains some ambiguity as to whether auxin signalling has been conserved under selective pressure through evolution, or whether it has arisen independently in different species. Nevertheless, the occurrence of this pathway almost throughout the plant kingdom is evidence of the significant role it plays in plant development. As a novel auxin signalling mechanism was identified in this project, it was necessary to determine whether this pathway is also conserved through evolution. To this end the yeast two-hybrid method used to detect IAA-sensitive interactions described previously (Chapter 3, Section 3.3.2) was adopted to determine whether this is observed in other species as well.

Among the species which are closely related to *Arabidopsis* within the Brassicaceae family, is the diploid species *C. rubella*. The *C. rubella* and *A. thaliana* progenitors diverged approximately 10 million years ago (mya) and although the genome sizes of the two differ, a high degree of co-linearity is found between the two genomes (Boivin et al., 2004). Possible explanations for the divergent characteristics of the two species include changes in gene expression levels (Slotte et al., 2013) and sequence changes in the intergenic and intronic regions (Boivin et al., 2004).

The *CrIND* and *ETT* genes from *C. rubella* were identified using the NCBI and Phytozome databases. A BLASTn search, using the *A.thaliana* counterparts of both genes, retrieved the *IND* and *ETT* orthologues in *C. rubella*. The search revealed that single copies of both genes exist in the *C. rubella* genome (Fig. 38, A,B).

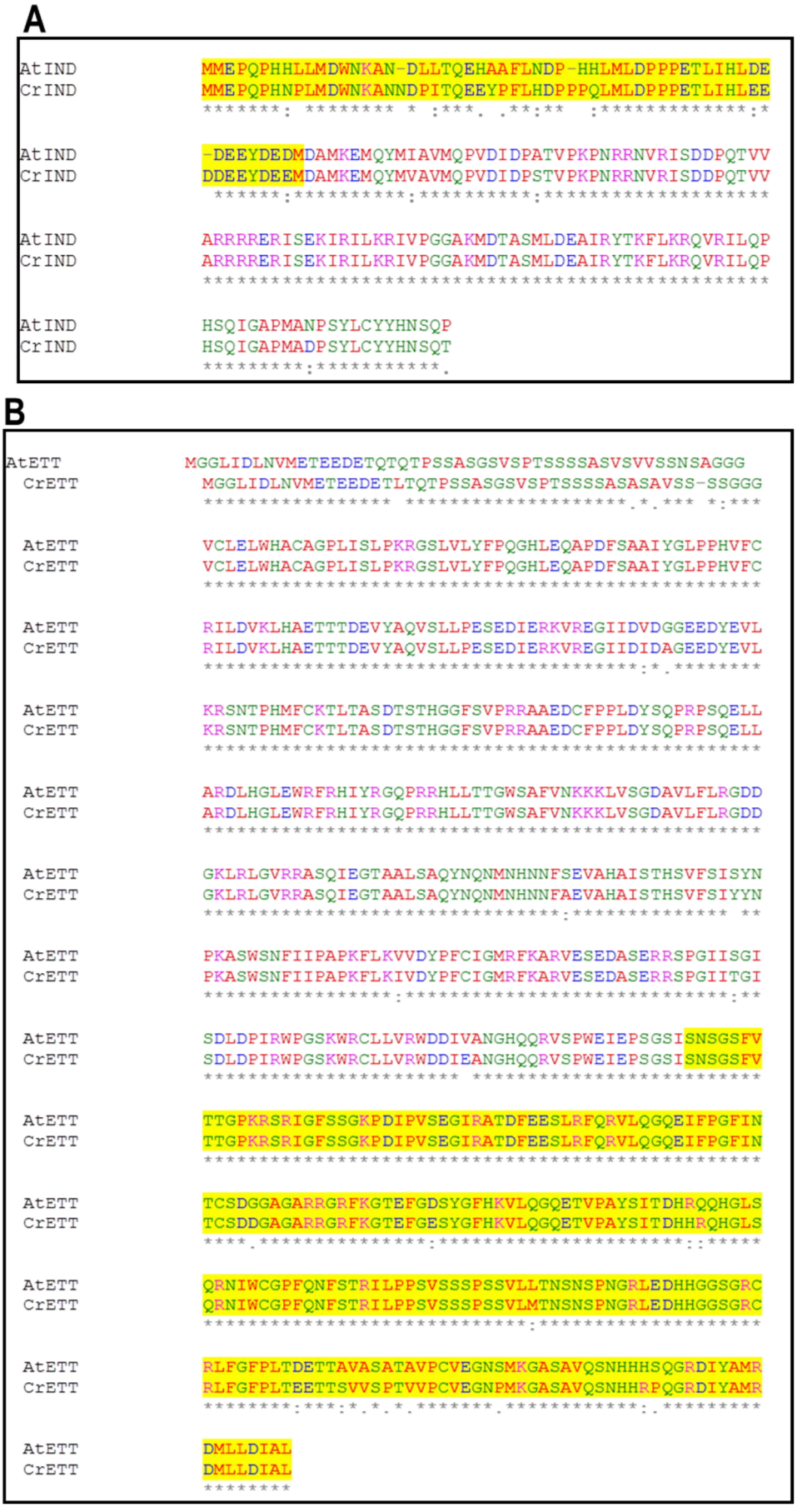


Figure 38 Multiple sequence alignment of IND and ETT proteins from *A.thaliana* and *C.rubella*. (**A**) Clustal alignment of AtIND and CrIND. The IND-ISdomain has been highlighted. **(B)** Custal alignment of AtETT and CrETT . The ES domain has been highlighted. Abb.: At: *Arabidopsis thaliana*, Cr: *Capsella rubella*

Concurrent with large-scale synteny and sequence similarity between the exonic regions of the two species (Boivin et al., 2004, Slotte et al., 2013) the *IND* and *ETT* genes of *C. rubella* and *A. thaliana* are highly conserved (Fig. 38, A,B). *Arabidopsis* ETT (AtETT) and *C. rubella* ETT (CrETT) proteins are 95.9% identical and share 97.5% similarity. *Arabidopsis* IND (AtIND) and *C. rubella* IND (CrIND) proteins share 89.5% identity and 94.2% similarity (EMBOSS-WATER Local Alignment). The high degree of homology with the *A. thaliana* orthologues suggested that these proteins are likely to interact particularly since both the IND-domain and the ES-domain could be easily identified (Fig. 38, B) and are also very similar between the two proteins: the IND domain is 74.6% identical and 83.1% similar between the two, while the ES domain is 94% identical and 96.3% similar.

The proteins were then cloned into the corresponding yeast vectors to test whether the two proteins would interact. As the interaction between AtIND and AtETT was strongest at room temperature (RT), the SD –LWHA selective plates were also kept at RT in all the following experiments. Preliminary results indicate that CrETT and CrIND proteins from *C. rubella* interact very strongly and yeast colony growth was visible after 2-3 days of plating (Fig. 39).

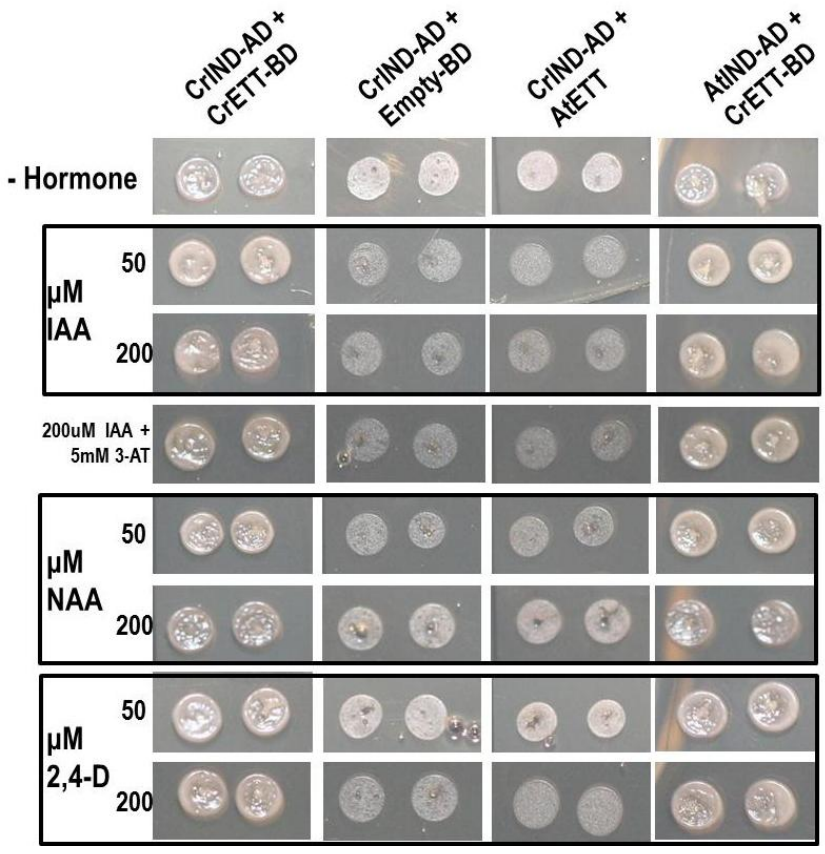


Figure 39 CrIND and CrETT interact in an IAA-insensitive manner. Yeast colonies were plated on SD –LWHA plates and kept at RT. Growth was observed after 2-3days of plating. The colonies are representative of 2 biological repeats.

This interaction was also very strong after the addition of 5mM 3-AT which supresses yeast growth and is used to detect false positives. The mechanism of this protein-protein interaction was likely to be similar as those between the *A.thaliana* proteins owing to the close related-ness shared by the two proteins. Further confirmation of the cross-species conservation of this interaction mechanism would be established by testing the ES domain by itself in the above assay.

The major aim of this experiment was to determine if this co-receptor complex functions within other Brassicaceae members as well. Thus, up to 200µM IAA was added to the yeast medium and the proteins were checked for an IAA-sensitive response. Unexpectedly the interaction between CrIND and CrETT was not IAA sensitive. Even at 200µM IAA, the interaction was still clearly visible (Fig. 39). The observation that IAA has no effect on any of the above interactions is rather surprising. As the *C. rubella* orthologues of both genes share a high degree of similarity with the *A. thaliana* genes, one would expect IAA to have a similar effect. One of the reasons why IAA failed to produce any effect on these interactions could be because of the strong yeast growth seen in this experiment which may mask any subtle effects IAA might have had. If the growth of the yeast strains is robust, then the IAA in the media may not be sufficient to supress the growth of the later colonies. This has been observed in other unrelated experiments as well and therefore is a possibility in this case as well. As this is a preliminary result it would require a repetition to clarify whether the lack of an IAA effect is due to experimental reasons or is due to the inherent structure of the proteins.

In addition to assessing the IAA sensitivity of the interaction, another aim of this experiment was to use the sequence divergence of the *IND* and *ETT* genes in the Brassicaceae as a tool to identify the key residues involved in the IAA-binding mechanism. For this purpose, cross-species interaction tests were carried out between AtIND-CrETT and CrIND-AtETT. A strong interaction between AtIND-CrETT was observed and interestingly, this interaction was also insensitive to IAA even at higher concentrations. NAA and 2,4-D also did not have any effect on the above interactions (Fig. 39).

Another interesting find was that CrIND was unable to interact with AtETT. This lack of an interaction is possibly due to a slightly different amino acid composition of the IND-IS domain of CrIND as the putative HEC and bHLH domains of the two proteins are almost identical (100% similarity- EMBOSS-WATER Local Alignment). A closer look at the

CrIND domain reveals that it is slightly longer (3 amino acids) and also has a few more Proline residues than its *A. thaliana* counterpart (Fig. 38, A). As Proline residues can affect the secondary structures of proteins, this might be the cause of the lack of interaction between CrIND and AtETT.

Thus, preliminary results indicate that the protein-protein interaction between IND and ETT is conserved between *A. thaliana* and its closely related species *C. rubella*. However, this interaction does not appear to function as a co-receptor complex for IAA in *C. rubella*, although this will need to be confirmed with further repetitions of the assay.

5.3.2 The proposed auxin-signalling mechanism may be conserved in B. rapa and B. oleraceae

The *Arabidopsis* and Brassica genera diverged approximately 40 mya (Beilstein et al., 2010). This was followed by a triplication event in the diploid Brassica genome as a result of which many of the genes are found in triplicate in these genomes. As *Arabidopsis* and *Brassica* spp. share a common ancestor, large syntenic regions can be found between the two genomes although the Brassica genomes show evidence of large scale chromosomal rearrangements (Ali et al., 2005)

Only a single copy of the *IND* gene is found in both the *B. rapa* and *B. oleraceae* (Girin et al., 2010) and both share close homology to *IND* from *Arabidopsis* (AtIND). The BolC.IND.a protein shares 73% identity and 82.4% similarity with AtIND, while BraA.IND.a and AtIND are 67.9% identical and share 79% similarity (EMBOSS-WATER Local Alignment). The HEC and bHLH domains of IND the proteins are largely identical within the family although BraA.IND.a is truncated at the C-terminal end (Fig. 40). Interestingly, the sequence of the IND-specific domain of the *Brassica* proteins appears to have diverged slightly from its *A.thaliana* orthologue as both IND proteins have a few amino acids extra within this domain, which are not found either in CrIND or AtIND (Fig. 40). This, however, does not appear to have altered IND's role in valve margin development, as mutants show an indehiscent phenotype (Girin et al., 2010).

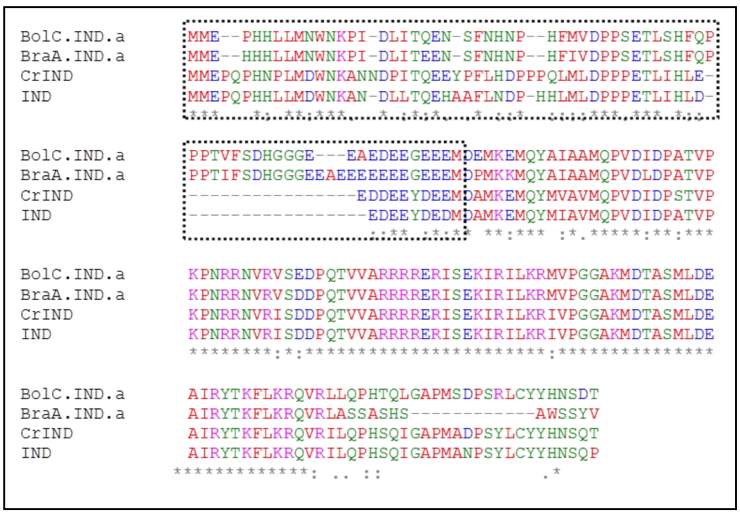


Figure 40 Clustal Alignment of the Brassicaceae IND genes. The boxed region corresponds to the IND domain of these proteins.

A BLAST search for the *ETT* gene in *B. rapa* reveals that two copies of the *ETT* gene exist in the *B. rapa* genome annotated as *B .rapa* ARF3-1 and *B. rapa* ARF3-2. These will hereafter be referred to as *BraA.ETT.a* and *BraA.ETT.b*, respectively, according to the standardized gene nomenclature for Brassica (Østergaard and King, 2008). *BraA.ETT.a* is more closely related to ETT from *Arabidopsis* (AtETT) (85.7% identity and 90.4% similarity) than *BraA.ETT.b* (78.3% identity and 82% similarity) (Fig. 41).

Phylogentic analysis reflects the evolutionary divergence of the *Arabidopsis*-type and *Brassica*-type genes. The AtIND and CrIND proteins are more closely related to one another than the IND proteins from *B.rapa* and *B.oleraceae* (Fig.42). Between the *Arabidopsis* and the *Capsella* lineage, however, the IND proteins from *A.thaliana* and *A.lyrata* are more closely related together and therefore might exhibit some conservation of function as well (Fig. 41). Interestingly the CrETT appears to more closely related to AtETT than AlETT which suggests that the *AlETT* sequence diverged after the initial separation of the *Arabidopsis* and *Capsella* genera, while *C.rubella* has retained the ancestral *AtETT* form (Fig. 42).

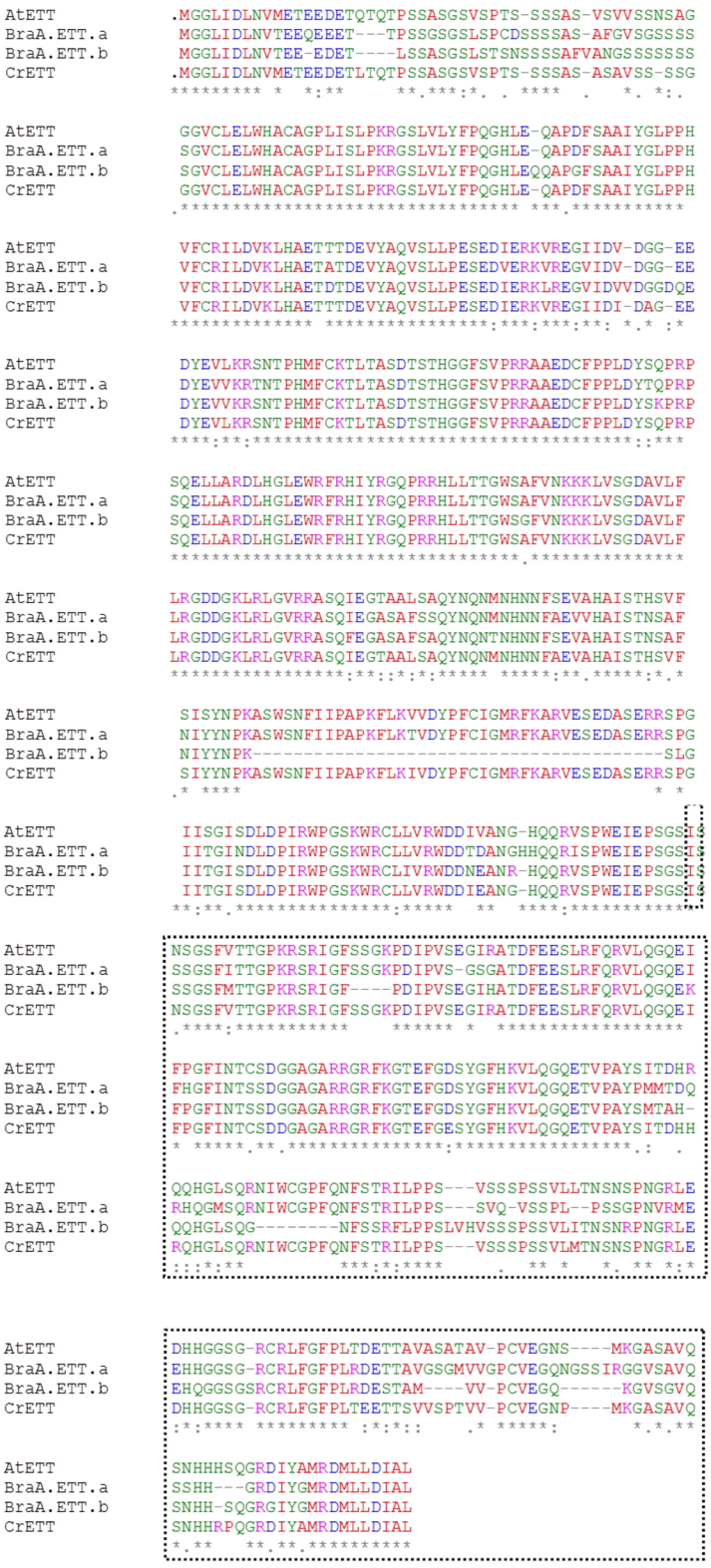


Figure 41 Clustal Alignment of ETT proteins from the Brassicaceae

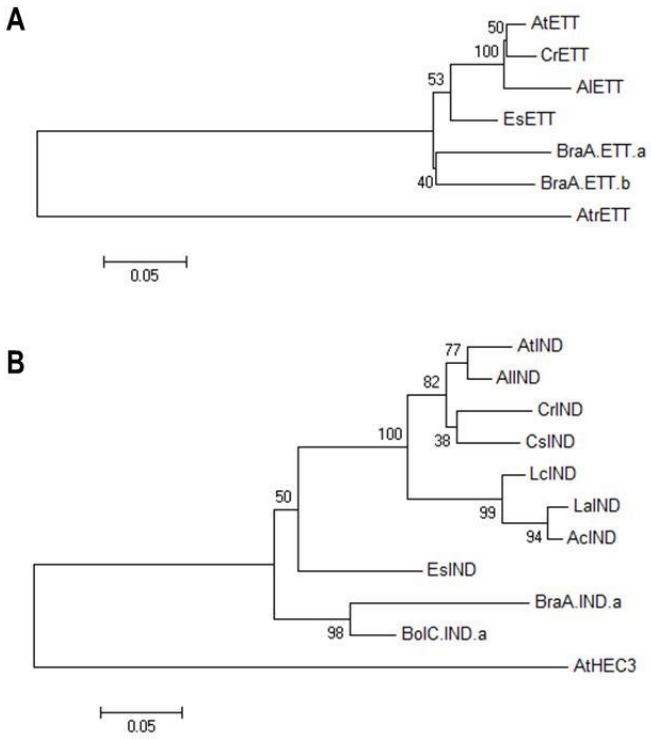


Figure 42 Phylogenetic analysis of IND and ETT proteins from the Brassicaceae. (A) Alignment of the IND protein sequences from the Brassicaceae family. AtHEC3 was used to root the tree. **(B)** Alignment of the ETT protein sequences from the Brassicaceae family. AtrETT was used to root the tree. Protein sequences were aligned using ClustalX. The phylogenetic tree was constructed using the Neighbour-Joining Method. Scale bar refers to the number of amino acid substitutions per site. Abbreviations: *At: Arabidopsis thaliana, Al: Arabidopsis lyrata, Ac: Aethionema carneum, Atr: Amborella trichopoda, Br: Brassica rapa, Bol: Brassica oleraceae, Cr: Capsella rubella, Cs: Camelina sativa, Es: Eutrema salsugineum, La: Lepidium appelianum, Lc: Lepidium campestre*.

As with the *C.rubella* genes, the *IND* genes from *B. rapa* and *B. oleraceae* and both the *ETT* genes from *B. rapa* were amplified and cloned into appropriate yeast vectors. During the cloning procedure, it was found that the cloned of *BraA.ETT.b* gene had a different sequence than the one obtained from the database. This gene had a 120bp insertion just after the DBD in the putative MR region of *BraA.ETT.b* (Fig. 43). A BLASTn search of this segment showed that it is ARF3 specific as it aligned with *ARF3* genes of other Brassica species. This segment is also not a target of alternative splicing, as the canonical AG/GA sites were not found bordering the insertion. Therefore, the most likely explanation is that the *BraA.ETT.b* gene is divergent between *B. rapa* var. pekinensis- the

gene sequence in the Brassica and NCBI databases- and *B. rapa* var. R-o-18- whose cDNA was used as the template for the cloning procedures in this experiment.

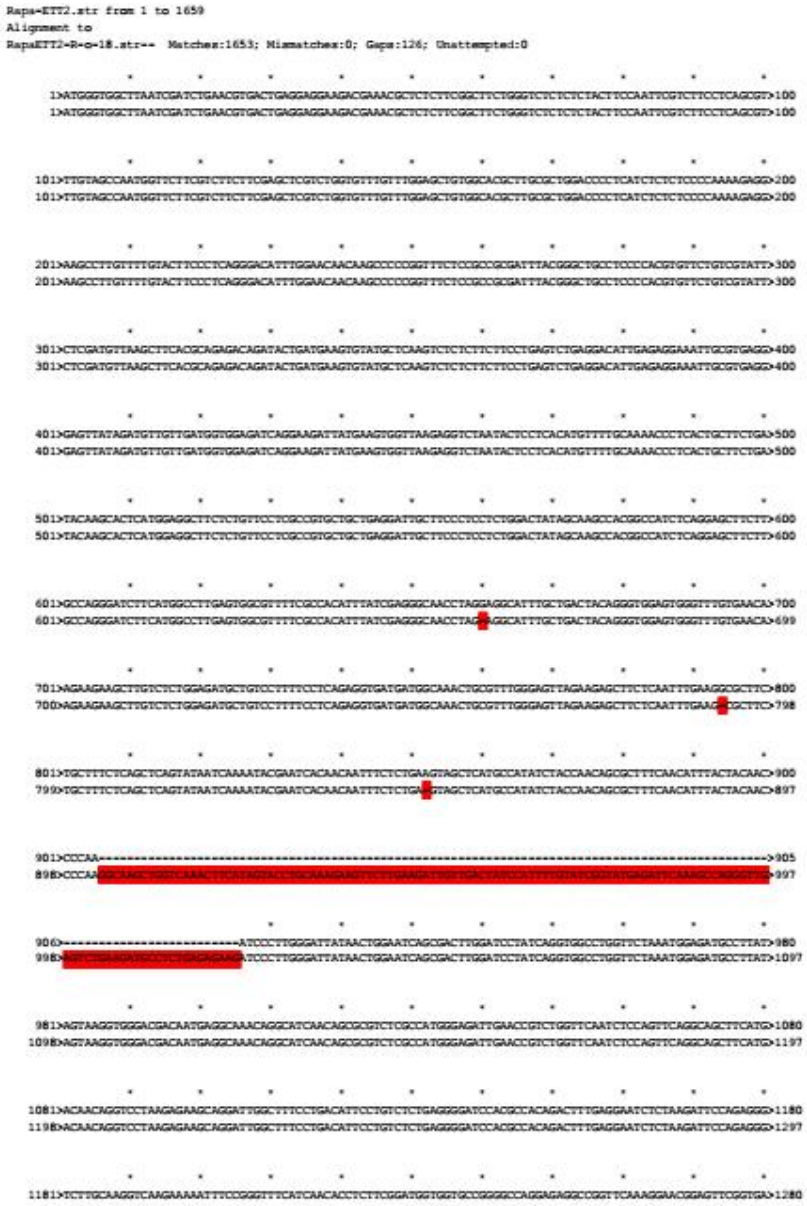


Figure 43 Sequence alignment of *BraA.ETT.b* from database and cloned *BraA.ETT.b*. The 120 bp insertion in the cloned *BraA.ETT.b* gene is highlighted in red. Top sequence: B.rapaETT2 var. pekinensis, Bottom sequence: *BraA.ETT.b* var R-o-18. Sequence alignment was carried out using ApE cloning software.

The yeast two-hybrid assay was setup at RT to identify potential interactions between the target proteins. Rather surprisingly, IND and ETT proteins from the *Brassica* genera did not interact (Fig. 44). Neither BraA.IND.a nor BolC.IND.a interacted with either of the BraA.ETT proteins. However, a cross-species interaction was detected between AtIND and both of the BraA.ETT (Fig. 45). Since the IND-IS domain of the Brassica proteins diverges from the AtIND-type sequence, it is possible that this plays an influential role in altering the mechanism of the interaction.

In order to ascertain whether the auxin-signalling mechanism is conserved across species, 200µM IAA was added to the yeast medium and the yeast growth was checked after a few days. The results revealed that the interactions between AtIND and both BraA.ETT.a and BraA.ETT.b are sensitive to IAA much like that is seen between AtIND and AtETT (Fig. 45). The weaker interaction between BraA.ETT.a and AtIND is affected from 50µM IAA and therefore appears to be hypersensitive, while with BraA.ETT.b, the interaction is affected from 100µM IAA onwards and the yeast growth is completely supressed at 200µM IAA (Fig. 45).

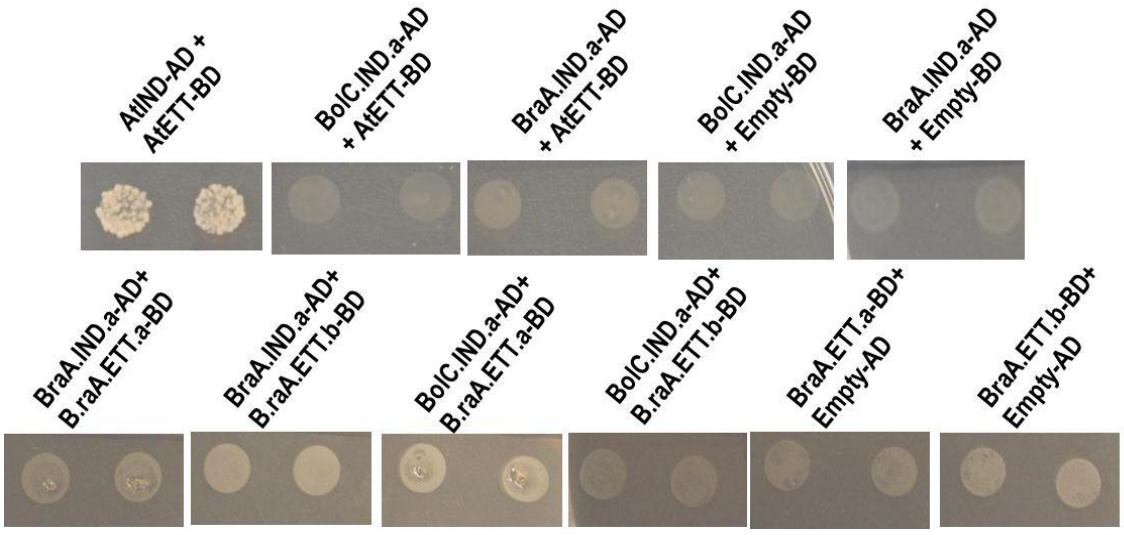


Figure 44 Yeast two-hybrid interactions between *Brassica* IND and ETT proteins. Yeast matings were plated on SD –LWHA plates and kept at RT. Growth was observed after 3-4 days.

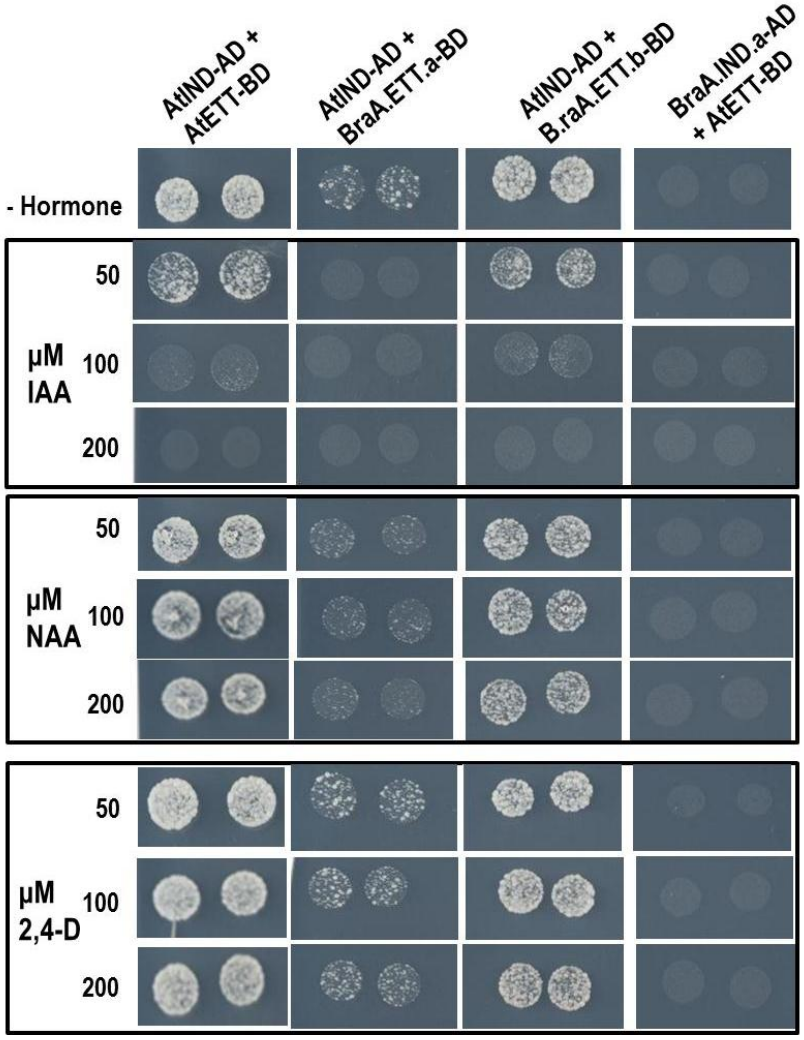


Figure 45 The interactions between AtIND and Brassica ETT are IAA sensitive. Yeast colonies were plated in SD-LWHA plates and kept at RT. Growth was observed after 3-4 days.

Thus, in *B.rapa*, both the copies of ETT have retained their ability to bind IAA and therefore could function as auxin co-receptors with other proteins in this species.

The heterodimerisation between IND and ETT, which is seen in both *A.thaliana* and *C.rubella*, does not appear to have been conserved in the slightly more distant relatives *B. rapa* and *B. oleraceae*. However, the novel IAA-signalling mechanism, which was discovered in *A.thaliana*, appears to have been preserved in *B. rapa* as both forms of the *ETT* genes in this species exhibited an IAA-sensitive response.

5.4 Discussion

5.4.1 The IND-ETT interaction in the Brassicaceae

IND and *ETT* are both involved in carpel development and although *IND* appears to have been acquired recently by the Brassicaceae family (*IND* is specific to the Brassicaceae family), *ETT* has more ancient origins as the both *A. trichopoda* and *C. aquatica* (Finet et al., 2010) have orthologues of *ETT*, which are expressed in carpel tissues.

As a novel interaction between AtIND and AtETT with possible roles in gynoecium patterning was found in this study, it was important to ascertain whether this protein complex could function to regulate carpel patterning within the Brassicaceae family. To this end, a yeast two-hybrid approach was first adopted to determine whether IND and ETT from different members of this family could interact.

The interaction between IND and ETT appears to be conserved in *C. rubella* as a very strong growth response was seen with the yeast strains expressing *CrIND* and *CrETT*. As the domains necessary for this interaction are very similar between the *A.thaliana* and *C.rubella* proteins, it was likely that the proteins would interact. AtIND could interact with CrETT probably as both proteins are highly conserved (95.9 % identical). What cannot be explained however is why CrIND did not interact with AtETT. CrIND and AtIND have slight differences in their N-terminal IND domains as a few extra proline residues were found, thus, a slightly divergent IND-IS domain sequence could be a possible reason for the non-interaction.

Although the expression domains of these two genes are not known in *C.rubella*, the biological relevance of this interaction is likely to be in the gynoecium. The cDNA obtained to clone the *CrETT* gene was from RNA isolated from *C. rubella* stage 14 fruits and so this protein is expressed in this organ. *CrIND* is also most probably a carpel/fruit specific gene although this has not yet been confirmed. An allelic series of mutants of *CrIND* in *C. rubella* has been obtained by Nicola Stacey in the lab and work is under-way to characterize these. This will provide further information as to the role of the *CrIND* gene in carpel formation in the Brassicaceae. In a similar manner, TILLING mutants in the *CrETT* gene could also be isolated to determine whether this protein also functions to regulate carpel patterning in *C.rubella*.

Surprisingly, the Brassica IND proteins did not interact with either AtETT or with BraA.ETT.a and BraA.ETT.b. Sequence alignments reveal that the IND domains of the proteins are divergent as BraA.IND.a and BolC.IND.a both have extra residues in this domain which is likely to affect the domain structure. The valve margin function of these IND proteins has been conserved, therefore, it would be of interest to know whether the inclusion of these extra residues has altered the gynoecium-specific function of IND.

Future work would involve transforming the *ind-2*, *ett-3* and *ind-2 ett-3* mutant with the *C.rubella IND* and *ETT* genes to identify any indications of functional conservation among the proteins in carpel development. Further, phenotypic analysis of the *ind-2* mutant transformed with the *B. rapa* and *B. oleraceae* genes might help uncover novel functions associated with the divergent IND-domain.

5.4.2 Conservation of the IND-ETT auxin co-receptor in the Brassicaceae

AtIND and AtETT were shown to function as an auxin co-receptor and are likely to function together to regulate style development (See Chapter 3 and 4). Although the fruit and carpel morphologies of the members Brassicaceae are quite diverse, the IND and ETT orthologues discussed above could potentially function as auxin co-receptors as well; thus to test whether this novel auxin-signalling mechanism is evolutionarily significant, the auxin sensitivity of the interactions mentioned in the previous section were tested in yeast two-hybrid assays.

The novel finding from these experiments was that the *ETT* orthologues in *B. rapa* appear to function as auxin receptors. Although the proteins did not interact with their *Brassica* IND counterparts, both BraA.ETT.a and BraA.ETT.b interacted with AtIND and this interaction was sensitive to IAA and not to 2,4-D and NAA. A yeast two-hybrid screen by Sara Simonini in the lab also identified other *A.thaliana* proteins that interact with ETT in an auxin-sensitive manner such as BABYBOOM (BBM) and PLETHORA5 (PLT5). Thus, these genes could be cloned from *B. rapa* and checked for the conservation of the auxin signalling pathway in this species. *In situ* analysis could be carried out to determine whether *BraA.ETT.a* and *BraA.ETT.b* genes are expressed in the same regions or whether their roles have sub-functionalized.

Both phylogenetic analysis and the results of these experiments suggest that this auxin signalling mechanism arose in the last common ancestor of *Arabidopsis* and *Brassica* spp.,

therefore, the fact that the interaction between CrIND and CrETT is not IAA-sensitive is rather surprising. The most plausible reason for this is technical - the strong yeast growth exhibited by this interaction would be resilient to the effect of IAA and thus no effect on yeast growth is observed. As these results are preliminary, a repetition would confirm whether this is the case.

Nevertheless, if the interaction between CrIND and CrETT is indeed IAA insensitive then this could be a useful tool to further study the biological relevance of this novel ETT-mediated auxin-signalling pathway by generating an IAA-insensitive version of the AtETT protein which is stable and can interact with IND (and other ETT interactors). For this purpose, an analysis of the sequence of the ETT-specific domain (ES domain at the C-terminus – see Chapter 3), was carried out. A preliminary analysis of the secondary structure of the ES domain using the online server Phyre2 revealed no recognizable motifs. A possible strategy therefore is to construct truncated versions of the ES domain to identify the region of the ES domain which confers the IAA response without compromising the overall protein stability. Aligning the sequences of the four ES domain of the three species reveals a large number of residues that are highly conserved and since the ETT proteins from *B. rapa* appear to have retained their auxin binding capacity, it is difficult to identify amino acids which can be mutated to obtain an IAA-insensitive AtETT protein. However, the first 95 amino acids of this domain appear to be highly conserved among all the species and therefore could be integral to protein stability (Fig. 46). A strategy that can be adopted is first, truncating the AtETT protein including these 95 amino acids and excluding the regions after and this would be followed by yeast two-hybrid tests for both protein stability and IAA-sensitivity. The results from this assay could provide some direction of how to proceed to obtain an IAA-insensitive ETT protein.

The findings from this study indicate that ETT may have retained its proposed function as a keyreceptor for auxin binding in members of the Brassicaceae family. Considering that the origins and carpel-specific functions of this protein can be traced to the ancient angiosperm clade (Finet et al., 2010) , it is possible that this novel mechanism might be conserved through evolution among the different plant families. The truncation of ETT occurred in the ANA grade angiosperms prior to the radiation of the extant angiosperms (Finet et al., 2010) it is possible that the truncated ARF4 proteins in the ANA grade angiosperms are progenitors of this ETT IAA-receptor.

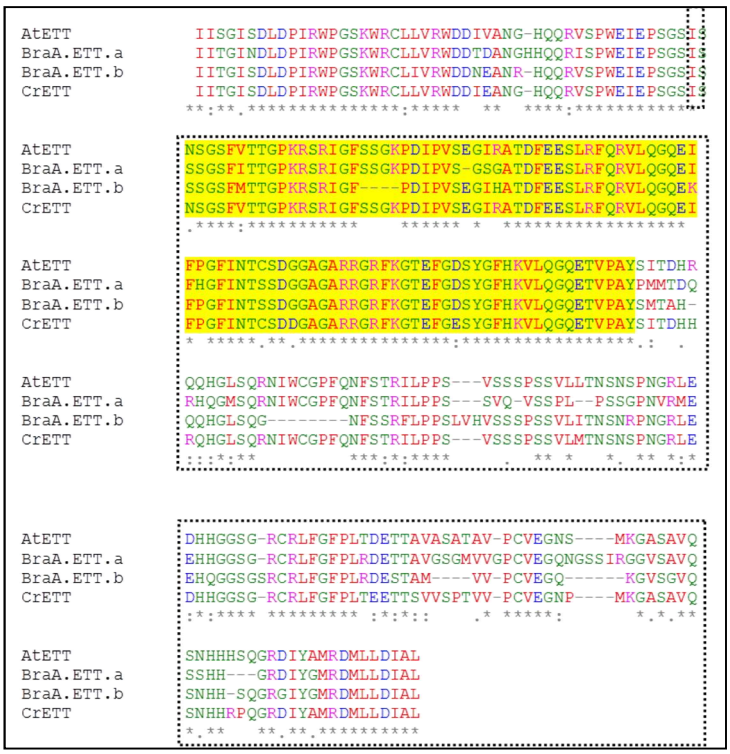


Figure 46 Clustal alignment of the ES domain of Brassica ETT proteins. The residues highlighted can potentially be mutated to obtain an IAA-insensitive version of the AtETT protein.The boxed alignments correspond to the putative ES domains of the proteins.

CHAPTER 6

CHAPTER 6- General Discussion

6.1 Project Aims and Summary

Studies on organ patterning in model organisms reveal the fundamental requirement of a morphogen and the transduction of this morphogen signal for organ development (Ashe and Briscoe, 2006, Schwank and Basler, 2010a). In the context of the *Arabidopsis* gynoecium, work from several laboratories indicates that the primary morphogenetic role is accomplished by auxin. However, the mechanism of transduction of the auxin signal to establish cell fate and tissue polarity in this organ, is an area which requires further investigation. The crucial mediators of this signal are transcription factors and although genetic analyses have revealed key players in the carpel (See Chapter 1), the cross-talk between hormonal and transcriptional pathways is less understood.

This project has investigated aspects of gynoecium development which have only recently begun to be addressed- first, the transcription factor regulatory networks that function to pattern the different domains of this organ and second, the hormonal regulation concurrent with transcriptional changes. To better elucidate the hierarchies of the transcriptional regulatory networks, a candidate-gene approach was adopted and these were then screened for protein-protein interactions using the yeast two-hybrid assay system and in plant cells by FRET-FLIM. In order to monitor changes in hormonal dynamics, reporter constructs in existence and novel constructs developed in this study were used.

The main findings and the conclusions from this study are as follows:

- A possible auxin co-receptor formed between IND and ETT operates in the gynoecium
- This complex has a role in style development
- The proposed auxin sensing mechanism may be conserved between *Arabidopsis* and *B. rapa*
- Cell-autonomous transcriptional repression of *IND* and *SPT* by ETT in the valves ensures correct apical-basal and adaxial-abaxial patterning in the carpel
- The establishment of tissue boundaries by ETT is not due to the hypothesized auxin gradient and functions through an unknown mechanism.

6.2 The IND-ETT Auxin Co-receptor

6.2.1 Mechanism of interaction and auxin binding

Protein-protein interactions between ARFs and bHLH proteins are known to occur and in the case of ARF8 and the bHLH protein BIGPETALp, a motif in DIII of ARF8 is responsible for the heterodimerisation (Varaud et al., 2011). The interaction involving IND and ETT however, relies on the non-canonical structure of ETT and therefore heterodimerisation between these two proteins involves a hitherto uncharacterized mechanism.

All three domains of the IND protein appear to be required for the interaction with ETT, while the ETT C-terminal ES domain is necessary for this interaction. However, this interaction is stabilised significantly by the IND-IS domain without which the interaction becomes substantially weaker in yeast two-hybrid assays. Since the function of IND in valve margin and style development both involve heteromeric interactions with the bHLH half of the protein, the function of the IS domain has received less attention and was essentially unknown (Sorefan et al., 2009, Girin et al., 2011). Thus, the results from this study show that the IS domain might serve as an additional dimerisation domain for the protein. Interestingly, it is the *Arabidopsis* and *Capsella* specific IS domain which facilitates dimerisation with ETT. The *Arabidopsis* and *B.rapa*-type IS domain differ in their sequence composition and therefore this divergence among the Brassicaceae IND, is likely to have affected the interaction with ETT.

The ETT ES domain serves the dual purpose of functioning both as a dimerisation domain and also as the auxin-binding region of the protein. ETT can interact with other proteins through its DNA binding domain (DBD), therefore it will be interesting to know whether IAA-dependent regulation of ETT functions when this domain is not in complex with its partner proteins. In this study, radioactive IAA-retention assays suggest that complexation is a requirement for auxin binding which is similar to the molecular-glue like mechanism seen in other auxin receptors (Tan et al., 2007, Jurado et al., 2010, Sauer and Kleine-Vehn, 2011). Nevertheless, this could be tested in yeast assays between ETT and a protein such as ATS, which dimerises with the DBD of ETT, to further clarify the IAA-binding mechanism. It has not been possible to identify the specific residues in the domain which are essential for the auxin binding mechanism and further, secondary structure prediction software used in this study, could not identify any distinct motif, making it difficult to

isolate the specific amino acids which form the back-bone of this interaction and/or auxin-binding. As the interaction between CrETT and AtIND was IAA-insensitive, the *Capsella* ES domain sequence could serve as a guide to identify these residues, however, further tests will be required to confirm this result following which the ES domain can be modified accordingly. Protein alignments among the ETT proteins from the Brassicaceae revealed that the first 95 amino acids in the ES domains of all the four ETT orthologues are highly conserved. This suggests that this region could either be integral for protein stability, or could be required for the IAA-binding. Thus, testing this conserved region in isolation, followed by progressive C-terminal deletions of the ES domain, might help limit the region required for auxin binding to a few specific amino acids in the absence of crystal structures.

This co-receptor complex also appears to have specificity only for IAA and not the other auxinic compounds tested. This makes it unique among the receptors identified so far as most can bind the synthetic auxin analogs 2,4-D and NAA although with varying sensitivities. There are however some exceptions to this, for instance AFB5 is only one among the F-Box protein IAA-receptors which can bind the synthetic auxin, picloram (Lee et al., 2014). As the ring structures of these compounds differ, both IBA and IAA were tested to ascertain whether the mechanism of binding was directed by the indole-ring. The results however indicate that both ends of the IAA molecule are involved in binding, and although IBA could affect the weaker interaction between the IND protein without the IS domain and ETT, at present it is difficult to conclude whether this is as a consequence of IBA binding to the proteins or due to inhibition of yeast growth due to chemical toxicity.

6.2.2 Biological Significance of the Novel Auxin Receptor Complex

The phenotype of the IAA-insensitive IND mutant (INDmut), coupled with the expression patterns of both proteins indicates a style-specific function for this co-receptor complex. Evidence suggests that the IND-ETT complex functions to ensure that the style of the carpel develops correctly by coordinating style elongation with the emergence of stigmatic tissue. IND has been shown to regulate both PIN1 and PIN3 polarity via PID and WAG2 (Sorefan et al., 2009), and therefore this coordinated development would most likely rely on the modulation of IND function to regulate auxin transport. This is also suggested by the phenotype of the IAA-insensitive INDmut which exhibits an apical tissue phenotype much like the *pid-8* mutant.

Thus, based on the mechanism of the interaction and the phenotype of the above mutants, the following model could be considered:

At early stages of style development, beginning around stage 9 the levels of auxin would be low, as indicated the by *DR5:GFP* expression (and *DII::VENUS* expression, Moubayidin and Ostergaard, 2014). *PID:GUS* expression in the style shows that the *PID* gene is repressed at this stage (Fig. 47). This suggests that the IND-SPT complex is active and repressing *PID*. Thus, with regards to the IND-ETT complex, two possible scenarios could exist. First, although IND-ETT and SPT do not form a trimeric complex in yeast, it is possible that the three proteins are part of a larger complex perhaps with the HECs and ETT, which is classified as a repressor, which helps to fine-tune the levels of *PID* expression. The second scenario would involve competition between the three molecules. This could not be confirmed in the yeast assays, but in general, the strength of the interaction between IND and SPT was stronger than that between IND and ETT. Therefore, depending on the stoichiometry of the molecules at this stage, IND and SPT could dimerise more readily than IND-ETT thus ensuring *PID* repression. Repression of *PID* at this stage would prevent apical-basal orientation of PINs in this region and promoter apolar organization. This would lead to increased concentration of auxin in the style thus forming the auxin maxima.

At the later stages of style development when the auxin levels reach the required threshold, auxin may function to strengthen the interaction between IND and ETT, like a molecular glue. This causes a conformational change in the complex and may lead to ETT repressing

IND activity. Some evidence for this comes from the experiments of Tiwari et al., 2003, who show that ETT functions as a repressor even in the presence of auxin.

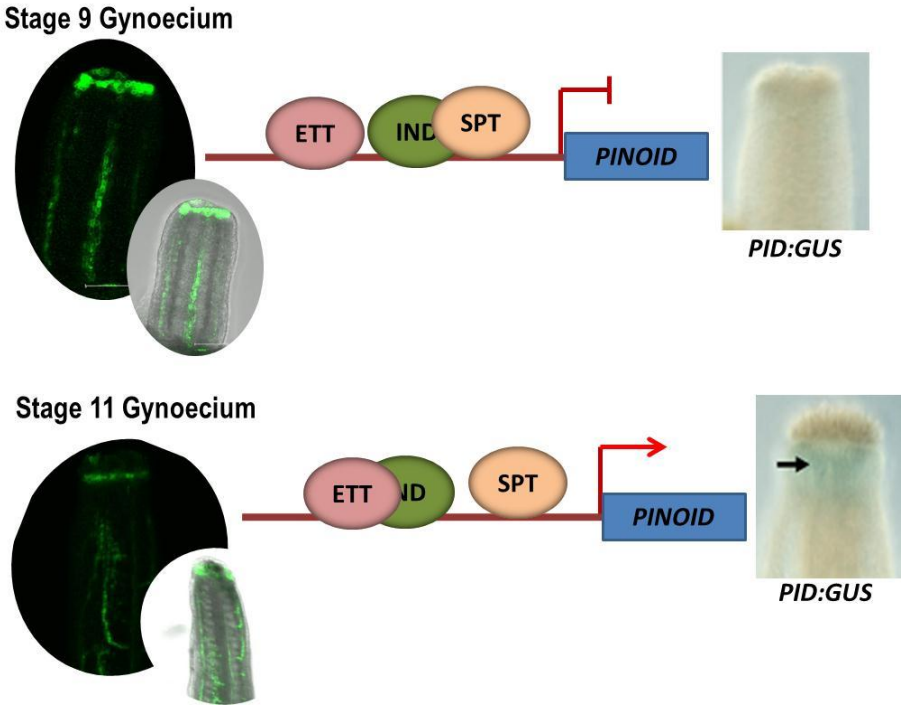


Figure 47 Model illustrating the function of the IND-ETT auxin receptor in style development. In the earlier stages of gynoecium development up to stage 9, IND and SPT repress *PID* expression. IND and ETT do not form a complex. At late stage 10-11, at a certain threshold concentration of auxin, ETT and IND form a complex allowing *PID* to be expressed. Confocal images show *DR5:GFP* distribution in gynoecia.

This repression of IND allows *PID* expression ensuring proper style development and fine tuning of IND activity to initiate stigmatic tissue development when the style is adequately developed.

6.2.3 Questions and Future Work

Several questions remain both regarding the mechanism of the interaction and also of how this relates to style development. It would first be necessary to confirm IAA-binding to the co-receptor by *in vitro* methods (See Chapter 3). All four of auxenic compounds tested here (IAA, NAA, 2,4-D and IBA) would also be tested in this assay to give a definite confirmation of direct auxin binding by this co-receptor complex. It would also be necessary to identify the residues or the regions, which comprise the auxin binding pocket of ETT. This region could then be tested with the other targets identified.

As regards the regulation of style development by these proteins, it would first be necessary to determine whether *PID* is also a target of ETT. This would involve ChIP assays coupled with mutant analysis, both of which are underway. Although ETT is a transcriptional repressor, it would be important to confirm whether it functions as a repressor in this context. Though this is suggested by the SPR assay conducted in this study, these experiments used refolded proteins, which may affect the protein's behaviour. Transient expression analysis in *Arabidopsis* protoplasts (+/- IAA) using a promoter segment of *PID* fused to a reporter gene, would help elucidate the mechanism of *PID* regulation *in vivo*.

Finally, it would also be relevant to determine the consequence of the change in the Brassica-type IS domain sequence in patterning. Transforming *Arabidopsis ind* mutants with the full-length *B.rapa* or *B. oleraceae IND* open reading frame as well as the IS domain in isolation would not only help to clarify its role, but would also be useful as a tool assess style development in the absence of ETT regulation of IND.

6.3 The Implications of the ETT Auxin-receptor Function in Gynoecium Patterning

A major question in gynoecium development is, how are the auxin levels in the carpel are interpreted into distinct boundaries? ETT has been suggested to be the molecule which adopts this role, however, no mechanism till date has been identified to explain how ETT might be able to do so. This study presents the first evidence that ETT is potentially capable of functioning as a receptor of auxin. The role of the IND-ETT complex in the style also suggests that a positive feedback exists between this receptor and auxin. One of the issues that needs clarification is if patterning and growth are coordinated by ETT, solely by the interpretation of existing auxin levels or whether it is also involved in setting up the right auxin thresholds by regulating auxin biosynthesis and transport, in the initial stages of carpel development.

In the absence of an auxin gradient and a protein gradient, the question arises as to how ETT is able to setup gene boundaries? As the proposed Gradient model of carpel development by Nemhauser et al.,2000 has proven to be inconsistent with recent findings, Hawkins and Liu, 2014 proposed the Early-action Model of gynoecium development wherein opposing auxin flows in the incipient carpel primordium is deemed significant for the specification of the adaxial-abaxial boundary at the very early stages of carpel

development which is considered necessary for establishing both the apical-basal patterning and growth. This model is consistent with observations in leaf development from which the angiosperm carpel is hypothesized to have evolved. In this study, the carpel adaxial-abaxial patterning defects in the *ett* mutant were recovered significantly in the *ind ett spt* triple mutant which gives support to this hypothesis. However, preliminary analyses of *IND::IND:GUS* expression in the *ett-3* mutant reveals that although the expression domain of the protein expands in the mutant, the timing of expression is detected at a stage slightly earlier in WT (around middle-late stage 8) and not in the very initial stages of carpel development. *IND* and *SPT* are however not the only marginal-tissue specific genes so the study of other genes such as the *HEC* genes, or genes upstream in the pathway (See Chapter 1) would be needed to validate the hypothesis.

The other mechanism proposed in this study which could contribute to the establishment of boundaries, is the regulation of gene expression by ETT which would then specify the subsequent carpel boundaries. This is suggested both by the stochastic nature of patterning and the misexpression of target genes in the *ett-3* mutant. Taking the example of the ectopic expression of *IND* and *SPT* in the *ett* mutant, early establishment of identity in these regions would interfere with the positional cues for establishing the other domains and therefore this would affect overall carpel patterning. This might also affect the auxin levels or flux in the developing regions which would affect growth and also the expression of genes in these regions. Preliminary results in this study show that PIN3:GFP distribution at stage 7-8 in the *ett-3* mutant is extended slightly into the main body of the carpel while in WT, PIN3 is restricted to the apical region. At stage 9-10, ectopic *PIN3::PIN3:GFP* expression is clearly visible and this appears to coincide with the ectopic stigmatic tissue regions. This suggests that auxin distribution in these mutants is perturbed and this would consequently affect carpel growth. Further experiments will be required to determine whether the mislocalized PIN3 pattern is due to the ectopic activity of the IND-SPT complex.

Finally, how does ETT regulate gene expression to specify cell fates? This study has shown that ETT is expressed uniformly in the carpel, however, it can cell-autonomously repress *IND* gene expression in the valves, while it does not do so in the style. In many other systems fine tuning the expression levels of morphogens to regulate target genes often involves the establishment of inverse gradients in the organs (Affolter and Basler, 2007, Schwank and Basler, 2010b). In the carpel, apart from auxin, cytokinin has also

shown to affect apical-basal patterning in the gynoecium and therefore, could have potentially acted as an inverse gradient, to coordinate the differential expression of genes. However, expression of the *TCS:GFP* reporter for cytokinin signalling (Muller and Sheen, 2008) suggests that cytokinin is restricted to the carpel marginal meristem, and the effects of cytokinin appear to be early in carpel development. One of the likely mechanisms which regulate ETT activity to fine-tune gene expression is through the TAS3 tasi-rna pathway. RNA DEPENDENT RNA POLYMERASE6 (RDR6) transgenic plants expressing *pETT::ETT* constructs and *ETT* genes with mutated splice-sites show various carpel defects such as overgrowth of internal tissues and defective medial tissues such as a split septum and style (Fahlgren et al., 2006). Thus, regulation of *ETT* through RNA-interference would ensure correct levels of ETT protein to affect target gene expression.

The ability of ETT to function as an auxin-receptor with different protein partners, also points to the possibility of inherent specificity in their response to IAA binding. The AUX/IAA protein and TIR/AFB co-receptor complexes show different degrees of affinities between different combinations among these two protein families. These differential binding strengths are likely to affect transcription rates. In a similar manner, the ETT-receptor might also have different affinities for its protein partners in the presence of auxin and this is also a possible mechanism which could fine tune gene expression patterns in the carpel.

6.3.1 Future Work

It would first be necessary to determine whether ETT is able to affect the auxin status early in carpel development or whether most of its function relies on its ability to act as an auxin sensor. For this, expression analysis of auxin biosynthetic genes in the *ett* mutant background would help determine their regulation states. GUS reporter constructs of these genes in the *ett* mutant could be used. Additionally, the *PIN1:GFP* and *PIN3:GFP* expression patterns at the very early stages of carpel primordium should also be visualized. At stage 7-8, PIN1:GFP localization in *ett* appears to be like WT, whereas PIN3:GFP shows a slightly altered distribution pattern in the mutant. Analysis of more samples would verify if this is indeed the case as this would suggest that ETT could be affecting auxin transport at the early stages of development. Next, to determine whether sequential establishment of tissue identity provides the positional cues for subsequent domain development, a system to induce *IND* expression in a few cells in the carpel would enable

us to assess whether this could perturb the patterning system. Using constructs employed for clonal analysis, such as the heat-shock inducible Cre-Lox system, to induce *IND* early in the carpel could help in testing this hypothesis. Alternatively, a DEX-inducible version of *IND* in WT or an inducible-*IND* version expressed under the *ETT* promoter can also be used for the same purpose. It would also be necessary to ascertain whether ectopic *PIN3* expression is coincident with *IND* expression and could be achieved. Colocalization of an optimized IND:YFP reporter with PIN3:GFP in the *ett-3* mutant would give some indication of this. Finally, to check binding affinities of ETT to its different protein partners, a qualitative analysis could be conducted using the yeast two-hybrid system along with the ONPG assay in yeast to quantitatively assess the strength of the interactions. This would be followed by SPR analysis of these interactions.

6.4 The Evolution of the ETT Auxin Receptor- Perspectives and Future Work

As the ETT-mediated auxin receptor system has been identified in both *Arabidopsis* and *Brassica*, it is likely that this receptor complex existed in the last common ancestor of the two. The fact that the IAA-sensitive interaction between IND and ETT was not seen in *Capsella* was unexpected, however, a repetition of the experiment would help verify whether this sensor has been lost in *Capsella* or whether it is an experimental issue.

It would therefore be interesting to trace the origins of this receptor complex. As *IND* is specific to the Brassicaceae other targets which might be conserved in other species like the PLETHORA proteins could be used to test the IAA response. As the truncation of *ETT* appears to have occurred in the ANA-grade angiosperms, with the sequence of *A. trichocarpa* now available, the sequence of these genes can be obtained and then tested in the yeast assay used in this study to determine if ETT-containing auxin receptor complexes also existed in the basal Angiosperms.

6.5 Concluding Remarks

The study of gynoecium development has significant implications spanning both fundamental biology and crop improvement. As the carpel is the precursor of the fruit, understanding the underlying mechanisms specifying the structures essential for pollination, fertilization and consequent fruit development, has agronomical significance. In the field of developmental biology, the complex tissue structure of the carpel presents a

unique model to understand the mechanisms in place which translate genetic regulatory inputs, into precise tissue and organ level patterning outputs.

Central to organ growth and patterning are the roles of plant hormones among which auxin has received the most attention due to its predominant role in all facets of plant development. The potential discovery of a new auxin receptor in this study has given more insight into the ways this simple molecule can effect multiple different responses for growth and development. This novel finding has posed new questions as to the mechanisms of organ patterning in plants. As ETT is expressed in both leaves and roots, it would also be necessary now, to understand how patterning is coordinated in these organs as well. Once it is better elucidated, in the future, this receptor could also be manipulated or could be a target of suitable chemicals to manipulate specific growth and patterning outputs to improve crops accordingly. But perhaps what this discovery highlights best is the unique ability of plant systems, to devise mechanisms which ensure both developmental plasticity and robustness in their growth and development, thus maximizing their chances of survival.

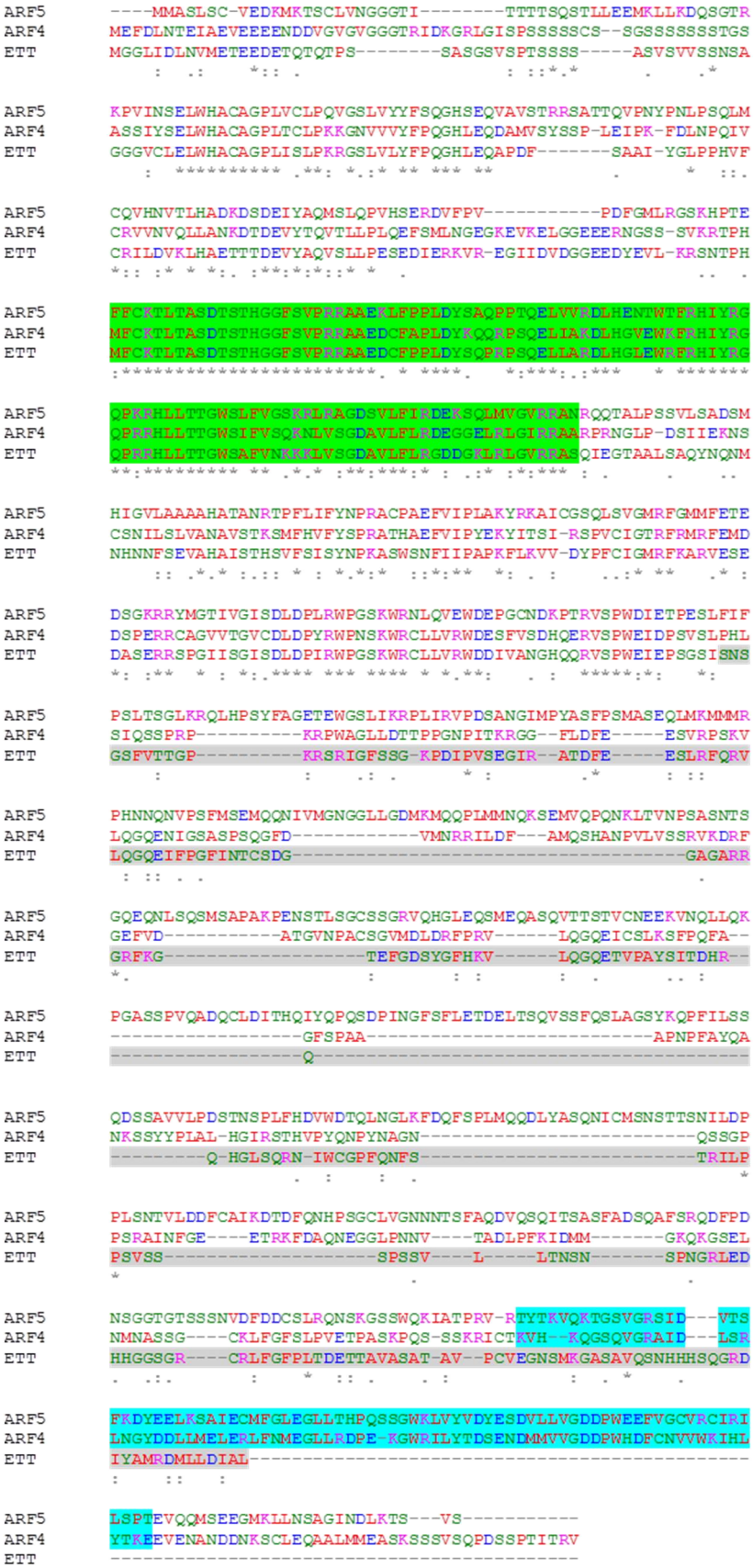
APPENDICES

APPENDIX I

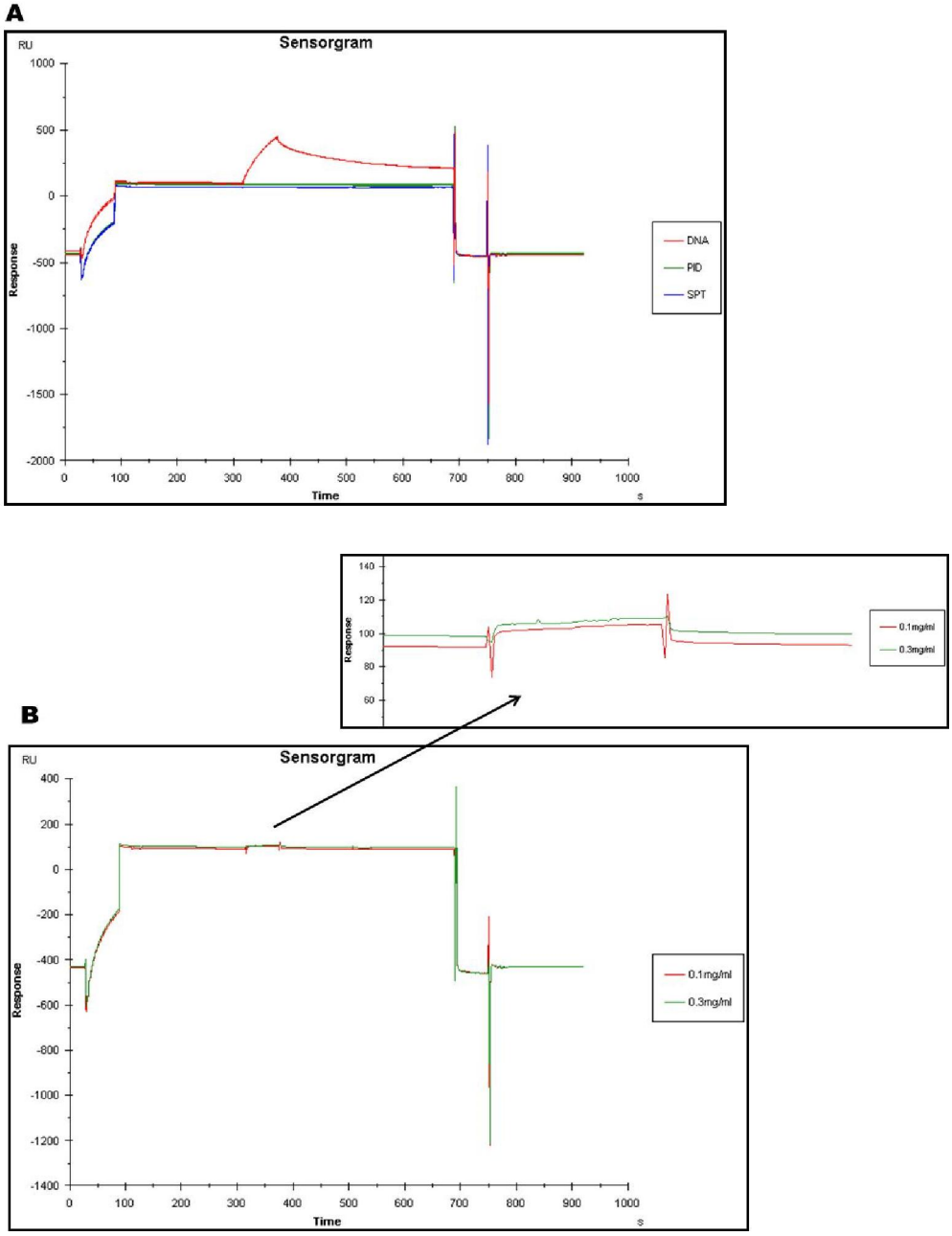
Supplementary figures for Chapter 3

Primer Name	Primer Sequence	Description
EboxY1H SPR1	AATTCTCTCACGCGTTGTCTCACGCGTTGTCTCACGC GTTGC	DNA oligo for SPR
EboxY1H SPR2	GCAACGCGTGAGACAACGCGTGAGACAACGCGTGAG AGAATTCCTACCCTACGTCCTCCTGC	DNA oligo for SPR with adapter
ETT-CT.1-SmaI Fw	TTAGcccgggaTCCAATTCAGGCAGCTTCGTAAC	Primer in ETT specific region which is after the conserved ARF region marked in blue. One extra nt. added to keep reading frame.
ETT-CT-SmaI Fw	ATATcccgggaCAAATCGAAGGCACCGCTGCTC	Primer where ETT-specific region starts immediately after the DBD. One extra nt. added to keep reading frame.
ETTdbd-PstI Rv	ATATctgcagCTAAGAAGCTCTTCTAACTCCCAGTCG	Primer where the DNA binding domain ends with STOP codon added
ETTIN-PstI Rv	CTGTctgcagCTAGAGAGCAATGTCTAGCAACATG	Primer at the end of the gene.
ETTpSPUTK Fw	GATTccatggGTGGTTTAATCGATCTGAAC	NcoI site + gene with no change at the start
ETTpSPUTK Fw.1	GCTTtcatgataGGTTTAATCGATCTGAAC	NcoI site has been replaced by a BspHI site which is compatible with NcoI. This was because ETT has 2 NcoI sites in the gene. Also in the Fw primer the second G in the gene has been modified to I
ETTseq Fw.3	GGCCTTCTCAGGAGCTTCTTGC	Sequencing primer in ETT CDS
HEC3-PstI Rv	ATCTctgcagCTAGATTAATTCTCCTACTCCTCTTC	HEC3 full length with stop codon ending in PstI site for cloning in PGBT9 or PGAD424
HEC3-SmaI Fw	GTACcccgggATGAATAATTATAATATGAACCCATC	HEC3 full length with SmaI for cloning into PGBT9 or PGAD424
IND-HEC Fw	CGAAGAGTACGATGAAGACATGgtgctatgaaggaaatgatgtacaag	Primers designed for extension overlap PCR to create IND-HEC3 Hec domain fusion protein
IND-Hec PstI Rv	TATActgcagCTAACGGCGGTTCGGCTTAGGGAC	Reverse primer for INDsp+Hec domain (+STOP)
IND-HEC Rv	TTGTACATCATTTCCTTCATAGCACCATGTCTTCATCG TACTCTTCG	Primers designed for extention overlap PCR to create IND-HEC3 Hec domain fusion protein
IND-HECbHLH Fw	GGTCCCTAAGCCGAACCGCCGTAACGTGAGGATCTCC	Extension overlap PCR for IND-HecbHLH domain fusion protein
IND-HecbHLH Rv	GGTCGTCGGAGATCCTCACGTTACGGCGGTTCGGCTTA	Extension overlap PCR for IND-HecbHLH domain fusion protein
IND-IS PstI Rv	CCTGctgcagCTACATGTCTTCATCGTACTCTTC	Reverse primer for IND-specific domain (+STOP) NB* Same as Sara Simonini's primer for cloning IS domain
INDpSPUTK Fw	GATTccatggAGCCTCAGCCTCACCATCTCCTCATG	NcoI site + modified IND gene. Gene starts with 2 ATG's, one has been removed so it now reads: MEPQPH
INDpSPUTK Fw.1	GATTccatggGACATCACCATCATCACCACatgatggagcctcagcc tcac	NcoI site + N-terminal 6xHis-Tag followed by native gene sequence
INDpSPUTK Fw.2	GATTccatggGACATCACCATCATCACCACGCTGCTGCTG CTatgatggagcctcagcctcac	NcoI site+ N-terminal His-tag+ 4 Ala+ native gene
NewETTpSPUTK Rv.1	gattTCATGActagagagcaatgtctagc	BspHI site- No tag
NewINDpSPUTK Rv	gattCCATGGtcagtggtgatgatggtgatgGGGTTGGGAGTTGTGGT AATAAC	
NewINDpSPUTK Rv.1	gattCCATGGtcaggttgggagttgtggtaataac	NcoI site + 6x His-Tag cloned in-frame with gene. Stop codon after His-tag. (C-terminal His-Tag)
NewSPTpSPUTK Rv	gagtCCATGGtcaagtaattcgatcttttagg	NcoI site- No tag
pGAD Fw.1	CTGGTTGGACGGACCAAACTG	pGAD424 Forward sequencing primer
pGAD Rv.1	GATCAGAGGTTACATGGCCAAG	pGAD424 Reverse sequencing primer
pGBT9 Fw.1	AGATTGGCTTCAGTGGAGACTG	pGBT9 Forward sequencing primer
pGBT9 Rv.1	CCTGACCTACAGGAAAGAGTTACT	pGBT9 Reverse sequencing primer
PIDnative SPR1	TTTTCTGTTTACCTCTCTCACGCGTTGAAAAGTGCAAT	DNA oligo for SPR
PIDnative SPR2	ATTGATTGCACTTTTCAACGCGTGAGAGAGGTAAAC AGAAAACCTACCCTACGTCCTCCTGC	DNA oligo for SPR with adapter
SPTpSPUTK Fw	GATTccatggttTCACAGAGAGAAGAAAGAGAAG	NcoI site + Second codon in gene changed to V from I.

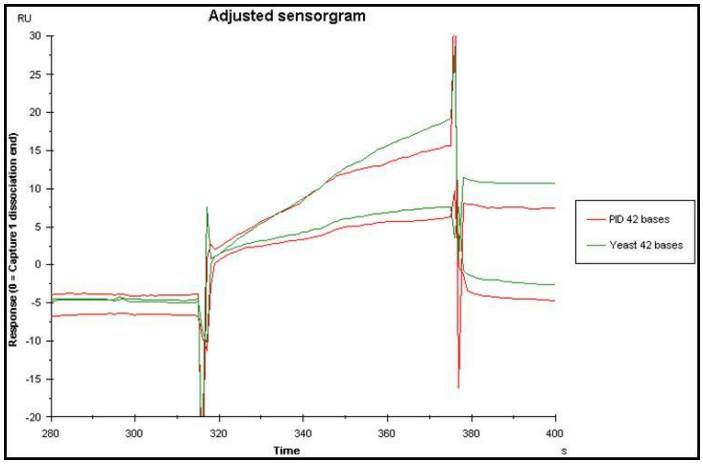
Appendix 1- Table 1 PCR primer sequences used for cloning and SPR in Chapter 3



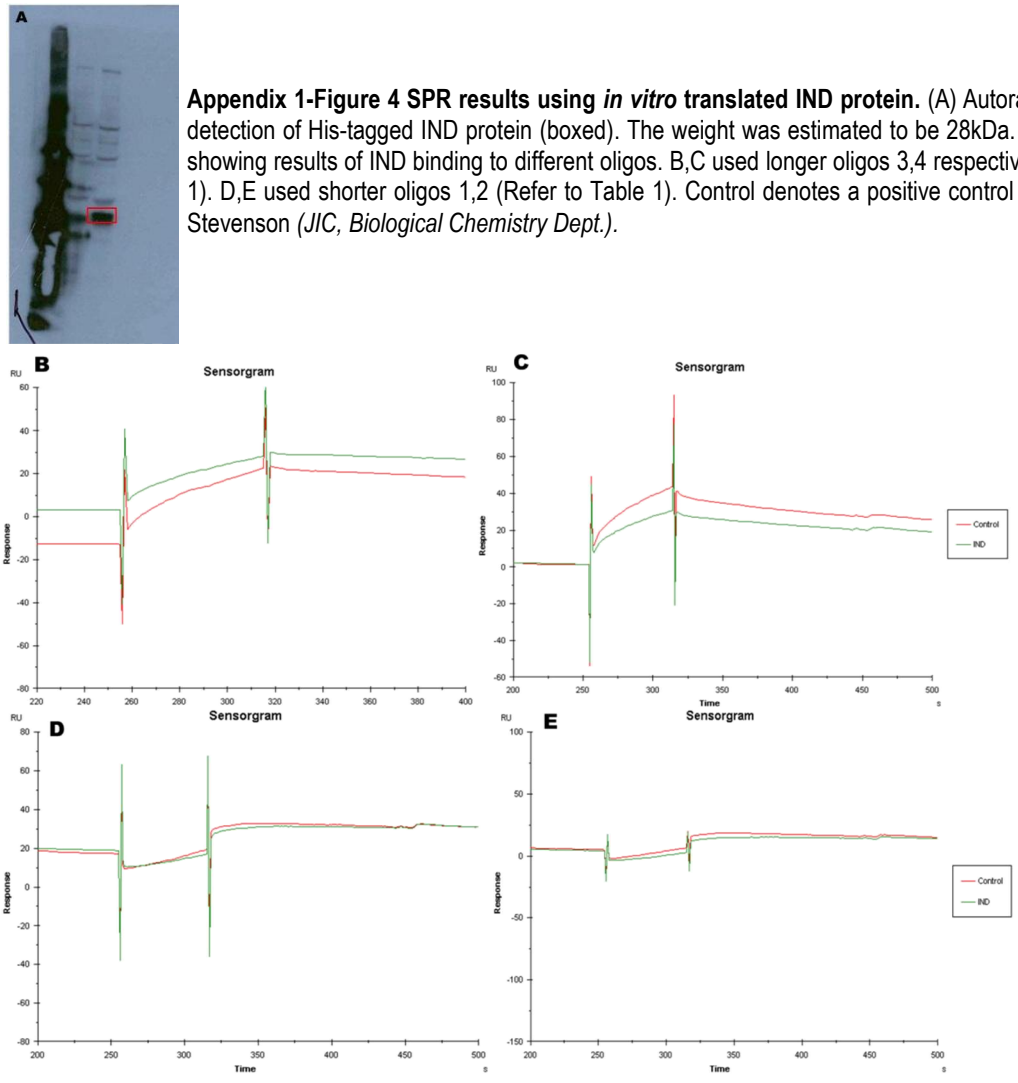
Appendix 1-Figure 1 Multiple Sequence Alignment ARF4, ARF5 and ETTIN proteins. The DNA Binding Domains of the proteins are highlighted in green. The ES domain of ETT is highlighted in grey. The PB1 dimerisation domains of ARF4 and 5 are highlighted in blue.



Appendix 1-Figure 2 SPR using commercially synthesized IND protein. (A) Sensogram showing results when 2nM and 200nM of IND protein was used to detect binding to the IND E-box. (B) Sensogram showing results when 0.1mg/ml and 0.3mg/ml of IND protein was used to detect binding to the IND E-box. The inset shows a possible weak binding response by the protein. *PID denotes E-box cis-element present in the PID promoter, SPT denotes E-box cis-element present in the SPT promoter, DNA is a positive control protein binding to its target cis-element (supplied by Clare Stevenson JIC, Biological Chemistry Dept.). Refer to Table 1 for oligo sequences.*



Appendix 1-Figure 3 Sensogram showing results of commercially synthesized IND protein binding behaviour to modified versions of DNA oligos. 0.1mg/ml and 0.3mg/ml of protein was used against 2 different DNA oligos which had longer nucleotide sequences than previously used. DNA oligo PID42 was 42 bases long and had 1 copy of the IND E-box with the flanking DNA sequences of the native *PID* promoter. Yeast42 was also 42 bases long with 3 copies of the IND E-box as was used for yeast one-hybrid experiments in *Girin et al., 2011.* For oligo sequences refer to Table1.

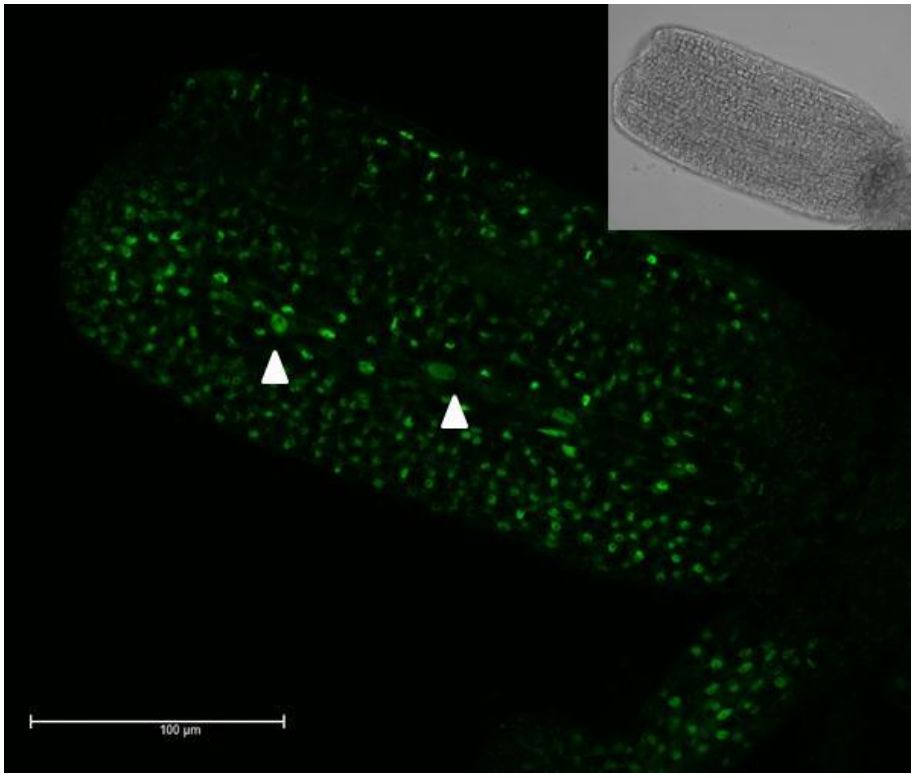


Appendix 1-Figure 4 SPR results using *in vitro* translated IND protein. (A) Autoradiogram showing detection of His-tagged IND protein (boxed). The weight was estimated to be 28kDa. (B-E) Sensogram showing results of IND binding to different oligos. B,C used longer oligos 3,4 respectively (refer to Table 1). D,E used shorter oligos 1,2 (Refer to Table 1). Control denotes a positive control supplied by Clare Stevenson *(JIC, Biological Chemistry Dept.).*

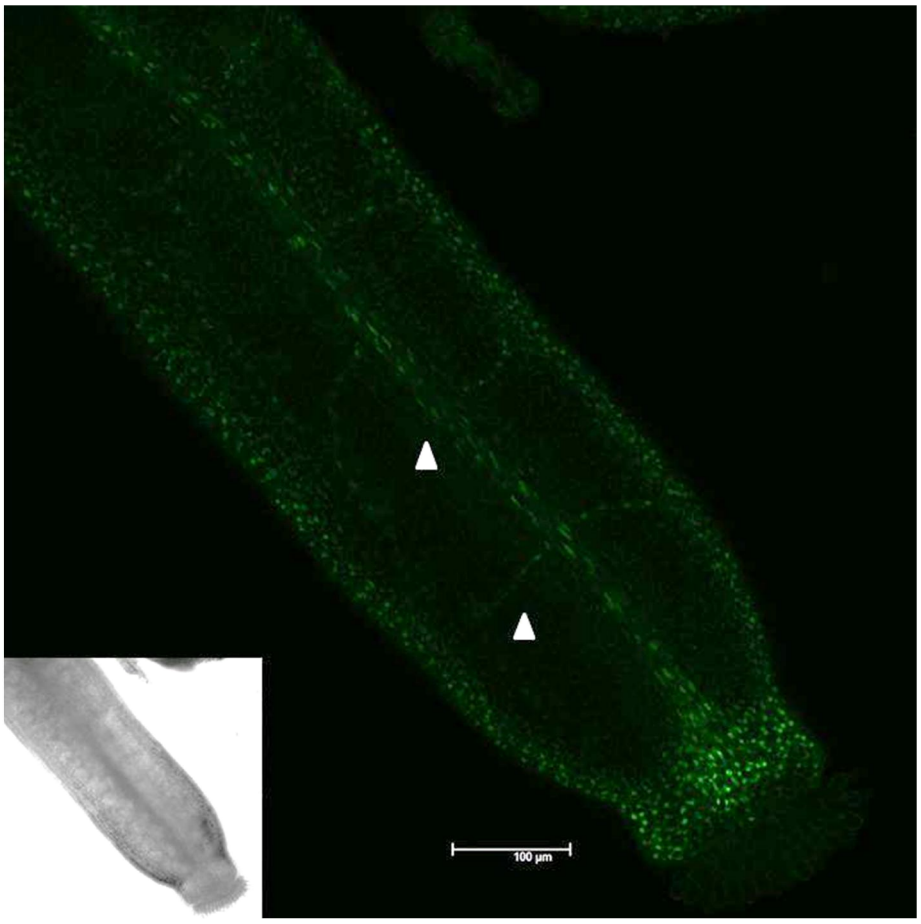
APPENDIX II

Primer Name	Primer Sequence	Description
dCAPS ett2 Fw	GAACAAGAAGAAGCTTGTCTCTGGTGATGCTGTGCTTTTCCCGA	dCAPS marker for ett-2 mutant genotyping (AvaI site created)
dCAPS ett3 Fw	TAAGTGGTATCAGCGACTTGGATCCAATCAGGTGGCCTGGTTCATCATG	dCAPS marker for ett-3 mutant genotyping (BspHI site created)
ett-2 Rv	AGCAGCGGTGCCTTCGATTTGAGAAG	primers for ett mutant genotyping
ett-3 Fw	CTTCTCAAATCGAAGGCACCGCTGCT	primers for ett mutant genotyping
ETTIN Fw	ATGGGTGGTTTAATCGATCTGAACGTG	Primers at ETTIN start (for genotyping)
ETTIN Rv	CTAGAGAGCAATGTCTAGCAACATGTC	Primers at ETTIN end (for genotyping)
ETTpMON Fw	TAGTTGGAATAGGTTCatgggtggtttaatcgatctgaac	Cloning for FRET-FLIM pMON999 CFP(Dolf Weijers)
ETTpMON Rv	AGTATGGAGTTGGGTTCgagagcaatgtctagcaacatgtc	Cloning for FRET-FLIM pMON999 CFP(Dolf Weijers) (-STOP)
IND.LV17Rv	TTATGGAGTTGGGTTCGAACgggttgggagttgtg	pPLV Adapter primer+INDgene (-STOP)
INDpMON Fw	TAGTTGGAATAGGTTCatgatggagcctcagcctc	IND cloning for FRET-FLIM in pMON999 YFP
INDpMON Rv	AGTATGGAGTTGGGTTCgggttgggagttgtg	IND cloning for FRET-FLIM in pMON999 YFP (-STOP)
pEGADFw	CTTCAAGATCCGCCACAACATCG	Sequencing primers
pEGADRv	TCCACCATGTTGACGGATCTC	Sequencing primers
pETT 3.7 SacI Fw.1	aattGAGCTCgcaccgcgtgtaccaacgc	Forward primer for ETT promoter -3736 bp from ATG. Use with pETT:ETT:pEGADRv to clone into pEGAD.
pETT 3.7seq Fw	GCGCATGTTCAACTCCAAGTACAAC	Sequencing primer for ETT promoter
pETT 3.7seq Fw.1	CCCCTGATCCTCGGGTTCTTTGTAATT	Sequencing primer for ETT promoter
pETT 3.7seq Rv	GGTTGTACTTGGAGTTGAACATGCGC	Sequencing primer for ETT promoter
pETTseq Fw.1	GTTTCAAATTTAGTCCTCGTTAGTC	Sequencing primers for ETT promoter
pETTseq Fw.2	GTGCATCAATCCCTAAAAGTATG	Sequencing primers for ETT promoter
PID Fw	ATGTTACGAGAATCAGACGGTGAGATG	Gene specific primers- genotyping
PID Rv	TCAAAAGTAATCGAACGCCGCTGGTTTG	Gene specifc primers- genotyping
pid-8_LP	gacgtcattagtcggcgcaac	dCaps marker for pid-8 genotyping. Digest with MseI Ref: Yunde Zhao PNAS 2007- NPY1.
pid-8_RP	cgttcgttggtacgcatgaatacgtggctt	dCaps marker for pid-8 genotyping. Digest with MseI Ref: Yunde Zhao PNAS 2007- NPY1.
pIND::IND.LV16 Fw	TAGTTGGAATGGGTTCGAAccttatgttaatatcaccgtag	Primer for LIC-Weijers plasmid- pPLV16 with YFP.Sequence in IND promoter same as Lars' plasmid LO151/152. Use with Rv primer IND.LV17Rv
pINDseq Fw.1	GGTGTATAGGAGTTGTCAAAAGAG	Sequencing primers for IND promoter
pINDseq Fw.2	TGTCACTATGATGTCATCACG	Sequencing primers for IND promoter
pPLV17Fw	ACGACTCACTATAGGGCGAATTG	Primer flanking LIC site for colony PCR
pPLV17Rv	AACTTGTGGCCGTTTACGTCG	Primer flanking LIC site for colony PCR
T-DNA LB Rv	AATATATCCTGCCGCCGCTGCCGC	RB from pSKI015- activation tagging vector for pid-9 genotyping
T-DNA Left Fw	GGTTTACCCGCCAATATATCCTGTC	Sequencing primers
T-DNA RB Fw	TGACAGGATATATTGGCGGGTAAAC	RB from pSKI015- activation tagging vector for pid-9 genotyping

Appendix 2- Table 1 DNA sequences of primers used in Chapter 4.



Appendix 2- Figure 1
Stage 9 gynoecia showing *pETT::ETT:GFP* expression in the lateral vascular bundles. Arrows indicate vascular localization of the reporter. Inset shows bright field image of the same. Scale Bars= 100µm.



Appendix 2- Figure 2
Stage 13 gynoecia showing *pETT::ETT:GFP* localization in vascular bundles. Reporter expression can be detected in the medial and funicular vascular bundles. Arrows indicate vascular localization of the reporter. Inset shows bright field image of the same.
Scale Bars= 100µm.

APPENDIX III

Primer Name	Primer Sequence	Description
BolIND-EcoRI Fw	gcacGAATTCATGATGGAGCCTCATCATCTCC	IND gene from Brassica oleraceae to clone into pGAD424/pGBT9 with EcoR1 site
BolIND-PstI Rv	gtcgCTGCAGTTAGGTATCCGAGTTGTGGTAATAAC	Same as above with PstI site
BraETT1 EcoRI Fw	gcctGAATTCATGGGTGGTTTAATTGATCTG	ETT-1 gene from Brassica rapa to clone into pGAD424/pGBT9 with EcoR1 site
BraETT1 PstI Rv	gataCTGCAGCTAGAGAGCAATGTCTAGCAAC	Same as above with PstI site
BraETT1seq	GGAGTGAGAAGAGCTTCTC	Sequencing primer
BraETT2 EcoRI Fw	gactGAATTCATGGGTGGCTTAATCGATCTG	ETT-2 gene from Brassica rapa to clone into pGAD424/pGBT9 with EcoR1 site
BraETT2 PstI Rv	gataCTGCAGCTAGAGAGCAATGTCTAGCAACATAT CTCTCATG	Longer primer as the ends of both ETT-1 and ETT-2 are the same except ETT-2 has C (which I have incorporated at the end of the primer) while ETT-1 has G. So the last nucleotide at the 3' end of the primer is different.
BraETT2seq	CGCTTCTGCTTTCTCAGCTCAG	Sequencing primer
BraIND-EcoRI Fw	gcacGAATTCATGATGGAGCATCATCATCTCC	IND gene from Brassica rapa to clone into pGAD424/pGBT9 with EcoR1 site
BraIND-PstI Rv	gtaaCTGCAGTCAGACATAGGAGCTCCAAGCTG	Same as above with PstI site
CapsellaETT Fw	ATGGGTGGTTTAATCGATCTG	Capsella ETT specific primer to clone into PCR8 using TA cloning. Then LR reaction into pGBTK (Gateway BD). BD vector has tag at N-terminal end, ATG is in frame with AAA-triplet of L1 site.
CapsellaETT Rv	CTAGAGAGCAATGTCTAGCAAC	Same as above
CapsellaIND-AD F	GGGGACAAGTTTGTACAAAAAAGCAGGCTatgatggagc	Gateway primer for cloning Capsella rubella IND into the pGADc vector-BP reaction
CapsellaIND-AD R	GGGGACCACTTTGTACAAGAAAGCTGGGTAggtttgggg	Gateway primer for cloning Capsella rubella IND into the pGADc vector- one extra nt at the end of gateway site to keep the C-terminal fusion readinf frame.

Appendix 3- Table 1 Sequences of the primers used in Chapter 5

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