Chapter 1. Transcription and modulation of transcriptional states in *Arabidopsis*

1.1 Transcription in *Arabidopsis* development

Variation at the level of transcription unarguably contributes enormously to differences between cells—it is, after all, the primary mechanism by which two cells with the exact same genome produce unique sets of proteins and thus acquire completely different morphologies, behaviors, and functions. Coordinated gene expression is particularly important for proper development and acquisition of specific cell fates. Of course, transcriptional networks do not occur in a vacuum, nor are they the absolute determinant of protein accumulation within a cell. The activity of transcription factors can be regulated by intracellular events as well as extracellular signaling, and there are several levels of post-transcriptional regulation that determine whether a protein is translated, stable, and properly modified. We focus here on some basic transcriptional networks in plants, as well as two mechanisms by which transcriptional states are modulated: extracellular signaling, and microRNA activity.

The proteins encoded by the plant homeotic MADS box genes are classic examples of transcription factors that cooperate to activate downstream targets. Organ identity in the flower is instructed by the overlapping expression patterns of floral homeotic genes. These genes encode members of a family of transcription factors called MADS box genes, which share homology with transcription factors in yeast and animals (Hayes et al., 1988; Jarvis et al., 1989). The floral homeotic proteins are classified into three groups (A, B, and C), based

on the organ type they are required to set up: sepals (A), petals (A + B), stamens (B + C), and carpels (C) (recently reviewed in (Eckardt, 2003; Lohmann and Weigel, 2002). Recent work has revealed that these proteins likely operate in complexes of 3 or 4 proteins to bind DNA and regulate expression of downstream targets (Honma and Goto, 2001). As has been the case with Hox proteins in animals, the identities of the downstream genes have long been elusive. More sophisticated experiments have made use of protein fusions with the glucocorticoid receptor (GR) – these allow a transcription factor to be held inactive outside the nucleus until treatment with dexamethasone, so that analysis of the resulting changes in gene expression (recently, through the use of microarrays) can accurately reflect the most immediate, direct targets. Such methods have successfully enabled the identification of direct targets of AGAMOUS (AG), the C class protein. In particular, AG is now known to activate the expression of *NOZZLE* (*NZZ*) in developing stamens (Ito et al. in preparation).

Several developmental processes in plants are set up and maintained by transcription factors which act in opposition to each other. These mechanisms can have the effect of reinforcing boundaries between different populations of cells. One such arrangement allows the stem cells in the shoot apical meristem to maintain their proliferative state, while still enabling organ primordia to form on the lateral edges.

SHOOT MERISTEMLESS (*STM*) is expressed in the shoot apical meristem and encodes a KNOTTED1-class homeodomain protein which functions to prevent differentiation of the stem cells. *stm* loss-of-function mutants arrest at the seedling stage, having no apical meristem between the cotyledons (Long et

al., 1996). STM represses transcription of *AS1* and *AS2*, two myb domain transcription factors which negatively regulate expression of two other genes in the same family as *STM*: *KNAT1* and *KNAT2*. This system of gene regulation resolves into a pattern in which STM, KNAT1, and KNAT2 are active in the center of the shoot apical meristem, while AS1 and AS2 function predominantly in lateral organ primordia (Byrne et al., 2002; Semiarti et al., 2001). Not surprisingly, ectopic expression of *STM* in leaf primordia results in transcription of *KNAT1* and *KNAT2* in leaf tissue, creating densely-staining cells which resemble meristematic cells (Lenhard et al., 2002).

It is thought that a similar strategy enables cells in lateral organs to acquire the appropriate fate, according to their position, particularly for abaxial-adaxial polarity and resulting organ outgrowth (the adaxial side is nearest the stem, and the abaxial side is farthest). The three HD-ZIP proteins PHAVOLUTA (PHV), PHABULOSA (PHB), and REVOLUTA (REV) are found on the adaxial side of lateral organs (McConnell et al., 2001). In contrast, the *KANADI* (*KAN*) genes are expressed in abaxial tissues. Although there is no evidence for direct transcription repression of *KAN1*, *KAN2*, and *KAN3* by the HD-ZIP proteins, or vice versa, there are data suggesting that such a mechanism might be in place. Dominant, gain-of-function mutations in *PHV*, *PHB*, and *REV* cause abaxialization of leaves, a phenotype similar to the triple loss-of-function mutant *kan1*; *kan2*; *kan3* (Emery et al., 2003; McConnell et al., 2001). Loss-of-function mutations in *KAN* result in ectopic expression of *REV* and *PHV* (Eshed et al., 2001), while *35S::KAN1*, *KAN2*, or *KAN3* mimics the triple mutant *phb*; *phv*; *rev* (Emery et al., 2003; Eshed et al., 2001).

1.2 Various inputs from outside the cell can alter the transcriptional program

An important kind of input affecting transcription is derived from information from outside the cell. Extracellular signals can convey information about the cell's external environment, and the identity and/or arrangement of its neighbors. Typical signaling pathways require the function of several proteins (often kinases or phosphatases) between the plasma membrane and the nucleus.

The predominant type of membrane-spanning receptor kinase found in plants contains an extracellular domain, a single-pass transmembrane domain, and an intracellular serine-threonine kinase domain. There are over 400 of these proteins, termed receptor-like kinases (RLKs), and they can be grouped on the basis of motifs in their extracellular domain (Shiu and Bleecker, 2001). A particularly large subset of this receptor family (200+) features proteins with extracellular leucine-rich repeats (LRRs); this motif is believed to be involved in protein-protein interactions. Several LRR-RLKs are known to function in various aspects of plant development, including plant architecture (ERECTA), floral organ abscission (HAESA), cell fate determination in stomatal precursors (TOO MANY MOUTHS), and regulation of cell division and growth in embryos and stamens (EXTRA SPOROGENOUS CELLS) (Canales et al., 2002; Jinn et al., 2000; Nadeau and Sack, 2002; Shpak et al., 2001; Torii et al., 1996; Zhao et al., 2002). In some cases, RLKs function by altering the transcription of downstream genes, rather than simply modifying cell structure or behavior directly.

One of the best-characterized LRR-RLKs contributes to the long-range signaling mediated by hormones. Unlike in animals, plant hormones are not produced in a particular tissue, but can be manufactured almost anywhere. The steroid hormone brassinolide (BL) is a well-studied example: it is known to be

required in several processes in plant development, including pollen tube growth, stem elongation, and photomorphogenesis (Li and Chory, 1999). The receptor for BL is termed BRASSINOLIDE-INSENSITIVE1 (BRI1) and encodes an RLK with 25 extracellular leucine-rich repeats (LRRs) (Li and Chory, 1997). Multiple experiments have confirmed that the extracellular domain binds BL, and that it does so with a high degree of specificity (Wang et al., 2001). A particularly elegant experiment showed that BL could induce a hypersensitive response in rice cell lines expressing a fusion protein containing the extracellular domain of BRI1 and the intracellular domain of XA21 (required in rice for resistance to particular strain of *Xanthomonas oryzae*) (He et al., 2000). Another interesting result is that BRI1 kinase activity is not required for BL binding (Wang et al., 2001).

Much of our understanding of RLK function at the biochemical level comes from research done on BRI1 signaling. Based on genetic results, BRI1 seemed unlikely to function as a homodimer, and a yeast two-hybrid screen did in fact reveal that BRI1 was capable of binding another RLK. This protein, named BAK1 for BRI1-associated kinase, interacts with BRI1 in yeast and co-precipitates with BRI1 *in vivo*. Furthermore, BAK1 and BRI1 show kinase activity if co-expressed in yeast, but not if expressed singly. The phenotypes of loss-of-function alleles of *BAK1*, and *BAK1* overexpression, are consistent with its function as part of a receptor complex for BL (Li et al., 2002; Nam and Li, 2002).

The BRI1 signaling pathway extends into the cell via inhibition of BIN1, which in turn negatively regulates two nuclear phosphoproteins, BZR1 and BES1, containing shared domains but no motifs conserved among transcription factors (He et al., 2002). Although the exact biochemical function of these two

proteins remains unclear, their activity shows strong positive correlation with transcript levels of genes upregulated by BL induction (Wang et al., 2002; Yin et al., 2002). This suggests that BZR1 and BES1 may act in the nucleus to enable BL-induced changes in gene expression, from a signal that is communicated across the plasma membrane by BRI1 and BAK1.

In addition to signals which act over long ranges, like hormones, there are extracellular signals which are more local, because they involve ligands which are either membrane-bound or secreted only a short distance. A well-studied example of such a mechanism is the CLAVATA1 (CLV1) / CLAVATA3 (CLV3) pathway. These genes were discovered by virtue of their similar, noticeable loss-of-function phenotypes: enlarged shoot apical meristems, larger floral meristems, and extra floral organs (most notably carpels) (Clark et al., 1993; Clark et al., 1995). This phenotype suggested that CLV1 and CLV3 were required for proper restriction of cell division in shoot and floral meristems. Molecular cloning of these genes revealed that *CLV1* encoded a LRR-RLK, whereas *CLV3* encoded a small secreted protein with no known motifs. *CLV1* and *CLV3* are expressed in overlapping domains within the meristem: the former is found in a central cone-shaped domain containing the stem cells, while the expression pattern of the latter is wider and below the top layers of the central zone (Clark et al., 1997; Fletcher et al., 1999).

The CLV3 protein has a cleavable signal sequence and has been shown to be localized to the extracellular space in transfected onion cells (Rojo et al., 2002; Sharma et al., 2003). Biochemical studies demonstrating that CLV3 and CLV1 belonged to the same protein complex have been retracted (Trotochaud et al., 2000); at this writing, the primary evidence that CLV3 is a CLV1 ligand is genetic.

Another possible candidate for a protein functioning in this proposed signaling complex is CLV2. Loss-of-function *clv2* alleles resemble weak alleles of *clv1* and *clv3*, suggesting that the requirement for CLV2 is not as stringent. However, recent work has indicated that alleles of *CLV1* more likely to be null alleles show a weaker phenotype than the alleles with seemingly subtle missense mutations (Dievart et al., 2003). These results indicate that the stronger *clv1* alleles may be interfering with the function of one or more additional receptors, perhaps by binding them in non-functional heterodimers, or by outcompeting them for downstream effectors.

When *CLV3* is overexpressed, the resulting plants have a terminated meristem phenotype which requires wild-type CLV1 function (i.e., *clv1* is epistatic to *35S::CLV3*). The *wuschel* (*wus*) loss-of-function mutant has a nearly identical phenotype (Laux et al., 1996). *WUS* was found to encode a novel homeodomain protein, and the gene is expressed in a sub-region of the *CLV1* expression domain (Mayer et al., 1998). Consistent with the phenotypic data, *WUS* is ectopically expressed in *clv1* and *clv3* mutants, but downregulated in *35S::CLV3*-containing plants (Brand et al., 2000). WUS therefore seems to be negatively regulated at the transcriptional level by CLV3/CLV1 signaling, although the transcription factor or factors which link CLV3 and CLV1 to the regulation of *WUS* expression have not yet been identified. WUS in turn activates the expression of *AGAMOUS* (*AG*) in floral meristems (Lenhard et al., 2001; Lohmann et al., 2001). In addition, *WUS* misexpression from the *CLV1* or *AINTEGUMENTA* (*ANT*) promoter is sufficient to turn on ectopic *CLV3* expression in the cells in nearby layers, suggesting that *WUS*, *CLV1*, and *CLV3* act in a negative feedback loop (Brand et al., 2000; Schoof et al., 2000). This type

of self-regulating pathway is necessary for maintaining the appropriate balance of cell proliferation and differentiation in a fluid, dynamic structure such as the meristem. Through the action of CLV3 and CLV1, cells are informed of the choices their neighbors are making, and can change transcriptional states to increase or decrease the size of the stem cell population.

1.3 MicroRNAs can modulate gene expression post-transcriptionally

An important mechanism for immediately modifying transcriptional output relies on developmentally regulated endogenous microRNAs (miRNAs), which act on specific target genes to destabilize their mRNA or prevent the mRNA from being translated. Such miRNAs were first discovered in *C. elegans* (Lau et al., 2001; Lee and Ambros, 2001; Olsen and Ambros, 1999; Pasquinelli et al., 2000; Reinhart et al., 2000), although a related type of small RNA, small interfering RNA (siRNA), was described earlier in plant post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999; Metzlaff et al., 1997; Smith et al., 1994; Vaucheret et al., 2001). The two kinds of small RNAs are both about 19-24 nucleotides long, and are processed from longer precursors by an enzyme called Dicer (Hutvagner et al., 2001; Ketting et al., 2001; Zamore et al., 2000). siRNAs are capable of guiding cleavage of the protein-coding target mRNA (Martinez et al., 2002; Yang et al., 2000). The two types differ in source and structure, however. siRNAs are double-stranded and generated in the presence of high copy number of a transgene (as in PTGS), or from an exogenous double-stranded precursor (injected dsRNA or a sense-linker-antisense transgene), whereas miRNAs are single-stranded and processed from an endogenous non-coding RNA folded into a stem-loop structure. Most animal

miRNAs actually act not by promoting cleavage of the target mRNA, but by blocking its translation (recently reviewed in (Pasquinelli and Ruvkun, 2002).

Mutations in genes known to act in miRNA metabolism cause a variety of developmental defects. For example, *carpel factory* (*caf*) mutants have extra carpels, defective ovules, abnormal leaf and floral organ shape, and indeterminate growth in the floral meristem (Jacobsen et al., 1999). Stronger alleles of the same gene (*sin1*, *sus1*) cause severe embryo defects (Golden et al., 2002; Ray et al., 1996; Schwartz et al., 1994). The CAF/SIN/SUS1 protein is an RNA helicase/ RNAse III which is homologous to Dicer. In fact, mature miRNAs do not accumulate to normal levels in *caf* mutants, although the abundance of precursors remain the same (Park et al., 2002). This suggests that CAF (recently renamed DICER-LIKE1, or DCL1) is required for processing plant miRNAs into their mature form. The *hen1-1* mutation causes a pleiotropic phenotype reminiscent of *caf*, and accumulation of mature miRNAs in *hen1-1* is similarly lacking. *HEN1* was found to encode a novel protein with homologs in fungi, bacteria, and metazoans (Park et al., 2002). Subsequent work has shown that HEN1 functions in both miRNA accumulation and some forms of PTGS (Boutet et al., 2003).

Work from several labs has resulted in the identification of 24 distinct miRNA sequences in *Arabidopsis*. Most sequences are found in multiple copies in the genome, sometimes quite near each other (for example, *MIR166c* and *d*) (Llave et al., 2002a; Park et al., 2002; Reinhart et al., 2002). Confirming the relevance of miRNAs to proper plant development, several miRNAs are spatially and/or temporally regulated, as detected on Northern blots (Park et al., 2002; Reinhart et al., 2002).

Animal miRNAs predominantly target heterochronic genes – genes which control the timing of developmental events (Abrahante et al., 2003; Grishok et al., 2001; Lee et al., 1993; Lin et al., 2003; Olsen and Ambros, 1999; Pasquinelli et al., 2000; Reinhart et al., 2000) – or genes required for proper morphogenesis (Brennecke et al., 2003; Xu et al., 2003). Similarly, the targets of plant miRNAs are likely to be transcription factors with crucial roles in developmental processes. Recent work to determine the candidate targets for miRNAs has shown that the list includes genes encoding 3 SCARECROW-like transcription factors, 5 NAC transcription factors, 5 MYB proteins, 5 HD-Zip proteins, AP2, and at least one AP2-like protein (Park et al., 2002; Reinhart et al., 2002; Rhoades et al., 2002). The majority of the miRNA-binding sites in the predicted target mRNAs are in the coding region, with the remainder located in the 3' untranslated region (Rhoades et al., 2002).

The first efforts to investigate plant miRNA function led to the surprising conclusion that, unlike the majority of animal miRNAs, plant miRNAs could behave like siRNAs by inducing cleavage of the target mRNA. Llave et al. focused on *MIR39*, which is predicted to target three *SCARECROW* (*SCR*) –like (*SCL*) genes. *MIR39* is expressed most strongly in inflorescences, and it is in this tissue that truncated transcripts (as well as the expected full-length transcripts) of two *SCL* genes can be detected. Further evidence to demonstrate a direct relationship was provided by co-transformation of *N. benthamiana* leaf tissue with *35S::SCL6* and *35S::MIR39* and detection of cleaved versus full-length *SCL6* transcripts. An additional insight was provided by careful analysis of the truncated transcripts: the cleavage event, while showing some preference for

particular base pairs, does not always occur at the same position (Llave et al., 2002b).

A study using wheat germ extract produced some additional exciting results. First, the extract was shown to include an RNA-induced silencing complex (RISC) pre-loaded with endogenous wheat miRNA. Using this extract in an *in vivo* assay, the authors observed cleaved wild-type *PHAVOLUTA* (*PHV*) mRNA, but not *PHV* mRNA containing a dominant mutation in the miRNA recognition sequence. This result confirmed the hypothesis, initially put forth by Rhoades et al. (2002), that the dominant alleles in *PHV* and *PHABULOSA* (*PHB*) are resistant to down-regulation by the activity of MIR165 and 166, the miRNAs predicted to bind *PHV* and *PHB* mRNAs (Tang et al., 2003). Since *PHV* and *PHB* are known to be required for proper polarity of lateral organs, these experiments uncovered a functionally relevant miRNA. Finally, this work showed that perfect miRNA:mRNA complementarity is not required for the cleavage-based mechanism of miRNA action.

MIR172 is the first published example of a plant miRNA acting through translational repression (rather than cleaving the target mRNA). *MIR172* is found in several copies in the genome and is predicted to target *AP2* and 3 genes in the same family. When this miRNA is overexpressed, the resulting phenotype resembles that of *ap2* loss-of-function alleles. *AP2* mRNA levels are unaffected, while AP2 protein is missing. In the *hen1* and *dcl1* mutant backgrounds, where miRNAs do not accumulate to wild-type levels, AP2 protein levels are higher than normal, but the *AP2* message remains at wild-type abundance. The same is true when a *35S::AP2* construct with mutations in the miRNA recognition site is transformed into plants (*35S::AP2ml*) (Chen, 2003). Overexpression of *MIR172*

results in a phenotype resembling an *ap2* loss-of-function mutant (with no change in *AP2* mRNA levels – only protein), and these plants also flower earlier than wild-type. This aspect of the phenotype suggests that *MIR172* does, in fact, target another of the *AP2*-like genes (in particular, At2g28550, which is known to block the transition to flowering) (Aukerman and Sakai, 2003; Chen, 2003).

Additional miRNAs have been shown to function by negatively regulating their predicted target mRNAs; for example, *miRJAW* is complementary to sequences in the transcripts of several TCP transcription factors, and has been shown to promote cleavage of *TCP4* (Palatnik et al., 2003).

A slight gap in our understanding of plant miRNA function stems from the fact that no loss-of-function alleles in miRNAs have been identified. The best genetic evidence for miRNA function, outside of the results from overexpressing miRNA-resistant target mRNAs, has been the *caf/dcl1* and *hen1* mutants mentioned above. Although these two mutants have a general defect in miRNA metabolism, the phenotypes do not always parallel that of the dominant gain-of-function alleles or overexpressed, mutated transgenes. For example, overexpression of miRNA-resistant *AP2* causes homeotic transformations in the third and fourth whorls, but for *hen1*, such phenotypes are only visible in a sensitized background (*hua1-1*; *hua2-1*) (Chen, 2003).

1.4 New insight into miRNA function in plant development

In this work, we describe a new miRNA, called *EARLY EXTRA PETALS* (*EEP1*), which plays a role in organ formation and patterning in the shoot and flower. Plants homozygous for the recessive *eep1* mutation have extra petals in the first ten flowers, and *eep1* enhances the floral phenotype of *pinformed* (*pin1*)

and *pinoid* (*pid*), as well as the *pid* phenotype in the shoot. The miRNA encoded by *EEP1* is closely related to published miRNAs *MIR164a* and *b* (Reinhart et al., 2002); all three are predicted to bind the mRNAs of six target genes in the NAC family of transcription factors (Rhoades et al., 2002). Included in this group of six genes are *CUC1* and *CUC2*, which mark boundaries between organs in the flower and between cotyledons in the developing embryo (Aida et al., 1997; Takada et al., 2001). Ubiquitous overexpression of *EEP1* from the cauliflower mosaic virus *35S* promoter leads to phenotypes resembling the *cuc1*; *cuc2* double mutant (Aida et al., 1997); furthermore, *CUC2* mRNA is significantly less abundant in *35S::EEP1* compared to wild type. These data suggest that *EEP1*can negatively regulate *CUC1* and *CUC2* post-transcriptionally, and that it acts via an mRNA cleavage-based mechanism, at least for *CUC2*. We discuss the possible implications of this negative regulation, as it relates to *pin1*, *pid*, and the interactions of each mutant with *eep1*.

1.5 Advances in functional characterization of proteins from the CLE family

In second part of this work, we describe a set of reverse genetic experiments designed to test whether several CLV3-like proteins might function as ligands for one or more RLKs. The genes encoding these proteins belong to a family of 25 *Arabidopsis CLV3/ERS* (*CLE*) genes, all of which code for predicted secreted or membrane-bound proteins of approximately 100 amino acids (Cock and McCormick, 2001). They share a small conserved domain in the C terminus – otherwise, there is little or no sequence conservation among them. We sought to investigate the function of five of these proteins, using overexpression, double-stranded RNA interference (dsRNAi), and reporter lines. We found that CLE11

and CLE12 were capable of mimicking CLV3 when ubiquitously overexpressed, in that they caused meristem termination and resulted in rare, defective flowers. CLE16 and CLE17, when overexpressed, cause defects in leaf shape but do not affect the meristem. We hoped to shed light on the endogenous functions of these proteins via the dsRNAi experiments, but they yielded no mutant phenotypes. Expression analysis indicates that the *CLE* genes are transcribed in restricted but overlapping patterns, predominantly in reproductive tissues. We discuss several explanations for the results, both positive and negative, that we have generated with these experiments.

1.6 References

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