Cis-regulatory Elements Controlling Expression of the Auxin Response Factor MONOPTEROS and its irrepressible variant $MP\Delta$

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

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The AUXIN RESPONSE FACTORS (ARFs) mediate changes in gene expression that are associated with auxin response in plants. One of the best-characterized ARFs is ARF5/MONOPTEROS(MP), for which strong loss-of-function and gain-of-function associated defects have been reported. Recent evidence has indicated that auxin response may be driven in part by MP-mediated control of its own expression. Here it is shown that removal of individual ARF-binding auxin response elements (AuxREs) from the MP promoter results in differential expression in specific tissues, while creation of supernumerary AuxREs is associated with auxin response-related phenotypic changes. The irrepressible MP variant MPΔ was used to track potentially expression-stage specific regions of the MP promoter and to determine in which tissues MPΔ can exert its gain-of-function effects independent of the activity of other, closely related ARFs.
Acknowledgements

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I dedicate this thesis to my grandmothers, Carol and Lucile.
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**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARF</td>
<td>AUXIN RESPONSE FACTOR</td>
</tr>
<tr>
<td>AuxRE</td>
<td>auxin response element</td>
</tr>
<tr>
<td>Basta</td>
<td>glufosinate-ammonium</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>Col-0</td>
<td>Columbia</td>
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<tr>
<td>DAG</td>
<td>days after germination</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<tr>
<td>GRE</td>
<td>G-box-related element</td>
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<td>hydrochloric acid</td>
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<td>indole-3-acetic acid</td>
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<tr>
<td>MS</td>
<td>Murashige and Skoog media</td>
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<tr>
<td>MRE</td>
<td>Myb-related element</td>
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</table>
MRE1  Myb-related element 1  
MYC2  Myc-related element 2  
NaCl  sodium chloride  
PCR  polymerase chain reaction  
rpm  revolutions per minute  
s  second  
SAM  shoot apical meristem  
SD  standard deviation  
SDS  sodium dodecyl sulfate  
T-DNA  transfer DNA  
Tris  Tris (hydroxymethyl) aminomethane  
vol  volume  
v/v  volume per volume  
w/v  weight per volume  
X-gluc  5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside  
ZRE  bZIP-related element
Chapter 1

Introduction
1.1 The phytohormone auxin

The phytohormone auxin is involved in a surprising number of processes in plants, being associated with patterning of vasculature and root meristem, initiation and positioning of lateral organs, apical-basal polarity, tropisms, and senescence (Davies, 2010). The most abundant auxin in higher plants is indole-3-acetic acid (IAA), which is synthesized in multiple locations from several precursors, including tryptophan (Zhao, 2010; Woodward and Bartel, 2005). Auxin changes the gene expression of individual plant cells, but its polar transport throughout the organism is also crucial to its developmental effects (Liu et al., 1993; Estelle, 1998; Friml et al., 2003).

Auxin can trigger certain cellular responses, e.g. cell elongation, extremely rapidly, and it is assumed that no gene expression change is involved in these responses (Fuente and Leopold, 1970; Christian et al., 2006). However, many other auxin functions, especially those controlling complex developmental programs, are executed through the regulation of auxin responsive genes (Hagen and Guilfoyle, 2002; Chapman and Estelle, 2009). The transduction of an auxin signal to gene expression has been a strong focus for plant biologists over the past two decades (Leyser, 2006). Genetic and molecular analysis of critical genes in those pathways has proven useful in identifying and characterizing the complexity of the genetic networks controlled by auxin and thereby creating a better understanding of how a single hormone governs so many aspects of plant development.

1.2 Polar auxin transport

Auxin is directed from cell to cell in an organized fashion, creating a “flow” of the hormone throughout the developing plant (Sachs, 1981; Okada et al., 1991; Liu et al., 1993). As a result, the hormone has developmental roles in tissues throughout the plant, all while
controlling its own direction, route, and strength as well as targeting and coordinating multiple instances of growth and development (Möller and Weijers, 2009; Vanneste and Friml, 2009). A shoot-to-root transport of auxin, especially pronounced in immature vascular tissues, has been observed for a long time, and is explained by a cellular model that is based on differential charge of the IAA molecule in the intra- and extracellular spaces (Rubery and Sheldrake, 1974). This model, called the chemiosmotic hypothesis, posits that the charged IAA anion is trapped in the cytoplasm and stringently requires specific membrane proteins to mediate its efflux from the cell. The targeted vesicle transport of such proteins towards specific parts of the plasma membrane, typically towards the root pole, creates a polar localization of these auxin efflux carriers; this mechanism is believed to be the primary force driving auxin flow (Benkova et al., 2003; Friml et al., 2003). Once these transmembrane proteins transport auxin into the extracellular space, the hormone is then absorbed by the nearest cell, either passively or through specific auxin influx carriers (Rubery and Sheldrake, 1974; Bennett et al., 1996; Marchant et al., 1999). This polarization in the distribution of plasma membrane proteins, when coordinated across multiple cells, creates a directional flow. Identification of auxin carrier proteins has helped provide experimental support for the chemiosmotic model and has allowed for (a) a better understanding of the cellular mechanisms underlying their directional transport and (b) a more detailed investigation of auxin’s developmental roles (Okada et al., 1991; Galweiler et al., 1998; Scarpella et al., 2006). The latter enormously benefited from the possibility to identify presumptive routes of auxin transport via in vivo visualization of fluorescently labelled auxin carrier proteins (Galweiler et al., 1998; Scarpella et al., 2006).

Auxin’s role in development involves transport between cells and predictable dynamic patterns, transport routes, and distributions prior to histologically recognizable cell specification (Vanneste and Friml, 2009). Genetic evidence suggests that these patterns generate the positional information to tissues to drive cell specification in biologically meaningful arrangements (Sabatini et al., 1999; Benkova et al., 2003; Friml et al., 2003;
Reinhardt et al., 2003; Scarpella et al., 2006). As auxin transport is primarily driven by the polar localization of influx and efflux carriers, the respective gene families became subject to intensive research. The PIN-FORMED (PIN) family of auxin efflux carrier (or facilitators) has been identified as a major factor in controlling polar auxin transport (Galweiler et al., 1998). The eight identified PIN proteins in Arabidopsis are characterized as either plasma membrane-localized (PIN1, 2, 3, 4, 7) and endoplasmic reticulum-localized (PIN5, 6, 8) (Mravec et al., 2009). Comparison of auxin distribution and PIN localization shows that this localization reflects the direction of auxin transport, and experimental modifications of PIN polarity have been shown to cause a corresponding change in the direction of auxin flow (Benkova et al., 2003; Wisniewska et al., 2006). These observations indicate that PIN localization is a crucial determinant of the direction of auxin transport.

The existence of specialized influx-promoting proteins was less predicted than those of efflux facilitators. Initial models of auxin transport proposed that the primary mechanism of auxin influx was passive transport of protonated auxin, IAAH, through the membrane (Rubery and Sheldrake, 1974). Upon exiting the acidic extracellular space and entering the comparatively basic cytoplasm, IAAH loses its hydrogen and becomes an IAA- anion, which cannot passively cross through the cell membrane. Consequently, the IAA anions must rely on the action of efflux carriers to exit the cell (Michniewicz et al., 2007). By contrast, the importance of influx carriers has become apparent mainly with genetic evidence. AUX1, an amino acid permease associated with auxin influx, has been shown to localize at the apical side of protophloem cells, where efflux carrier PIN1 localizes basally (Kleine-Vehn et al., 2006). Additionally, aux1 mutants show phenotypic features that reflect deficiencies in auxin transport, and these deficiencies are rescued by membrane-permeable auxin variants (Maher and Martindale, 1980).
1.3 The role of auxin response in embryo patterning

The cell divisions during early embryogenesis can be highly stereotyped, as for example in the genetic model plant species *Arabidopsis*, which could suggest that they are specified by rigid genetic programs, leaving little room for self-organizing controls that are dependent on transport and dynamic distribution of a low-molecular weight compound like IAA. However, despite this apparent rigidity, experimental and genetic studies have shown that (a) the embryo pattern is highly robust and not at all dependent on a specific sequence of cell divisions, (b) that embryos can even be formed from somatic cells and this process typically involves the application of external auxin signals (Torres-Ruiz and Jurgens, 1994; Raghavan, 2006), and finally, (c) the most severe and lasting interferences with even basic features of embryo patterning, such as apical-basal polarity, can all be linked to genetic defects in auxin signal transduction or auxin transport (Meinke, 1985; Okada et al., 1991; Berleth and Jurgens, 1993; Mayer et al., 1993). Hence, contrary to what a mere description of *Arabidopsis* embryogenesis may suggest, it turns out that not defined cell division patterns, but rather dynamic auxin-flow configurations are instrumental for generating the basic outline of a seedling during embryogenesis (Capron et al., 2009).

The following outline will therefore integrate reported features of auxin signaling and transport into a description of *Arabidopsis* embryo development. For auxin signaling, the artificial *DR5* promoter, comprising concatamers of prototypical Auxin Response Elements (AuxREs, see below) linked to suitable reporter genes, has been widely used as a general transcriptional readout of auxin signal transduction, though it may not accurately reflect the genuine distribution of auxin in all situations (Ulmasov et al., 1997b). Likewise, the translational fusion of fluorescent protein domains to PIN protein transgenes, most often PIN1, is widely used as a proxy for predominant auxin-flow routes, because it does not only define concrete lines of cells that are also identified as auxin-flow routes by other means, but its polar localization towards the same end of all cells along such a line.
indicates the direction of auxin flow (Galweiler et al., 1998; Scarpella et al., 2006).

After fertilization, the single-celled zygote elongates and divides asymmetrically, producing a small, cytoplasmically dense embryonic apical cell and a larger, highly vacuolated, extraembryonic basal cell (Mansfield and Briarty, 1991; Capron et al., 2009). Shortly after this division, the apical cell begins to show expression of a DR5 reporter, indicating auxin activity in the cell (Friml et al., 2003). Furthermore, efflux carrier PIN7 in the basal cell has been shown to polarly localize at the apical plane of the plasma membrane, indicating the transport of auxin towards the apical cell (Friml et al., 2003). The apical cell undergoes two longitudinal divisions and one transverse, forming a globular 8-cell proembryo known as the octant (Capron et al., 2009). The four upper tier (u.t.) cells of the octant will form the plants apical structures, while the four lower tier (l.t.) cells will form nearly all basal structures. Meanwhile, the basal cell undergoes multiple transverse divisions to form a filament called the suspensor, which connects the developing proembryo to the mother plant (Capron et al., 2009). As a notable exception, the uppermost descendant of the initial basal cell will become part of the embryo, and hence seedling, by forming the centre of the distal root meristem of the embryo (Jürgens, 2001).

During these early stages of embryogenesis, the three major tissue identities, vascular, ground, and epidermal, become patterned. As the octant cells divide to form the early globular embryo, the four innermost lower tier cells begin to display elevated levels of auxin reporter signaling (Friml et al., 2003). These cells are the provascular cells, the initials of the stele. Auxin has long been known play a significant role in vascular differentiation based on experiments which showed that exogenous auxin application results in formation of new vascular strands (Sachs, 1991), and according to PIN1 localization, these cells are the first to show a strong and consistent apical-basal auxin flow, reflecting the newly emerging embryo axis (Steinmann et al., 1999). Therefore, the presence of auxin in the provascular cells strongly suggests that auxin patterns the identity of these cells. Auxin
reporter activity is observed to increase in the provascular cells until around the 32-cell stage, at which point activity shifts basipetally towards the hypophysis, the basal-cell descendant bordering the basal plane of the provascular cells (Friml et al., 2003). The PIN1 reporter protein localizes to the basal planes of these provascular cells at this stage, suggesting the polar transport of auxin towards the hypophysis (Steinmann et al., 1999). The hypophysis then undergoes an asymmetric, transverse division, forming a small, lens-shaped upper cell and larger lower cell (Scheres et al., 1994). Unlike the rest of the suspensor, the progeny of these cells will become incorporated into the developing embryo. The lens cell is the precursor of the quiescent centre (QC), a small group of ultimate root stem cells, which form an organizing centre in the root meristem, while the lower cell descendants eventually form the columella, the cells capping the tip of the root (Capron et al., 2009). Through its contribution to hypophysis specification (Weijers et al., 2006), auxin completes the orientation of the apical-basal axis, and patterns the cell groups that will govern the growth of the apical and basal structures of the maturing plant.

1.4 Auxin and root patterning

Formation of the root during *Arabidopsis* embryogenesis relies on the accumulation of auxin at or near the hypophysis, driven by transport from the adjacent apically provascular cells of the proembryo (Friml et al., 2003). As development progress, auxin appears to be continuously transported through the vascular cylinder of the root to the root apex. The auxin maximum at the root apex continuously stabilizes patterned cell state acquisition, including stem cell niches, of the cells of the root meristem, ensuring its continued mitotic activity (Capron et al., 2009). Inhibition of auxin transport, both through auxin transport inhibitors and *pin* knockout mutants, causes defects in the organization of root meristem tissues (Jiang and Feldman, 2005). Severe disruption of auxin transport or response pathways can lead to complete failure to form or to continuously grow a root (Meinke,
Auxin is also associated with formation of lateral roots. Exposure to exogenous auxin has long been known to induce formation of a supranormal number of lateral roots (Torrey, 1950). Lateral root initiation begins with the asymmetric division of a pericycle founder cell, which then continues to divide to form an entirely new meristem that arises from the interior of the primary root and generates a lateral root (Casimiro et al., 2001). Expression analysis of the $DR5$ reporter has indicated that an increased concentration of auxin exists in these cells immediately prior to asymmetric division, and continues to be present in the developing meristem, indicating auxin’s potential role in specifying these pericycle cells to create lateral roots (Benkova et al., 2003). Additionally, auxin plays a role in gravitropic response of the root by establishing a gravity-induced auxin gradient in specialized cells in the columella root cap, initiating elongation of cells on the opposite side of the gravitational force, which pushes the root tip downward towards the direction of gravity (Blancaflor et al., 1998; Mullen et al., 1998; Swarup et al., 2005). Many auxin transport and response mutants show varying degrees of lateral root formation and root gravitropism defects (Marchant et al., 1999; Okushima et al., 2005; Weijers et al., 2005).

### 1.5 Auxin and lateral organ initiation

Auxin has long been known play a role in development of lateral organs from the shoot apical meristem. Inhibition of auxin transport leads to pin-shaped shoots devoid of lateral organs (Okada et al., 1991). Knockout mutants of the auxin efflux carrier $PIN-FORMED1$ ($PIN1$) display an identical phenotype, giving the protein family its name (Okada et al., 1991; Galweiler et al., 1998). New primordia are initiated by an auxin maximum generated in the ring of tissue called the peripheral zone, which surrounds the centre of the meristem termed the central zone (Reinhardt et al., 2000). Analysis of PIN1 localization and auxin mutant phenotypes led to the formation of a model where auxin is focused in the
epidermis of the peripheral zone towards selected spots (Benkova et al., 2003; Reinhardt et al., 2003). At these spots, auxin is postulated to be internalized into the inner layers of the plant, thereby defining the site of the new primordium initiation. The internalized auxin patterns a new vein as it drains through a canal of cells to the central vein of the shoot, giving the developing lateral organ a direct link to the vasculature of its shoot. Thus, the primordia function as an auxin sink, reducing the concentration of auxin in the surrounding area. Due to the sink activity of current primordia, auxin can only be focused at a certain distance away from the two youngest primordia.

Upon reaching the peripheral zone, auxin begins to move towards the point of highest concentration (Reinhardt et al., 2000). PIN1 plays an essential role in the process, becoming localized to the plane of nearby cells with higher auxin concentration (Benkova et al., 2003; Heisler et al., 2005; Vieten et al., 2005). Increased auxin concentration not only strengthens the polarization of PIN1 proteins in cells, but also increases the expression of the $PIN1$ gene itself. Thus, it is theorized that auxin activates the expression of $PIN1$ and increases its own concentration at points of high concentration on the meristem, explaining how auxin positions and initiates the formation of a lateral organ. The repetition of this pattern as the shoot grows is theorized to govern the precise spiral phyllotactic positioning of the lateral organs of Arabidopsis (Reinhardt et al., 2003). Computational models using this experimental evidence for assumption and parameters are able to create multiple different phyllotactic patterns observed in plants, though production of auxin in the peripheral zone needs to be assumed in order to generate the spiral phyllotaxis observed in Arabidopsis (Smith et al., 2006). Nevertheless, these models support auxin’s role in organizing phyllotaxis in plants.
1.6 Auxin and vascular patterning

Plants rely on a network of vasculature to allow transport of water and solutes throughout the organism (Esau, 1965). The continuity of this network is crucial to its function, and thus the development of vascular tissue has been closely studied for decades. Auxin was implicated to play a role in vascular development in early experiments showing exogenous application of auxin to wounded stems results in the formation of a new vascular strand extending from the site of application towards the root (Sachs, 1969). These observations were used to create the canalization hypothesis, which suggested that the polar, directional flow of auxin, through a continuous file of cells, patterns the formation of vascular strands (Sachs, 1981). Additionally, auxin response expression markers show increased auxin concentration in developing vascular tissue and interruption of auxin transport leads to discontinuity and irregularities in vascular strands and loss of patterning (Mattsson et al., 1999; Sieburth, 1999; Avisian-Kretchmer et al., 2002; Mattsson et al., 2003).

Due to its two-dimensional nature and complex vasculature, the leaf has been a very useful system for clarifying the how auxin initiates vascular development and controls the patterning of vasculature as a whole. Vascular patterning of the leaf appears to be a self-organizing process driven by auxin transport, highly comparable to the patterning of lateral organ emergence from the shoot meristem. Early in leaf development, no vascular tissue is present, and instead beneath the epidermis there are layers of indistinguishable cells called the ground meristem (Esau, 1965). At this stage, auxin appears evenly distributed throughout these cells. As the leaf matures, auxin becomes directed towards the centre of the organ, forming a channel of cells that will become the midvein (Leyser, 2005; Scarpella et al., 2006). Meanwhile, auxin in the epidermis is transported towards convergence points along the leaf margin, where it is internalized and directed towards the midvein. This process initiates the formation of secondary veins. As these secondary veins form, auxin in the ground meristem moves towards nearby cells with higher concentration,
creating focused channels of cells with high auxin flux, and depleting auxin from cells surrounding these channels (Scarpella et al., 2004). As this process progresses, channels meet one another to form loops while new channels form in any space with available auxin. As the leaf grows, the auxin-depleted ground meristem becomes differentiated into mesophyll starting from the distal end, causing the process of new vein branching to stop as the availability of procambium-capable meristem cells decreases. Due to this halt in the canalization process, the highest order channels end up freely ending, not having enough time to form a second connection. Auxin in the channels of procambial cells activate auxin responsive genes to begin differentiation into vascular tissue (Scarpella et al., 2006; Wenzel et al., 2007).

Experimental evidence suggests that PIN1 plays a crucial role in the polar auxin transport that governs leaf vascular patterning. Knockout mutants in PIN1 display vascular patterning phenotypes similar to those of wild type plants treated with auxin transport inhibitors (Okada et al., 1991; Mattsson et al., 1999). Similar to its function in the shoot apical meristem, PIN1 is polarized towards the direction of auxin flux, and its expression is activated by auxin as well (Galweiler et al., 1998; Benkova et al., 2003; Heisler et al., 2005; Vieten et al., 2005; Scarpella et al., 2006). These properties create a self-regulated system that gradually directs auxin into concentrated, one cell wide channels and leads to the basipetal draining of the hormone, connecting the developing leaf to the total vasculature of the plant itself (Scarpella et al., 2006).

1.7 Auxin signaling

Auxin initiates a gene regulatory response primarily through the activity of two families of nuclear proteins: Aux/IAAs and auxin response factors (ARFs) (Tiwari et al., 2003). The interactions between members of these two families are posited to activate the first gene expression changes of auxin response. The Aux/IAA family consists of 29 genes,
all encoding small, nuclear-localized proteins consisting of four conserved domains (Abel et al., 1994; Gray et al., 2001; Reed, 2001). Domain I is a strong repression domain, domain II interacts with specific F-box proteins within larger complexes, and domains III and IV are homotypic protein-protein interaction domains (Gray et al., 2001; Tiwari et al., 2004). Aux/IAA genes were first identified due to their strong, rapid increase in expression in the presence of auxin. Aux/IAA proteins have very short half-lives, and their presence is reduced by auxin exposure, but increased by protease inhibitors (Abel et al., 1994; Ramos et al., 2001). The ARF family consists of 23 genes encoding DNA-binding transcription factors, most of which also consist of four conserved domains (Umasov et al., 1997a). One is a B3-like DNA-binding domain, while domain II is termed the middle region, the composition of which defines whether the protein is transcriptionally activating or repressing. Domains III and IV of ARFs are conserved with domains III and IV of Aux/IAAs, and the shared nature facilitates interactions between the two protein families (Kim et al., 1997; Umasov et al., 1999; Tiwari et al., 2003). ARFs bind to conserved cis-regulatory elements called auxin response elements (AuxREs), which are typically characterized by a TGTCTC motif (Umasov et al., 1997a). In the absence of auxin, Aux/IAA proteins are bound to ARFs, conferring repression of activity of auxin-response genes near DNA-bound ARFs (Umasov et al., 1997b; Tiwari et al., 2001).

Though Aux/IAAs were first identified due to their rapid increase in expression due to auxin, later experiments also showed that auxin dramatically decreases the half life of Aux/IAAs by increasing their degradation rate (Zenser et al., 2001). This response was shown to be caused by the activity of an Arabidopsis ubiquitin ligase complex called SCF\textsuperscript{TIR1} (Gray et al., 2001). SCF ligases are composed of 4 subunits: an SKP1-like protein, a cullin, RBX1, and an F-box protein (Gray et al., 1999). Involvement of SCF in auxin response first became apparent due to discovery that disruption of the gene encoding the F-box protein TIR1 caused auxin insensitivity (Ruegger et al., 1998; Gray et al., 1999). Further analysis has shown that the substrate for TIR1 appears to be
Aux/IAAs; this interaction creates a binding site for auxin, and auxin binding causes
the complex to ubiquitinate the bound Aux/IAA, marking the protein for degradation
(Gray et al., 2001; Dharmasiri et al., 2003; Dharmasiri et al., 2005b; Kepinski and Leyser,
2005; Tan et al., 2007). TIR1 is member of a family of proteins called auxin signaling
F-BOX proteins (AFB) (Dharmasiri et al., 2005a). The other three proteins in the family,
AFB1, AFB2, and AFB3, are also components of SCF complexes and act similar to
TIR1 in auxin reception and response. Knockout mutants of TIR1 and other members of
the AFB family give progressively stronger auxin insensitivity as more are knocked out,
with quadruple mutants of tir1 afb1 afb2 afb3 displaying a severe embryonic phenotype
resembling that of other strong auxin insensitivity mutants (Dharmasiri et al., 2005a;
Dharmasiri et al., 2005b). Analysis of TIR1 and Aux/IAA crystal structures has shown
that TIR1 contains a single binding pocket for both Aux/IAAs and auxin (Tan et al.,
2007). When auxin binds to the pocket, it enhances the TIR1-Aux/IAA interaction. By
strengthening this interaction, auxin increases the likelihood of ubiquitination of Aux/IAA
by SCF$^{TIR1}$, thus initiating Aux/IAA degradation (Guilfoyle, 2007b; Kepinski, 2007; Tan
et al., 2007). Reduced Aux/IAA levels removes repression of ARFs, allowing them to
initiate auxin responsive gene expression (Tiwari et al., 2003).

1.8 Auxin response factors

Auxin response factors, or ARFs, are a family of transcription factors that regulate
expression of auxin response genes (Guilfoyle and Hagen, 2001; Tiwari et al., 2003). As
stated earlier, ARFs generally contain four conserved domains. The so-called “middle
region” is either a transcriptional activation or repression domain depending on its amino
acid profile. Glutamine-rich middle regions are associated with five ARFs: ARF5/MP,
ARF6, ARF7/NPH4, ARF8, and ARF19 (Guilfoyle and Hagen, 2001). These five are
also the only transcriptionally activating members of the gene family, and all other ARFs
that have been analyzed do not have glutamine-rich middle regions and seem to act as transcriptional repressors (Ulmasov et al., 1999; Tiwari et al., 2003). ARFs appear to be under extensive transcriptional control, evidenced by the distinct and dynamic expression patterns of many individual ARFs throughout development (Hardtke et al., 2004; Okushima et al., 2005; Weijers et al., 2005; Rademacher et al., 2011). However, many of these expression domains overlap with each other, and many cell types show expression of both repressing and activating ARFs (Rademacher et al., 2011). The complexity of transcriptional control of ARFs could help explain how auxin plays a role in a number of seemingly distinct processes.

NONPHOTOTROPIC HYPOCHOTYL4 (NPH4), or ARF7, was originally identified by genetic screens for Arabidopsis mutants with abnormal tropic response to light. Unlike the other nph mutants, nph4 mutants displayed defects in both phototropic and gravitropic responses (Liscum and Briggs, 1995; Liscum and Briggs, 1996). Later experiments revealed its involvement in auxin differential growth response, and it was identified as a member of the ARF family (ARF7) (Stowe-Evans et al., 1998; Harper et al., 2000). nph4 mutants have epinastic or hyponastic rosette leaves, but are otherwise morphologically normal and fertile. They show defects in hypocotyl gravitropism and phototropism, indicating that NPH4 may be involved in auxin growth response in aerial tissues (Stowe-Evans et al., 1998). NPH4 is expressed throughout the embryo from the heart stage onward, and in seedlings shows strong expression in the hypocotyl, petioles, leaves, and cotyledons, as well as root vascular tissue and lateral root primordia (Okushima et al., 2005; Wilmoth et al., 2005). In adult plants, NPH4 expression is found in all aerial tissues, with stronger expression in young organs and in pollen and ovules (Hardtke et al., 2004).

ARF19 is a closely related transcriptional activator that is weakly expressed in aerial vascular tissue, but is strongly expressed throughout the root tissue (Okushima et al., 2005; Wilmoth et al., 2005). arf19 mutants do not have any noticeable morphological differences from wild-type plants if untreated, but experiments have shown auxin and
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ethylene insensitivity in roots (Okushima et al., 2005; Li et al., 2006). *nph4 arf19* double mutants display severely reduced lateral root formation and loss of root and hypocotyl gravitropism (Okushima et al., 2005; Wilmoth et al., 2005). This evidence indicates that NPH4 and ARF19 have some redundancy in their roles in auxin response processes, especially with regards to root primordia, where both display expression and where double mutants have the most prominent defects (Okushima et al., 2005; Wilmoth et al., 2005; Li et al., 2006).

MONOPTEROS (MP)/ARF5 is another transcriptional activator closely related to NPH4 and ARF19 (Okushima et al., 2005). MP plays a vital role in early embryogenesis by establishing the apical-basal axis, and throughout development in processes like initiation of new organs from the shoot apical meristem and patterning of vascular tissue (Berleth and Jurgens, 1993; Hardtke and Berleth, 1998; Schuetz et al., 2008). *mp* mutants have a very strong embryonic phenotype, failing to differentiate the basal embryonic domain into hypocotyl and primary root (Berleth and Jurgens, 1993). Instead, *mp* seedlings only develop an undifferentiated mass of cells at the basal domain, and are therefore unable to grow past the seedling stage. *mp* mutants also have highly reduced and discontinuous vasculature (Berleth and Jurgens, 1993). MP has been shown to be crucial to establishment of the hypophysis, the precursor to the root meristem cell (Hardtke and Berleth, 1998). In the early globular embryo, auxin is directed towards the lower tier of the embryo, activating the expression of *MP* (Weijers et al., 2006). MP then promotes *PIN1* expression and promotes its localization to the basal end of these cells, directing auxin to the hypophysis below (Weijers et al., 2006). Auxin accumulation alone, though, is not sufficient to rescue basal domain patterning in *mp* mutants. MP in the lower tier proembryo cells also activates the expression of a mobile transcription factor, TMO7/PRE3, which migrates from these cells into the adjacent hypophysis (Schlereth et al., 2010). There, it helps initiate the specification of the hypophysis as the precursor of the root and basal structures. *TMO7* has been shown to be a downstream target of
MP, and RNAi suppression of the gene leads to a loss of basal structure formation similar to that of mp mutants (Schlereth et al., 2010).

MP is also involved in initiation of lateral organs from the shoot apical meristem (SAM) and in the organization of vascular tissue patterning, processes that are both driven by polar auxin transport (Scarpella et al., 2006; Schuetz et al., 2008). mp mutants are capable of producing adventitious roots under hormonal stimulation, and thus researchers have used this capability to grow mp plants to adulthood (Przemeck et al., 1996). Mature mp plants are able to form vegetative lateral organs such as the rosette leaves but are unable to form floral organs, creating pin-shaped inflorescences which are indicative of defects in the reproductive SAM (Przemeck et al., 1996). This phenotype bears a strong resemblance to that of pin1. However, exogenous auxin application to the SAM does not rescue floral development in mp mutants as it does in pin1 mutants, indicating that mp mutants are deficient in auxin sensitivity (Reinhardt et al., 2000; Reinhardt et al., 2003). mp pin1 double mutants have even more severe SAM defects, failing to form any lateral organs and instead forming a leafless dome of tissue (Schuetz et al., 2008). Subsequent examination has shown MP directly upregulates expression of PIN1 and other PIN genes (Wenzel et al., 2007; Krogan et al., 2016). Consistent with this, MP and PIN1 expression domains overlap, and their expression becomes refined into specific cells that synchronize with the refinement of auxin distribution in both leaf vascular development and meristematic leaf initiation (Vieten et al., 2005; Scarpella et al., 2006; Wenzel et al., 2007; Schuetz et al., 2008). Essentially, MP appears to be a critical component of the self-organizing properties of auxin.

1.9 Protein-protein interactions between ARFs and Aux/IAAs

ARF transcriptional activity is controlled by their interaction with repressor Aux/IAA proteins. Suspension culture experiments as well as genetic evidence indicates that
activating ARFs are repressed upon protein-protein interaction with Aux/IAAs through domains III and IV (Tiwari et al., 2001; Tiwari et al., 2003). Many aux/iaa mutants display dominant auxin response-related growth defects and auxin insensitivity, and all of these mutants contain gain-of-function mutations in domain II (Gray et al., 2001). Domain II of Aux/IAAs is a highly conserved motif that facilitates binding of Aux/IAAs to SCF ubiquitin ligase complexes (such as SCF$^{TIR1}$, making it essential for auxin-driven Aux/IAA degradation) (Tiwari et al., 2003). Mutations in domain II compromise the ability of ubiquitin ligases to apply the tag, making the mutant protein consistently stable, irrespective of auxin concentration. As the mutant Aux/IAA protein concentration does not decrease in the presence of auxin, these Aux/IAA proteins remain bound to ARFs and repress their activity (Zenser et al., 2001). This can result in defects similar to those of some arf knockout mutants (Gray et al., 2001). For example, a gain-of-function mutation in the BODENLOS (BDL) gene causes failure to develop a primary root or hypocotyl, resembling the phenotype of mp mutants (Hamann et al., 1999). BDL encodes an Aux/IAA and proved to repress auxin response in carrot protoplast assays and bind to MP in a yeast two-hybrid assay (Hamann et al., 2002). Additionally, BDL is also expressed in the proembryo cells adjacent to the hypophysis during the globular embryo stages, the same cells where MP expression is elevated (Hamann et al., 2002; Weijers et al., 2006). Specification of hypophysis is reliant on the auxin-induced degradation of BDL in the proembryo cells, which allows MP proteins to activate expression of downstream targets like TMO7 that determine hypophysis fate (Hamann et al., 2002; Weijers et al., 2006; Schlereth et al., 2010). Other IAAs have also been identified due to auxin growth phenotypes and shown to interact with other ARFs. For example, gain-of-function iaa19 mutants, which have non-phototropic hypocotyls like loss-of-function nph4 mutants, and gain-of-function iaa14 mutants, which have highly reduced lateral root formation like loss-of-function nph4 arf19 mutants (Watahiki and Yamamoto, 1997; Tatematsu et al., 2004; Fukaki et al., 2005). Yeast two-hybrid assays confirm that IAA19 interacts with
NPH4, and IAA14 interacts with both NPH4 and ARF19 (Tatematsu et al., 2004; Fukaki et al., 2005).

$MP_\Delta$ is an artificial $MP$ variant that deletes the portion of the $MP$ coding sequence containing domains III and IV (Krogan et al., 2012). Supporting the model of Aux/IAA repression of ARFs, $MP_\Delta$ acts as a gain-of-function allele, showing phenotype and gene expression changes consistent with MP overexpression and overactivity (Krogan et al., 2012). Furthermore, these changes are epistatic over Aux/IAA gain-of-function alleles. Without the C-terminal dimerization domains, $MP_\Delta$ is irrepressible, conferring constant activation of $PIN1$ expression, and possibly other downstream targets, independent of auxin concentration. This results in a distinct vascular phenotype in cotyledons and leaves, reflecting the failure to properly restrict $PIN1$ expression, and by extension auxin distribution, into specific, organized domains (Krogan et al., 2012). Hemizygotes have disrupted and irregular leaf and cotyledon venation patterning, while in homozygotes patterning of these organs is reduced to numerous parallel strands and highly narrowed cotyledons and leaves. Additionally, homozygotes have strong floral defects and are infertile (Krogan et al., 2012).

1.10 ARF control of gene expression

ARFs bind to cis-regulatory elements called Auxin Response Elements (AuxREs), which were first identified in the promoters of auxin responsive genes (Li et al., 1994; Liu et al., 1994; Ulmasov et al., 1995). As mentioned earlier, AuxREs are defined by a TGTCTC motif, of which the first four base pairs are essential for ARF binding (Ulmasov et al., 1997a). AuxREs have been shown to function primarily as composite elements, either as repeats of the TGTCTC motif, such as in the synthetic $DR5$ promoter, or in conjunction with binding sites of other transcription factors, such as those of the bZIP, MYB, and Myc families (Ulmasov et al., 1997a; Ulmasov et al., 1997b; Shin et al., 2007; Varaud et al.,...
Due to their shared C-terminal domains, ARFs are able to bind each other as well as Aux/IAAs, and this dimerization allows cooperative binding to adjacent AuxREs and potentiation of transcriptional activation (Ulmasov et al., 1999; Tiwari et al., 2003). MP has been shown to have a strong dimerization preference for itself and NPH4, and NPH4 has far stronger dimerization preference for MP over any other ARF (Hardtke et al., 2004). It is theorized that homo- and heterodimerization of activating ARFs may help recruit additional ARFs to the promoters of auxin response genes, potentiating their transcription activating effects (Tiwari et al., 2003). This may explain the strong activating nature of DR5, which has evenly spaced AuxRE repeats that may facilitate binding of dimerized activating ARFs (Ulmasov et al., 1995). Recent analysis of MP crystal structure has revealed that MP can also dimerize through its DNA-binding domain, and that this interaction is necessary for its developmental functions (Boer et al., 2014). This interaction creates a set of molecular calipers that bind AuxREs of specific distances apart, which may allow for greater binding specificity of MP.

1.11 General Research Objectives

Recent evidence has suggested that auxin not only acts through the abundance of Aux/IAA proteins regulating the activity of ARFs, but that the expression of ARFs themselves, at least in some cases, is also modulated by auxin. For example, auxin-responsive expression of MP may be driven by MP activation of both its own gene and the gene of its Aux/IAA repressor, BDL (Lau et al., 2011). It is posited that at low auxin concentration, MP and BDL levels are both low, and any free floating MP is bound by BDL. However, once a certain threshold of auxin concentration is reached, the rate of BDL degradation increases to a point that MP is able to outcompete BDL and rapidly increase its own expression (Lau et al., 2011). This model is supported by the fact that the MP promoter contains several full and incomplete (TGTC) AuxREs, nine of which are within 500 base
pairs upstream of the transcription initiation site (Lau et al., 2011). Also, AuxREs in the *MP* coding region have been shown to contribute to the auxin sensitivity of *MP* expression (Bhatia et al., 2016). The demonstration that expression of an irrepressible variant of *MP*, *MPΔ*, impacts on the expression of *pMP::GFP* (Krogan et al., 2012) lead me to hypothesize that the transcriptional regulation of ARFs through *cis*-regulatory elements upstream of their transcriptional initiation site have a role in auxin responses. As ubiquitous expression of *MP* has been shown to be detrimental in a number of patterning processes (Hardtke et al., 2004), individual regulatory regions upstream of *MP* could, for example, be crucial for the tightly regulated tissue specificity of *MP* expression and for its differential expression in shoot and root. These differential roles of *MP* in shoots and roots, in turn, might be reflected in differential genetic interactions that *MP* might have with its closest paralogs in Arabidopsis, *NPH4/ARF7* and *ARF19*.

I will explore the roles of MP and of *cis*-regulatory regions relevant for its expression in controlling traits in shoots and roots by determining the effects MP promoter alterations on MP expression and on phenotypic traits.

1) To determine the relevance of individual AuxREs and other elements in the *MP* promoter to *MP* expression and function.

2) To explore the options for targeting *MP* activity to individual organs or stages through manipulations of the *MP* promoter regulatory regions.

3) To investigate the epistatic relationship between an irrepressible *MP* variant and the ARFs *NPH4/ARF7* and *ARF19* in order to better understand their specific and possibly differential roles and potentials in shoots and roots.
Chapter 2

Materials and Methods
2.1 Plant material

All seed lines used in this thesis are in the Columbia (Col-0) background of *Arabidopsis thaliana*. Experiments using *mp* mutant backgrounds used the loss-of-function allele *mp*1058, containing a T-DNA insertion disrupting the coding sequence of the gene (Alonso et al., 2003). Experiments involving *nph4* backgrounds used the neutron-generated loss-of-function allele *nph4*-1 (Harper et al., 2000). Experiments involving *arf19* backgrounds used *arf19*-4, another T-DNA insertion loss-of-function allele (Alonso et al., 2003).

All *Arabidopsis* growth conditions followed guidelines set by Weigel and Glazebrook (2002) unless otherwise specified, including growth environment, composition and use of growth media, cultivation of plants in soil, seed collection, storage, and sterilization. Growth medium was composed of a solution of Murashige and Skoog (MS) basal salts (Sigma-Aldrich, St. Louis, MO, USA), sucrose (Bioshop, Burlington, ON, Canada), and agar (Bioshop, Burlington, ON, Canada), titrated to a pH of 5.6-5.8. Selection was performed using 2.5 mg/L hygromycin B (Bioshop, Burlington, ON, Canada) or 10 mg/L Basta (glufosinate-ammonium) herbicide (Bayer, Leverkusen, Germany).

A number of *Arabidopsis* transgenic lines were made available from the Berleth lab for analysis in this thesis. The constructs in these lines are listed below.

- **pMP::GUS**: An *MP* promoter fragment of 3000 bp upstream of the *MP* transcription initiation site driving expression of a *GUS* reporter in a pPLV15 vector (De Rybel et al., 2011).

- **pMP-mAuxRE126::GUS**: An *MP* promoter fragment of 3000 bp upstream of the *MP* transcription initiation site containing two point mutations in an AuxRE located 126 base pairs upstream of the start codon, changing the TGTCTC motif to AATCTC. The modified promoter is fused to a *GUS* reporter gene within a pPLV15 binary vector (De Rybel et al., 2011).
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\textit{pMP-mAuxRE503::GUS} An \textit{MP} promoter fragment of 3000 bp upstream of the \textit{MP} transcription initiation site containing two point mutations in an AuxRE located 503 base pairs upstream of the start codon, changing the TGTCTC motif to AATCTC. The modified promoter is fused to a \textit{GUS} reporter gene within a pPLV15 binary vector (De Rybel et al., 2011).

\textit{pMP-multiAuxRE::MP} A genomic fragment of the full length \textit{MP} gene, including 3281 bp of upstream sequence, containing 3 point mutations at 477, 483, and 501 base pairs upstream of the transcription initiation site, creating three novel AuxREs within the \textit{MP} promoter. The 3’ end of the \textit{MP} coding sequence is fused in frame to a \textit{GUS} reporter gene within a pPLV15 binary vector (De Rybel et al., 2011).

\textit{pMP::MP:GUS} A genomic clone of the full \textit{MP} gene, including 3281 bp of upstream sequence, fused to a \textit{GUS} reporter gene in a pBI101 binary vector (Arabidopsis Biological Resource Center, Columbus, OH, USA, Krogan, 2006).

\textit{MPΔ} A full length (including 3281 bp upstream promoter) genomic \textit{MP} clone with a portion of the 3’ coding sequence removed and replaced with a shorter \textit{MP} cDNA fragment, all within a pE-GAD binary vector (Cutler et al., 2000). The cDNA fragment deletes a section of the coding sequence at the 3’ end which encodes two C-terminal domains necessary for dimerization interactions (Krogan et al., 2012).
A construct identical to $MP\Delta$, except in a pPLV01 vector (De Rybel et al., 2011), and of the 3281 bp of sequence upstream of the transcription initiation site only the most proximal 515 bp were retained.

A construct identical to $MP\Delta$, except in a pPLV01 vector (De Rybel et al., 2011), and of the 3281 bp of sequence upstream of the transcription initiation site only the most proximal 700 bp were retained.

A construct identical to $MP\Delta$, except in a pPLV01 vector (De Rybel et al., 2011), and of the 3281 bp of sequence upstream of the transcription initiation site only the most proximal 940 bp were retained.

A construct identical to $MP\Delta$, except in a pPLV01 vector (De Rybel et al., 2011), and of the 3281 bp of sequence upstream of the transcription initiation site only the most proximal 1178 bp were retained.

A construct identical to $MP\Delta$, except in a pPLV03 vector (De Rybel et al., 2011), and of the 3281 bp of sequence upstream of the transcription initiation site only the most proximal 1300 bp were retained.

A construct identical to $MP\Delta$, except in a pPLV01 vector (De Rybel et al., 2011), and of the 3281 bp of sequence upstream of the transcription initiation site only the most proximal 1500 bp were retained.
2.2 Microtechniques and microscopy

For visualization of cotyledon and first leaf vasculature, plants were grown on in light MS/suc/agar plates for 7 days, after which seedlings were cleared in ethanol:acetic acid solution (3:1, v/v) overnight at room temperature. Samples were then stored in 70% ethanol at room temperature.

For histochemical visualization of β-glucuronidase (GUS) activity, protocol and conditions were adopted from Krogan (2006). After Plant tissue was incubated at 37°C in 100 mM sodium phosphate buffer pH 7.6 (Sigma-Aldrich, St. Louis, MO, USA), 10 mM EDTA, 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc) (Bioshop, Burlington, ON, Canada), supplemented with 5 mM of potassium ferrocyanide and potassium ferricyanide (Sigma Aldrich, St. Louis, MO, USA). Tissue was stained for two hours, after which the enzymatic reaction was terminated and the tissue samples cleared by fixing in ethanol:acetic acid (3:1, v/v) overnight at room temperature. Samples were then stored in 70% ethanol at room temperature. In order to ensure uniformity of staining conditions, samples were stained in parallel using a single reaction batch.

For general microscopic visualization, samples were mounted on microscope slides in chloral hydrate:glycerol:water solution (8:3:1) w/v/v). Leaves were mounted adaxial side down to prevent trichomes from interfering with visualization. Images were captured using a Leica MZ FLIII dissecting stereomicroscope at 16x magnification (Leica Microsystems, Wetzlar, Germany). Images of non-enzymatic (vascular visualization) samples were taken with dark field illumination, while images GUS-stained samples were taken with bright field illumination.

Images of live seedlings, rosettes, and mature plants were acquired with a Canon EOS D60 digital camera (Canon Inc., Tokyo, Japan).

All images were assembled using GNU Image Manipulation Program (The GIMP Development Team, Free Software Foundation, Boston, MA, USA) and Libreoffice Draw.
(The Document Foundation, Berlin, Germany). Leaf and rosette measurements were taken using ImageJ software (National Institutes of Health).

2.3 Root assays

For assessment of root gravitropic response, the protocol utilized by Weijers et al. (2005) was adopted. Seedlings were grown vertically on MS/suc/agar plates, with the orientation of gravity clearly marked on each plate. At 7 days after transfer to light, plates were turned 90° and grown for an additional 24 hours, after which the angle of the root tip to the original gravity vector was measured using ImageJ software (National Institutes of Health).

For assessment of root growth response to auxin, seedlings were grown vertically on MS/suc/agar plates for 5 days, after which seedlings were transferred to plates containing 1 µM indole-3-acetic acid (IAA) and images were taken. Three days later, new images were taken and the change in root length was assessed using ImageJ (National Institutes of Health).

2.4 Genomic DNA extraction

To collect template for PCR and genotyping, a rosette leaf or seedling was placed in a centrifuge tube with calcium beads and 200 µL of DNA extraction buffer (250 mM Tris-HCl, pH 7.5 (Sigma-Aldrich, St. Louis, MO, USA), 250 mM NaCl (Sigma-Aldrich, St. Louis, MO, USA), 25 mM EDTA (Bioshop, Burlington, ON, Canada), and 0.5% (w/v) SDS (Bioshop, Burlington, ON, Canada)), and the plant material was then homogenized using a BeadBeater (BioSpec Products Inc., Bartlesville, OK, USA). The mixture was spun down for 1 min at 13,000 rpm in a microcentrifuge, after which the supernatant was transferred into a new tube. 200 µL of isopropanol was added to the supernatant, and the
tube was incubated at room temperature for 5 minutes. The tube was then spun down again for 5 min at 13,000 rpm, after which the supernatant was discarded. The DNA pellet was washed with 70% (v/v) ethanol, dried, and resuspended with 20 µL of double distilled water. 1 µL of the solution was used a DNA template for a 20 µL PCR reaction.

2.5 Polymerase Chain Reaction

PCR reactions were a total volume of 50 µL, consisting of 50-100 ng of template DNA, 200 µM of each dNTP (Thermo Fisher Scientific, Waltham, MA, USA), 100 ng of each primer (Eurofins Scientific, Luxembourg), 1x PCR buffer (Thermo Fisher Scientific, Waltham, MA, USA), one unit of Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and nuclease-free double-distilled water to bring the solution to 50 µL. A typical PCR cycle consisted of an initial 3 minute denaturation at 95°C, followed by 30 cycles of a 30 second denaturation step at 95°C, a 30 second annealing step at 57°C, and an extension step of one minute per kb of expected PCR product at 72°C. A final extension step of 10 minutes at 72°C completed the PCR.

2.6 Determination of genotypic backgrounds

In order to distinguish mp1058 homozygous individuals from those carrying one or two MP alleles, primers BS1354-F (5’ GAGATGGCCTGGTTCTAAGGTTGCC 3’) and BS1354-R (5’ GCCAGTTCAACATCTCGGTTATGC 3’) were used to amplify a 2600 bp product specifically from the wild type MP allele, while primers Lba1 (5’ TGGTTCACG-TAGTGGGCCATCG 3’) and Afl-ins-R (5’ ATCGGAACACACATCAATGC 3’) were used to amplify a 900 bp product corresponding to the T-DNA insertion associated with mp1058 (Alonso et al., 2003). The PCR protocol followed that listed above, with a 3 minute extension step. mp1058 was propagated as a heterozygous line due to the infertility
of the homozygote. Heterozygous lines were identified either by PCR or through progeny, which segregate 25% rootless seedlings.

In order to distinguish \textit{nph4-1} homozygous individuals from those carrying one or two \textit{NPH4} alleles, primers NPH4.5 (5’ TCCTGCTGAGTTTGTGGGTCTCCTT 3’) and NPH4.6 (5’ GGGGCTTGCTGATTCTGTTTTGTA 3’) were used to amplify an 823 bp product specifically from the wild type \textit{NPH4} allele. \textit{nph4-1} homozygous individuals do not support amplification of the fragment. The PCR protocol followed that listed above, with a 60 second extension step. Homozygous lines were established by identifying homozygotes through PCR and propagating these individuals.

In order to distinguish \textit{arf19-4} homozygous individuals from those carrying one or two \textit{ARF19} alleles, primers ARF19LP (5’ CATCTCTGCAAAACCAGTACCC 3’) and ARF19PR (5’ TTTTTCAGGTTGCAGCAGCATCG 3’) were used to amplify a 913 bp product specifically from the wild type \textit{ARF19} allele, while primers Lba1 (5’ TG-GTTCACGTAAGTGCCCCATCG 3’) and ARF19PR were used to amplify a 600 bp product corresponding to the T-DNA insertion associated with \textit{arf19-4}. The PCR protocol followed that listed above, with a 60 second extension step. Homozygous lines were established by identifying homozygotes through PCR and propagating these individuals.

In order to determine the presence of the \textit{MP\textDelta} transgene, \textit{MP\textDelta}(+)+L (5’ CTGGCGTAATAGCGAGAGAGGAGGTTAGAGTCTG 3’) and \textit{MP\textDelta}(+/-)R were used to produce a 432 bp band confirming \textit{MP\textDelta} insertion. \textit{MP\textDelta}(-)+L (5’ CAGGGAGCAGGCTAGAGTCTGTGTCAGCAGATGCAGTCCAGCAGAGGAGGAGGAGGTTAGAGTCTG 3’) and \textit{MP\textDelta}(+/-)R (5’ GAGAGAACGGAGGAGCAGAGGCTAGAGTCTGTGTCAGCAGATGCAGTCCAGCAGAGGAGGAGGTTAGAGTCTG 3’) were used to produce a 317 bp band corresponding to the wild type locus at the \textit{MP\textDelta} insertion site. \textit{MP\textDelta}, as well as other genotypes containing it, was propagated as a hemizygous line due to the infertility of the homozygote. The transgene construct carries a gene conferring Basta resistance, so hemizygous lines were identified by 75% Basta resistant seedling progeny, while homozygous lines displayed 100% resistance. For root assays, \textit{MP\textDelta} lines were grown on Basta selection, and resistant seedlings were analyzed for phenotype to assess zygosity.
MP\Delta truncated promoter lines were confirmed for single insertion in the T2 generation through 3:1 segregation of Basta resistant progeny, and these lines were then propagated. The subsequent progeny were then assessed for 100% Basta resistance to confirm homozygosity. The exception to this is pMP1300::MP\Delta, which was previously confirmed for single insertion and homozygosity.

pMP::GUS, pMP-mAuxRE126::GUS, and pMP-mAuxRE503::GUS were assessed for presence of the transgene through identifying lines which segregated hygromycin-resistant seedlings.

pMP-multiAuxRE::MP lines were assessed for 75% hygromycin-resistant progeny to confirm single insertion hemizygosity.

2.7 Identification of promoter elements

AuxRE, GRE, MRE1, and MYC2 elements were taken from Berendzen et al. (2012) and identified using Pattern Locator (Computational Microbiology Laboratory, Department of Microbiology, University of Georgia, Athens, GA, USA).
Chapter 3

Functions of AuxREs in the

*MONOPTEROS* promoter
Chapter 3. Functions of AuxREs in the MONOPTEROS promoter

3.1 Introduction

Auxin response factors (ARFs) influence gene expression through interaction with cis-regulatory elements called auxin response elements (AuxREs). One particular ARF, ARF5/MONOPTEROS (MP), plays a critical role in early embryonic developmental processes like establishing the apical-basal axis, root initiation, and meristem initiation and maintenance, as well as processes later in development such as vascular patterning and phyllotaxis (Berleth and Jurgens, 1993; Scarpella et al., 2006; Vidaurre et al., 2007; Schuetz et al., 2008). Recent evidence suggests that MP may activate its own expression as well as the expression of its repressor, BDL (Lau et al., 2011). Mathematical modeling shows that this could create a genetic switch, which would allow MP expression to exponentially increase once a threshold of auxin concentration is reached. The first 515 base pairs of the MP promoter immediately upstream of the transcription initiation site contain four canonical and five putative AuxREs, however, the relevance of those potential ARF binding sites for MP expression is not clear and were explored on the basis of in vitro mutagenized constructs available in the lab. These constructs were analyzed with the goal of giving greater insight as to whether individual AuxREs have significant and/or distinct roles in MP expression, and possibly by extension self-regulation. To investigate part of this question, two GUS constructs, each with one of the four canonical AuxREs within 515 base pairs of immediate MP promoter being mutated, were analyzed for expression changes relative to unmutated control constructs (Figure 3.1). If the AuxREs in this region indeed have an important role in MP expression, then it would follow that addition of unnatural AuxREs to the promoter region could potentially lead to misregulation of MP. Overexpression of MP has been previously shown to have disruptive effects on developmental patterning (Hardtke et al., 2004), therefore it appears possible that phenotypic changes may become apparent if misregulation occurs. To investigate this possibility, a second experiment was performed in which one canonical and two incomplete
AuxREs were generated in addition to the existing ones through mutagenesis, and linked to full length MP coding sequence to assess for changes in phenotype (Figure 3.1).

3.2 Results

3.2.1 Exploration of MP expression upon AuxRE mutations in the MP promoter

The importance of two AuxREs in the MP promoter for expression of MP was investigated using three GUS reporter gene constructs, each comprising 3000 base pairs of Arabidopsis genomic sequence upstream of the MP transcriptional start site. One, pMP-mAuxRE126::GUS, carried a mutation in an AuxRE 126 base pairs upstream of the transcription initiation site, the other, pMP-mAuxRE503::GUS, in an AuxRE 503 base pairs upstream of the MP transcription initiation site (Figure 3.1A, B). In both cases, the mutation altered the initial TG of the canonical AuxRE motif TGTCTC, which had been shown to be absolutely essential to ARF binding, into AA (Ulmasov et al., 1997a). Therefore, the respective AuxRE in either construct was most likely functionally eliminated. A third construct containing the unmutated MP promoter driving a GUS reporter, termed pMP::GUS, was used as a control.
Figure 3.1: Overview of analyzed constructs. (A) \textit{pMP-mauxRE::MP} (B) \textit{pMP-\textit{mAuxRE126::GUS}} (C) \textit{pMP-mAuxRE503::GUS}. Arrows indicate base changes.
All seedlings were grown simultaneously to 5 days after germination (DAG), at which point they were stained in parallel for two hours using a single batch of 5 mM GUS staining solution. Figure 3.2 shows typical examples from at least 10 samples of each category from two transgenic lines. In lines from all three constructs, GUS staining was visible in vasculature of cotyledons and leaves (Figure 3.2A-F). As the most pronounced genotype-specific effect, *pMP-mAuxRE126::GUS* has little to no expression in the hypocotyl (Figure 3.2I-J), as opposed to both *pMP::GUS* and *pMP-mAuxRE503::GUS* (Figure 3.2G-H, K-L). These features indicate a region-specific requirement of the AuxRE at position 126 for hypocotyl expression of *MP*, while it appears completely dispensable in cotyledons and leaves. A quantitatively robust requirement for a concrete AuxRE was only apparent for AuxRE126 in hypocotyl expression (see Discussion).
Figure 3.2: Representative GUS expression patterns of AuxRE deletion lines at 5 DAG. Cotyledon (A-C), first leaf (D-F), hypocotyl (G-L), and root (M-R) staining in 5 DAG seedlings of pMP::GUS (A, D, G-H, M-N), pMP-mAuxRE126::GUS (B, E, I-J, O-P), and pMP-mAuxRE503::GUS (C, F, K-L, Q-R). Scale bars: (A-F) 0.5 mm, (G-R) 0.2 mm.
3.2.2 Growth consequences of additional AuxRE in the MP promoter

Using three point mutations in a single construct, designated as \( pMP-multipAuxRE::MP \), one full AuxRE and two incomplete AuxREs were effectively added to the genuine 3281 base pair \( MP \) promoter as part of a construct driving a full length \( MP \) coding sequence, immediately downstream of the AuxRE that was modified in the \( pMP-mAuxRE503::GUS \) construct from the previous section (Figure 3.1C). The incomplete AuxREs deviated from the canonical AuxRE repeat only with regard to the last two bases of the TGTCTC motif, which has been shown to have relatively little effect on ARF binding and expression levels (Ulmasov et al., 1997a). It is therefore possible that the added AuxREs might provide additional binding sites for ARFs and possibly for MP itself, which through the attached \( MP \) coding sequence might change \( MP \) activity in those plants, with possible phenotypic consequences.

When inspected at 7 DAG, control plants of the Col-0 wild type had developed two cotyledons and two first leaves which were both easily discernable (Figure 3.3H). The same was true for Col-0 plants carrying an \( MP::MP:GUS \) transgene, which comprises a functional \( MP:GUS \) translational fusion under the control of the same 3281 bp promoter, and can therefore be expected to express the \( MP \) wild type gene in four copies (Figure 3.3I). By contrast, the progeny of hemizygous \( pMP-multipAuxRE::MP \) individuals displayed a range of phenotypes, the most dramatic being the reduction in the number of rosette leaves formed at this stage (Figure 3.3A-F). Although genotypes of individual plants were not assessed, a near-quarter segregation (23% and 28%, in two experiments) of hygromycin-sensitive plants confirmed normal segregation in the progeny of single-insert hemizygous plants in both investigated lines (Figure 3.4). It can therefore be inferred that among the hygromycin-resistant plants, a third carried two copies and two thirds carried one copy of the \( pMP-multipAuxRE::MP \) transgene. In contrast to wild type, which nearly invariantly produces two rosette leaves at this stage, most (approximately 70%
of all transgenics) of the hygromycin-resistant plants in each of the transgenic lines had produced fewer leaves and approximately 40% (43% and 39%) of these plants had not produced any leaf at this stage.
Figure 3.3: Representative phenotype classes of $pMP$-multiAuxRE::MP seedlings at 7 DAG on hygromycin. (A-E) Phenotype classification of progeny of $pMP$-multiAuxRE::MP hemizygotes (A) Class 1 - Long hypocotyl, 2 first leaves (B) Class 2 - Long hypocotyl, <2 first leaves (C) Class 3 - Short hypocotyl, 2 first leaves (D) Class 4 - Short hypocotyl, <2 first leaves (E) hygromycin-sensitive. (F-J) Aerial view of 7 DAG (F) Col-0, (G) $MP$::GUS, (H) $pMP$-multiAuxRE::MP class 1, and (I-J) $pMP$-multiAuxRE::MP class 2 seedlings. Scale bars: 2 mm.
Hygromycin-resistant transgenic plants could also be classified by whether or not they had abnormally long hypocotyls (Figure 3.3A-D), which was the case in a minority of the transgenic plants in both lines (27% and 30%) (Figure 3.4). Plant classes were further divided by whether they showed strongly delayed leaf production (<2 leaves by 7 DAG, Figure 3.3B,D), and remarkably these long hypocotyl plants were more likely to display strongly delayed leaf production (88% and 79% versus 57% and 50%), suggesting that the two defects may have a common basis (Figure 3.4).
Figure 3.4: Distribution of phenotype classes for progeny of two independent \( pMP\text{-multiAuxRE::MP} \) hemizygous lines. (A) Table of percentage of phenotype classes in progeny. (B) Pie charts showing distribution of phenotype classes for each line. Control \( MP::MP::GUS \) and wild type distributions are not shown, as 100% of seedlings would fit in class 3 - short hypocotyl with two first leaves.
At 21 DAG, the phenotype classes lose distinction, and develop a range of rosette abnormalities. Wild type and $MP::MP:GUS$ plants typically arrange leaves in a highly organized fashion to create a radially symmetrical rosette (Figure 3.5I-L). In contrast, 75% of $pMP$-$multiAuxRE::MP$ rosettes showed strong asymmetry about the shoot meristem (Figure 3.5A-D). Individuals that showed moderate to negligible asymmetry still displayed deviations from wild type, such as inconsistent leaf shapes (Figure 3.5E-H) or a low number of rosette leaves (nearly all wild type and $MP::MP:GUS$ plants at this stage have 8-12 distinct, $>1$ mm long rosette leaves) (Figure 3.5I-L). Around 43% of $pMP$-$multiAuxRE::MP$ also showed meristematic abnormalities. The shoot meristem is easily distinguished in Col-0 plants, as it is positioned in the centre of the rosette and new leaves can be clearly observed arising from it in an organized pattern (Figure 3.5Q-T). This subset of $pMP$-$multiAuxRE::MP$ plants, however, did not have an easily discernable meristem, and instead displayed disorganized clusters of leaves seeming to originate from an ambiguous point, with no clear pattern or organization (Figure 3.5M-P).
Chapter 3. Functions of AuxREs in the *MONOPTEROS* promoter

Figure 3.5: Representative rosette phenotypes of 21 DAG *pMP-multiAuxRE::MP* plants. (A-H) *pMP-multiAuxRE::MP* rosettes, (I-J) Col-0 rosettes, (K-L) *MP::MP:GUS* rosettes, (M-P) Close-up of *pMP-multiAuxRE::MP* meristems, (Q-R) Close-up of Col-0 meristems, (S-T) Close-up of *MP::MP:GUS* meristems. Scale bars: (A-L) 10 mm (M-T) 5 mm.
Chapter 3. Functions of AuxREs in the *MONOPTEROS* promoter

3.3 Discussion

3.3.1 *MP* expression domain changes from promoter AuxRE deletions

The diversity of responses to the simple IAA molecule has been tentatively assigned to the complexity of the *ARF* and *Aux/IAA* gene families relaying these signals. For example, *ARFs* could have tissue specific expression profiles which would already target specific auxin responses to individual tissues or cell types (Guilfoyle, 2007a). Strictly regulated expression domains of *ARFs* in embryo development have been demonstrated, and *ARFs* and their interacting *Aux/IAA* partners have to be expressed in the same cells to form a circuit (Weijers et al., 2005). Furthermore, *MP* and *NPH4* constitute a good example of how expression specificity can have functional relevance. When both are knocked out, the phenotype of the double mutant is far more severe than that of *mp* single mutants, indicating a redundant, highly related function of *NPH4* in patterning (Hardtke et al., 2004). This function was not expected based on the *nph4* single mutant phenotype, which is restricted to defects in hypocotyl elongation. The question, why redundant *MP* function does not mask the *nph4* phenotype also in this domain, is easily answered by ectopically expressing *MP* throughout the hypocotyl, which makes *NPH4* function obsolete. Hence, restricted expression of *MP* in the hypocotyl is a pre-requisite for normal *NPH4* function in tropic responses in the hypocotyl.

Despite the conceptionally and experimentally supported importance of region-specificity in *ARF* gene expression, relevant *cis*-regulatory elements conferring region-specificity have not been identified. The finding in Figure 3.2, indicating a requirement of an AuxRE at position 126 for normal hypocotyl expression of MP is therefore a novelty and may therefore be an entry point to address this question in more detail. For example, it seems worthwhile to explore the sequence context of this AuxRE in *MP* with that of the spatially co-regulated *IAA12/BDL* gene to track a region-specific module within the *MP* promoter.
If \textit{MP} does directly influence its own expression, then it can be assumed that it does so by binding to one or more of the auxin response elements present in its promoter. A large cluster of AuxREs is situated in the first 515 base pairs of the promoter sequence. The lack of other significant expression changes after deletion of these single AuxREs may indicate a redundancy among AuxREs in the \textit{MP} promoter. It also may be the case that other expression changes caused by loss of a single AuxRE are too small to be visible through GUS staining. Instead, one could alter more AuxREs, as the result of AuxRE126 elimination supports the hope to identify more robust regulatory effects through this strategy.

### 3.3.2 Added AuxREs cause changes in \textit{MP} expression that result in auxin response-related abnormalities

The addition of canonical AuxRE sequences to the \textit{MP} promoter at the chosen position had subtle, but reproducible phenotypic effects. This finding may be fortunate and may not be obtained at many other positions. The effects are seen in auxin-related traits, phyllotaxis and hypocotyl/petiole elongation, but do not lend themselves to a simple explanation. Nevertheless, as such effects are observed upon amplification of certain AuxREs of \textit{MP}, but not upon simple duplication of the \textit{MP} gene, this first addition of an AuxRE to the promoter may be seen as a proof-of-concept for this strategy and should be followed by detailed expression analysis to pin down the regulatory effects of AuxRE alterations.

Given the difference between expression changes in the different AuxRE deletion GUS lines, it appears that different elements elicit different levels of expression change when deleted, and that deletion of a single element can cause expression changes that affect the threshold of auxin response. This appears to imply that some elements in this cluster play a more significant regulatory role than others. Therefore, it seemed unclear
whether addition of three AuxREs to this cluster would have any significant effect on expression. However, clear changes in phenotype occurred in these lines. The elongated petiole phenotype resembles those of wild type seedlings in low light conditions, which is an auxin-driven growth response. Additionally, a significant number of seedlings from these lines have apparent phyllotaxis defects. Among presumed homozygous individuals, around 25-30% grow only a single first leaf while 50-60% fail to form any leaves in the first week of growth. A number of seedlings producing two leaves have clear asymmetry in shape and size between the two. Additionally, the majority of rosettes from these lines were asymmetrical. Failure to generate two first leaves, or even any first leaves at all, indicates some sort of disruption of shoot apical meristem patterning.

Further evidence lies in the asymmetrical and inconsistent organization of the rosette in the majority of adult plants. Auxin is heavily involved in meristem patterning, evidenced by significant patterning defects observed when polar auxin transport is inhibited. Knockouts of \textit{PIN1}, which encodes an auxin efflux carrier vital to SAM function, form pin-shaped inflorescences and few lateral organs (Okada et al., 1991). \textit{mp} knockout plants also form pin-shaped inflorescences if induced to form a root and grow to adulthood, and have defects in symmetry evident in their formation of a single cotyledon in majority of individuals (Berleth and Jurgens, 1993; Przemeck et al., 1996). \textit{MP} has also been shown to directly regulate \textit{PIN} gene expression and bind to promoter elements in \textit{PIN} genes, further solidifying its role as a factor in SAM patterning (Wenzel et al., 2007; Krogan et al., 2016). It seems plausible, then that these observed defects in symmetry are related to the activity of the \textit{pMP-multiAuxRE::MP} transgene. Also notable is that four copy lines of wild type \textit{promoter::MP} do not show any phenotype distinctions from wild type. This could mean that the abnormalities of \textit{pMP-multiAuxRE::MP} lines are due to misexpression of \textit{MP} ectopically, rather than simple overexpression of the gene. Since \textit{MP} is both induced by auxin and plays a role in directing polar auxin transport, ectopic expression of \textit{MP} may be disrupting the organized formation of auxin maxima in
the SAM, the process that specifies the growth of a new lateral organ and organizes the
phyllotactic patterning of lateral organs as the shoot progresses (Reinhardt et al., 2000).
Though it remains unclear if it is MP or other factors that are causing these changes, the
results do show the significance of AuxREs in the MP promoter and their ability to cause
changes in expression that influence auxin transport and response.
Chapter 4

Characterization of *MONOPTEROS*

promoter regions


4.1 Introduction

The auxin response factor \textit{MONOPTEROS/ARF5 (MP)} is a key regulator of \textit{Arabidopsis} embryonic development, vascular patterning, and meristem function (Berleth and Jurgens, 1993; Przemeck et al., 1996; Hardtke and Berleth, 1998; Mattsson et al., 2003; Schuetz et al., 2008). Like other transcriptionally active auxin response factors (ARFs), MP activity is controlled by a family of repressor proteins called Aux/IAAs, which share a dimerization domain (Ulmasov et al., 1999; Tiwari et al., 2003). Auxin facilitates the degradation of these proteins, freeing MP to activate auxin-inducible genes. The transgene \textit{MP}∆ removes the dimerization domain from the gene coding sequence, creating an MP variant that cannot be bound by Aux/IAAs and is therefore functionally irrepressible (Krogan et al., 2012). \textit{MP}∆ appears to have partial \textit{MP} function, as it can rescue root development of \textit{mp} mutants and activate auxin-inducible genes. \textit{MP}∆ homozygotes have a strong phenotype from seedling to adult stages, the most characteristic being highly narrowed cotyledons and leaves, reduced vascular complexity, and infertility (Krogan et al., 2012). \textit{MP}∆ hemizygotes have less severe narrowing and vascular defects, and are fertile as well. Given the less severe phenotype of the hemizygote, it appears that expression levels of the transgene are somewhat correlated with the severity of the phenotype. These properties make \textit{MP}∆ a useful genetic tool for roughly assessing expression levels of \textit{MP} in specific tissues.

Naturally occurring AuxREs have been shown to function as composite elements, requiring nearby constitutive elements to confer auxin-responsive gene expression (Ulmasov et al., 1995; Guilfoyle et al., 1998). \textit{MYB77}, a member of the MYB transcription factor family, has been shown to interact with \textit{NPH4/ARF7} and synergistically promote expression of downstream targets (Shin et al., 2007). \textit{myb77} mutants show a reduction in lateral root formation, similar to the phenotype of \textit{nph4 arf19} double mutants (Okushima et al., 2005). A recent bioinformatic analysis found that auxin-inducible promoters of
Arabidopsis and several other plant species are enriched in bZIP related elements (ZREs) and Myb-related elements (MREs), often in bipartite and tripartite modules with AuxREs (Berendzen et al., 2012). These modules were defined as instances when individual motifs are less than 100 base pairs from each other. The results of this study indicate that ARFs may require the binding of other regulatory factors in order to activate transcription. In particular, the study found that G-box related elements (GREs) tended to be associated with auxin-inducible genes, especially when in combination with AuxREs (Ulmasov et al., 1995; Berendzen et al., 2012). Other associated elements were Myb-related elements (MREs), such as Myb-related element 1 (MRE1) and Myc-related element (MYC2). Though this study did not find an enrichment of these elements among genes of the ARF family, the 3281 bp of upstream sequence which represents the functional MP promoter contains a number of AuxREs, ZREs, and MREs. The presence of these elements may support the model of MP self-regulation, but their functional significance has not been assessed yet.

In order to gain a better understanding of the significance of different MP promoter regions and elements, MP\(\Delta\) was utilized in several constructs, each under the control of a different size MP promoter fragment. The originally described MP\(\Delta\) is under the control of the aforementioned 3.3 kb fragment of the MP promoter. The significant difference between hemizygous and homozygous MP\(\Delta\) individuals suggests that the phenotype severity is associated with the expression level of MP\(\Delta\) (Krogan et al., 2012). Therefore, any expression changes due to shortened promoter length could be predicted to result in distinct phenotypes. By assessing the resulting phenotypic changes from these varying lengths of promoter, this chapter aims to identify specific regions of the MP promoter that are significant to expression, and possibly gain a better understanding of the significance of the elements within these regions.
Chapter 4. Characterization of MONOPTEROS promoter regions

4.2 Results

4.2.1 ARF associated binding elements within the MP promoter

Multiple different constructs were generated consisting of varying truncations of the MP promoter driving expression of MP\(\Delta\). The six truncations used in this experiment deleted most sequences of the standard 3281 bp fully functional promoter fragment, and retained only the sequence between the transcriptional start site and positions immediately upstream of it, whereby their length in base pairs is given by the numbers 515, 700, 940, 1178, 1300, and 1500 (Figure 4.1). Plant lines expressing these constructs were compared to standard MP\(\Delta\) lines (Krogan et al., 2012), which is expressed from the 3281 bp promoter. Homozygous individuals from all lines were isolated for analysis, and all genotypes below are homozygous unless otherwise noted.

Using Pattern Locator (see Materials and Methods), AuxRE, GRE, MRE1, and MYC2 elements were identified throughout the 3281 bp promoter (Figure 4.1). The 515 promoter fragment contains a large cluster of nine AuxREs. One of these AuxREs overlaps with a MYC2 element (Figure 4.1K). Between 515 and 700 base pairs, there are no auxin response-associated elements present, but the segment does contain 55 base pairs of the 69 base pair long Arabidopsis UCE-like element 6 (ULE6) (Kritsas et al., 2012). The remaining 14 base pairs of the element stretch into the 700 to 940 segment, where there are also two GRE motifs, the closest ones to the start site (Figure 4.1I). These motifs lie 119 base pairs away from an upstream partial AuxRE; too far, based on parameters by Berendzen et al. (2012), for the elements to be considered part of a transcriptional module. This AuxRE and another one at 78 base pairs further upstream are located between 940 and 1178 base pairs from the start site (Figure 4.1H). Between 1178 and 1300 is another AuxRE 32 base pairs further upstream from the previous one. From 1300 to 1500, none of the elements examined here were found. The full length promoter used
in MPΔ is 3281 base pairs, and between 1500 and 3281 base pairs are several putative transcriptional modules, including GRE-AuxRE (Figure 4.1A,D), MYC2-AuxRE (B,F), MRE1-AuxRE (C,E), and a possible tripartite GRE-AuxRE-MYC2 (G).
Figure 4.1: Visualization of $MP$ promoter truncations driving $MP\Delta$ and associated putative AuxRE constitutive elements. (A) GRE-AuxRE (B) MYC2-AuxRE (C) MRE1-AuxRE (D) GRE-AuxRE (E) MRE1-AuxRE (F) MYC2-AuxRE (G) GRE-AuxRE-MYC2 (H) UCE-like element 6 (ULE6) (I) MYC2-AuxRE.
4.2.2 Cotyledon morphology and venation patterns associated with $MP\Delta$
under truncated promoter variants

The shortest truncation transgene, $pMP515::MP\Delta$, containing only the initial 515 base pairs of the promoter, did not show any marked morphological differences from wild type at 7 days after germination (DAG) (Figure 4.2A, D). Wild type cotyledons had a rounded oval shape, with vasculature generally forming four distinct loops in a consistent arrangement, occasionally only forming three loops or incomplete lower loops (Figure 4.1A-D). $pMP515::MP\Delta$ cotyledons displayed a shape and venation pattern virtually indistinguishable from wild type (Figure 4.1E-H).
Chapter 4. Characterization of *MONOPTEROS* promoter regions

(figure continued on following page)
Figure 4.1: Phenotype range of cotyledon shape and vasculature of MPΔ truncated promoter lines at 7 DAG. (A-D) Col-0, (E-H) pMP515::MPΔ, (I-L) pMP700::MPΔ, (M-P) pMP940::MPΔ, (Q-T) pMP1178::MPΔ, (U-X) pMP1300::MPΔ, (Y-Z,AA-BB) pMP1500::MPΔ, (CC-FF) MPΔ. Scale bars 0.5 mm.
Chapter 4. Characterization of MONOPTEROS promoter regions

$pMP700::MP\Delta$ cotyledons were slightly elongated, with irregularly shaped (often narrow and elongated) vascular loops, with little consistency in pattern between individual cotyledons (Figure 4.1I-L). Cotyledons of $pMP940::MP\Delta$ displayed similar shape and venation pattern disruption. Vascular loops in $pMP940::MP\Delta$ were smaller but rounder than those of $pMP700::MP\Delta$, and incomplete loops were observed more frequently (Figure 4.1M-P). Like $pMP700::MP\Delta$, patterning was highly inconsistent between cotyledons.

$pMP1178::MP\Delta$ plants displayed cotyledons that were distinctly narrower than the shorter truncations and wild type cotyledons. Additionally, vascular patterning was highly simplified. All veins were essentially parallel to the midline, with small spaces in between (Figure 4.1Q-T). $pMP1300::MP\Delta$ cotyledons were slightly narrower and more vascularized than $pMP1178::MP\Delta$ and $pMP1300::MP\Delta$. The veins of the cotyledons were also very tightly arranged parallel to the midline, with very little space visible in the bundle of vasculature (Figure 4.1U-X). $pMP1500::MP\Delta$ cotyledons were very similar to those of $pMP1178::MP\Delta$ in both shape and vascular pattern (Figure 4.1Y-Z,AA-BB). The phenotypes of $pMP1178::MP\Delta$, $pMP1300::MP\Delta$, $pMP1500::MP\Delta$ cotyledons appeared essentially as strong as those of $MP\Delta$ (Figure 4.1CC-FF).

4.2.3 First leaf morphology and venation patterns associated with $MP\Delta$ under truncated promoter variants

Like the cotyledons, $pMP515::MP\Delta$ first leaves were largely indistinguishable from wild type in both shape and vascular patterning (Figure 4.1A-D, E-H). First leaves of $pMP700::MP\Delta$ displayed moderate elongation compared to wild type and $pMP515::MP\Delta$ (Figure 4.1I-L). Leaf vasculature still formed distinct loops like wild type, but irregularities were evident. Additionally, the midvein was more vascularized, as it was clearly wider with more vascular strands.
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(figure continued on following page)
Figure 4.1: Phenotype range of leaf shape and vasculature of $MP\Delta$ truncated promoter lines at 7 DAG. (A-D) Col-0, (E-H) $pMP515::MP\Delta$, (I-L) $pMP700::MP\Delta$, (M-P) $pMP940::MP\Delta$, (Q-T) $pMP1178::MP\Delta$, (U-X) $pMP1300::MP\Delta$, (Y-Z,AA-BB) $pMP1500::MP\Delta$, (CC-FF) $MP\Delta$. Scale bars 0.5 mm.
pMP940::MPΔ first leaves were largely indistinguishable from pMP700::MPΔ, showing a similar degree of elongation and vascular patterning irregularities (Figure 4.1M-P). Some of the least elongated pMP1178::MPΔ first leaves were only slightly more elongated than those of pMP940::MPΔ (Figure 4.1Q-R), but others displayed a more severe phenotype (Figure 4.1S-T). The majority of pMP1178::MPΔ first leaves were highly elongated with noticeably simplified and irregular vascular patterning. Additionally, the midvein was widened and composed of multiple vascular strands.

First leaves of pMP1300::MPΔ were highly variable, ranging from rounded leaves with vascular loops (Figure 4.1U-V) to needle-shaped, unexpanded leaves with mostly tightly-packed, parallel vascular strands (Figure 4.1W-X). In expanded leaves with visible vein loops, vasculature was highly simplified (Figure 4.1U,V). Notably, unlike any of the other transgenic truncation lines, nearly all pMP1300::MPΔ first leaves were asymmetrical along the midline (Figure 4.1U-W).

pMP1500::MPΔ first leaves were also variable in degree of elongation, ranging from similar to pMP1178::MPΔ leaves (Figure 4.1Y) to the needle-shaped leaves with parallel vasculature resembling those of MPΔ (Figure 4.1AA-BB). Unlike pMP1300::MPΔ, pMP1500::MPΔ leaves were generally bilaterally symmetrical.

4.2.4 Rosette morphology of MPΔ truncated promoter variants

At 7 days after germination (DAG), the MPΔ truncated promoter variants did not differ significantly in size (Figure 4.2). As the rosette developed, pMP515::MPΔ measured on average slightly larger (<10%) than wild type at both 14 and 21 days (Figure 4.3). The longer promoter variants, however, showed more significant size differences as development progressed. pMP700::MPΔ rosettes were around 20% smaller than wild type at 14 days, and around 40% smaller at 21 days (Figure 4.3). pMP940::MPΔ plants were nearly the same size as wild type at 14 days, but by 21 days they were 24% smaller than wild type
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at the same stage (Figure 4.3). *pMP1178::MPΔ* plants had a higher variation in size than other genotypes, but the mean rosette diameter at both 14 and 21 days was smaller than *pMP940::MPΔ* (Figure 4.3). *pMP1300::MPΔ* and *pMP1500::MPΔ* followed a similar pattern, each promoter variant generating plants with smaller 14 day and 21 day old rosettes than the preceding shorter variant line (Figure 4.3). *MPΔ* plants had the smallest rosettes of all the analyzed genotypes at both stages (1.26 cm at 14 days and 1.73 at 21 days) (Figure 4.3).
Figure 4.2: Rosette phenotypes of 7 DAG (1st column), 14 DAG (2nd column), and 21 DAG (third column) MPΔ truncated promoter plants. (A-C) Col-0, (D-F) pMP515::MPΔ, (G-I) pMP700::MPΔ, (J-L) pMP940::MPΔ, (M-O) pMP1178::MPΔ, (P-R) pMP1300::MPΔ, (S-U) pMP1500::MPΔ, (V-X) MPΔ. Scale bars: 1st column 2 mm, 2nd column 5 mm, 3rd column 10 mm.
Figure 4.3: Graph showing rosette diameter of $MP\Delta$ truncated promoter genotypes at 14 days (blue) and 21 days (red) after germination. Error bars represent standard deviation ($n=8$ for each genotype).
Phyllotaxis did not appear to be significantly interrupted in any of the analyzed genotypes. First leaves arise from the meristem approximately 180° from each other, therefore any phyllotactic defects would theoretically result in deviation from this angle. Measured at 14 days after germination, the first leaves of wild type, $MP\Delta$, and all the truncated promoter variants fell between 150° and 170° apart, and no significant differences were found between the genotypes (Figure 4.4). By 21 days after germination, all the genotypes display rotationally symmetrical rosettes, despite differences in leaf shape and size (Figure 4.2).
Figure 4.4: Graph showing angle of first leaf pair of $MP\Delta$ truncated promoter lines at 14 days after germination. Error bars represent standard deviation ($n=8$ for each genotype).
At 14 days after germination, the rosette has around 4-5 distinct leaves, and the first leaf pair are clearly the largest leaves of the rosette (Figure 4.5A). Genotypes were compared by calculating the ratio of the height of each first leaf (defined as the length of a line from the point furthest from the petiole to the base of the leaf lamina) to the width (defined as the length of a line connecting the furthest points on the leaf border from the midline) (Figure 4.5B). $pMP515::MP\Delta$ first leaves and cotyledons appeared identical to wild type, consistent with the cleared cotyledons and first leaves of the genotype in the previous sections (Figure 4.2A,D). There was no significant difference between the mean dimensional ratios of Columbia (Col-0) and $pMP515::MP\Delta$ leaves ($t(30)=0.046$, $p=0.964$, Figure 4.5B).

First leaves of $pMP700::MP\Delta$ plants were significantly narrower than those of wild type and $pMP515::MP\Delta$ (Figure 4.2H, $t(30)=8.335$, $p<0.0001$). 14 day old rosettes of $pMP940::MP\Delta$ were very similar in appearance to $pMP700::MP\Delta$, but first leaves were slightly, but significantly, less narrow based on dimensional measurements ($t(31)=2.9068$, $p<0.01$, Figure 4.5C, 4.2J-K). First leaves of $pMP1178::MP\Delta$ were significantly narrower than $pMP700::MP\Delta$, and by extension $pMP940::MP\Delta$ ($t(27)=2.81$, $p<0.01$, Figure 4.2N, 4.5C). $pMP1300::MP\Delta$ first leaves were significantly more elongated than both $pMP1178::MP\Delta$ ($t(27)=8.21$, $p<0.0001$) and $pMP1500::MP\Delta$ ($t(30)=5.45$, $p<0.0001$, Figure 4.5C). $MP\Delta$ first leaves were narrower than all other genotypes, significantly so for all except $pMP1300::MP\Delta$ ($t(29)=0.40$, $p=0.69$, Figure 4.2W, 4.5C).
Figure 4.5: Analysis of 3rd and 4th leaf dimensions of $MP\Delta$ truncated promoter variant lines. (A) Illustration of 3rd and 4th leaf locations Col-0, $pMP700::MP\Delta$, and $MP\Delta$, from left to right. Scale bars: 5 mm. (B) Illustration of measurement methodology for height ($h$) and width ($w$) of aforementioned leaves. (B) Mean $h/w$ ratio for all genotypes pictured in figure 4.2. Error bars show standard deviation. Asterisks indicate statistical significance (***, ** = very strong statistical significance).
By 21 days after germination, wild type plants had a rotationally symmetrical rosette with 7-10 visible mature leaves (Figure 4.2C). Like earlier stages, \( pMP515::MP\Delta \) mature rosette leaves were indistinguishable from wild type (Figure 4.2F). Mature rosette leaves of \( pMP700::MP\Delta \) plants were smaller and narrower, with uneven margins (Figure 4.2I). The leaves of 21 day old \( pMP940::MP\Delta \) rosettes were less narrow than \( pMP700::MP\Delta \) and had smooth, rounded margins (Figure 4.2L). \( pMP1178::MP\Delta \), \( pMP1300::MP\Delta \), and \( pMP1500::MP\Delta \) all had similar rosette leaves, much smaller and narrower than wild type, with uneven and irregular leaf margins (Figure 4.2O, R, U). \( MP\Delta \) leaves were similar, but more severe in each phenotypic feature than any of the other genotypes (Figure 4.2X).

4.2.5 Floral development of \( MP\Delta \) truncated promoter variants

Plants homozygous for \( MP\Delta \) form inflorescences and flowers, with all floral organs present. However, petals are elongated and fail to open fully, and the flowers are invariably fail to develop seeds, instead forming very reduced siliques (Krogan et al., 2012, Figure 4.6H). In order to see to what extent distal promoter elements were required to sustain this phenotypic effect of \( MP\Delta \) expression, 20 homozygous plants of each of the lines carrying \( MP\Delta \) under control of truncated promoters were grown parallel to \( MP\Delta \) plants. In contrast to \( MP\Delta \) plants, plants of all other genotypes formed inflorescences consisting of flowers with no morphological defects (Figure 4.6A-G). These flowers consistently formed seed-bearing siliques, and none displayed \( MP\Delta \)-like reduced siliques.
Figure 4.6: Mature inflorescences, flowers, and siliques of mature MPΔ truncated promoter plants. (A) Col-0, (B) pMP515::MPΔ, (C) pMP700::MPΔ, (D) pMP940::MPΔ, (E) pMP1178::MPΔ, (F) pMP1300::MPΔ, (G) pMP1500::MPΔ, (H) MPΔ.
4.3 Discussion

4.3.1 Distribution of AuxREs in the MP promoter

Although MP is also regulating its own expression, and other ARFs may bind to AuxREs in the MP promoter, there is no simple correlation between the importance of an MP upstream region and the number of AuxREs in it. For example, a proximal promoter element, comprising no less nine AuxREs, is nevertheless insufficient to drive even basal MP expression (Figure 4.1E-H). Whereas a slightly longer element, comprising 185 additional base pairs, but no additional AuxREs, provides sufficient expression to an $MP\Delta$ transgene to cause phenotypic alterations at vegetative stages (Figure 4.1I-L). This hints to other non-AuxRE cis-regulatory elements in the section of 515-700 bp which are highly important for MP expression.

Further, the promoter region between 1500 and 3281 bp turned out to be essential for $MP\Delta$ expression selectively in reproductive organs. This finding provides a formidable tool to target MP and derived activities with impact on phenotype, such as for example $MP\Delta$, to specific stages and organs (see below).

4.3.2 Non-AuxRE cis-acting elements and their impact on MP activity

Since $MP\Delta$ has massive phenotypic effects at many stages of development, multiple promoter deletions were used to assess $MP\Delta$ activity in specific stages of development, to enable targeting of $MP\Delta$ activity and in order to better characterize the transcriptional control of MP itself. MP and PIN1 have been shown to play a key role in ovule specification (Galbiati et al., 2013), and $MP\Delta$ homozygotes display ovule abnormalities that are suspected to be the cause of their infertility (Caragea, A, unpub.). Therefore, the fertility of lines expressing truncated promoter variants of $MP\Delta$ indicates that these distal
regions are necessary for expression of the transgene in the developing reproductive organs. AuxREs in genuine promoter contexts have been shown to require coupling elements to confer auxin-responsive gene expression (Ulamasov et al., 1995; Hagen and Guilfoyle, 2002). bZIP transcription factors have been shown to be required for auxin responsiveness of AuxRE elements in the auxin-inducible soybean GH3 gene, and a gene encoding a member of the MYB family of transcription factors was shown to act synergistically with NPH4/ARF7 to promoter expression of an auxin-inducible reporter (Ulamasov et al., 1995; Shin et al., 2007). Bioinformatic analysis of auxin-responsive reporters has shown an enrichment of bZIP-related and Myb-related elements in close proximity to AuxREs in the promoters of auxin-response genes, further implicating their potential role in auxin-responsive gene expression (Berendzen et al., 2012). The lack of these elements in the sequence of 1500 upstream of the start site, and relative enrichment in the 1781 bp further upstream, means that distal elements more than 1.5 kb from the transcription initiation site may be involved in expression of MP during floral development.

Despite the nine suspected AuxREs present in the first 515 bp of the MP promoter, the promoter fragment alone was unable to activate expression of MPΔ enough for the presence of the transgene to cause any change in phenotype. Additionally, the lengthening of the promoter fragment to include the following upstream 185 bp, within which there are no additional AuxREs or relevant ZREs/MREs, was sufficient to drive expression enough to cause slight disruptions in shape and vasculature of cotyledons and leaves that expression is expected to cause. The irrepresible activity of MPΔ is posited to expand the procambium within the early leaf, leading to an overall increase in vasculature, as well as misregulation of PIN1, which disrupts the self-organizing capability of auxin flow and thus leads to abnormal venation patterns (Krogan et al., 2012). Given the difference between the severity of pattern disruption between MPΔ in a hemizygous background and homozygous background, it is possible that increase expression levels of MPΔ are correlated with broader expansion of procambium and greater disruption of
PIN1 expression domains, thus leading to abnormal auxin transport patterns. It appears then that within this 185 base pair segment is something that is facilitating stronger expression of MPΔ, and sequence analysis showed that present in the segment is the majority of a conserved UCE(ultraconserved element)-like element (ULE), ULE6. Kritsas et al. (2012), who identified the element, compared the genomes of Arabidopsis thaliana and Vitis vinifera (grapevine), species which diverged 115 million years ago (Fawcett et al., 2009), looking for conserved sequences with the parameters of being over 55 bp in length, at least 85% sequence similarity, a copy number less than 5, and location in non-protein-coding regions. Their analysis found 36 such sequences, 22 being in areas upstream or downstream from a gene (the other 14 being intronic). Gene Ontology (GO) analysis shows that these ULEs have a significant enrichment for being near genes involved in development, and a significant functional enrichment for genes associated with transcription factor activity and nucleic acid binding (Kritsas et al., 2012). These annotations all describe MP (Berardini et al., 2015). With the results here showing a change in phenotype in the 700 bp promoter line that is not present in the 515 promoter line, there is some indication that ULE6 may have a role in MP expression. Another region of interest is the 200 base pairs between 1300 and 1500 base pairs upstream of the MP transcription initiation site. The results of figure 4.5 indicate that addition of this segment to the promoter results in a decrease in MPΔ activity, giving the possibility of repressive regulatory elements present in the sequence.
Chapter 5

Epistatic relationship between $MP\Delta$
and the activities of ARF7/NPH4 and ARF19
5.1 Introduction

*NON-PHOTOTROPIC HYOCOTYL 4 (NPH4)*, also known as *ARF7*, is a close relative of *MP* associated with differential growth response in the hypocotyl (Ulmasov et al., 1999; Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Harper et al., 2000). Double loss-of-function *mp nph4* double knockout mutants have a more severe phenotype than either single mutant, only growing as a small, poorly polarized club-shaped seedling with minimal vascular tissue and no discernible apical structures like cotyledons and leaves (Hardtke et al., 2004). A very small fraction of *mp* single mutants exhibit this phenotype; most *mp* mutants form at least one cotyledon, and often grow leaves as well. This supports other evidence that *NPH4* and *MP* have some overlap in function, as *NPH4* clearly plays a role in patterning the apical region, but *MP* masks its function in wild type plants. *MP* and *NPH4* share many of the same expression domains, and their protein products preferentially dimerize with themselves and then with each other (Hardtke et al., 2004). Finally, by sequence similarity, the two ARFs are highly related, despite their divergent single-mutant phenotypes.

Another ARF that overlaps with *NPH4* in function and expression is *ARF19* (Okushima et al., 2005). *arf19* loss-of-function mutants look fairly normal, but *nph4 arf19* mutants have severely reduced lateral root development, root auxin sensitivity, and root gravitropism, which is not observed in either of the two single mutants (Wilmoth et al., 2005; Okushima et al., 2005). Though wild type expression of *MP* is unable to supplant *NPH4* and ARF19 function, overexpression under a constitutively active 35S promoter gives *MP* the ability to rescue the *nph4 arf19* double mutant phenotype (Stamatiou, 2007). Triple *nph4 arf19 mp* mutants have a similar phenotype to that of *nph4 mp* mutants (Stamatiou, 2007).

*MPΔ* is able to partially rescue *mp* basal structures and primary root formation, indicating that it does retain some MP function despite a presumed inability to dimerize
Chapter 5. MPΔ epistatic relationship with other ARFs

with other ARFs (Krogan et al., 2012). It is not clear, however, if this incomplete rescue is independent of the activity of other ARFs as it is in the case of MP overexpression. As these genes are contributors to root formation, this rescue may be impossible or weaker in nph4, arf19, and nph4 arf19 knockout mutant backgrounds. Additionally, because of the overlapping expression patterns of the three ARFs, it is also possible that MPΔ activity is sufficient to rescue root gravitropic response and auxin insensitivity in nph4-1 arf19-4 double mutants.

5.2 Results

5.2.1 MPΔ cotyledon and leaf phenotypes in arf loss-of-function mutants

For clarity, the genotypes in this chapter which reference nph4-1 and arf19-4 are all assumed to be homozygous for these alleles, while MPΔ and mp1058 are denoted as hem for hemizygous (in the case of MPΔ), het for heterozygous (in the case of mp1058), and hom for homozygous backgrounds. The centralized and overabundant vascular phenotype in cotyledons was observed in all MPΔhom individuals regardless of their nph4-1 and/or arf19-4 mutant backgrounds (Figure 5.1C,E,I,M). Even in hemizygous MPΔ individuals, where one might expect easier distinction of quantitative changes, the MPΔ effect was fully epistatic over all influences from NPH4 or ARF19 activity (Figure 5.1B,E,H,L). MPΔhem nph4-1 arf19-4 cotyledons shared the same traits as the nph4-1 background, while homozygous cotyledons were notably smaller than MPΔhem cotyledons.

Essentially similar epistatic relationships were observed when the venation patterns in the first pair of vegetative leaves was compared (Figure 5.2). First leaves of MPΔhem individuals regardless of their nph4 or arf19 genotypes also showed similar defects in venation patterning at 7 days after germination from the seed coat (DAG) (Figure 5.2B, E, H, K). MPΔhem nph4-1 leaves were slightly reduced in size and width compared
to the other backgrounds (Figure 5.2H). \( MP\Delta hom \) leaves regardless of \( nph4 \) or \( arf19 \) backgrounds displayed the characteristic narrow leaf shape with all vascular strands parallel to the midline (Figure 5.2C, F, I, L). However, in \( nph4-1 \) and \( nph4-1 arf19-4 \), \( MP\Delta hom \) was not fully epistatic, as these backgrounds reduced leaf length, which was not observed in \( arf19-4 \) backgrounds (Figure 5.2I, L). These observations were based on a sample size of 20 individuals for each genotype.
Figure 5.1: Cleared 7 DAG cotyledons of non-transgenic (1st column), \( MP\Delta hem \) (2nd column), and \( MP\Delta hom \) (3rd column) seedlings in Col-0 (A-C), \( arf19-4 \) (D-F), \( nph4-1 \) (G-I), and \( nph4-1 arf19-4 \) (G-I) backgrounds. Scale bars: 1 mm.
Figure 5.2: Cleared 7 DAG leaves of non-transgenic (1st column), $MP\Delta$hem (2nd column), and $MP\Delta$hom (3rd column) seedlings in Col-0 (A-C), arf19-4 (D-F), nph4-1 (G-I), and nph4-1 arf19-4 (G-I) backgrounds. Scale bars: 0.5 mm.
5.2.2 Effect of $MP\Delta$ of auxin responses on root development in $arf$ loss-of-function mutants

The same epistatic relationships as in 5.2.1 were investigated with regard to auxin responses on root development traits. The first assay measured growth of the primary root in 4 DAG seedlings after exposure to 1 $\mu$M IAA media for 4 days (see Materials and Methods for details). Auxin exposure has been previously shown to significantly slow root growth in wild type plants, and this response is weakened in auxin insensitive mutants (Stamatiou, 2007). Consistent with this, root growth was less inhibited in nph4-1 plants and far less inhibited in nph4-1 arf19-4 double mutants compared to Col-0 plants (Figure 5.3A). Remarkably, this indication of auxin insensitivity was essentially not affected regardless of presence and dosage of $MP\Delta$ activity (Figure 5.3A). In all genetic backgrounds, $MP\Delta$ activity did not seem to affect this trait regardless of hemi- or homozygous dosage. Furthermore, nph4-1 single mutant response changes were also below the significance threshold given by large standard deviation error bars in Figure 5.3A. Therefore, it required elimination of both NPH4/ARF7 and ARF19 activity to turn roots auxin insensitive in this assay, but then this insensitivity was independent of $MP\Delta$ activity.

A second assay examined root gravitropic response of these genotypes. Seedlings were grown on media vertically for 3 days after germination before being turned 90°; 24 hours later, the angle of the root tip against the original vertical vector was measured. In this assay, an angle of 90° would signify perfect gravitropism, and a fraction of it was considered a quantitative measure of auxin-mediated gravitropic response (Weijers et al., 2005). Wild type, $MP\Delta$hem, and $MP\Delta$hom mean angles were all around 75°, as were arf19-4 and the $MP\Delta$ genotypes in that background (Figure 5.3B). The mean angle in nph4-1 was slightly lower, around 67°, and $MP\Delta$hem and $MP\Delta$hom increased to 71° and 78°, respectively, in the nph4-1 background (Figure 5.3B). As previous work has
suggested, \textit{nph4-1 arf19-4} had strongly reduced gravitropic response, with a mean angle of 49° (Okushima et al. (2005), Figure 5.3B). \textit{MPΔhem nph4-1 arf19-4} displayed an small increase to 52°, and \textit{MPΔhom nph4-1 arf19-4} increased slightly further to 57° (Figure 5.3B), but neither of these increases were significant.
Figure 5.3: Root assays of $MP\Delta$ arf mutants. (A) Root growth of 4 DAG seedlings after transfer to 1 µM IAA media for 3 days. Each value represents the mean change in root length over 3 days. Error bars represent standard deviation. (B) Root gravitropic response of 3 DAG seedlings after being turned 90° and analyzed 24 hours later. The values represent the angle of the root with respect to the original gravity vector.
5.2.3 Primary root initiation in MPΔ and arf loss-of-function mutants

Since mp\textsuperscript{1058} homozygotes are almost invariably rootless, approximately 25% of the progeny of an mp\textsuperscript{1058} heterozygote will be rootless. MPΔ has previously been shown to rescue root initiation in around 60% of mp\textsuperscript{1058} mutant seedlings (Krogan et al., 2012). As there is no linkage between the MP locus and the MPΔ insertion site (Caragea, A, unpub.), in an unselected sample of progeny from an MPΔ\textit{hem} mp\textsuperscript{1058}\textit{het} line, only around 10% will be rootless, which was reflected by the results here (Figure 5.4). MPΔ\textit{hem} arf\textsuperscript{19-4} mp\textsuperscript{1058}\textit{het} progeny show a rootless percentage of 13% (Figure 5.4). This percentage was not significantly different from the 10% rootless proportion observed in MPΔ\textit{hem} mp\textsuperscript{1058}\textit{het} lines (z=1.24, P=.215). MPΔ\textit{hem} nph\textsuperscript{4-1} mp\textsuperscript{1058}\textit{het} and MPΔ\textit{hem} nph\textsuperscript{4-1}\textit{hom} arf\textsuperscript{19-4}\textit{hom} mp\textsuperscript{1058}\textit{het} lines show rootless progeny of 21% and 19%, respectively (Figure 5.4). Both of these proportions are very significantly higher than MPΔ\textit{hem} mp\textsuperscript{1058}\textit{het} (z=3.9, 3.3, P<.001).
Figure 5.4: Proportions of rootless individuals in the seedling progeny of $mp^{1058het}$, $MP\Delta hem$ arf19-4 $mp^{1058het}$, $MP\Delta hem$ nph4-1 $mp^{1058het}$, and $MP\Delta hem$ nph4-1 arf19-4 $mp^{1058het}$ lines. P-values represent results of a two-proportion z-test (Lee, 2010).
5.2.4 Generation of apical patterns by MPΔ in arf loss-of-function mutants

\(mp^{1058}\) homozygotes were identified as rootless individuals in the progeny of \(mp^{1058}\) heterozygotes from the various MPΔ arf loss-of-function lines, and were confirmed for homozygosity of the \(mp^{1058}\) allele using PCR (see Materials and Methods). These individuals were also assessed for the presence of MPΔ using PCR, but distinction between one or two copies of the transgene was not assessed because the entire pool of rootless seedling progeny was being analyzed. Therefore, the use of MPΔ in the genotypes below refers to the presence of either one or two copies of the MPΔ transgene. Basta selection was not used in order to ensure that there were no external factors influencing phenotype. The \(nph4-1\ arf19-4\ mp^{1058} homo\) triple mutant does not produce any cotyledons (Stamatiou, 2007) and therefore any deviation from this in MPΔ \(nph4-1\ arf19-4\ mp^{1058} homo\) plants (identified by rootlessness and confirmed through PCR) would indicate that MPΔ activity is sufficient to trigger apical development even in the nearly complete absence of activity of an entire ARF subclade. As shown in Figure 5.5, MPΔ \(nph4-1\ arf19-4\ mp^{1058} homo\) plants still showed significantly more advanced development of apical structures than the \(nph4-1\ arf19-4\ mp^{1058} homo\) at 5 DAG (Figure 5.6, 5.5). MPΔ \(nph4-1\ arf19-4\ mp^{1058} homo\) individuals were more likely to form two fully distinct cotyledons than \(mp^{1058} homo\), but had a higher proportion of fused cotyledons compared to the other mutant backgrounds (Figure 5.6). MPΔ also appeared to restore some ability to form vascular strands in these mutants, and they displayed abundance of vasculature resembling the MPΔ genotype (Figure 5.5). Despite the reduced percentage of root rescue in the seedling progeny of the MPΔhem \(nph4-1\ arf19-4\ mp^{1058} het\) line (Figure 5.4), a small number of MPΔ \(nph4-1\ arf19-4\ mp^{1058} homo\) were observed to be successful in establishing a primary root (Figure 5.5C). These individuals were identifiable by their otherwise strong resemblance to MPΔ \(nph4-1\ arf19-4\ mp^{1058}\) rootless individuals (reduced size and/or single or fused cotyledon), and again MPΔ presence and \(mp^{1058}\) homozygosity was confirmed by PCR. Growth of
these roots halted around 1-2 mm, and of 10 individuals transferred to soil, none grew into adult plants.
Figure 5.5: Comparison of phenotype and vasculature of 5 DAG nph4-1 arf19-4 mp^{1058}hom (A,D) and MPΔ(hom/hem) nph4-1 arf19-4 mp^{1058}hom (B-C,E-F). Scale bars: (A-C) 0.5 mm, (D-F) 0.25 mm
Figure 5.6: Cotyledon phenotype distributions of rootless seedling progeny of $mp^{1058\text{het}}$ (n=38), $MP\Delta$hem $arf19-4\; mp^{1058\text{het}}$ (n=31), $MP\Delta$hem $nph4-1\; mp^{1058\text{het}}$ (n=45), and $MP\Delta$hem $nph4-1\; arf19-4\; mp^{1058\text{het}}$ (n=51) lines.
5.3 Discussion

The structural and function relationship of the three ARFs, MP, NPH4, and ARF19, is clearly complex, with perfect asymmetric redundancy in many patterning processes, contrasted by unique functions of MP, based on its particular protein structure (Boer et al., 2014), as opposed to unique functions for the others, being based on exclusion of MP from the respective tissues (Hardtke et al., 2004). The MPΔ transgene is expressed from a bona fide full length MP promoter (3281 bp), but as it only partially normalizes mp mutant traits, its effects on traits of the other two ARFs demanded investigation.

By and large, the very limited or missing ability of MPΔ to normalize root specific traits in nph4 arf19 double mutants is consistent with the fact that it is within these tissues that those mutant traits are also not masked by MP wild type activity. In these areas, very restricted MP expression domains leave room for the unique functions exerted by those two genes. Most strikingly, however, this is contrasted by the epistatic relationships in the shoot.

5.3.1 MPΔ shoot phenotypes are largely independent of NPH4 and ARF19 activity

The distinct changes to cotyledon and leaf vasculature observed in MPΔ hemizygous and homozygous individuals did not appear to be affected by the absence of NPH4 or ARF19. Even in np4-1 arf19-4 mp1058 het triple knockouts, the presence of MPΔ gave rise to abundant, parallel vascular bundles in the midveins of cotyledons. This indicates that the overproduction of vasculature by MPΔ is largely independent of NPH4, ARF19, and MP activity.

Even more strikingly, MPΔ could boost relatively normal apical development, including abundant vasculature, even in the nph4 arf19 mp triple mutant, i.e. a genotype which
is considered to be nearly completely unresponsive to auxin (Hardtke et al., 2004). It will justify further explorations to find out how auxin-based patterning can operate, if the only known relevant ARF activity in this process is an ARF variant, whose activity cannot be modulated by known mechanisms.

5.3.2 $MP\Delta$ does not compensate for loss of $NPH4$ and $ARF19$ activity in the root

Auxin insensitivity phenotypes of $nph4-1\ arf19-4$ double mutants, such as lack of gravitropic response and lateral root formation, are rescued by overexpression of $MP$ through a constitutively active $35S$ promoter. If $MP$ is a significant factor in activation of its own expression, it might be expected that the irrepressible activity of $MP\Delta$ may upregulate expression of genomic $MP$ enough to rescue auxin sensitivity of $nph4-1\ arf19-4$ mutants in a similar fashion. The fact that this was not observed, however, supports results from the previous chapter suggesting that ARF activity may not be a significant activator of $MP$ expression. It is also possible though, that the irrepressible activity of $MP\Delta$, which would activate downstream targets of $MP$ constitutively, may simply be too much for full-length $MP$ to influence gene expression, and thus auxin-responsive formation of lateral roots, in a significant fashion. $35S::MP$ has not yet been introduced into an $MP\Delta\ nph4-1\ arf19-4\ mp^{1058}het$, and until that point this explanation remains conjecture.
Chapter 6

General Conclusions
6.1 The role of $MP$ in the context of other $ARF$ functions

The adaptive flexibility and reiterative organization of plant development has suggested that positioning of organs and certain tissues may be controlled by self-organizing processes, and that dynamic auxin distribution patterns have a key role in this self-organization (Leyser, 2011). Examples of auxin-dependent site selections include, for example, the phyllotactic arrangement of lateral shoot organs, the venation patterns in leaves or the positioning of lateral roots (Reinhardt et al., 2000; Casimiro et al., 2001; Scarpella et al., 2006). The developmental roles of auxin appear to be primarily mediated through changes in gene expression (Dharmasiri et al., 2005a). Based on current models of auxin perception, the ARF family of transcription factors are the first transcription factors to become active upon cellular reception of auxin (Chapman and Estelle, 2009). Activation of ARFs through the auxin-mediated degradation of Aux/IAA regulators can occur very quickly and can then influence the expression of all kinds of genes, including those of ARFs, as well as genes in auxin synthesis, metabolism, and transport (Chapman and Estelle, 2009). An increasing number of mathematical models have demonstrated how interconnections between those parameters, including feedback controls, can generate stable, but highly adaptive, reiterative patterns, very similar to those observed in nature (Reinhardt et al., 2003; Jönsson et al., 2006; Lau et al., 2011; Pinon et al., 2013) Obviously, ARFs, their expression patterns, and their influence on each others’ expression pattern are important elements in any such mechanism.

While functional analyses have shown that ARFs are involved in all kinds of developmental processes, it seems that only a subset of the large $ARF$ family is involved in auxin-driven feedback controls that define the localization of cellular events at early stages of organ development (Rademacher et al., 2011; Li et al., 2016). Although subtle modulating contributions may have been overlooked and entire stages and organs not investigated, it is well possible that for several main patterning events, decisive roles of $ARFs$ are
restricted to the five members of the traditionally called “activating” ARF{s, comprising \textit{ARF5, 6, 7, 8, and 19} (Ulmasov et al., 1999; Tiwari et al., 2003). However, even within this limited set of ARF{s, information critical to their potential roles in feedback controls is missing. For example, expression domains of only a few ARF genes have been explored beyond in silico profiles, and where this has happened, static rather than dynamically self-regulating expression patterns have been published (Stamatiou, 2007; Rademacher et al., 2011). Further, little is known about the transcriptional regulation of ARF genes. What auxin-independent controls are involved through what \textit{cis}-regulatory elements and which AuxRE{s in their promoters have influence on their expression? Finally, how much is their activity regulated by auxin through interacting Aux/IAA proteins?

Within the subclade of “activating” ARF{s there is some functional dissection. The roles of these five ARF{s in self-regulatory mechanisms seem to be very unequal, as earlier work by Stamatiou (2007) has shown that with regard to vascular and meristem patterning, all of these 5 ARF activities are linked in asymmetric functional redundancy. In the absence of MP/ARF5, each of the other four ARF{s (6, 7, 8, and 19) provide incremental contributions to both patterning processes, but these contributions are overshadowed by ARF5 activity in wild type development. This can be demonstrated using an \textit{arf6 7 8 19 quadruple mutant}, where the remaining MP activity is sufficient to sustain normal vascular and meristem development (Stamatiou, 2007). This extra-ordinary role of MP in the subclade is matched by structural peculiarities, as Boer et al. (2014) have shown that MP can multimerize through its DBD. Multimers of MP generated through this binding can act as molecular calipers on exactly spaced AuxRE{s of specific sets of target genes, which could explain its peculiar role. Further, rendering MP, but not ARF10 or ARF19 (Goldstein, R, unpub.) constitutively active (MP\textsubscript{\Delta}) has dramatic influences on certain patterning processes, in that it dramatically increases expression from the MP promoter (Caragea, A, unpub.) and from the promoter of the auxin efflux facilitator \textit{PIN1} (Krogan et al., 2012). Nevertheless, despite the extraordinary role of MP among
other ARFs and its obvious self-regulation, nearly nothing has been known about the significance of individual AuxREs in the MP promoter, about the possibility of targeting the MPΔ activity by promoter alterations, or about the independence of MPΔ activity from other ARFs.

6.2 Insights into MP regulation may help explain self-regulatory feedbacks

The apparent special role of MP in patterning processes that seem to involve self-regulatory feedback mechanisms puts emphasis on the need to better understand MP regulation. For example, MP seems to be required for maintaining procambial cell fate within the ground meristem of a developing leaf. The absence of MP activity shifts the balance between procambial and mesophyll cell fate in favour of the latter (Berleth and Jurgens, 1993), while irrepressible MP activity leads to a strong expansion of procambium and hence vasculature in the leaf (Krogan et al., 2012). Further, narrow lines of procambial cells form in areas of elevated auxin flows (Mattsson et al., 2003), which are preceded by larger areas of elevated auxin response and expression of the auxin-inducible auxin efflux facilitator PIN1 (Scarpella et al., 2006). As PIN1 has also been shown to be a direct transcriptional target of MP (Krogan et al., 2012), it is tempting to speculate that the gradual confinement of auxin to increasingly narrow flow channels is mediated by Aux/IAA interaction with MP. As auxin becomes more and more concentrated in pre-procambial channels, MP becomes highly active within those channels, but increasingly repressed by Aux/IAA proteins in surrounding areas, from which auxin has been drained away (Wenzel et al., 2007; Krogan et al., 2012). A recently designed inducible MPΔ variant, MPΔ:GR, can be used to alter, for example, self-regulatory processes in leaf development at any stage (Caragea, 2015). It turns out that introducing irrepressible MPΔ activity at stages when the narrowing of MP expression domains has already advanced, can revert this
gradual confinement to lead to $MP$ expression across the developing lamina (Caragea, A, unpub.). This finding demonstrates $MP$ self-regulation as well as the fact that interaction of $MP$ with Aux/IAA proteins is necessary for the normal restriction of $MP$ domains and procambium formation.

Mathematical models have also been used to demonstrate that combinations of $ARF$ and Aux/IAA activities can generate stable cell state switches as functions of auxin concentration. As shown by Lau et al. (2011), even a simplified module comprising just $MP$ and its negative regulator $BDL/IAA12$ is able to act as a quantitative switch, in which auxin signals beyond a certain level may lead to ongoing on-states. Both the experimental findings as well as the conceptual requirements presented in this study support a regulation of $MP$ of its own expression as well as that of $BDL/IAA12$, the latter being independently supported by Krogan et al. (2014). In summary, mathematically refined conceptual considerations have long postulated feedback mechanisms as they are now being experimentally confirmed for regulatory interactions between $MP$ and its target genes, among them $MP$ itself, certain Aux/IAA genes, and certain $PIN$ genes.

### 6.3 Regulation of $MP$ and its impact on developmental traits

In this context, it appeared important to expand these studies to assign specific roles to individual AuxREs in the $MP$ promoter. This has been partially achieved in studies presented here through changes in reporter or actual $MP$ gene expression in correlation to altered AuxREs. Specifically, the study presented here has shown that

1. An AuxRE at position 126 bp upstream of the transcription initiation site is specifically required for $MP$ expression in the hypocotyl, and traits normally associated with reduced auxin signaling, like hypocotyl and petiole elongation in the light, were surprisingly correlated to the addition of AuxREs, whereas doubling apparent $MP$ gene dosage through an extra pair of the $MP$ gene had no such effect.
2. Distal regions in the $MP$ promoter regulatory regions located between 3281 and 1500 bp upstream of the transcription initiation site were selectively important only for $MP$ expression in reproductive organs, while proximal regions are sufficient to sustain all $MP$ expression relevant at vegetative stages.

3. Factors other than AuxREs may be contributors to $MP$ expression. The initial 515 bp upstream of the transcription initiation site, containing nine total AuxREs, was not sufficient to cause phenotypic changes when driving the $MP$ CTD deletion variant $MP\Delta$, whereas the addition of the following 185 base pairs, which only contain an uncharacterized ultra-conserved element, results in phenotypic changes.

4. The ability of an irrepressible $MP$ variant, $MP\Delta$, to compensate for the lack of multiple ARF activities differs dramatically between shoot and root. While $NPH4/ARF7$ and $ARF19$ are largely dispensable in the presence of $MP\Delta$ activity to produce cotyledons and even leaves, epistasis of $nph4\ arf19$ double mutants over $MP\Delta$ in several root traits demonstrate their genuine functions beyond redundancy with $MP$.

5. In addition to involvement in apical patterning (Hardtke et al., 2004), $NPH4/ARF7$ has some involvement in basal domain patterning and embryonic root initiation that is hidden by $MP$ activity in wild type plants, but revealed by increased rootless segregation of $MP\Delta\ nph4\ mp$ individuals compared to $MP\Delta\ mp$.

Although the study might have been more comprehensive with regard to the definition of AuxRE functions if more mutated AuxREs had been tested in a single reporter gene setup, this would have been at the expense of new findings on the morphogenetic potential of $MP$. In light of the dramatic changes that $MP\Delta$ imposes on several developmental processes, it was important to define to what degree it can override the influences of other ARF activities in individual processes and to what extent one can target the $MP\Delta$ action to individual stages and organs based on $MP$ promoter design. The fact that even within
the limited number of mutated AuxREs, functionally relevant ones were identified, makes further surveys much more rewarding, and the epistatic roles that mutations in other ARFs have in the $MP\Delta$ background suggests processes in which these ARFs and their regulation may have pivotal roles.

Finally, a number of new MP target genes have recently been published (Scacchi et al., 2010; Schlereth et al., 2010; DeRybel et al., 2013; Grunewald et al., 2013; Robert et al., 2015). Mutations in at least one of these targets, $PIN1$, have also been shown to be at least partially epistatic over $MP\Delta$ (Caragea, 2015). It will be interesting to see, in this and other upcoming examples, through which effector genes $MP$ executes its phenotypic effects and to what degree $MP$ self-regulation is a necessary prerequisite for precision and robustness of patterning processes.

### 6.4 Relevance of AuxREs and other sequence contexts for $MP$ expression and phenotypic effectiveness

Only the first four of the six core hexamers defining an AuxRE are strictly required, at least in concatamers comprising multiple AuxREs (Ulmasov et al., 1997a). As any sequence of four base pairs has probability of $1/256$ and even the hexamer has a probability of $1/1024$ to arise by chance, it is clear that functional auxin inducible promoter regions require sequence context beyond AuxREs. The functional redundancy between members of the $ARF$ and $Aux/IAA$ families presents some difficulty in identifying expression changes for a single gene like $MP$. $MP\Delta$, however, eliminates the relevance of $Aux/IAAs$ and provides clear phenotypic changes that, based on the appearance of hemizygous and homozygous individuals, appear to increase in severity along with expression levels of the transgene (Krogan et al., 2012). Here it was shown that the initial 515 bp upstream of the $MP$ gene was insufficient for significant $MP\Delta$ expression, as the phenotypic changes associated with the transgene containing the full length standard $MP$ promoter were not present to
any appreciable degree. Inclusion of 185 bp more upstream sequence, which contains no AuxREs, does create a visible phenotypic change. A very recent study identifies further AuxREs relevant for auxin inducibility even within the *MP* coding sequence (Bhatia et al., 2016), but for neither of the two gene classes has the relationship between regulatory elements controlling auxin-inducibility and others conferring region specific patterns been worked out. In this regard, it will be interesting to identify more region-specific AuxRE functions, such as AuxRE126.

Despite the apparent inactivity of the 515 bp promoter when driving *MPΔ*, there is evidence that the AuxREs within this region are important. Lau et al. (2011) found that the fragment was sufficient to mediate auxin inducibility, and mutation of all nine possible AuxREs in the sequence removed the ability of *MPΔ* to activate *MP* expression. It was shown here that removal of an individual AuxRE (AuxRE126), but not another (AuxRE503), caused a change in expression of *MP* in a distinct tissue (hypocotyl) while expression mirrored wild type in other tissues. It was also shown that addition of AuxREs to the *MP* promoter, driving expression of full length *MP*, leads to petiole elongation in seedlings: an auxin-related growth response. The findings further strengthen support for the significance of *ARF* activity in *MP* expression, and also indicates that certain AuxREs may have specific and distinct significance toward the expression domains of *MP*.

The distal regions of the *MP* promoter (1500 to 3281 bp upstream of the *MP* transcription initiation site) include the presence of several AuxREs, but are also notably more enriched for specific bZIP and Myb-related binding elements than the more proximal regions of the promoter (Berendzen et al., 2012). The apparent relevance of this promoter region for *MP* expression in reproductive organs may support other evidence showing involvement of coupling elements in AuxRE-driven transcription. However, without a more detailed analysis of the significance of individual elements, these assumptions remain largely speculative.
6.5 Future Directions

The ability of $MP$ activity to mask all other “activating” $ARF$s with regard to their patterning functions, as well as another study (Bhatia et al., 2016) demonstrating an instructive role of $MP$ expression domains in shaping subsequent patterning events, highlight the uniqueness of $MP$ in the $ARF$ family. From this, it follows that a better understanding of $MP$ expression and of $MP$ target gene identity should be decisive contributions to move the entire field of auxin-mediated patterning. Natural extensions of this study should therefore comprise

- A comprehensive mutagenesis experiment including all AuxREs within 3 kb upstream and even downstream (Bhatia et al., 2016) of the $MP$ transcriptional start site. All constructs should be linked to a fluorescent protein reporter to allow for live imaging of the dynamic expression pattern in combinations with other markers or even in mutant background.

- Promoter mutants showing altered expression in experiment one should be tested when driving $MP$ and $MP\Delta$ expression in $mp$ mutant background to see to what extent they are relevant for phenotypic traits controlled by $MP$.

- AuxREs and their sequence context that are identified as developmentally important inputs into $MP$ expression should be used as tags to identify protein and protein complexes binding specifically to them.

- AuxREs and their sequence context chosen in experiment three should also be multimerized en bloc to see whether they can enhance $MP$ expression profiles and phenotypic effects.

- As $MP$ upstream regions comprise binding motives for transcription factors other than $ARF$s, experiments analogous to the ones above should be conducted for those
binding sites as well.

- Bioinformatics should be used to compare functional regions identified in the *Arabidopsis MP* promoter with those in other species. Previous experiments have already shown that *MP* can exert similar effects in other dicotyledonous species (Ckurshumova, W, unpub.), suggesting similar roles for *MP* in those species. Bioinformatics could determine what sequence features are typically associated with similar roles of *MP* in species with similar *MP* functions as opposed to those, e.g. monocots, where *MP* might have entirely different roles.
Bibliography


