Organogenesis *in vitro* under Altered Auxin Signaling Conditions

by

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Abstract

The ratio of auxin to cytokinin determines de novo organogenesis in plants. Relatively little is known about the effect of genetically altered auxin signaling on in vitro organogenesis. Here, callusogenesis, shoot, and root formation were studied in loss- (LOF) and gain-of-function (GOF) alleles in two phylogenetically related Auxin Response Factors (ARFs), MONOPTEROS (MP/ARF5) and NON-PHOTOTROPHIC HYPOCOTYL 4 (NPH4/ARF7). Reduced MP activity greatly diminished shoot regeneration, and partially diminished callusogenesis and root formation. LOF in NPH4 strongly decreased callusogenesis, and mildly decreased shoot and root regeneration in particular categories of explants. By contrast, organogenesis responses were strongly increased in aerial explants carrying the GOF transgene dMP. Thus, both MP and NPH4 seem to act as positive regulators of certain organogenesis processes and the GOF dMP transgene may be of interest for stimulating organogenesis in plant species with poor regeneration properties. Also, organogenesis in vitro may reveal unknown developmental ARF functions.
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<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2-iP</td>
<td>gamma, gamma-Dimethylallylamino purine</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ARF</td>
<td>AUXIN RESPONSE FACTOR</td>
</tr>
<tr>
<td>AuxRE</td>
<td>auxin response element</td>
</tr>
<tr>
<td>B5</td>
<td>Gamborg B5 media</td>
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<tr>
<td>Bar</td>
<td>Basta resistance gene</td>
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<td>Basta</td>
<td>glufosinate-ammonium</td>
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<td>bp</td>
<td>base pair</td>
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<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Centigrade</td>
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<tr>
<td>CIM</td>
<td>callus initiation medium</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<tr>
<td>Col-0</td>
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<tr>
<td>DAG</td>
<td>days after germination</td>
</tr>
<tr>
<td>DMSO</td>
<td>methylsulfoxide</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>
</tr>
<tr>
<td>E</td>
<td>Einstein</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>f</td>
<td>forward</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<td>G</td>
<td>guanine</td>
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<tr>
<td>GFP</td>
<td>GREEN FLUORESCENT PROTEIN</td>
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<tr>
<td>GOF</td>
<td>gain-of-function</td>
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<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<td>HCL</td>
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<tr>
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<td>indole-3 butyric acid</td>
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<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
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</table>
L  litre
LB  left border
LOF  loss-of-function
m  meter
M  molar
min  minute
MES  2-(4-Morpholino)ethanesulfonic acid
MP  MONOPTEROS
MS  Murashige and Skoog media
NaCL  sodium chloride
NPH4  NON-PHOTOTROPIC HYPOCOTYL 4
dNTP  deoxyribonucleotide triphosphate
ORF  open reading frame
PCR  polymerase chain reaction
r  reverse
RB  right border
RFLP  restriction fragment length polymorphism
RIM  root initiation medium
RNA  ribonucleic acid
rpm  revolutions per minute
RT  room temperature
s  second
SAM  shoot apical meristem
SE  standard error of the mean
SDS  sodium dodecyl sulfate
SIM  shoot initiation medium
T  thymine
Tris  Tris (hydroxymethyl) aminomethane
UTR  untranslated region
v/v  volume per volume
w/v  weight per volume
wt  wild type
Chapter 1

Introduction

1.1 Plant hormone signals in plant regeneration

Plant tissue culture and molecular genetics are core techniques for the genetic engineering of plants (Li and Gray, 2005). The methodology is based on the unique ability of plants to regenerate into complete plants from their parts (organs, tissues, or cells) (Schwarz et al., 2005). Plant tissue culture is also an increasingly important tool for studies in plant development, especially when integrated with techniques of molecular biology and genetics (Zuo et al., 2002; Zhang and Lemaux, 2004; Caponetti et al., 2005; Gray, 2005; Mordhorst et al., 2005; Zhang and Lemaux, 2005).

In 1957, Skoog and Miller demonstrated that the ratio of auxin to cytokinin in a culture medium determines the outcome of developmental processes in tissue culture, and results in callus, shoot, or root formation (Skoog and Miller, 1957). During the last two decades, the main foci of tissue culture research were on cytokine signaling and shoot meristem genes, in light of their effect on de novo shoot meristem formation (Howell, 2003; Zhang and Lemaux, 2004; Zhang and Lemaux, 2005). However, auxin response genes may also play a crucial role in organogenesis and shoot regeneration processes in vitro, given their key role in most developmental processes in planta (Berleth et al., 2004; Teale et al., 2006), and given the requirement for auxin and cytokinin balance in the culture medium in vitro (Skoog and Miller, 1957). Thus, the study of auxin response genes may provide insight into the molecular basis of de novo organogenesis in tissue culture.

Auxin signaling operates through a family of transcription factors called auxin response factors (ARFs), which regulate expression of the primary auxin responsive genes (Berleth et al., 2004; Teale et al., 2006). Two ARFs with partially overlapping functions in embryo shoot meristem patterning, ARF5 (MONOPTEROS) (MP/ARF5) and ARF7 (NONPHOTOTROPHIC HYPOCOTYL 4) (NPH4/ARF7) (Hardtke et al., 2004), have been tested in this research to determine their roles during callus formation and organogenesis in vitro.
1.1.1 The balance of auxin to cytokinin in a culture medium determines the type of organogenesis

Various types of developmental responses and organogenesis in vitro can be triggered by particular ratios of auxin and cytokinin in a culture medium (Skoog and Miller, 1957). Generally, high cytokinin to auxin levels promote de novo shoot regeneration; while auxin alone, or auxin in combination with low cytokinin levels, triggers de novo root formation. An intermediate ratio of auxin to cytokinin is required for callus proliferation (Skoog and Miller, 1957; Gaba, 2005). However, it is still unclear, how the ratios of these hormones direct the variations of organogenesis responses (Howell, 2003; Zhang and Lemaux, 2004; Zhang and Lemaux, 2005). Yet, it is clear that a molecular understanding of the signal transduction pathways of both hormones will aid in the research on plant regeneration properties.

1.2 The auxin signal transduction pathway

Physiological responses which are directed by auxin are central to plant structure and functioning. The fate of developing tissues may be determined by their sensitivity to auxin, to the concentration of active auxin, and to the relative concentrations of other phytohormones (reviewed in Teale et al., 2006). Natural auxin (mainly indole-3-acetic acid, IAA) is a key regulator of remarkably diverse developmental processes in plants, including root initiation, embryo and fruit development, organogenesis, vascular tissue differentiation, root and embryo patterning, photo- and gravitropism, apical dominance, and phyllotaxy. On the cellular level, auxin mediates cell division, elongation, and differentiation, although how exactly it is involved in each process is not completely understood (reviewed in Hagen and Guilfoyle, 2002; Berleth et al., 2004; Davies, 2004; Paciorek and Friml, 2006; Teale et al., 2006).

Auxin plays a central role within the plant transduction network, frequently acting in conjunction with other signals and signaling transduction pathways to regulate complex developmental processes. There is evidences of auxin ‘cross-talk’ with other pathways: cytokinin, ethylene, ABA (abscisic acid), GA (gibberellic acid), brassinosteroids, jasmonic acid, and with the light signaling system (reviewed in Swarup et al., 2002). That is why many “auxin-response” mutants exhibit pleiotropic signaling defects: they are a reflection of the existence of wide ‘cross-talk’ among signaling pathways (Hobbie and Estelle, 1994; Swarup et al., 2002).
The complex and diverse processes mediated by auxin operate through the signaling cascades that recruit specific transcriptional factors to regulate executor genes, the ones which carry out the required responses. The specificity of the processes is provided by the diversity of the transcription factors and by the auxin distribution. Both the auxin distribution and the expression profiles of certain classes of transcription factors contribute to the specificity of various auxin responses (Vogler and Kuhlemeier, 2003).

1.2.1 Biosynthesis, transport, and distribution of auxin in planta

Auxin is actively transported basally from the sites of its biosynthesis in the aerial parts to the roots (Davis, 2004). According to Ljung et al. (2001), all plant organs can synthesize auxin, with the highest rate of auxin synthesis in young leaves. Patterns of auxin distribution are not only dependent on auxin synthesis and catabolism, but also on auxin transport, processes which in turn are regulated through the cells’ capacity for auxin influx and efflux. Auxin active transport and redistribution involve many proteins. Among them, the family of polarly localized plasma-membrane proteins, PIN-FORMED (PIN) proteins, which mediate auxin efflux (Petrasek et al., 2006; Teale et al., 2006; Vieten et al., 2007; Vanneste and Friml, 2009), is intensely studied. Several other protein families involved in auxin transport are known, among them, the presumed influx carrier (AUX1), and two more proteins mediating auxin transport (reviewed in Leyser, 2006; Vieten et al., 2007; Vanneste and Friml, 2009).

Several reporters were created to visualize the spatial patterns of auxin responses as an indication for the distribution of auxin. Among them, the DR5 reporter is the one most commonly used (Ulmasov et al., 1997; Benkova et al., 2003). DR5 is a composite, synthetic auxin responsive element (AuxRE) which consists of 5-7 tandem repeats of the auxin-responsive TGTCTC element (Ulmasov et al., 1997). To visualize auxin responses, the DR5 element can be fused to either the β-glucuronidase (GUS) reporter gene (Ulmasov et al., 1997), or to the green fluorescent protein (GFP) gene sequence (Friml et al., 2003). Routes of auxin transport are typically monitored through a translational fusion of the PIN1 auxin efflux carrier protein to GFP (PIN1::PIN1-GFP transgene construct) (Benkova et al., 2003; Friml et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Gordon et al., 2007).
Several reporters were created to visualize auxin distribution and routes of auxin flow. One of the most commonly used reporters to monitor sites of auxin accumulation is DR5::GUS/GFP, a composite of 5-7 repeats of the auxin response element (AuxRe) TGTCTC, found in promoters of auxin response genes (Ulmasov et al., 1997).

1.2.2 Auxin-mediated regulation of gene expression in *Arabidopsis*

Active auxin transport by PIN proteins influences the cellular auxin concentration that then triggers the transcriptional control of the expression of auxin dependent genes. The gene regulatory auxin responses in *Arabidopsis* are mediated by two major protein families: Aux/IAA proteins, and auxin response factors (ARFs) (reviewed in Berleth et al., 2004; Teale, 2006; Guilfoyle and Hagen, 2007; Vanneste and Friml, 2009). A key feature of the auxin response system is the ubiquitin-mediated proteolysis of Aux/IAA proteins (reviewed in Berleth et al., 2004; Gray 2004; Weijers et al., 2005; Vanneste and Friml, 2009). A summary of auxin signaling is represented in Fig. 1.1.

1.2.3 Aux/IAA genes and proteins

The 29 members of the Aux/IAA protein family are small, short-lived nuclear proteins, which are transcriptional regulators of early auxin-response genes. Aux/IAA proteins are encoded by the primary auxin response genes. Aux/IAA proteins consist of four domains (I-IV). Domain I repress the transcription factors to which it becomes attached. Domain II contains a “degron” sequence, through which AUX/IAA proteins are targeted towards the SCF/TIR ubiquitin ligase. Interaction between AUX/IAA proteins and SCF/TIR is enhanced by auxin. Through this mechanism, auxin promotes the ubiquination and degradation of Aux/IAA proteins (Gray et al., 2001; Berleth et al., 2004; Vanneste and Friml, 2009). Domains III and IV play an important role in homo- and hetero-dimerization with other Aux/IAA proteins and ARFs (Ulmasov et al., 1999b; Berleth et al., 2004; Vanneste and Friml, 2009). The functions of Aux/IAA dimers are not clear (Paciorek and Friml, 2006). Heterodimerization of Aux/IAA proteins with ARFs results in the ARFs’ repression (Ulmasov et al., 1999b; Berleth et al., 2004; Vanneste and Friml, 2009).

When the concentration of auxin in a cell is low, Aux/IAA proteins are available to bind to the corresponding ARFs, resulting in the inhibition of ARF. When the auxin concentration in a cell increases, the Aux/IAA proteins are targeted for degradation, which results in the ARFs’ de-
**Figure 1.1** Model for the regulation of auxin signaling by auxin levels and auxin-induced Aux/IAA protein degradation

Low auxin levels result in the binding of the Aux/IAA proteins to ARFs via shared dimerization domains III and IV (shown in yellow and green in both Aux/IAA and ARF) and a repression of ARF function. The repression of ARFs is mediated via domain I (red) of Aux/IAA protein. The ARF proteins are bound to the promoter part of the target genes via their DNA binding domain (DBD, in purple).

Increased auxin levels promote Aux/IAA protein degradation and de-repression of ARF protein function. Auxin-induced Aux/IAA protein degradation occurs via the ubiquitin-26S proteasome protein degradation pathway (on the right). Ubiquitin molecules are conjugated to the Aux/IAA protein through the concerted action of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugated enzyme (E2), and ubiquitin protein ligase (E3). The SCF-type ligases represent the largest family of E3 enzymes in *Arabidopsis*. They consist of four subunits, three of which, SCP1-like protein (ASK1/2 here), Cullin (CUL1/AXR6 here), and an F-box protein (the TIR1 protein here) gave the name of this E3 complex – SCF or SCFTIR1. The TIR1 protein acts as an auxin receptor. The binding of auxin to TIR1 promotes substrate-specific binding and enzymatic activity of TIR1 to the substrate Aux/IAA protein, and its ubiquitination and degradation. The conserved domain II (in blue) of Aux/IAA proteins is necessary for the interaction with the TIR1 subunit, and the mutation in this domain results in increased stability of the Aux/IAA protein and auxin resistance.

Disassociation of Aux/IAA from ARF allows for the dimerization of ARFs via their shared domains III and IV, resulting in further potentiation of ARF activity.

(Figure modified from Berleth et al., 2004; the legend based on Berleth et al., 2004; Hagen, 2004, Kepinski et al., 2004, Dharmasiri et al., 2005, Krogan, 2006).
IAA

REPRESSION

HIGH AUXIN

ACTIVATION

POTENTIATION

LOW AUXIN

E1

E2

E3

SCF^{TIR1}

complex

ARF

Aux/IAA

RBX1

CUL1/ARF6

ASK1/2

TIR1

26S

AAA

AAA

AAA

AAA

AAA

AAA

AAA

AAA

AAA
repression and their activation as transcriptional factors. As long as the auxin concentration remains high, Aux/IAA proteins turn over at a high rate due to the targeted proteolysis (Hagen et al., 2004). The rapid, auxin-dependent turnover of Aux/IAA proteins makes them highly responsive to changes in auxin signaling (Gray and Estelle, 2000).

Some AUX/IAA protein interactions with ARFs have been investigated. IAA28 interacts with ARF5, 6, 7, 8 and 19 (Rybel et al., 2010); IAA14/SLR and IAA13/SHY2 interact with NPH4/ARF7 and ARF19 (Peret et al., 2009, and references therein). IAA12/BDL interact with MP/ARF5 (Hamann et al., 2002; Weijers et al., 2005; Rybel et al., 2010). Gain-of-function (GOF) mutations in AUX/IAA proteins typically affect the “degron” in domain II and lead to stable AUX/IAA, whose abundance is no longer affected by auxin (Gray et al., 2001; Weijers et al., 2005). GOF mutants of iaa12/bdl, and, to a lesser degree, with constitutively repressed MP/ARF5 function, cause phenotypes similar to those found in mp mutants (Hamann et al., 2002; Hardtke et al., 2004; Weijers et al., 2005). GOF mutations in iaa28 and iaa14/slr cause a lack of lateral roots, similar to that found in nph4 arf19 double mutants (Rogg et al., 2001; Okushima et al., 2005; Peret et al., 2009; Rybel et al., 2010).

1.2.4 ARF genes and proteins

ARFs are transcription factors encoded by a gene family of 23 members. ARFs are characterized by a conservative amino-terminal DNA binding domain (DBD), which interacts directly with the auxin response element (AuxRE). AuxREs (TGTCTC) are found in the promoters of most primary auxin-response genes. ARFs comprise another, only moderately conserved domain, called the middle domain, which determines the functional properties of an ARF as a transcriptional activator or repressor. Carboxy-terminal domains III and IV are similar to those in AUX/IAA proteins, and mediate dimerization with Aux/IAAs or other ARFs (Ulmasov et al., 1999a, Ulmasov et al., 1999b; Berleth et al., 2004). Recently Shin et al. (2007) showed that ARF activity can also be modulated by interaction with other factors, such as MYB77, which increase the combinatorial possibilities in auxin-dependent transcriptional regulation.

Whether through differential expression, differential affinities for target promoters, or both, individual ARFs have a distinct function in developmental processes (Vogler and Kuhlemeier,
**Figure 1.2** MP/ARF5 and dMP protein structures and functions:

A) When the auxin concentration is low, the Aux/IAA protein, BDL/IAA12, binds to MP/ARF5 through their shared dimerization domains III and IV. As a result, MP/ARF5 does not function as a transcription activator and the expression of early auxin response genes downstream of MP/ARF5, is repressed.

B) The proposed model suggests that, when the dimerization domains III and IV are truncated, as in the dMP protein, no binding of the Aux/IAA protein occurs. As a result, dMP functions constitutively as a transcriptional activator regardless of auxin levels.

*AuxRE* – auxin response element; DBD (purple) – DNA binding domain; MR (light blue) – middle region; III (yellow) and IV (green) – the shared dimerization domains in MP/ARF5 and BDL/IAA12; I (red) and II (blue) – domains I and II in BDL/IAA12 protein.
Inhibition of the MP/ARF5 function and expression of early auxin gene by the binding of Aux/IAA protein. 

Constitutive dMP/dARF5 function and early auxin gene expression when Aux/IAA protein is not able to bind to dMP/dARF5.
2003). However, the precise functions and downstream gene targets of most ARFs are still not known (Okushima et al., 2005; Guilfoyle and Hagen, 2007).

A systematic reverse genetics approach with \( ARF \) genes failed to reveal an abnormal phenotype of most ARF mutations, which suggested that most of the \( ARF \)s have largely redundant functions (Okushima et al., 2005; Teale, 2006). The analyses of \( ARF1 \) to \( ARF10 \) have shown that their mRNAs are ubiquitously expressed in most major organs, as well as in cultured undifferentiated \( Arabidopsis \) cells (Ulmasov et al., 1999b). Participation in multiple transduction pathways was confirmed for several ARFs. For example, ARF19 participates in both auxin and ethylene signaling (Li et al., 2006), and ARF6 and ARF8 promote jasmonic acid production (Nagpal et al., 2005). Unlike \( Aux/IAA \) genes, \( ARF \) genes do not belong to the group of early auxin response genes. ARFs have been found to be permanently expressed and bound to promoters of the target genes containing the AuxRE sequence. ARF functions are activated when the auxin concentration increases and Aux/IAA proteins dissociate from ARFs, allowing a homo- or hetero-dimerization of ARFs with each other (Berleth et al., 2004; Hagen et al., 2004). Interestingly, there is a growing body of information on posttranscriptional regulation of ARFs’ mRNA levels by micro-RNAs, and by small interfering RNAs. This posttranscriptional regulation of ARFs seems to be as important as their transcriptional regulation (Guilfoyle and Hagen, 2007).

1.2.5 Auxin response factors \( MONOPTEROS (MP/ARF5) \) and \( NON-PHOTOTROPIC HYPOCOTYL 4 (NPH4/ARF7) \)

Loss-of-function mutations have been identified by single mutant phenotype in only 4 of 23 ARFs, \( arf2/hss, arf3/ett, arf5/mp, \) and \( arf7/nph4 \) (Okushima et al., 2005). They include those in \( MONOPTEROS (MP/ARF5) \) and \( NON-PHOTOTROPIC HYPOCOTYL 4 (NPH4/ARF7) \). Both belong to the group of transcriptional activators with corresponding middle domains (Ulmasov et al., 1999a; Ulmasov et al., 1999b). Both ARFs, MP and NPH4, are capable of homo- and hetero-dimerization with each other (Hardtke et al., 2004). Especially in their DNA binding domains, \( MP \) and \( NPH4 \) have a high degree of sequence similarity, and they have partially redundant functions (Liscum and Reed, 2002; Hardtke et al., 2004; Remington et al., 2004; De Smet et al., 2010).
Loss of function (LOF) mutations in *MP* interfere with embryonic patterning, and with the formation of embryonic hypocotyl and primary roots (Mayer et al., 1991; Berleth and Jurgens, 1993). LOF mutations in *MP* also severely reduce auxin sensitivity, auxin transport, and vascular development (Li et al., 2006; Przemeck et al., 1996; Mattsson et al., 2003; Schuetz et al., 2008). Finally, they result in the formation of “pin-like” inflorescences, which lack leaves and flowers (Berleth and Jurgens, 1993; Przemeck et al., 1996). The expression of PIN1 (auxin transport) and DR5 (auxin response) reporters is defective in *mp* mutant leaves, suggesting that these responses were diminished in *mp* mutants (Mattsson et al., 2003; Wenzel et al., 2007).

LOF mutations in *NPH4* lead to hypocotyls that no longer bend towards the light, as well to changes in leaf shapes and in other aspects of post-embryonic morphology (Stowe-Evans et al., 1998). LOF *nph4* mutants exhibit auxin resistance, and severely impaired expression of many primary auxin-dependent genes (Okushima et al., 2005). Expression of the auxin-responsive reporter DR5 was also significantly reduced in *nph4* mutants (Stowe-Evans et al., 1998; Wang et al., 2005). Also, Watahiki and Yamamoto (1997) demonstrated that *nph4* mutants showed a 15 to 20 fold increase in auxin resistance (2,4-D) in the aerial parts, whereas the root parts were of normal sensitivity to auxin (Watahiki and Yamamoto, 1997; Okushima et al., 2005). In the presence of exogenous IAA, the excised *nph4* roots formed fewer lateral roots, and the *nph4* excised hypocotyls formed fewer adventitious roots than did wild-type roots and hypocotyls (Wilmoth et al., 2005). However, almost no adventitious or lateral roots were formed in *nph4 arf19* double mutants (Okushima et al., 2005; Wilmoth et al., 2005).

The developmental functions of *MP* and *NPH4* are far more related than is suggested by their single mutant phenotypes. Both *MP/ARF5* and *NPH4/ARF7* can act in the regulation of embryonic pattern formation (Hardtke et al., 2004). Although the single mutant phenotype of *NPH4* implicates the gene only in conditional responses during post-embryonic development, *NPH4* is already expressed in embryos, and LOF mutations in *NPH4* dramatically enhance the *mp* embryo phenotype (Hardtke et al., 2004).

*MP* is expressed in broad domains in embryos and in emerging organs. The early functions of the *MP* gene are related to vascular and body patterning, and to organ initiation (Hardtke and Berleth, 1998; Hardtke et al., 2004; Schuetz et al., 2008; Cole et al., 2009; De Smet et al., 2010). However, upon organ maturation, the expression of *MP* is gradually narrowed to the central
domains, and then to the vascular tissues (Hardtke et al., 2004). By contrast, NPH4 is fairly ubiquitously expressed throughout plant development (Hardtke et al., 2004; Okushima et al., 2005).

Selective deletion of domains III and IV of MP results in a GOF version of MP that is associated with the dominant enhancement of auxin-related traits. Therefore, it was named \textit{dMP} (for \textit{dominant MONOPTEROS}) (Krogan et al., 2006). Those traits included increased adventitious root formation, when \textit{dMP} cotyledons were rooted in a liquid medium containing IBA (Krogan et al., 2006). By contrast, adventitious root formation in LOF \textit{mp} cotyledons was strongly decreased (Mattsson et al., 2003). Also, \textit{MP} together with \textit{PIN1} is involved in the process of embryonic shoot meristem formation and patterning, through control of the expression of the meristem genes \textit{CUC2} and \textit{STM} (Aida et al., 2002). It is also involved in the regulation of meristem size (Schuetz et al., 2008); and in the regulation of the activity of some A-type ARABIDOPSIS RESPONSE REGULATORS (A-type ARRs, negative cytokinin response regulators) in SAM (Zhao et al., 2010). The \textit{MP/ARF5} protein interacts with \textit{BODENLOS (IAA12/BDL)} resulting in inhibition of the \textit{MP/ARF5} function (Fig.1.2 A; Hardtke et al., 2004; Weijers et al., 2005). However, \textit{dMP} is partially epistatic over \textit{bdl} (Krogan et al., 2006).

1.3 Roles of cytokinins

Cytokinins were discovered by their ability to stimulate plant cell division (del Pozo et al., 2005, and references therein). Later, Skoog and Miller introduced their hypothesis, according to which plant morphogenesis is under the control of the auxin to cytokinin ratio (Skoog and Miller, 1957). Cytokinins control key aspects of plant growth and development, such as cell division, shoot meristem initiation, control of the stem cell pool in the shoot apical meristem, leaf and root differentiation, vascular patterning, chloroplast biogenesis, photomorphogenesis, fertility, seed development, stress tolerance, and senescence (reviewed in Muller and Sheen, 2007a; Muller and Sheen, 2007b). Together with auxin, cytokinin can reprogram terminally differentiated cells to stem cells, and initiate \textit{de novo} shoot formation in culture (Gaba, 2005; Zhang and Lemaux, 2005). The most common cytokinin in plants is zeatin (Davies, 2004). Although the important sites of cytokinin biosynthesis seem to be in the root meristems (Davies, 2004; Nordstrom et al., 2004), it has been shown recently that cytokinin biosynthesis occurs in certain quantities in all types of aerial organs. The major subcellular compartments of cytokinin biosynthesis are
plastids. The major steps in cytokinin signaling are reviewed in detail in Maxwell and Kieber (2004), and in Muller and Sheen (2007 a, b).

Many studies were done to illustrate the interaction of auxin and cytokinin signaling pathways, and their mutual control of growth and differentiation in plants (Coenen and Lomax, 1997; Nordstrom et al., 2004; Moubayidin et al., 2009; Zhao et al., 2010). The existence of complex and multilevel networks of synergistic, antagonistic, and additive interactions between these two plant hormones and their signaling systems have been demonstrated in a number of studies (Klee and Estelle, 1991; Hobbie and Estelle, 1994; Coenen and Lomax, 1997; Nordstrom et al., 2004; Moubayidin et al., 2009; Zhao et al., 2010). One of the indicators of interaction, between auxin and cytokinin signaling pathways, is the number of mutants cross resistant to both hormones (Hobbie and Estelle, 1994; Hobbie et al., 1994; Coenen and Lomax, 1997; Swarup et al., 2002; Moubayidin et al., 2009). Auxin signaling mutants, such as aux1, axr1, axr2, axr3, iaa28, and shy2, have demonstrated pleiotropic signaling defects affecting other signaling pathways, including cytokinin signaling (Hobbie and Estelle, 1994; Swarup et al., 2002). Also, several mutants, initially characterized as affected in cytokinin perception, have been shown to have altered auxin responses (Smalle et al., 2002; Tajima et al., 2004; Laxmi et al., 2006). The molecular basis of auxin-cytokinin cross talk is largely unclear (Gray, 2004; Hartig and Beck, 2006), although important advances in its understanding have been recently made (reviewed in Moubayidin et al., 2009).

1.4 Cell proliferation and callusogenesis in plant tissue culture

Plant callusogenesis is a process also observed under natural growth conditions, where two types of triggers can lead to the development of callus tissues – tumor formation in response to infection by Agrobacterium tumefaciens, and wounding with subsequent regeneration of damaged tissues (Frank et al., 2000; Gordon et al., 2007). Callus proliferation can be induced in vitro, when the culture medium contains a certain ratio of auxin to cytokinin (Skoog and Miller, 1957). Cell proliferation during callusogenesis leads to disruption of the ordered morphology of differentiated tissues (Cary et al., 2002).

The mechanisms leading to callus proliferation are not well understood (Cary et al., 2002; Zhang and Lemaux, 2005). Generally, cell-cycle genes, such as cyclins and cyclin dependent kinases (CDKs), play a major role in the mediation of hormonal effects, or in response to wounding
signals, leading to cell proliferation (Hemerly et al., 1993; Soni et al., 1995; Shaul et al., 1996; Riou-Khamlichi et al., 1999; Sugiyama, 1999; Richard et al., 2002; Che et al., 2007). It has been shown that the level of \( CDK \) expression correlates with competence for cell proliferation (Hemerly et al., 1993; Zhang et al., 2005); and, \( CDK \) expression and cell proliferation are increased upon wounding in tissue culture (Hemerly et al., 1993).

Callus initiation, callus growth on callus inducing medium (CIM), and subsequent shoot formation on SIM were completely prevented in the \( Arabidopsis \) root explants cultured during the first 1 to 2 days on the CIM supplied with reversible cell cycle inhibitors aphidicolin (APH) or hydroxyurea (HU), which block G1-S transition (Che et al., 2007). Overexpression of \( cyclin D3 \) (CycD3) resulted in cytokinin-independent callus growth, but these calluses failed to regenerate shoots (Riou-Khamlichi et al., 1999). Richard et al. (2002) found that, during the initiation of a cell suspension culture, the different members of the \( CDK \) or \( CYC \) families were differentially up-regulated by mitogenic factors, including auxin and cytokinin. Moreover, \( cis \)-acting regulatory elements specific for particular plant hormones, such as \( AuxREs \) for auxin, were found in the promoters of the cell cycle genes (Richard et al., 2002). Auxin responsive elements (AuxREs) were found in the promoter regions of many cyclin genes, indicating that they might be primary auxin responsive genes (Perrot-Rechenmann, 2010).

Gordon et al. (2007) studied the dynamics of the auxin and cytokinin responses, and the pattern of the distribution of hormonal responses during developmental processes in tissue culture. According to their data, auxin responses, visualized by DR5 and PIN1 driven reporters, were strongly increased only during the initiation of callus proliferation on CIM, but they were downregulated after 5 days on CIM. They were not observed within the large callus outgrowths after 1 week on CIM, and were not detected after 10 days on CIM. Conversely, the expression of the cytokinin signaling marker \( pARR5::GFP \), and of \( CUP-SHAPED COTYLEDON 1 \) and 2 (\( CUC1 \) and \( CUC2 \)), were detected in an already established culture after 8 days on CIM. The signal expanded throughout the callus after 2 weeks of induction (Gordon et al., 2007). No expression of \( WUSCHEL \) (\( WUS \)), \( SHOOT MERISTEMLESS \) (\( STM \)), \( CLAVATA3 \) (\( CLV3 \)), \( FILAMENTOUS FLOWER \) (\( FIL \)), or \( REVOLUTA \) (\( REV \)) reporters was detected during 2 weeks on CIM, indicating that expression of these key SAM genes require incubation on SIM (Cary et al., 2002; Gordon et al., 2007). Interestingly, both the \( CUC2 \) and \( ARR5 \) reporters were expressed at the site of callus formation, even on the medium lacking cytokinin, and containing auxin as the
sole hormone. By contrast, the culture of explants with the hormone kinetin as the sole hormone did not lead to callus proliferation and CUC2 reporter expression, suggesting that auxin containing medium is required for callus proliferation and the CUC2 gene expression (Gordon et al., 2007).

Global analysis of gene expression during the first 4 days of callus proliferation on CIM demonstrated that more than 250 genes were significantly upregulated, and about 500 genes were downregulated, during the early stages of callus formation, usually associated with the stage of acquisition of competence for organogenesis (Che et al., 2006). A dramatic increase in the expression of genes involved in hormone responses, particularly Aux/IAA genes, was detected on the second day of CIM incubation. These genes were downregulated after the transfer of explants on SIM (Che et al., 2002). When cells proliferated and formed callus tissue (after 10 days of CIM incubation), the main categories of upregulated genes were stress-related transcription factors and stress-related proteins (Che et al., 2006). However, the authors concluded that the genes associated with the acquisition of competence, and those specific for callus formation, were more difficult to categorize compared to those that are part of the characterization of gene expression during shoot formation on shoot inducing medium (SIM), or root formation on root inducing medium (RIM) (Che et al., 2006).

Based on the requirement of a high auxin concentration in CIM (Skoog and Miller, 1957), mutations in auxin signaling most likely can interfere with callusogenesis processes. Thus, explants of the auxin-resistant mutant axr1-3 required more auxin for callus induction, and tolerated more auxin to grow calluses, while sensitivity to kinetin was normal in tissue culture (Lincoln et al., 1990; Kubo and Kakimoto 2000; Sieberer et al., 2003). Another well known auxin-resistant mutant, aux1, also required higher auxin concentrations for callus initiation, and demonstrated a tendency to form roots rather than calluses on CIM (Kakani et al., 2009). Altered auxin transport in pin1 mutants resulted in the proliferation of callus-like tissues, instead of lateral roots, when exogenous auxin was applied to the roots (Benkova et al., 2003).

1.5 de novo organogenesis in plant tissue culture

Plant tissues can potentially develop into somatic embryos, shoots, and roots. The specificity of the regeneration is determined by the hormone composition of the culture medium (Schwarz et al., 2005, and references therein). Primordia originate de novo from callus cells. The cell, or
cells, that can be direct progenitors are stimulated to undergo a number of rapid cell divisions, resulting in the formation of a meristemoid. A meristemoid represents an aggregate of small, isodiametric, thin-walled, micro-vacuolated cells. Early in their development, meristemoids are capable of developing into different types of organ primordia (Schwarz et al., 2005, and references therein; Atta et al., 2009). The capabilities of plants for de novo shoot and root organogenesis are among the most important qualities required for successful micropropagation in vitro (Schwarz et al., 2005).

Organogenesis in vitro can be direct or indirect. In direct organogenesis, explants are fully competent to respond to inductive hormones, and do not require a callus proliferation phase (Schwarz et al., 2005). Recently, the capability of Arabidopsis thaliana roots and hypocotyl explants for direct shoot organogenesis has been described (Atta et al., 2009). In general, however, the explants of most plants, including Arabidopsis, can undergo organogenesis only following a callus growth phase on CIM. During this phase, it is hypothesized that genetic or developmental factors blocking the competence to respond to the inductive signals (such as inducing shooting or rooting hormone ratio in tissue culture medium) are overcome. As a result, many efforts to induce organogenesis focus on overcoming these blocks to the competence to respond to the inductive signals (Cary et al., 2002, and references therein).

According to the most commonly accepted model (Christianson and Warnick, 1985; Schwarz et al., 2005), regeneration occurs in three sequential phases (Fig. 1.3): first, when explants are cultured on CIM, they acquire competence to respond to organogenesis stimuli during the following stage (on SIM or RIM); second, the determination phase, shortly after calluses are transferred either to SIM or RIM, when the type of organogenesis (shoot or root regeneration) is determined, and, third, the organogenesis phase, during which meristems are forming, and shoots/roots emerging. The third phase, the formation of organ primordia, and organ emergence, is considered to be hormone independent (Christianson and Warnick, 1985). Ozawa et al. (1998) argued that “competence for shoot regeneration is assumed to be always acquired additively, over and above the competence for root redifferentiation”. Shown first in leaf explants of Convolvulus arvensis (Christianson and Warnick, 1983, 1984, 1985, 1988), the organogenic phases were widely confirmed for other plant species and cultivars, including pine, tobacco, apple, Arabidopsis, mangosteen, etc. (Flinn et al., 1988; Attfield and Evans, 1991; Hicks, 1994;
According to the model of Christianson and Warnick (1985), de novo regeneration occurs in three sequential phases: the first phase, the acquisition of competence, i.e. of the competence to respond to the stimuli for organogenesis during dedifferentiation on callus inducing medium (CIM); the second phase, called the determination phase, where the type of organogenesis is determined depending on the type of medium; and, the organogenesis phase, when meristems are fully developing shoots or roots are emerging. The first phase, the acquisition of competence, occurs on auxin-rich CIM. The second, the determination of either shoots or roots, requires either a cytokinin-rich shoot initiation medium (SIM), or an auxin-containing root initiation medium (RIM). The third phase, the formation of organ primordia and organ emergence, is considered to be hormone independent.

(Figure modified from Howell et al., 2003)
During in vitro organogenesis, a high cytokinin level in SIM initiates and promotes the processes of meristemoid specification, and the “canalization” of a developmental program for the formation of shoot primordia (Schwarz et al., 2005). In Arabidopsis, the origin of the shoot meristem in vitro has recently been shown to be initiated from dividing xylem pericycle cells, or from lateral root meristem-like protuberances, which are formed during culture on CIM (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010). The pericycle cells remain more meristematic than the surrounding cells, and retain the expression of cell cycle genes. In particular, the xylem pericycle cells have a higher capacity to re-enter the cell cycle, and to form new meristems when compared to the other cells (Atta et al., 2009).

Many authors have indicated that the ability for shoot regeneration differs dramatically among Arabidopsis ecotypes. In particular, the Columbia (Col) ecotype, widely used in research, has a low shoot regeneration potential (Akama et al., 1992; Cary et al., 2002; Zhao et al., 2002; Chatfield and Raizada, 2008). As a rule, Arabidopsis ecotypes more capable of shoot regeneration, such as C24, and Wasilewskija, need less time on SIM to be committed to regeneration, and to form green foci and shoots (Cary et al., 2002; Zhao et al., 2002). The regeneration potential also varies depending on the types of explants, and on explant age - partly because of the different endogenous hormone concentrations (Akama et al., 1992; Zhao et al., 2002; Gordon et al., 2007).

1.5.1 Roles of shoot apical meristem genes during de novo shoot regeneration

Several key genes, many encoding transcription factors, are known to have critical roles in the initiation and maintenance of a shoot apical meristem (SAM). Some are expressed as early as the embryonic heart stage, and usually play crucial roles in post-embryonic meristem maintenance (Aida et al., 1999; Long et al., 1996; Cary et al., 2002). The SHOOT MERISTEMLESS (STM) gene of Arabidopsis encodes a specific, knotted-like 1 (KN1-like), type homeodomain protein (Barton and Poethig, 1993; Zhang and Lemaux, 2005). The STM gene is required for SAM formation and maintenance (Long et al., 1996). It promotes cell division, and suppresses cell...
differentiation in the SAM (Lenhard et al., 2002). The WUSCHEL (WUS) gene is a member of another subtype of homeodomain transcription factors, required for specifying and maintaining a stem cell population in the SAM (Mayer et al., 1998; Zhang and Lemaux, 2005). The WUS and STM genes are activated independently, but mutations in WUS results in the loss of expression of STM and vice versa (Lenhard et al., 2002). The CLAVATA3 (CLV3) gene encodes a small protein, which appears to be a ligand for CLV1 receptor kinase (Fletcher et al., 1999). CLV1 and CLV3 restrict cell proliferation activity in SAM (Fletcher et al., 1999). The CLV3 gene is activated by WUS, and functions in a negative feedback loop that antagonizes WUS activity, and thereby controls the size of the central stem cell population (Gallois et al., 2004, and references therein). The redundant genes CUP-SHAPED COTYLEDON 1 and 2 (CUC1 and CUC2) encode transcription factors with similarities to the NO APICAL MERISTEM (NAC) protein. The latter acts in the development of embryos and flowers in petunias (Aida et al., 1997, and references therein; Takada et al., 2001). These types of proteins are known to play important roles in establishing SAM and in separating cotyledons (Aida et al., 1997, and references therein; Takada et al., 2001). Loss of function mutations in STM, WUS, or CUC1 and CUC 2 (in double mutants) block SAM formation in planta (Cary et al., 2002; Hibara et al., 2003). All four genes are also used as reporters of shoot meristem initiation in vitro (Cary et al., 2002; Zhang and Lemaux, 2005; Gordon et al., 2007). De novo shoot organogenesis can be a useful model for the study of developmental processes in the SAM (Cary et al., 2002; Che et al., 2002; Che et al., 2006; Gordon et al., 2007).

The dynamics of gene expression of STM, CUC1, CUC2, WUS, and CLV1 during shoot regeneration have been studied (Cary et al., 2002; Daimon et al., 2003). They showed that CUC1 and CUC2 were strongly expressed at 3 to 6 days on SIM, before the expression of other markers, and prior to shoot commitment, or any visible green foci or shoot meristem organization. The increase of the WUS gene expression occurred after 6 days on SIM, and gradually increased until 15 days on SIM. STM and CLV1 transcript levels rose later than those of WUS, CUC1, and CUC2, and were significantly higher only after 11 -15 days on SIM, at about the time of shoot commitment (Cary et al., 2002).

A live imaging approach enabled visualization of the expression of CUC1 and CUC2 via a GFP fluorescent marker (Cary et al., 2002). This showed that the initially broad expression during preculture on auxin rich CIM, and the first days on SIM, was progressively localized to the sites
of presumptive shoot formation at later stages, finally coinciding with green foci formation and
the development of SAMs (Cary et al., 2002; Gordon et al., 2007). The authors hypothesized that
“restriction of gene expression to the new pattern might be a critical event in shoot commitment”
(Cary et al., 2002). Similar results were obtained by another group, when they analyzed the
expression patterns of the CUC1, CUC2, and STM genes on CIM, and on SIM (Daimon et al.,
2003).

Gordon et al. (2007) studied the dynamics and patterns of expression of several major SAM
genes, such as CUC2, WUS, STM, PIN1, REV, CLV1, and FIL, as well as cytokinin (ARR5) and
auxin (DR5) reporters, during de novo SAM formation in Arabidopsis tissue culture. The authors
demonstrated that CUC2 and PIN1 were expressed within the same domains in the callus tissues
on CIM and in the promeristems on SIM. The distribution of the signals from the hormone
reporters ARR5 and DR5 showed that shoot meristems can be initiated in areas of low auxin and
high cytokinin responses (Gordon et al., 2007). At later stages of meristem development, the
DR5 signal was detected following the PIN1 reporter upregulation at the sites of future leaf
primordia formation. In contrast to DR5, the cytokinin responsive ARR5 reporter was expressed
in the areas of shoot meristem initiation, and within the developing meristems; but, was
downregulated in leaf primordia, and in the areas of callus where no shoots or root meristems
were formed (Gordon et al., 2007). Based on the results, Gordon et al. (2007) suggested that de
novo shoot organogenesis can be broken down into distinct events: callus induction, cytokinin-
induced specification of cell identity within the callus, radial patterning within shoot progenitors,
and meristem morphogenesis. They hypothesized that the non-homogenous distribution of
cytokinin and auxin may be a key factor for the partition of the cell identity within a callus on
SIM. They also hypothesized that the hormone gradient must be gradually reorganized from the
disrupted initial conditions, during shoot induction in culture (Gordon et al., 2007).

In summary, the expression of key meristem genes during de novo SAM formation occurs during
late CIM (4 days) or early SIM (3-6 days) incubation times in Arabidopsis, which appears to be
related to the acquisition of competence on CIM, and to the shoot commitment stage during the
first days on SIM (Che et al., 2002; Che et al., 2006; Christianson and Warnick, 1985; Gordon et
al., 2007).
1.5.2 Mutations affecting shoot regeneration in Arabidopsis

Cytokinins mediate de novo shoot regeneration in tissue culture (Skoog and Miller 1957; Gaba, 2005). Accumulating evidence supports the idea of molecular interactions between cytokinin signaling, the cell cycle, and shoot meristem developmental pathways (Riou-Khamlichi et al., 1999; Sieberer et al., 2003; Zhang and Lemaux, 2004; Zhang and Lemaux, 2005). Key SAM genes, and the genes of cytokinin signaling are among the primary candidates for study in tissue culture to determine their possible effects on the efficiency of shoot regeneration in vitro (Zhang and Lemaux, 2004; Zhang and Lemaux, 2005). Among the genes involved in meristem initiation and maintenance, CUC1, CUC2, STM, WUS, ENHANCER OF SHOOT REGENERATION 1 and 2 (ESR1, and ESR2) showed a significant effect on shoot regeneration. Typically, mutations in these genes affect shoot meristem initiation, but not the callus initiation stages (Barton and Poethig, 1993; Aida et al., 1997; Banno et al., 2001; Daimon et al., 2003; Gordon et al., 2007). An elevation of endogenous cytokinin levels generally results in increased shoot regeneration in cytokinin overproducing mutants, and often in shoot formation on cytokinin-free or hormone-free media (Chaudhury et al., 1993; Kunkel et al., 1999; Frank et al., 2000; Kakimoto, 2001; Catterou et al., 2002; Zubko et al., 2002; Sun et al., 2003; Zhang and Lemaux, 2004; Zhang and Lemaux, 2005). However, genes belonging to other signaling pathways can be implicated in shoot regeneration as well, and some of them will be discussed in the following sections.

1.5.3 Mutations in auxin signaling genes affecting shoot regeneration

Although SIM contains auxin (Skoog and Muller, 1957), relatively limited information is available about the direct effects of auxin, and auxin signaling genes on shoot regeneration in tissue culture. Typically, auxin signaling genes are not among those reviewed in the context of shoot regeneration in vitro (Zuo et al., 2002b; Howell, 2003; Zhang and Lemaux, 2004; Zhang and Lemaux, 2005). Nevertheless, auxin response marker levels are highly elevated in shoot meristems in planta (Kakani et al., 2009). Evidence of the effects of mutations in auxin signaling genes on shoot regeneration has been demonstrated (Coenen and Lomax, 1998; Chatfield and Raizada, 2008; Kakani et al., 2009). Also, many auxin signaling genes are implicated in cross talk with other signaling pathways (Hobbie and Estelle, 1994). In addition, impairment of auxin genes can affect coordinated interaction among multiple developmental processes (Gordon et al., 2007; Kakani et al., 2009).
For example, the *AUXIN RESISTANT1 (AUX1)* gene encodes a permease-like membrane protein, facilitating auxin influx into plant cells. Loss of function *aux1* mutants result in resistance to auxin, ethylene, and cytokinin (Hobbie and Estelle, 1994). The *AUX1* gene is highly expressed within the SAM, in the root tips of plants (Swarup et al., 2004), and in callus culture (Kakani et al., 2009). Remarkably, *aux1* LOF mutants regenerated roots instead of shoots on SIM (Kakani et al., 2009).

Loss of the *PIN1* gene function, encoding the auxin efflux protein and facilitating polar auxin transport, resulted in a moderate decrease of shoot regeneration (Gordon et al., 2007). However, the *PIN* gene family consists of eight members with partly redundant functions. Therefore an effect on shoot regeneration could be more prominent if multiple *PIN* mutations were tested in tissue culture.

The *ALTERED AUXIN RESPONSE (AXR1)* gene encodes a subunit of the protein RUB1 which is required for auxin-dependent ubiquitin-mediated degradation of Aux/IAA proteins (Gray et al., 2001; del Pozo et al., 2002). The *AXR1* gene is highly expressed in growing cells throughout plant organs. Loss of *AXR1* function results in decreased auxin responses, auxin resistance in all plant organs, reduced apical dominance, fewer lateral roots and root hairs, low fertility, impaired callusogenesis, and abnormal expression of the *Aux/IAA* and *SAUR* genes (Lincoln et al., 1990; del Pozo et al., 2002). Cross-resistance to cytokinin was also reported for *axr1* mutants (Hobbie and Estelle, 1994). Remarkably, the *axr1* mutants were dramatically impaired in shoot regeneration, which was abolished in strong *axr1* alleles, and reduced to only 2-3% in weaker alleles (Chatfield and Raizada, 2008). Similar to *aux1* mutants, *axr1* mutants also developed roots rather than shoots on SIM (Chatfield and Raizada, 2008).

Constitutively auxin-overproducing *superroot1 (sur1)* explants demonstrated auxin-autonomous growth, proliferation, and root formation on a hormone free medium. Unlike the wild type, they were capable of shoot regeneration on a medium with cytokinin as the sole hormone (Boerjan et al., 1995). In contrast, *superroot2 (sur2)* explants, which also demonstrated elevated auxin levels, failed to produce shoots on a cytokinin containing medium (Delarue et al., 1998). The authors discussed the difference between the *sur1* and *sur2* phenotypes. They proposed that, while *sur1* constantly overproduces auxin, *sur2* accumulates auxin as a result of an impaired mechanism of auxin conjugation, and possibly of impaired auxin transport (Delarue et al., 1998).
1.6 Regeneration of adventitious roots *in vitro*

In tissue culture, regeneration of adventitious roots occurs in response to high auxin and low cytokinin levels, or when auxin is the sole hormone in the root inducing medium (RIM) (Miller and Skoog, 1957; Gaba, 2005). Rooting is often a critical step in plant propagation, because many species are extremely recalcitrant to adventitious root formation (de Klerk, 2002; Schwarz et al., 2005). Although roots have been routinely induced in response to auxin in plant propagation (Callis, 2005; Schwarz et al., 2005), the molecular mechanisms of this event are not completely clear (Schwarz et al., 2005; Rose et al., 2006).

Three temperature-sensitive mutants of *Arabidopsis*, shoot redifferentiation 1, 2, and 3 (*srd1, srd2*, and *srd3*), defective in shoot regeneration, were isolated as tools for the study of organogenesis (Yasutani et al., 1994). Genetic analysis indicated that mutations resulted from single, nuclear, recessive mutations in three different genes located on chromosome 1, designated *SRD1*, *SRD2*, and *SRD3*. Products of these *SRD* genes function at different stages of shoot regeneration (Ozawa et al., 1998). Using *srd1, srd2, srd3* mutants, Ozawa et al. (1998) were able to break down more precisely the time frame for acquiring organogenesis competence in *Arabidopsis* calluses. They demonstrated that the competence to form organs is acquired sequentially: first roots, and then shoots. They also showed that competence to form roots depends on the type of explant. While hypocotyl explants required CIM preincubation before transfer to RIM, no CIM stage was required for root explants (Ozawa et al., 1998).

Histological examination of adventitious root formation was performed on leaf explants in *Medicago truncatula* (Rose et al., 2006), and in tobacco (Attfield and Evans, 1991). In both studies, root primordia were formed from procambial cells in veins, which function as pluripotent stem cells, with the ability to form either root primordia or vascular tissues in response to added auxin (Attfield and Evans, 1991; Rose et al., 2006). Rose et al. (2006) concluded that pools of stem cells exist in vascular tissues, which - in combination with auxin and other factors, such as ethylene and other hormones--drive the diversity of plant developmental responses.

Cytokinin inhibits root formation *in planta* (Aloni et al., 2006; Riefler et al., 2006; Kyozuka, 2007), and *in vitro* (Sugiyama, 1999; Kubo and Kakimoto, 2000). Excessive development of roots on explants cultured on cytokinin containing media can be attributed either to a low
cytokinin to auxin ratio, to an impairment of cytokinin signaling, or to a low cytokinin sensitivity (Kubo and Kakimoto, 2000; Kakani et al., 2009). When cytokinin responses were elevated, opposite traits were observed. Such explants typically failed to produce roots, as upon overexpression of the CYTOKININ-INDEPENDENT1 (CKII) gene or the ISOPENTENYLTRANSFERASE (IPT) gene, which mediate cytokinin responses (CKII) and cytokinin biosynthesis (IPT) (Kakimoto, 1996; Kunkel et al., 1999; Kakimoto, 2001; Sun et al., 2003).

Auxin promotes the initiation of root regeneration, but the growth of already formed root primordia is largely a hormone independent process (Christianson and Warnick, 1985; reviewed in Schwarz et al., 2005). Auxin overproducing explants of sur1 mutants were capable of root initiation on a hormone free medium (Boerjan et al., 1995). Also, Frank et al. (2000) isolated several lines of autotrophic calluses producing roots on a hormone free medium. A molecular analysis of these lines demonstrated that these rooting calluses had either auxin overproduction, or had increased levels of Aux/IAA genes, such as IAA1, IAA2, or IAA9 (Frank et al., 2000). The authors suggested that loss-of-function mutations of auxin response repressors of the AUX/IAA family can result in a constitutive auxin response, and root overproduction (Frank et al., 2000). Auxin insensitivity often causes impairment in root regeneration (Sugiyama, 1999, and references therein). Thus, auxin-tolerant rac mutants in tobacco are defective in primary root formation, and regeneration of adventitious roots. RAC can be involved in the auxin transduction pathway, modifying the levels of auxin sensitivity required for adventitious root formation (Sugiyama, 1999, and references therein).

Che et al. (2006) demonstrated - using the gene profiling method - that molecular signatures for callus formation and root regeneration are quite similar, because both represent developmental processes induced by an auxin-rich medium. Competence for root regeneration correlated with an increased expression of IAA genes on CIM. Many IAA genes were downregulated shortly after transfer on SIM, at the same time as loss of root commitment occurred (Cary et al., 2002; Che et al., 2006). The main difference between callusogenesis and root formation was in the expression of root specific genes associated with the formation of the cell wall, and with vascular development (Che et al., 2006).
The similarity of callus induction and root development pathways was also reflected in auxin deficient root initiation defective (rid1 and rid2) mutants, which were impaired in both callus and root formation (Konishi and Sugiyama, 2003). However, one of the mutants, rid3, was specifically affected only in adventitious root growth, but not callus formation (Konishi and Sugiyama, 2003). Interestingly, the rid3 mutants were also significantly affected in shoot initiation, and in the expression of some SAM genes. This demonstrated that the regeneration of adventitious roots and shoots in culture can also share partly overlapping developmental pathways, presumably at the stages of acquiring competence, and of promeristemoid formation (Christian and Warnick, 1985; Schwarz et al., 2005; Tamaki et al., 2009). Recently, it has been shown that lateral root meristem (LRM)-like structures are formed during CIM incubation; when calluses are transferred on RIM or SIM, these LRM-like primordia convert to either root or shoot meristems (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010). The enhanced adventitious root (on RIM) and shoot (on SIM) regeneration in increased organ regeneration 1(ire1) mutants also supports the model of shared developmental pathways during root and shoot regeneration (Cary et al., 2001).

1.7 General Research Objectives

In this study, the two related ARFs, MP/ARF5 and NPH4/ARF7, were investigated with regard to their roles in callusogenesis and de novo organogeneses (shoot and root formation). Mutations in the MP/ARF5 gene result in the formation of rootless seedlings (Mayer et al., 1991; Berleth and Jurgens, 1993), and severely reduced auxin sensitivity and auxin transport (Przemeck et al., 1996; Mattsson et al., 2003; Li et al., 2006; Schuetz et al., 2008). Moreover, MP is upstream of several meristematic genes, such as CUC and STM, during embryonic meristem formation (Aida et al., 2002); and, of the ESR1/DRN gene during cotyledon development (Cole et al., 2009). These genes are also known to be required for shoot regeneration in vitro (Barton and Poethig, 1993; Banno et al., 2001; Daimon et al., 2003; Tamaki et al., 2009). Thus, MP/ARF5 may possibly be involved in de novo organogenesis. NPH4/ARF7 plays a central role in the modulation of auxin-dependent differential growth (Stowe-Evans et al., 1998; Watahiki et al., 1999; Hardtke et al., 2004). nph4 mutants exhibit severely reduced auxin sensitivity, severely impaired expression of many primary auxin dependent genes, but they have normal responses to other hormones, including cytokinin (Stowe-Evans et al., 1998; Okushima et al., 2005; Wang et al., 2005).
The general research objectives addressed in this thesis are therefore:

1) To explore the effect of altered auxin signaling on callusogenesis, and shoot and root regeneration efficiency *in vitro*;

2) To investigate the impact of explant organ origin on callusogenesis and organogenesis responses in the genotypes of *Arabidopsis thaliana* with altered auxin signaling;

Each objective is investigated for both loss- and gain-of-function alleles of specific ARFs:

a) LOF mutations in *MP/ARF5* (*mpG12*) and *NPH4/ARF7* (*nph4-1*);

b) *MP* GOF transgene *dMP*, which should no longer be regulated by AUX/IAA proteins (Fig. 1.2; Krogan, 2006)
Chapter 2
Materials and Methods

2.1 Plant material

All genotypes used in this research were in the Columbia (Col-0) background of *Arabidopsis thaliana*. The severe allele *mpG12*, induced by gamma-ray mutagenesis (Hardtke and Berleth, 1998) was used as a *MP* LOF mutant. The *dMP* transgene line, generated by Krogan (2006), was used in the experiments as a GOF mutation in the *MP* gene. The *dMP* line was obtained as a result of the insertion into the Col-0 background of a truncated copy of the *MP* gene, which lacks regulatory and dimerization domains III and IV (Krogan, 2006). For the *nph4* LOF allele, I used the presumed null allele *nph4-1*, generated by fast neutron mutagenesis (Harper et al., 2000). For all mutant alleles and the *dMP* transgene line only homozygous genotypes were used for the experiments. Some characteristics of the genotypes are represented in Table 2.1; Fig. 2.1.

2.2 General growth conditions

For the experiments and seed propagation, all seeds were dry-sterilized. For this procedure, a small amount of seeds was placed inside open Eppendorf tubes into an excicator. Sterilization of the seeds occurred by chlorine gas produced as a result of the reaction when 3 ml of concentrated hydrochloric acid were added to 100 ml of a 3% bleach solution inside the closed excicator. After 4 hours of sterilization in chlorine gas, the seeds were aseptically transferred into a sterile laminar box and sown on tissue culture dishes (100x20 mm, Sarstedt, USA) containing 0.5X Murashige and Skoog (MS) salts (Sigma, USA), 0.5mg/L morpholino ethane sulfonic acid (MES), 1X Gamborg’s vitamin solution, 1.5% (w/v) sucrose, pH 5.7 (KOH), solidified by 4-5 g/L Bacto-agar (BioShop, Canada) for the purpose of seed propagation, or solidified by 2.4 g/L Phytogel (Sigma) for the tissue culture experiments. The density of the seeds was about 1-2 seeds per 1 cm². For seed stratification, plates with seeds were kept in the dark in a refrigerator at 4°C for 4 days. After stratification, the seeds were germinated and grown at 24-26°C under constant light (100μEm⁻²sec⁻¹). “Days after germination” (DAG) are defined as the number of days the seeds were cultivated in light conditions after stratification.
Table 2.1: Main characteristics of the mutant genotypes, *mpG12*, *dMP*, and *nph4-1*

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Type of mutation, and the strength of the allele</th>
<th>Type of mutagenesis</th>
<th>Character of molecular changes in the mutated gene sequence</th>
<th>Phenotype in homozygous seedlings in normal condition</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. mpG12</td>
<td>recessive, LOF, presumed null allele</td>
<td>gamma-ray mutagenesis</td>
<td>the molecular lesion in the allele <em>G12</em> has not been fully characterized. It is associated with multiple RFLPs</td>
<td>rootless seedlings, reduced vasculature, pin like inflorescences</td>
<td>Hardtke and Berleth, 1998; Hardtke et al., 2004</td>
</tr>
<tr>
<td>2. dMP</td>
<td>dominant, GOF insertion of transgene</td>
<td>insertion of transgene</td>
<td><em>dMP</em> transgene express the truncated MP protein lacking the C-terminal part from the middle of domain III (absence of dimerization domains III and IV presumably prevent the Aux/IAA and ARF proteins binding the following up or down regulation of dMP protein function)</td>
<td>elongated cotyledons and narrow shape of leaves, increased vasculature, infertile flowers</td>
<td>Krogan, 2006</td>
</tr>
<tr>
<td>3. nph4-1</td>
<td>recessive, LOF, presumed null allele</td>
<td>mutagenesis by fast neutrons</td>
<td>A alteration of the coding sequence as a result of the combination of inversion with internal deletion between exons 11 and 12</td>
<td>epinastic or hyponastic rosette leaves in seedlings</td>
<td>Stowe-Evans et al., 1998; Harper et al., 2000</td>
</tr>
</tbody>
</table>
Figure 2.1 Seedling (A-D), and inflorescence (E-G) phenotypes in the LOF mutants *nph4-1* (B), *mpG12* (D, F), and the GOF transgene line *dMP* (homozygous) (C, G) compared to wild type (A, E). Genotypes as indicated on top right.

(Modified from Krogan, 2006; Hardtke et al., 2004)
For the propagation of the dMP transgene line, dMP seeds were sown on ½ MS medium without sucrose, supplemented with 10 μM Basta. This allowed selective growth of homozygous and heterozygous dMP seedlings, while wild type seedlings were eliminated by Basta. The dMP homozygous seedlings can be distinguished from heterozygous dMP seedlings based on their elongated cotyledons and narrowed first leaves (Table 2.1; Fig. 2.1). Only heterozygous dMP plants are fertile and can be used for propagation; and only homozygous dMP seedlings were used for the experiments. Seedlings intended for propagation were transferred into Promix BX soil at 12 DAG, and grown under fluorescent light (100 μE/m²/s²) in an 8-hour dark cycle, at 22°C.

2.3 Hormone stocks

Several auxin and cytokinin plant hormones were used for the experiments. The auxins 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma), indole-3-butyric acid (IBA, Sigma), and 3-indoleacetic acid (IAA, Sigma) were dissolved in liquid dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ml. Cytokinin stock solutions, such as kinetin (Sigma) and 6-(gamma,gamma-Dimethylallylamino)purine (2-iP, Sigma), were used as commercially prepared (Sigma) hormone solutions, dissolved in water (sterile filtered, concentration 1 mg/ml). All stock hormone solutions were stored at -20°C.

2.4 Plant DNA extraction

A crude, rapid DNA isolation was done to collect templates for PCR analyses and genotyping. For crude DNA isolation a small rosette leaf or a small piece of tissue culture was flash-frozen in liquid nitrogen, and ground in a 1.5 ml Eppendorf tube with a micropestle on ice. Then 500 μL of extraction buffer [250mM Tris-HCl, 250mM NaCl, 25mM EDTA, 0.5% (w/v) SDS] were added, and the tube was vortexed for 5 sec. To discharge debris the tube was centrifuged for 1 minute in a table-top centrifuge at 14000 rpm/RT. The supernatant was transferred into a new Eppendorf tube and 300 μl of Isopropanol was added to 300 μl of supernatant, vortexed, and left to sit for 2 minutes at RT. Then the mixture was centrifuged at 14000 rpm for 5 minutes at RT, and the supernatant was discarded. The pellets were washed once with 750 μL of ice-cold 70% (v/v) ethanol, air-dried, and resuspended with a pipette tip in 30 μL of water. The mix was briefly centrifuged one more time at 14000 rpm/RT for 1 minute, and the supernatant was carefully
removed and stored at -20°C. 1μL of crude DNA extract was used as a template per 25μL or 50μL of PCR mix.

2.5 General procedure for polymerase chain reaction (PCR)

PCRs were performed in Biometra UNO-Thermoblock (Germany). The PCR mix (25μL total volume) contained 1 μl of DNA template, 2.5 μL Taq buffer (supplied with polymerase), 2.5 μL of MgCl₂, 2.5 μL of each primer (Sigma-Genosys), 2.5 μL of dNTPs (Fermentas), and Taq DNA polymerase 1 unit. Double distilled autoclaved water was added to a total volume of 25 μL was reached, and then all was overlaid with light mineral oil. The general thermocycling regime was performed as follows: 94°C for 90s, 40 cycles of (94°C for 30s, 55°C for 30s, 72°C for 2min), 72°C 10min; hold at 4°C.

2.6 Genotyping

Several screening methods can be applied to distinguish between plant samples containing dMP insertion (homogenous or heterogeneous dMP genotypes) and those of wild type. First, only the seeds containing the dMP transgene (homogenous or heterogeneous dMP seedlings) can survive on Basta plates, while wild type seedlings will not germinate, or will die soon after germination (see Section 2.2, General growth conditions). The homogenous and heterogeneous dMP seedlings can be distinguished based on a difference in their phenotypes, where homozygous dMP seedlings had elongated cotyledons and narrowed first leaves (Table 2.1; Fig. 2.1).

To differentiate DNA samples containing dMP (homo or heterozygous insertion) from wild type the PCR primers for the Basta resistance gene (Bar) were designed. The primers were designed at the beginning and the end of the Bar gene and gave a PCR product of 403bp. The primer sequences are:

BST-3-F: 5’-GGTCTGCACCATCGTCAACCACA-3’
BST-3-R: 5’- CCAGTTCCGTGCTTGAAAGCC-3’

The thermocycling regime was performed as follows: 94°C for 2min, 35 cycles of (94°C for 30s, 62°C for 30s, 72°C for 1 min), 72°C for 5 min; hold at 4°C.
Homogenous mpG12 seedlings do not form primary roots and hypocotyls (Table 2.1; Fig. 2.1). This phenotypic trait allows clear distinction of the homogenous mpG12 seedlings from the heterogeneous (MP/mpG12) or wild type. Additionally, PCR diagnostics were performed on the genomic DNA to verify a genotypic identity of individual samples (plant or tissue culture origin). To distinguish between mpG12 homozygous samples and those with at least one wild type MP copy, primers BS1354-F (5’-GAGATGGCCTGGTTCTAAGTGGC-3’; corresponding to a central portion of MP ORF) and BS1354-R (5’-GCCAGTTCAACATCTCGGTTATCG-3’; corresponding to a region of MP 3’-UTR) were used in diagnostic PCR reactions. The presence of at least one wild type MP gene copy will support amplification of a 2660bp product. In homozygous mpG12 samples there is no amplification of any PCR products. The thermocycling regime was performed as follows: 94°C for 90s, 45 cycles of (94°C for 30s, 60°C for 30s, 72°C for 3min), 72°C 10min; hold at 4°C.

Genotyping of nph4 homozygous individuals was based on PCR diagnostics of plant and tissue culture samples. To distinguish between nph4-1 homozygous individuals and those with at least one wild type NPH4 copy, primers NPH4.5 (5’- TCCTGCTGAGTTTGTGGTTCCTT -3’) and NPH4.6 (5’- GGGGCTTGCTGATTCTGTTTGTTA -3’) were used in diagnostic PCR reactions. The presence of at least one wild type NPH4 gene copy supports amplification of a 823bp product, whereas the genomic DNA of nph4-1 homozygous individuals does not support amplification of any PCR products. The thermocycling regime was performed as follows: 94°C for 90s, 45 cycles of (94°C for 30s, 63°C for 30s, 72°C for 2min), 72°C 10min; hold at 4°C.

2.7 Microtechniques and microscopy

To introduce explants in tissue culture, seedlings were dissected under a Hund Wetzlar stereomicroscope (GmbH, Germany) in aseptic conditions in a flowhood. Samples were viewed under dark field illumination with a Leica MZFLIII stereomicroscope (Leica Microsystem, Wetzlar, Germany), equipped with a Canon EOS D60 digital camera (Canon inc., Tokyo, Japan). All images were assembled using Adobe Photoshoop 7.0 (Adobe Systems, Mountain View, CA, USA). The fresh weight of the samples was measured using Sartorius analytical balances (Sartorius, GmbH, Germany).
2.8 Plant tissue culture experiments

Cotyledons, leaves, petioles, hypocotyls, and adventitious roots were used as explants in tissue culture experiments (Table 2.2). Because *mpG12* seedlings lack hypocotyls and primary roots, no *mpG12* hypocotyl explants could be tested. To obtain root explants in *mpG12* seedlings, a special rooting procedure was applied to *mpG12* and the rest of the genotypes to obtain adventitious roots (see Section 2.8.1 for more details). The main steps of the tissue culture experiments are summarized in Fig. 2.2.

At least three independent replicas of the experiment were taken unless otherwise stated. Student’s *t*-test was used to evaluate the significance of the comparison between the mean responses, such as fresh weight of calluses, number of shoots or roots per callus, etc. For the purposes of this research, each of the evaluated genotypes was compared against wild type via Student’s *t*-test (Microsoft Excel, 2003).

2.8.1 Rooting of dissected *mpG12*, *dMP*, *nph4-1* and wild type seedlings

Seeds of *mpG12*, *nph4-1*, *dMP*, and wild type were germinated as described above (see for details Section 2.2, General growth conditions). 5-6 DAG seedlings were dissected under a Hund Wetzlar stereomicroscope (GmbH, Germany) in aseptic conditions. In rootless *mpG12* seedlings, peg parts (short hypocotyl-like outgrowths below the cotyledon node) were cut with a sharp scalpel. Seedlings of other genotypes were similarly dissected by cutting off the roots with a part of the hypocotyl. Dissected seedlings were placed in tissue culture dishes (100x20 mm, Sarstedt, USA) with root inducing medium (RIM), containing 1 mg/L IBA (Table 2.3), and cultured at 24-26°C under constant light (100μEm⁻²sec⁻¹). After 7-9 days on RIM, about 5-10% of the *mpG12* dissected seedlings, and most of the seedlings of the other genotypes formed adventitious roots. Rooted seedlings were transferred on hormone-free ½ MS medium, to restore normal root morphology; and, after 14 days of culture, they were dissected and their adventitious roots were used for tissue culture experiments.
Figure 2.2 General scheme of the tissue culture experiments and scored traits, unless otherwise specified in the description of the particular experiment.
2.8.2 Preparation of explants for tissue culture

For the tissue culture procedure, seedlings of a particular age (Table 2.2) were aseptically dissected into explants under a Hund Wetzlar stereomicroscope (GmbH, Germany) in aseptic conditions. The types of explants, the genotypes, and the age of the seedlings at the time of explant dissection (DAG) are summarized in Table 2.2.

Each culture dish (100x20 mm, Sarstedt, USA) was divided into sections corresponding to the genotypes, and explants of each genotype were cultured together. About 18-20 explants per culture dish were used during callus initiation on CIM, 12 explants per culture dish were used for shoot initiation on SIM, and about 16 explants per dish were used for root initiation on RIM. The culture dishes were sealed with Parafilm (Parafilm® M, USA). Tissue culture was maintained under fluorescent light (100 μE/m²/s²), at 24-25°C.

Table 2.2. Types of explants, genotypes, approximate sizes, and DAG at the moment of dissection and introduction in tissue culture.

<table>
<thead>
<tr>
<th>Explants</th>
<th>Cotyledons</th>
<th>Leaves</th>
<th>Petioles</th>
<th>Hypocotyls</th>
<th>Adventitious roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (DAG)</td>
<td>8</td>
<td>11</td>
<td>11</td>
<td>9</td>
<td>26-28</td>
</tr>
<tr>
<td>Size (mm)</td>
<td>4-5</td>
<td>4-5</td>
<td>3-5</td>
<td>4-6</td>
<td>5-7</td>
</tr>
<tr>
<td>Genotypes</td>
<td><em>mpG12,</em></td>
<td><em>mpG12,</em></td>
<td><em>mpG12,</em></td>
<td><em>nph4-1,</em></td>
<td><em>mpG12,</em></td>
</tr>
<tr>
<td></td>
<td><em>nph4-1,</em></td>
<td><em>nph4-1,</em></td>
<td><em>dMP,</em></td>
<td><em>dMP,</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>dMP,</em></td>
<td><em>dMP,</em></td>
<td><em>dMP,</em></td>
<td>*wt (Col-0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>*wt (Col-0)</td>
<td>*wt (Col-0)</td>
<td>*wt (Col-0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For shoot and root regeneration experiments, explants were precultured for 6 days on CIM and then transferred on SIM or RIM. For callusogenesis experiments explants were left on CIM for 6 more days (12 days in total). Results of shoot or root regeneration were scored after 21 days on SIM or RIM. For these purposes explants were screened directly in tissue culture dishes under a Hund Wetzlar stereomicroscope (GmbH, Germany) or under a dark field illumination with a Leica MZFLIII stereomicroscope (Leica Microsystem, Wetzlar, Germany), equipped with a
Canon EOS D60 digital camera (Canon inc., Tokyo, Japan). The schema of the experiments is shown in Fig. 2.2.

2.8.3 Preparation of tissue culture media

The tissue culture procedure followed the protocol suggested by Valvekens et al., (1988). Following the protocol, Gamborg B5 (B5) salt base (Sigma, USA) was used for the preparation of CIM and SIM. For both media, CIM and SIM, B5 salt base was supplemented with 2% sucrose, 0.5 g/L MES, 1x Gamborg’s vitamin solution (Sigma, USA), and pH 5.7 (KOH) before autoclaving. The hormone compositions varied depending on the type of the tissue culture medium (Table 2.3). The media were solidified with 2.4 g/L Phytogel (Sigma) and sterilized for 20 min at 121°C and 15 psi in the autoclave (Hirayama, Japan). The liquid hot medium was poured into tissue culture dishes (about 20 ml/dish) and stored at -4°C till the time of the experiment.

Table 2.3. The hormone composition of tissue culture media: CIM, SIM, and RIM.

<table>
<thead>
<tr>
<th></th>
<th>CIM</th>
<th>SIM</th>
<th>RIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>0.5 mg/L</td>
<td>IAA - 0.15 mg/L</td>
<td>IBA - 1 mg/L</td>
</tr>
<tr>
<td>Kinetin</td>
<td>0.05 mg/L</td>
<td>2-IP - 5 mg/L</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3
Callusogenesis under conditions of altered auxin signal transduction

3.1 Background and rationale

Callus proliferation is induced in vitro at a certain relative concentration of auxin and cytokinin in the culture medium (Skoog and Miller 1957). Cell proliferation during callusogenesis leads to disruption of the ordered morphology of differentiated tissues (Cary et al., 2002). The mechanisms leading to callus proliferation are not well understood (Cary et al., 2002; Zhang and Lemaux, 2005). Generally, cell-cycle genes, such as cyclins and cyclin dependent kinases (CDKs), play a major role in the mediation of hormonal effects, or in the response to wounding signals, leading to cell proliferation (Hemerly et al., 1993; Soni et al., 1995; Shaul et al., 1996; Riou-Khamlichi et al., 1999; Sugiyama 1999; Richard et al., 2002; Che et al., 2007). Cis-acting regulatory elements, specific for the binding of hormone transcription factors, such as AuxREs for auxin signaling, have been found in the promoters of some cell cycle genes (Richard et al., 2002; Perrot-Rechenmann, 2010).

Auxin responses, visualized by DR5 and PIN1 driven reporters (reviewed in Chapter 1.2), were strongly increased during the initiation of callus proliferation. Then they were downregulated after 5 days on CIM (callus inducing medium). After 10 days on CIM, DR5 and PIN1 reporters were not detectable in the callus culture (Gordon et al., 2007). Among the other reporters associated with proliferating callus growth, are the meristem genes CUC1 and CUC2, and the ARR5 gene, which is involved in cytokinin signaling (reviewed in Chapter 1.4; Cary et al., 2002; Gordon et al., 2007). The expression of the corresponding reporter genes was observed after 8 days on CIM, and continuously increased during CIM cultivation (Gordon et al., 2007). When callusogenesis was induced on the medium supplemented with auxin as the sole hormone, CUC2 and ARR5 reporters were still expressed. In contrast, only weak callus proliferation occurred, and no expression of the callus proliferation reporters, CUC1, CUC2 and ARR5, was observed, when the explants were cultured on the medium supplemented with cytokinin only (Gordon et al.,
This observation emphasizes the importance for callus proliferation of the presence of auxin, rather than cytokinin, in the culture medium.

As described in Chapter 1 (1.2.5), the auxin response factors MP (ARF5) and NPH4 (ARF7) belong to the group of the transcriptional activators whose potency is enhanced in the presence of auxin (Ulmasov et al., 1999a; Ulmasov et al., 1999b; Berleth et al., 2004). Among other described defects (Hardtke et al., 2004), the loss of MP or NPH4 function severely reduces auxin sensitivity. The expression of the auxin-responsive reporter DR5 and auxin-inducible genes was significantly reduced in nph4 and mp mutants (Stowe-Evans et al., 1998; Watahiki et al., 1999; Hardtke et al., 2004; Li et al. 2006; Przemeck et al., 1996; Mattsson et al., 2003; Okushima et al., 2005; Wang et al., 2005; Schuetz et al., 2008).

The NPH4 gene is expressed throughout the Arabidopsis life cycle, and NPH4 transcripts are generally present in all major organs (Hardtke et al., 2004). The MP gene is expressed in broad domains in embryos and in emerging organs. Later, upon organ maturation, the expression of MP is gradually narrowed to central domains and then to vascular tissues (Hardtke and Berleth, 1998; Hardtke et al., 2004). The mRNAs of both genes, MP and NPH4, were found to be ubiquitous and uniformly expressed in Arabidopsis cells in suspension culture (Ulmasov et al., 1999b; reviewed in Chapter 1.2.4). The genotype dMP generated by the deletion of protein interaction domains in the MP gene is described in the General Introduction (Chapter 1.2.5; Fig. 1.2; Krogan, 2006). As these interaction domains are required to bind negative regulators of ARF function, it seems likely that dMP represents a constitutively active MP gene.

In summary, the MP and NPH4 genes play important roles during many auxin-triggered processes in plant development. Because callus proliferation is triggered by the hormone auxin, changes in MP and NPH4 function in the loss-of-function mutants may have an impact on callusogenesis.

3.2 Specific research objectives

The investigation of the possible influence of altered auxin signaling through LOF and GOF alleles of ARF5/MP and ARF7/NPH4 on callusogenesis was the purpose of this part of the project. To this end, the loss-of-function mutants mpG12 (Hardtke and Berleth, 1998), and nph4-1 (Harper et al., 2000), and the gain-of-function transgene line dMP (Krogan, 2006) with
truncated dimerization domains III and IV (reviewed in Chapter 1.2.5; Fig. 1.2), were tested to determine the influence of these genotypes on callus formation in vitro. For a comprehensive comparison of callus proliferation among the genotypes, different types of explants (cotyledons, leaves, petioles, hypocotyls, and adventitious roots) were cultured on CIM for 12 days (for details see Material and Methods, Chapter 2.8). Afterward, the patterns of callusogenesis and the fresh weights of the calluses were scored. The frequencies of the “whole” type of calluses, and the fresh weights of the calluses (mean weights of calluses ± SE) were determined based on the pooled data of all replicas of the experiments. All genotypes used in the experiments were in the Col-0 background and were compared to the Col-0 wild type. Explants of approximately similar size and age (DAG) were used for the callusogenesis experiments. This minimized the variations in the fresh weights of the calluses on CIM (for details see Chapter 2.8.2; Table 2.2).

3.3 Results

3.3.1 Types of calluses on CIM

Callus proliferation on CIM depended on the genotype, and on the type of organ used as explant (Fig. 3.1). When aerial explants were compared, two major types of calluses were detected after 12 days on CIM. The first type was characterized by even callus proliferation across the whole explant surface (Fig. 3.2; 3.3). As a result, this pattern of callusogenesis was called the “whole” type. In the second type, callus proliferation was limited to some patches on the explant surface, with some areas free of callus proliferation (Fig. 3.2; 3.3). Often, in the latter type of calluses, cell proliferation occurred mainly near the wounded areas (cut parts) of the explants, with or without a combination of local patches along the explant surface. Because of the localized character of the callus proliferation, this second type of callus was called the “patchy” type. No noticeable difference in the callusogenesis patterns was observed in the adventitious root explants after 12 days on CIM. The adventitious root explants were relatively evenly covered by callus cells along their axes (similar to the “whole” type described for callusogenesis in aerial organs) (Fig. 3.4).
Figure 3.1. Fresh weight of calluses (mean numbers ± SE) (A), and percentage of the “whole” type of calluses (B) after 12 days on CIM; n, total number of explants; bars represent SE.
Figure 3.2 Callus initiation and callus proliferation in cotyledon (A-D) and leaf (E-H) explants after 6 (A, C, E, G)) and 12 (B, D, F, H) days on CIM. Origin of explant as shown on top right; days on CIM and types of callus growth (“whole” versus “patchy”) as indicated, bottom right; arrows indicate sites of callus proliferation
3.3.2 Callusogenesis of cotyledon explants after 12 days on CIM

Callus proliferation, indicated by the fresh weights of the cotyledon derived calluses, differed among the genotypes. The fresh weights of the dMP calluses were similar to those of the wild type calluses (the wild type calluses 52±1.4 mg, n=137; dMP 52±1.5 mg, n=133) (Fig. 3.1 A). In contrast, the fresh weights of the nph4-1 and the mpG12 calluses were significantly less than those of the wild type calluses (nph4-1 13±6 mg, n=135, P<0.001; and mpG12 16±6 g, n=133, P<0.001) (Fig. 3.1 A). Thus, there were two distinct phenotype classes: the dMP calluses growing like the wild type ones versus the strongly impaired callus growth in nph4-1 and mpG12 (Fig. 3.1 A).

The distribution of the callusogenesis types (the “whole” versus the “patchy” types) in the cotyledon explants also dramatically differed among the genotypes (Fig. 3.1 B; 3.5 A). All wild type (n=137) and all dMP cotyledon explants (n=133) had the “whole” type of callusogenesis (Fig. 3.1 B). In contrast, mpG12 and nph4-1 showed mostly the “patchy” type of callusogenesis. The “whole” type calluses were found in only 4% of the mpG12 explants (n=135), and in 8% of the nph4-1 explants (n=133) (Fig. 3.1 B). The impaired callus proliferation in the arf mutants, indicated by the lower fresh weights of the calluses, was combined with a strong prevalence of the “patchy” type calluses in these genotypes. In contrast, the dMP and the wild type calluses, which defined normal callus growth, were exclusively the “whole” type.

3.3.3 Callusogenesis of leaf explants after 12 days on CIM

The fresh weights of the leaf calluses also differed among the genotypes. As with the cotyledon explants, the fresh weights of the calluses derived from the leaf explants were similar between wild type and dMP (wild type 60±2.4 mg, n=161; and dMP 65±1.6 mg, n=161). However, the fresh weights of the nph4-1 calluses (18±1 mg, n=174; P<0.001), and of the mpG12 calluses (40±0.6 mg, n=167; P<0.001) were significantly less than those of wild type (Fig. 3.1 A). Also, the fresh weights of the mutant genotypes, nph4-1 and mpG12, differed significantly: the mean fresh weight of the nph4-1 calluses was only about half that of mpG12 (nph4-1 18±1 mg, n=174; mpG12 40±0.6 mg, n=167; P<0.001, for the comparison of the mean responses between nph4-1 and mpG12) (Fig. 3.1 A).
The distribution of the callusogenesis types (the “whole” versus the “patchy” types) in the calluses derived from leaf explants also differed among the genotypes (Fig. 3.1 B; 3.5 B). In wild type, the “whole” type calluses formed in only 54% of the explants (n=161). The frequency of the “whole” type calluses among the dMP explants (94%, n=161) was much higher compared to that of the wild type ones (Fig. 3.1 B). The frequencies of the “whole” type calluses were significantly lower in the mutant genotypes. Only 33% of the mpG12 (n=167), and only 7% of the nph4-1 (n=174) leaf explants developed the “whole” type calluses (Fig. 3.1 B). The rest of the mpG12 and nph4-1 leaf explants were only partly covered by callus patches (Fig. 3.5 B).

Thus, analysis of the fresh weights and of the distribution of callusogenesis types in the leaf explants once again revealed differences in callus proliferation among the genotypes. Compromised callus proliferation was detected in the nph4-1, and, to a lesser degree, in the mpG12 leaf explants. Increased callus proliferation was found in the dMP explants, as indicated by the frequency of the “whole” type calluses.

3.3.4 Callusogenesis of petiole explants after 12 days on CIM

The fresh weights of the petiole derived calluses showed genotype and organ-specific differences. The fresh weights of the dMP calluses were almost twice those of the wild type calluses (wild type 22±1 mg, n=108; dMP 38±3 mg, n=73; P<0.001) (Fig. 3.1 A). The fresh weights of the nph4-1 and mpG12 calluses were significantly lower than those of the wild type ones (nph4-1 13±1 mg, n=105, P<0.001; mpG12 18±1 mg, n= 62; P<0.05) (Fig. 3.1 A). Further, between the mutants, the fresh weights of the nph4-1 calluses were significantly less than those of the mpG12 ones (nph4-1 13±1 mg, n=105; mpG12 18±1 mg, n= 62; P<0.001 for the comparison of the mean responses between nph4-1 and mpG12).

There were also difference among the genotypes in the distribution of the callus types in the petiole explants among the genotypes (Fig. 3.1 B; 3.6 A). Almost all wild type (95%) and dMP (97%) petiole explants developed the “whole” type of calluses (Fig. 3.1 B). By contrast, only 27% of the nph4-1 petiole calluses were of the “whole” type. However, the frequency of the “whole” type calluses in the mpG12 petiole explants was relatively high (79%), which was closer to the frequency in wild type than to that in nph4-1.
Figure 3.3 Callus initiation and callus proliferation in petiole (A-D) and hypocotyl (E-H) explants after 6 and 12 days on CIM. Origin of explant as indicated on top right; days on CIM
and types of callus growth (“whole” versus “patchy”) as indicated, bottom right; arrows indicate sites of callus proliferation.

To summarize, the distribution of the fresh weights and of the types of calluses in the petiole explants indicated the restricted character of callus proliferation in the *nph4-1* explants; and, to a lesser degree, in the *mpG12* petiole explants. By contrast, callus proliferation was elevated in the *dMP* explants, as indicated by the fresh weights of the *dMP* calluses, the vast majority of which were of the “whole” type.

### 3.3.5 Callusogenesis of hypocotyl explants after 12 days on CIM

The fresh weights of the hypocotyl calluses also showed genotype related differences. However, these differences were weaker compared to those of the other organs. The fresh weights of the *dMP* calluses were significantly higher than those of the wild type ones (wild type 19±1 mg, n=137; *dMP* 23±1 mg, n=92; P<0.001). The hypocotyl explants were the only type of aerial explants where the fresh weights of the *nph4-1* calluses were not less than those in wild type (*nph4-1* 19±1 mg, n=132) (Fig. 3.1 B). No *mpG12* hypocotyl explants could be tested, because of the *mpG12* seedlings’ lack of a hypocotyl.

The distribution of the callus types in the hypocotyl explants differed among the genotypes (Fig. 3.1 B; 3.6 B). Most of the wild type explants (82%, n=137) and most of the *dMP* explants (97%, n=92) had the “whole” type of calluses (Fig. 3.1 B). In contrast, there were only 35% of the “whole” type calluses in *nph4-1* (n=132) (Fig. 3.1 B).

To summarize, the comparison of the callus growth and type data suggests that the “whole” type calluses are not just the result of vigorous growth. Specifically, the fresh weights of the *nph4-1* calluses were similar to those of the wild type ones in hypocotyl explants. The “patched” calluses dominated in this group. Among the genotypes, callus proliferation was once again greatest in *dMP* calluses.

### 3.3.6 Callusogenesis of adventitious root explants after 12 days on CIM

Because *mpG12* seedlings lack primary roots (Berleth and Jurgens, 1993), the procedure to initiate adventitious roots in *mpG12* and in the other genotypes preceded the callusogenesis
Figure 3.4 Callus initiation and callus proliferation in adventitious root explants after 6 (A, C, E, G) and 12 (B, D, F, H) days on CIM. The genotypes and days on CIM as indicated on top right; arrows indicate sites of callus proliferation.
Figure 3.5 Cotyledon (A) and leaf (B) explants after 12 days on CIM. Genotypes indicated within plate sectors; arrows point at “patchy” type calluses
Figure 3.6 Petiole (A) and hypocotyl (B) explants after 12 days on CIM. Genotypes indicated within plate sectors; arrows point at the “patchy” type calluses.
Figure 3.7 Adventitious root explants after 12 days on CIM. Genotypes indicated within plate sectors.
experiments (see Chapter 2.8.1 for details). Seedlings of all the genotypes were dissected by cutting off the lower part of their hypocotyl (or the low part of the peg in the mpG12 seedlings). The dissected seedlings were re-rooted on the auxin (IBA) containing medium, and then transferred to the hormone-free 1/2MS medium to allow the outgrowth of adventitious roots. These adventitious roots were used as explants in the experiments described below (for details see Chapter 2.8).

In this category, root explants, the distribution of the fresh weights of the calluses among the genotypes was different compared to those of the aerial organs (Fig. 3.1A). The fresh weights of the calluses were: wild type 56±0.4 mg, n=116; the similar nph4-1, 57±0.4 mg, n=111; and, the significantly lower (by more than half) mpG12, 26±0.2 mg, n=110 (P<0.001). In contrast to the growth of calluses on all the aerial organs, the growth of the dMP calluses on the root explants was slightly, but significantly, lower than that of the wild type calluses (dMP 4±0.4 mg, n=114; P<0.01) (Fig. 3.1 A).

Thus, in this category of root explants, callus growth among the genotypes was quite different than that in the aerial explants. No genotype related difference in callus types was detected in this category, and the calluses of all the genotypes looked relatively similar after 12 days on CIM, resembling the “whole” type calluses of the aerial explant group.

3.3.7 Callus proliferation in aerial explants after 6 days on CIM

The difference in phenotypic classes between genotypes observed after 12 days on CIM in calluses derived from aerial explants (the “whole’ or the “patchy’’ types of calluses) raises the question of the origin of this difference. Two distinct possibilities can be considered. First, the difference in callus types might simply reflect the difference in callus growth rate. In this case, two callus types could be initiated as local callus patches along the explant surface, and around the wounded areas. Later, these areas would merge to form the “whole” calluses, when callus proliferation was strong, or stay “patchy”, when the callus growth rate was low. Alternatively, the two types could reflect genuinely different growth patterns observable right from the beginning of callus growth. To distinguish between these two possibilities, the explants were visually examined after 6 days on CIM.
When aerial explants (cotyledons, leaves, petioles, and hypocotyls) were examined after 6 days on CIM, clear differences were detected between explants later developing the “whole” type or the “patchy” type calluses. Most of the explants (more than 90%), which later developed the “whole” type of calluses, were getting visibly friable and partly translucent. Their original dense green color was changing to whitish or yellowish green (Fig. 3.2; 3.3). Callus proliferation in these explants occurred evenly across the entire explant surface. Further, when cotyledon and leaf explants were examined, the callus proliferation was denser along the veins and the petiole parts (Fig. 3.2). All these changes made the explants developing into the “whole” type calluses look quite distinct from those developing into the “patchy” type calluses (Fig. 3.2; 3.3).

By contrast, in the majority of the explants (more than 90%) which later developed the “patchy” type of calluses, relatively large areas of the explants retained their original appearance after 6 days on CIM. In these areas, the tissues did not look friable and translucent. The color of the explants did not change from the original dense green to whitish or yellowish shades. Initiation of callus proliferation in these explants was limited to the wounded (cut) areas (in all organ types of the aerial explants), and to some locally restricted patches of proliferating cells on the explant surface, when the cotyledon and leaf explants were examined (Fig. 3.2; 3.3). The rest of the explant tissues stayed “untouched” and mainly preserved their original color and structure (Fig. 3.2; 3.3).

In summary the results indicated that the differences in the callus types (the “whole” type versus the “patchy” type) were due to the distinctive initiation of callus proliferation in the explants, rather than to the differences in the callus growth rate, even though there were differences in the callus growth rates.

3.4 Discussion and Conclusions

The results in this chapter indicate genotype and organ related differences in callus proliferation responses among the genotypes. These differences affect both the callus growth rate, as well as the pattern of proliferation.

In the calluses derived from the aerial explants (cotyledons, leaves, petioles, and hypocotyls), two major phenotypic classes of callus type were observed: the “whole” type, with even callus proliferation along the entire explant surface; and the “patchy” type, where callus proliferation
occurred as isolated patches, often localized to the wounded areas. The callus types and the callus weights differed among the tested genotypes, falling into two major groups. The first group was comprised of the dMP and wild type genotypes. Callusogenesis in dMP was either equal to, or sometimes greater than that in wild type, as indicated by the fresh weights and the frequencies of the “whole” type calluses. The second group was represented by the arf mutants, nph4-1 and mpG12. In these, callusogenesis was partly impaired and significantly weaker compared to that in wild type. This was indicated by the lower fresh weights of the nph4-1 and the mpG12 calluses, and usually by much lower frequencies of the “whole” type calluses. Also, of these arf genotypes, callusogenesis compromise was greater in nph4-1 than in mpG12, when their aerial explants were cultured on CIM. The differences in the callus types (the “whole” type versus the “patchy” type) were as a result of the distinct initiation of callus proliferation (throughout the explant surface in the “whole’ type, or in local patches in the “patchy” type of calluses), rather than as a result of differences in the callus growth rate.

The callusogenesis responses were different when calluses derived from the adventitious root explants were compared. Although no distinct callus types were detected, genotype related differences in callusogenesis were indicated by the fresh weights of the calluses. Dissimilar to calluses derived from the aerial explants, no impairment in callusogenesis was detected in nph4-1 root explants. These data are compatible with previous observations that only the aerial organs, but not the roots, of nph4 plants were resistant to toxic concentrations of the synthetic auxin 2,4-D (Watahiki and Yamamoto, 1997). In contrast to nph4-1, callusogenesis in mpG12, as indicated by the fresh weights of the calluses, was significantly weaker compared to that of wild type. Also, the callus weights were slightly, but significantly, lower in the dMP calluses. Generally, however, in this category of root explants, differences in callusogenesis among the genotypes were more subtle than those in the calluses derived from the aerial explants. Given the artificial character of the procedure for the induction of adventitious roots (the long exposure to an auxin containing medium followed by culture on a hormone free medium), the observed differences in callus weights might reflect the genotype specific response to preculture conditions during adventitious root induction, rather than a true genotype related difference in callus proliferation.

In nature, callusogenesis is a part of the mechanism of protection facilitating the repair of wounded or damaged tissues (Frank et al., 2000). This mechanism involves auxin signaling, which is crucial for callus initiation (Frank et al., 2000; Gordon et al., 2007). Thus, it is plausible
that auxin insensitive *arf* mutants are impaired in callusogenesis. The relative degree of impairment may reflect the degree to which a given *ARF* gene is involved in auxin signaling in a specific organ or tissue. It may also reflect the degree to which a particular *ARF* gene is related to the regulation of the cell-cycle genes mediating cell proliferation responses (Hemerly et al., 1993; Richard et al., 2002; Che et al., 2007; Perrot-Rechenmann, 2010).

The *dMP* transgene represents a gain-of-function allele of *MP*, possibly a constitutively active allele (Krogan, 2006). Callusogenesis was strong in the *dMP* transgene line in aerial, but not in root explants. In this case, one may speculate that this reflects the enhanced auxin signal transduction features of *dMP* tissue (Krogan, 2006).

To summarize, loss of *MP* and particularly *NPH4* functions in *mpG12* and *nph4-1* mutants resulted in the impairment of callus proliferation in particular categories of explants. The presumably elevated *MP* function in the *dMP* genotype may increase callus proliferation in aerial explants, and decrease the same in adventitious root explants which needs further evaluation.
Chapter 4
Shoot regeneration under conditions of altered auxin signal transduction

4.1 Background and rationale

Shoot regeneration in vitro is triggered by a high cytokinin to auxin ratio in the tissue culture medium (Skoog and Muller, 1957; Gaba, 2005). Although the phytohormone auxin is always used in the composition of a shoot inducing medium (SIM), relatively little is known about the effect of auxin on shoot regeneration in tissue culture. Typically, the role of auxin in this process is only discussed in the context of the ratio of auxin to cytokinin (Howell 2003; Zhang and Lemaux, 2004; Gaba, 2005; Zhang and Lemaux, 2005). However, several factors can contribute to the effect of auxin signaling on shoot regeneration. First, auxin plays a key role in shoot meristem patterning (Reinhardt et al., 2000); auxin levels are high in shoot meristems in planta (Kakani et al., 2009). Therefore, proper auxin signaling might be required for de novo shoot meristem assembly (Coenen and Lomax, 1998; Gordon et al., 2007; Chatfield and Raizada, 2008; Kakani et al., 2009). Second, many genes linked to auxin signaling may also be implicated in other hormone pathways, due to existing cross talk between signaling pathways (Hobbie and Estelle, 1994; Swarup et al., 2002). As a result, auxin gene impairment may affect coordinated interactions among multiple developmental processes (Gordon et al., 2007; Kakani et al., 2009). Third, shoot regeneration is severely impaired in a number of auxin signaling mutants, such as aux1 (Kakani et al., 2009) and axr1 (Chatfield and Raizada, 2008), and mildly impaired in pin1 (Gordon et al., 2007). Thus, genes involved in auxin signaling, auxin homeostasis, and auxin transport may affect processes of de novo shoot regeneration in vitro (reviewed in Chapter 1.7.5).

The auxin response factors MP (ARF5) and NPH4 (ARF7) interact with each other and have partly overlapping functions. They are both transcriptional activators (Ulmasov et al., 1999a; Ulmasov et al., 1999b, Berleth et al., 2004; Hardtke et al., 2004). Mutations in the MP gene affect the formation of the embryonic root meristem and result in rootless seedlings (Berleth and Jurgens, 1993), and in severely reduced auxin sensitivity, auxin signaling and auxin transport (Li et al. 2006, Przemeck et al., 1996, Mattsson et al., 2003, Schuetz et al., 2008). Moreover, in
*planta*, *MP* is involved in the regulation of several meristematic genes, such as the *CUC* genes and *STM*, during embryonic meristem formation (Aida et al., 2002). *MP* is also linked to the *ESR1/DRN* gene during cotyledon development (Cole et al., 2009). Because all these genes are known to be crucial for *de novo* shoot meristem formation (Zhang and Lemaux, 2005), the *MP* gene may have a significant effect on shoot regeneration.

Regarding *nph4* mutants, they exhibit auxin resistance in the aerial parts of plants, indicated by their resistance to toxic concentrations of auxin, and by the low signal of the DR5 auxin response reporter. Further, the levels of many primary auxin response genes are severely affected in *nph4* mutants (Stowe-Evans et al., 1998, Wang et al., 2005). Notably, *nph4* mutants represent a case of auxin-resistant mutants that are not cross-resistant to other plant hormones, including cytokinin (Watahiki and Yamamoto, 1997, Wang et al., 2005).

The genotype *dMP*, generated by the deletion of protein interaction domains in the *MP* gene has been described (Krogan, 2006; see also Chapter 1.2.5; Fig. 1.2). As these interaction domains are required for the binding of regulatory proteins, it seems likely that *dMP* represents a constitutively active *MP* gene.

In this chapter, the roles of the *MP* and *NPH4* genes in *de novo* shoot regeneration are explored. The extent to which shoot regeneration in each of the investigated genotypes is further influenced by the origin of the tissue sample is also explored.

### 4.2 Specific research objectives

Loss-of-function mutants *mpG12* (Hardtke and Berleth, 1998), and *nph4-1* (Harper et al., 2000), and the gain-of-function transgene line *dMP* with truncated dimerization domains III and IV (reviewed in Chapter 1.2.5; Fig. 1.2; Krogan, 2006) were tested to determine the influence of three genotypes on shoot regeneration *in vitro*. All genotypes used in the experiments were in the Col-0 background and were compared to the Col-0 wild type. Explants of different organs (cotyledons, leaves, petioles, hypocotyls, and adventitious roots) were first cultured on CIM for 6 days and then transferred to SIM (Chapter 2.8). The frequency of shoot regeneration was based on the pooled data of all replicas of the experiments, and the mean number of shoots per shoot regenerating callus (mean ± SE) was compared among the genotypes after 21 days on SIM.
4.3 Results

4.3.1 Shoot regeneration in cotyledon derived calluses

Calluses derived from cotyledon explants were generally not capable of shoot regeneration, with the notable exception of dMP calluses (Fig. 4.1 and 4.2 A). In all other genotypes, including wild type, shoot regeneration was almost absent (Fig. 4.1 A). There could be more than one shoot per shooting callus in dMP (5±0.49), but the small number of shooting calluses in the other genotypes precluded any statistical evaluation of this feature (Fig. 4.1 B). Also, because of small numbers, it was not feasible to statistically compare the mean number of shoots per shoot regenerating callus among all the genotypes. Nevertheless, 2 shoots were scored in the wild type single shoot regenerating callus, and 5 shoots in the nph4-1 shoot regenerating callus. The mean number of shoots in the mpG12 calluses was 1.6±0.6, range 1 to 4 in 5 shoot regenerating calluses (Fig. 4.1 B). The mean number of shoots in the dMP calluses was relatively high (5±0.49, range 1 to 20 in 171 calluses) (Fig. 4.1 B).

4.3.2 Shoot regeneration in leaf derived calluses

Calluses derived from leaf explants showed a clear difference among the genotypes in their ability for shoot regeneration (Fig. 4.1 and 4.2 B). Generally, shoot regeneration in calluses derived from leaf explants was stronger in all tested genotypes compared to those in cotyledon derived calluses (Fig. 4.1 A). In wild type, 24% of the calluses generated shoots (59 out of 245, where n represents the total number of explants in five independent experiments). Shoot regeneration in nph4-1 was 21% (50 out of 241 calluses), which was only slightly less than that in wild type (Fig. 4.1 A). Shoot regeneration in mpG12 was only 9.5% (17 out of 179 calluses), which was much lower than that in wild type, and also in nph4-1 (Fig. 4.1 A). Whereas shoot
Figure 4.1 Percentage of shoot regenerating calluses (A), and mean number of shoots per shoot regenerating callus (B) after 21 days on SIM (following 6 days on CIM to produce the calli).

In (A) “n” is the total number of explants used in the experiments; in (B) “n” is the number of shoot regenerating calluses. Bars indicate SE.
**Figure 4.2** Calluses derived from cotyledon (A) and leaf (B) explants after 21 days on SIM (following 6 days on CIM to produce the calli). Genotypes indicated within plate sectors; arrows indicate shoot regenerating calluses.
regeneration in mpG12 was reduced, it was very high in dMP (87%, or 229 out of 264 calluses) (Fig. 4.1 A).

The number of shoots per shoot regenerating callus also showed differences in shoot regeneration among the genotypes (Fig. 4.1 B). In wild type, the mean number of shoots per shoot regenerating callus was 2.2±0.22, range 1 to 8 in 59 calluses. The mean number of shoots was different in nph4-1 and in mpG12. In nph4-1 calluses, the mean number of shoots per shoot regenerating callus was 2.1±0.21, range 1 to 7 in 50 calluses. This was comparable to that in wild type (Fig. 4.1 B). However, mpG12 calluses had only 1.1±0.08 shoots per callus, range 1 to 2 in 17 mpG12 calluses (P<0.001 for the comparison of the mean responses between mpG12 and wild type, and between mpG12 and nph4-1 calluses) (Fig. 4.1 B). In contrast, in dMP calluses, the mean number of shoots was much higher than that in wild type (6.2±0.27, range 1 to 24 in 229 calluses, P<0.001) (Fig. 4.1 B).

Collectively, these data indicate a strongly elevated shoot regeneration capability in dMP calluses derived from leaf explants compared to that in wild type. This was indicated by a difference in the proportion of shoot generating calluses, and in the mean number of shoots formed by shoot regenerating calluses. The arf mutant genotypes, nph4-1 and mpG12, were very different in their shoot regeneration ability. Shoot regeneration in nph4-1 was relatively similar to that in wild type, whereas shoot regeneration in mpG12 was much lower than that in wild type. These differences between the arf mutants were indicated by both the frequency of the shoot regenerating calluses, and the mean number of shoots per callus.

4.3.3 Shoot regeneration in calluses derived from petiole explants

Calluses derived from petiole explants showed clear genotype related difference in their ability for shoot regeneration on SIM (Fig. 4.1 and 4.3 A). In addition, their shoot regeneration profile generally resembled that of leaf derived calluses (Fig. 4.1). In wild type, 20% of calluses (22 out of 108) regenerated shoots (where n represents the total number of explants in four independent replicas of the experiment). Shoot regeneration in the arf mutants, nph4-1 and mpG12, was generally lower than that in wild type, but distinctly different in these two genotypes. Thus, shoot regeneration in nph4-1 was slightly less than that in wild type, and 16%, or 22 out of 140

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Figure 4.3 Calluses derived from petiole (A) and hypocotyl (B) explants after 21 days on SIM (following 6 days on CIM to produce the calli). Genotypes indicated within plate sectors; arrows indicate shoot regenerating calluses.
calluses, regenerated shoots (Fig. 4.1 A). Shoot regeneration in mpG12 was much lower than that in wild type, and only 8%, or 9 out of 118 calluses, developed shoots (Fig. 4.1 A). Once again, shoot regeneration in dMP was very strong compared to that in wild type, and a majority of dMP calluses (71%, or 105 out of 148) formed shoots (Fig. 4.1 A).

The mean number of shoots per shoot regenerating callus also showed differences in shoot regeneration ability among the genotypes (Fig. 4.1 B). In wild type, the mean number of shoots per shoot regenerating callus was 2.7±0.42, range 1 to 7 in 22 calluses. The mean number of shoots was significantly lower in nph4-1 and mpG12 calluses compared to that in wild type. In nph4-1, the mean number of shoots was 1.8±0.28, range 1 to 6 in 21 calluses (P<0.1) (Fig. 4.1 B). The mpG12 calluses had 1.4±0.43 shoots per callus, range 1 to 5 in 9 calluses (P<0.05) (Fig. 4.1 B). In this category of calluses, the mean number of shoots in nph4-1 and mpG12 did not differ significantly. In dMP calluses the mean number of shoots per shoot regenerating callus was 5.9±0.39, range 1 to 24 in 230 calluses (P<0.001) (Fig. 4.1 B).

Collectively, these data showed strongly elevated shoot regeneration ability in dMP calluses derived from petiole explants. This was indicated by the higher frequency of shoot regenerating calluses, and by the higher number of shoots formed in the shoot regenerating calluses. Shoot regeneration in the arf mutant genotypes, nph4-1 and mpG12, was generally lower than that in wild type. Shoot regeneration was slightly decreased in the nph4-1 calluses, and greatly decreased in the mpG12 calluses. Thus, the presumably elevated MP function in the dMP calluses resulted in greatly increased shoot regeneration. Further, the loss of the MP function in the mpG12 mutants resulted in greatly decreased shoot regeneration. Meanwhile, the loss of the NPH4 function in the nph4-1 mutants had a more moderate effect on shoot regeneration, when the calluses derived from petiole explants were compared on SIM.

4.3.4 Shoot regeneration in calluses derived from hypocotyl explants

The genotype related difference in shoot regeneration ability was relatively moderate when calluses derived from hypocotyl explants were compared on SIM (Fig. 4.1 and 4.3 B). Generally, shoot regeneration in calluses derived from these explants was strongest among all the types of explants (cotyledon, leaf, petiole, and adventitious roots) (Fig. 4.1). All the tested genotypes (wild type, dMP, and nph4-1) had a relatively high rate of shoot regeneration on SIM (Fig. 4.1).
No \textit{mpG12} calluses could be tested in this category, because of the lack of hypocotyls in the \textit{mpG12} seedlings. In wild type, 63\%, or 97 out of 153 calluses, regenerated shoots (where \( n \) represents the total number of explants in four independent replicas of the experiment). Shoot regeneration in \textit{nph4-1} was slightly higher than that in wild type, and 74\%, or 109 out of 147 \textit{nph4-1} calluses, formed shoots (Fig. 4.1 A). Once again, shoot regeneration in \textit{dMP} calluses was stronger compared to that in wild type, and a majority of \textit{dMP} calluses (88\%, or 127 out of 145) produced shoots (Fig. 4.1 A).

The mean number of shoots per shoot regenerating callus indicated the difference in shoot regeneration ability among the genotypes more clearly (Fig. 4.1 B). In wild type, the mean number of shoots per shoot regenerating callus was 4.14±0.23, range 1 to 10 in 97 calluses. The mean number of shoots was significantly less in the \textit{nph4-1} calluses compared to that in the wild type ones (3.4±0.19 shoots per callus, range 1 to 10 in 109 calluses, \( P<0.05 \)). In the \textit{dMP} calluses, the mean number of shoots was much higher than that in the wild type calluses (7.68±0.39, range 1 to 24 in 127 calluses, \( P<0.001 \)).

Collectively, these data showed the increase in shoot regeneration in \textit{dMP} calluses derived from hypocotyl explants. This was indicated by a greater frequency of shoot regenerating calluses, and by a higher mean number of shoots formed by the shoot regenerating calluses. Shoot regeneration in the \textit{arf} mutant genotype \textit{nph4-1} gave mixed results. On the one hand, the frequency of shoot regenerating calluses in \textit{nph4-1} was higher than that in wild type. On the other hand, the average number of shoots per shoot regenerating callus was significantly lower than that in wild type. Thus, when compared to wild type, \textit{dMP} calluses again showed increased shoot regeneration on SIM. \textit{nph4-1} calluses demonstrated a moderate increase in the frequency of shoot regenerating calluses, which produced significantly fewer shoots per callus compared to those in wild type.

4.3.5 Shoot regeneration in calluses derived from adventitious root explants

Shoot regeneration in calluses derived from adventitious root explants partly differed from shoot regeneration profiles in other types of explants (cotyledon, leaves, petioles, and hypocotyls) (Fig. 4.1 and 4.4). In this category of calluses, the strongest shoot regeneration was observed in wild type, where 49\%, or 120 out of 247 calluses, regenerated shoots (where \( n \) represents the total
Figure 4.4 Calluses derived from adventitious root explants after 21 days on SIM (following 6 days on CIM to produce the calli). Genotypes indicated within plate sectors; arrows indicate shoot regenerating calluses.
number of explants in four independent replica of the experiment). Shoot regeneration in the arf mutants, nph4-1 and mpG12, was less than that in wild type, and also differed between these arf genotypes. Thus, in nph4-1, 29%, or 70 out of 238 calluses, regenerated shoots, while in mpG12 only 7%, or 15 out of 228 calluses, did so (Fig. 4.1 A). This category of calluses was the only one where the frequency of shoot regeneration in the dMP calluses was lower than that in the wild type calluses. Only 26%, or 67 out of 254 dMP calluses, formed shoots (Fig. 4.1 A).

The mean number of shoots per shoot regenerating callus also indicated differences among the genotypes (Fig. 4.1 B). In wild type, the mean number of shoots per shoot regenerating callus was 3.1±0.19, range 1 to 10 in 120 calluses. The mean number of shoots was significantly less in the nph4-1 and mpG12 calluses compared to that in the wild type calluses. In nph4-1 calluses, the mean number of shoots was 2.25±0.22, range 1 to 12 in 70 calluses (P<0.01) (Fig. 4.1 B). The mean number of shoots per shoot regenerating callus was even less in the mpG12 calluses (1.7±0.24, range 1 to 4 in 15 calluses, P<0.001) (Fig. 4.1 B). In the dMP calluses, the number of shoots per shoot regenerating callus was slightly and insignificantly higher than that in the wild type calluses (3.5±0.51 shoots per callus). However, the maximum number of shoots scored in the dMP calluses was more than twice that in the wild type calluses, ranging from 1 to 23 in 67 dMP shoot regenerating calluses (Fig. 4.1 B).

Thus, the dMP transgene did not affect shoot regeneration in calluses derived from adventitious root explants. However, once again, the loss of the MP function in the mpG12 mutants resulted in strongly decreased shoot regeneration. The loss of the NPH4 function in nph4-1 mutants also resulted in decreased shoot regeneration, yet had a less prominent effect on shoot regeneration than the loss of MP function. This is based on the comparison on SIM of calluses derived from adventitious root explants.

4.3.6. Effect of duration of CIM preculture on shoot regeneration on SIM in calluses derived from adventitious root explants

To determine the effect of the duration of CIM preculture on subsequent shoot regeneration on SIM, adventitious root explants of the tested genotypes were precultured on CIM for different periods of time (6, 9, 12, or 16 days on CIM), and then transferred on SIM for shoot regeneration. The results of the experiment showed genotype related changes in the efficiency of
Figure 4.5 Percentage of shoot regenerating calluses (A) and mean number of shoots per shoot regenerating callus (B) in calluses derived from adventitious root explants after 21 days on SIM depending on the time on CIM (6, 9, 12 and 16 days).

In (A) “n” represent the total number of explants used in the experiments; in (B) “n” is the number of shoot regenerating calluses. Bars indicate SE.
shoot regeneration depending on the time on CIM (Fig. 4.5). The results of a single experiment are presented below.

In the experiment, when the adventitious root explants were exposed to CIM for 6 days (a standard protocol, see Materials and Methods, Chapter 2.8), shoot regeneration was strongest in the wild type calluses, and 64%, or 46 out of 72 calluses, formed shoots. Shoot regeneration among all other genotypes was much lower compared to that in wild type. Specifically, shoot regeneration in the arf mutants, nph4-1 and mpG12, was lower than that in wild type. Thus, 33% (22 out of 67) nph4-1 calluses, and only 16% (11 out of 68) mpG12 calluses regenerated shoots (Fig. 4.5 A). In dMP, the frequency of shoot regenerating calluses was also lower than that in wild type, and only 25% (18 out of 73 calluses) formed shoots (Fig. 4.5 A).

The number of shoots per shoot regenerating callus differed slightly among the genotypes, where the only significant difference was detected between wild type and mpG12. In wild type, the mean number of shoots per shoot regenerating callus was 2.55±0.28, range 1 to 9 in 46 calluses. In dMP, the mean number of shoots was 4.38±1.3, range 1 to 20 in 18 calluses. In nph4-1, the mean number of shoots was 2.9±0.54, range 1 to 12 in 22 shoot regenerating calluses (Fig. 4.5 B). In mpG12, the mean number of shoots was significantly less than that in wild type (1.64±0.3, range 1 to 4 in 11 calluses, P<0.05) (Fig. 4.5 B).

When the time on CIM was extended to 9 days, the shoot regeneration profile changed significantly. Generally, shoot regeneration increased, and became relatively similar in all the genotypes. An exception was mpG12, where shoot regeneration stayed consistently low (Fig.4.5). At 9 days, 86%, or 30 out of 35 wild type calluses; 85%, or 29 out of 34 dMP calluses; 81%, or 29 out of 36 nph4-1 calluses; and only 16%, or 5 out of 32 mpG12 calluses, regenerated shoots (Fig. 4.5 A). Also, in this experimental condition, the mean number of shoots per shoot regenerating callus generally increased in all the genotypes, except for mpG12. In wild type, the mean number of shoots per shoot regenerating callus was 4.87±0.5, range 1 to 12 in 31 calluses. In dMP, the mean number of shoots was 13.31±1.23, range 1 to 26 in 29 calluses (P<0.001). In nph4-1, the mean number of shoots was 7.1±0.78, range 1 to 16 in 29 calluses (P<0.05) (Fig.4.5 B). In mpG12, the mean number of shoots per shoot regenerating callus was significantly less than that in wild type (1.2±0.19, range 1 to 2 in 5 calluses, P<0.001) (Fig. 4.5 B).
Extension of the time on CIM to 12 days affected shoot regeneration in the genotypes differently. In wild type, shoot regeneration was strongly reduced compared to that at 9 days on CIM, and only 27% (6 out of 22) wild type calluses regenerated shoots. Shoot regeneration was also reduced in the dMP and nph4-1 calluses. However, these reductions were not as strong as in the wild type calluses. Specifically, 54% (13 out of 24) dMP calluses, and 68% (17 out of 25) nph4-1 calluses regenerated shoots (Fig. 4.5 A). Shoot regeneration in mpG12 calluses was also lower, with only 8%, or 2 out of 24 calluses, regenerating shoots (Fig. 4.5 A).

The mean number of shoots per shoot regenerating callus was also reduced compared to that of the previous experimental settings (9 days on CIM) in all the genotypes, except mpG12. In wild type, the mean number of shoots per shoot regenerating callus was 3±1, range 1 to 8 in 6 calluses. The mean number of shoots per shoot regenerating callus was significantly higher in dMP compared to that in wild type (9.85±2.27, range 1 to 30 in 13 calluses, P<0.05) (Fig. 4.5 B). The mean number of shoots in nph4-1 was insignificantly higher than that in wild type (4.88±0.7, range 1 to 10 in 17 calluses) (Fig. 4.5 B). The number of shoots scored in 2 out of 24 mpG12 shoot regenerating calluses was 1 and 2, which was consistent with all the data for shoot regeneration in this genotype (Fig. 4.5 B).

A further drop in shoot regeneration was observed when the time on CIM was extended to 16 days (Fig. 4.5). Only 8%, or 1 out of 12 wild type calluses, regenerated shoots. A decrease in shoot regeneration was also detected in dMP and nph4-1. However, shoot regeneration in these genotypes was still higher than that in wild type. Only 36%, or 5 out of 14 dMP calluses, and 33%, or 4 out of 12 nph4-1 calluses, were still capable of shoot regeneration. Shoot regeneration ability in mpG12 did not change, and 8%, or 1 out of 12 calluses, regenerated shoots.

Because of a generally low number of shoot regenerating calluses, a statistical analysis could not be performed to discriminate the differences in the mean number of shoots per shoot regenerating callus in this category. In wild type, the single shoot regenerating callus produced 2 shoots. In dMP, the mean number of shoots was 5.4±2.4, range 1 to 10 in 5 calluses (Fig. 4.5 B). In nph4-1, the mean number was 2±0.4, range 1 to 3 in 4 calluses (Fig. 4.5 B). In mpG12, the single shoot regenerating callus produced one shoot (Fig. 4.5 B).

Taken together, these data indicate that the increased time on CIM affected shoot regeneration in wild type, dMP, and nph4-1, but not in mpG12. While the extension of CIM preculture from 6 to
9 days generally had a stimulating effect on shoot regeneration in wild type, *nph4-1*, and *dMP*, the longer extension (12 or more days on CIM) inhibited shoot regeneration on SIM. Interestingly, *dMP* and *nph4-1* were less sensitive to the increased time of CIM preculture, while shoot regeneration in wild type dropped sharply after 9 days on CIM. Thus, the efficiency of shoot regeneration underwent dynamic changes in response to the duration of CIM preculture in wild type, *dMP*, and *nph4-1*. However, shoot regeneration was consistently low and relatively independent of the duration of CIM preincubation in *mpG12* calluses derived from adventitious root explants.

### 4.3.7 Greening of calluses derived from adventitious root explants on SIM

Genotype related differences were detected in the timing of greening--when adventitious root calluses, originally whitish after CIM, turned green on SIM. After transfer to SIM, the calluses increased in size, and turned green. The period of time required for greening was clearly different in the genotypes. In wild type, the first evidence of greening appeared after 5 days on SIM. This was visualized as green spots embedded in the whitish color of the calluses. Later, these green areas increased in size, and, after 10 days on SIM, the whole callus turned green (Fig. 4.6). In the intermediate stage (8 days on SIM), wild type calluses usually had a mixed color (green and whitish). About 56% of wild type calluses (29 out of 52) were mostly whitish (the whitish color covered more than 50% of the area), while the rest, 44% (23 out of 52), were mostly green (more than 50% of the callus area was green) (Fig. 4.7). A similar picture was observed for the *nph4-1* calluses, with different proportions between the whitish and greenish calluses. 81% of the *nph4-1* calluses (42 out of 52) were mostly whitish, while the rest (19%, or 10 out of 52) were mostly green (Fig. 4.7). The *dMP* and *mpG12* calluses formed two distinct phenotypic groups. Most of the *dMP* calluses were clearly whitish (98%, or 55 out of 56 calluses in total). However, most of the *mpG12* calluses (98%, or 50 out of 51 calluses) were bright green in color (Fig. 4.6; 4.7).

Thus, the *mpG12* calluses were the ones which turned green on SIM faster, more evenly and intensively than the calluses of wild type and other genotypes. Greening of the *dMP* calluses was delayed, and it was less intensive compared to those in wild type. Greening of the *nph4-1* calluses was slightly less intensive compared to that of the wild type ones. No genotype related
Figure 4.6 Greening of calluses and root outgrowth in calluses derived from adventitious root explants on SIM after 8 (A), and 13 (B) days on SIM (following 6 days on CIM to produce the calli). Genotypes indicated within plate sectors.
Figure 4.7 Percentage of green versus whitish calluses after 8 days on SIM in adventitious root derived calluses; n, number of explants.
difference in greening was established for calluses derived from other organs than roots, because they were already greenish at the time of their transfer on SIM.

### 4.4. Discussion and Conclusions

The results in this chapter indicate that the efficiency of shoot regeneration depends on the genotype, the type of organ used as the explant, and the duration of preculture on CIM. As shown earlier (Akama et al., 1992; Chatfield and Raizada, 2008), the Columbia ecotype of *Arabidopsis thaliana* is relatively recalcitrant to shoot regeneration, especially when cotyledons were used to induce shoot regeneration *in vitro*. In the experiments here, shoot regeneration in wild type varied dramatically depending on the type of organ used as the explant. In wild type, the frequency of shoot regenerating calluses was negligible in cotyledon derived calluses, moderate in leaf and petiole derived calluses, and relatively high in hypocotyl and adventitious root derived calluses. A similar dependence of shoot regeneration efficiency on the type of explant was clearly detected in *nph4-1* calluses. However, shoot regeneration in *dMP* and *mpG12* calluses derived from these explants differed from those in wild type. Shoot regeneration in *mpG12* was very low regardless of the organ derivation of the callus. In contrast, shoot regeneration in *dMP* was strong in calluses derived from aerial organs (cotyledons, leaves, petioles, and hypocotyls), where it was significantly and consistently higher than that in wild type. No elevation of shoot regeneration was recorded in the *dMP* calluses derived from adventitious root explants.

These data indicate that the presumably elevated *MP* function in *dMP* promotes shoot regeneration in calluses derived from aerial explants. Consistent with this, loss of *MP* function in *mpG12* mutants lead to greatly reduced shoot regeneration. This was indicated by the low frequency of shoot regenerating calluses, and by the low number of shoots per callus in *mpG12*. Loss of *NPH4* function in *nph4-1* mutants had a relatively modest effect on shoot regeneration. The frequency of shoot regenerating calluses and the number of shoots per callus were only slightly lower in *nph4-1* compared to those in wild type.

*MP/ARF5* and *NPH4/ARF7* have a sequence similarity, interact with each other, and have partially redundant functions (Liscum and Reed, 2002; Hardtke et al., 2004; discussed in General Introduction, Chapter 1.2.5). As shown here, during *de novo* shoot initiation, the *NPH4* gene
could not completely substitute for the loss of MP function. The MP gene is implicated in the regulation of several key shoot meristem genes, such as CUC2 and STM (Aida et al., 2002), and the ESR1/DRN gene (Cole et al., 2009). Moreover, MP is involved in the control of cytokinin signaling through the regulation of activity A-type ARR15 and ARR7 (Zhao et al., 2010). The meristem genes, such as CUC, STM, and ESR1/DRN, and some of the ARR genes are implicated in de novo shoot regeneration (reviewed in Chapter 1.6.1; Hwang and Sheen, 2001; Osakabe et al., 2002; To et al., 2004; Zhang and Lemaux, 2005). Thus, the observed strong impairment of shoot regeneration in mpg12 mutants, as well as the consistently elevated shoot regeneration in dMP calluses derived from aerial explants, may be explained by changes in the expression levels of these genes in the mpg12 and dMP genotypes. The failure of dMP to promote shoot regeneration from root derived calluses may be explained by the fact that MP, and hence dMP, are not normally expressed in mature roots (Dr. W. Curshumova, personal communication). However, low shoot regeneration in mpg12 may contradict the suggestion of the irrelevance of MP to shoot regeneration in root derived calluses. Future studies are required to determine whether dMP expression in root derived calluses is similar to that in calluses derived from other organs.

No data demonstrating the involvement of NPH4 in the regulation of shoot meristem genes were found in the literature. Instead, it has been shown that NPH4/ARF7 plays a central role in the modulation of auxin-dependent differential growth in the aerial parts of Arabidopsis plants (Stowe-Evans et al., 1998, Watahiki et al., 1999; reviewed in Chapter 1.2.5). Auxin insensitivity has been shown for aerial organs, but not for the roots of nph4 mutants (Watahiki and Yamamoto, 1997). However, here shoot regeneration in nph4-1 calluses derived from adventitious root explants, with presumably normal auxin sensitivity and callusogenesis (Chapter 3.3.6), was significantly lower than that in the wild type calluses. This could reflect the partially redundant function that NPH4 has with MP in forming the apical part of embryos, including the shoot meristem (Hardtke et al., 2004). The contribution of NPH4 could be subtle, and completely dispensable, in calluses cultured for more than 9 days.

In summary, decreased MP/ARF5 and NPH4/ARF7 levels in mpg12 and nph4-1 mutants had a different impact on de novo shoot regeneration in these genotypes. While shoot regeneration in nph4-1 was only slightly decreased compared to that in wild type, shoot regeneration in mpg12 was consistently, and markedly, reduced in calluses derived from any type of explant in all sets
of experiments. In contrast, elevated MP activity in $dMP$ had a strong stimulating effect on shoot regeneration when aerial organs were used as explants. Thus, $MP$ played an important role in *de novo* shoot meristem initiation.
Chapter 5
Root regeneration under conditions of altered auxin signal transduction

5.1 Background and rationale

In tissue culture, regeneration of adventitious roots occurs in response to a high auxin to low cytokinin ratio, or when auxin is present as a sole hormone in the root inducing medium (RIM) (Miller and Skoog, 1957; Gaba, 2005). Rooting is often a critical step in the plant propagation industry, because many species are extremely recalcitrant regarding adventitious root formation (de Klerk, 2002; Schwarz et al., 2005). Although adventitious roots have been routinely induced in response to auxin (Callis, 2005; Schwarz et al., 2005), the molecular mechanisms of this event are unclear (Schwarz et al., 2005; Rose et al, 2006).

In tissue culture, the acquisition of competence by cells for either shoot or root formation occurs during the preincubation stage on CIM (Christian and Warnick, 1985; Che et al., 2007) - the stage when promeristemoid structures are formed (Schwarz et al., 2005). The specification of organ development depends on subsequent hormonal stimuli on SIM or RIM. Ozawa et al. (1998) - using the temperature-sensitive mutants, srd1, srd2 and srd3 -demonstrated that the competence to form organs is acquired progressively, first roots and then shoots (Ozawa et al., 1998).

*In vitro* experiments with leaf explants have shown that root primordia are formed from the procambial-like cells of veins. These function as pluripotent stem cells, with the ability to form either root primordia or vascular tissues in response to added auxin (Attfield and Evans, 1991; Rose et al., 2006). Unlike the hormone auxin, cytokinin inhibits root formation *in planta* (Aloni et al., 2006) and *in vitro* (Sugiyama 1999; Kubo and Kakimoto, 2000). Auxin insensitivity causes impairment in root regeneration in culture. The auxin-tolerant rac mutants in tobacco are defective in primary root formation and the regeneration of adventitious roots (Sugiyama, 1999, and references therein). By contrast, cytokinin insensitivity promotes root and root primordia formation even on cytokinin containing media (Kubo and Kakimoto, 2000; Kakani et al., 2009). Che et al. (2006) demonstrated that molecular signatures for callus formation and root
regeneration are quite similar, since both are triggered by auxin-rich media. They also showed that competence for root regeneration correlates with the increased expression of IAA genes on CIM.

Besides their other features (for details see Chapter 1.2.5), ARF5/MP and ARF7/NPH4 are implicated in root formation. However, mp seedlings lack primary roots (Berleth and Jurgens, 1993). Increased auxin responses and increased adventitious root formation have been shown for cotyledon explants of the GOF MP line, dMP (Krogan et al., 2006). However, root formation from cultured cotyledons of mp mutants is strongly decreased (Mattsson et al., 2003). Also, mp mutants show auxin insensitivity and reduction of DR5 expression in leaf primordia (Mattsson et al., 2003). The nph4 mutants exhibit auxin resistance in the aerial parts of plants. They have severely impaired expression of many primary auxin-dependent genes and the auxin-responsive reporter DR5 (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Wang et al., 2005). Also, NPH4, together with ARF19, regulates lateral and adventitious root formation (Tatematsu et al., 2004; Wilmoth et al., 2005; Okushima et al., 2007). Wilmoth et al. (2005) showed that, in the presence of exogenous IAA, the excised nph4 roots formed fewer lateral roots. The nph4 excised hypocotyls formed fewer adventitious roots than did wild type roots and hypocotyls. By contrast, nph4 arf19 double mutants mainly lacked lateral roots.

In this chapter, the roles of the MP and NPH4 genes in de novo root regeneration in tissue culture are explored. The extent to which root regeneration in each of the investigated genotypes is further influenced by the origin of the respective tissue sample is also explored.

5.2 Specific research objectives

This project investigates the possible influence on de novo root regeneration of auxin signaling through ARF5/MP and ARF7/NPH4. To this end, the loss-of-function mutants mpG12 (Hardtke and Berleth, 1998), and nph4-1 (Harper et al., 2000), and the gain of function transgene line dMP with truncated dimerization domains III and IV (reviewed in Chapter 1.2.5; Fig. 1.2; Krogan, 2006) were tested to determine the influence of these genotypes on adventitious root regeneration in vitro. All the genotypes used in the experiments were in the Col-0 background and were compared to the Col-0 wild type. Explants of different organs (cotyledons, leaves, and adventitious roots) were first cultured on CIM for 6 days and then transferred to RIM (reviewed
in Chapter 2.8; Valviken et al., 1988). Additionally, root formation in calluses derived from adventitious root explants, and cultured on SIM after 6 days on CIM, was compared among the genotypes. The frequency of root regeneration, a calculation based on the pooled data of all replicas of the experiments, was compared among the tested genotypes after 21 days on RIM or on SIM, as was the mean number of roots per root regenerating callus (mean +/- SE).

5.3 Results

5.3.1 Root regeneration in cotyledon derived calluses on RIM

There were some genotype related differences in root regeneration frequency, and in the mean number of roots per root regenerating callus (Fig. 5.1; 5.2 A; 5.3). In wild type, only 67% of calluses (63 out of 89) regenerated roots (Fig. 5.1 A). Among the rest of the genotypes, the proportion of root regenerating calluses was higher than that in wild type, and relatively similar: 96% of dMP calluses (84 out of 88); 91% of nph4-1 calluses (76 out of 85); and, 93% of mpG12 calluses (71 out of 77) regenerated roots (Fig. 5.1 A).

The mean number of roots per root regenerating callus was quite similar among wild type, nph4-1, and mpG12 calluses. It was significantly higher only in dMP (Fig. 5.1 B). In wild type, the mean number of roots was 8.6±0.69, range 2 to 25 roots per calluses in 63 root regenerating calluses. In nph4-1, the mean number of roots was 8.72±0.53, range 1 to 22 roots in 76 calluses. In mpG12, this number was 8±0.5, range 1 to 20 in 71 calluses (Fig. 5.1 B). The mean number of roots was significantly higher in dMP, equal to 17.3±0.82, range 3 to 35 in 84 root regenerating calluses (P<0.001) (Fig. 5.1 B).

To summarize, in calluses derived from cotyledon explants, root regeneration in nph4-1 and mpG12 was not impaired. It was either equal to that in wild type (in the mean number of roots per root regenerating callus), or even exceeded that in wild type (in the frequency of root regenerating calluses). Moreover, root regeneration in dMP calluses was significantly increased compared to that in wild type.
Figure 5.1 Percentage of root forming calluses (A), the mean numbers of roots per root forming callus (B) in calluses derived from cotyledon, leaf and adventitious root explants after 21 days on RIM (following 6 days on CIM to produce the calli);

In (A) “n” is the total number of explants used in the experiments; in (B) “n” is the number of root regenerating calluses. Bars represent SE.
Figure 5.2 Cotyledon (A) and leaf (B) derived calluses after 21 days on RIM (following 6 days on CIM to produce the calli). Genotypes indicated within plate sectors; arrow in A points at a callus without roots
Figure 5.3 Root initiation in cotyledon explants after 6 days on RIM (following 6 days on CIM to produce the calli).

Genotypes, bottom right, arrows indicate root outgrowth.
5.3.2 Root regeneration in leaf derived calluses on RIM

Root regeneration in calluses derived from leaf explants was generally lower than that in cotyledon derived calluses (Fig. 5.1; 5.2 B). In wild type, only 48% (30 out of 62) calluses regenerated roots. Once again, this frequency was the lowest one among the genotypes (Fig. 5.1 A). In the arf mutants, nph4-1 and mpG12, the frequency of root regenerating calluses was higher than that in wild type: 58% of nph4-1 calluses (40 out of 69); and, 71% of mpG12 calluses (48 out of 68) regenerated roots (Fig. 5.1 A). The highest frequency of root regenerating calluses was scored again in dMP, where 94% of calluses (63 out of 67) formed roots (Fig. 5.1A).

The mean number of roots per root regenerating callus was similar among wild type, nph4-1, and mpG12 calluses (Fig. 5.1B). However, the mean number of roots was significantly higher in dMP (Fig. 5.1 B). In wild type, the mean number of roots per root regenerating callus was 4.35±0.35, range 2 to 10 per callus in 30 calluses. In nph4-1, the mean number of roots was 4.3±0.31, range 2 to 8 in 40 calluses. In mpG12 the mean number was 4.2±0.26, range 2 to 9 in 48 calluses (Fig. 5.1 B). The mean number of roots was significantly higher in dMP, equal to 7.3±0.39, range 2 to 13 in 63 calluses (P<0.001) (Fig. 5.1 B).

To summarize, in leaf derived calluses, root regeneration in the arf mutants, nph4-1 and mpG12, was not lower than that in wild type. However, in dMP, root regeneration was significantly greater than that in wild type.

5.3.3 Root regeneration in calluses derived from adventitious roots on RIM

Since root explants are able to produce roots on RIM without CIM preincubation (Ozawa et. al, 1988), all explants in this experiment formed roots. The genotype related differences were manifested only in the number of roots per root regenerating callus (Fig. 5.1; 5.4; 5.5). In wild type, the mean number of roots was 22.12±1, range 2 to 50 in 111 calluses. The mean number of roots in dMP calluses was comparable to that in wild type (19.86±0.95, range 1 to 45 in 99 calluses) (Fig. 5.1 B). By contrast, the mean number of roots per callus was significantly lower in nph4-1 (11.57±0.59, range 2 to 30 in 100 calluses, P<0.001); and especially in mpG12 calluses (7.99±0.42, range 1 to 18 in 103 calluses, P<0.001). The difference in the mean number of roots per callus between nph4-1 and mpG12 was also statistically significant (P<0.001) (Fig. 5.1 B).
**Figure 5.4** Calluses derived from adventitious root explants after 21 days on RIM (following 6 days on CIM to produce the calli). Genotypes indicated within plate sectors.
In summary, quantitative differences in root formation in this category of explants occurred only with regard to the number of roots per callus. In the *arf* mutant genotypes, the number of roots per callus in *nph4-1* was only half that of wild type; and in *mpG12* it was even less. In *dMP* root calluses—contrary to *dMP* cotyledon and leaf derived calluses—no increase in root regeneration was observed. The number of roots per callus was comparable in wild type and *dMP*.

### 5.3.4 Root formation in adventitious root explants on SIM

Genotype related differences in root initiation on RIM in adventitious root calluses were expressed only in the number of roots per callus (Fig. 5.1). In light of that finding, this study explored whether greater distinctions would become apparent in the presence of a root suppressing stimulus (Fig. 4.4; 5.6; 5.7). To that end, root formation in adventitious root calluses on cytokinin-rich SIM was investigated. As one might expect, rooting on SIM was much less than on RIM in all genotypes and was particularly low in *mpG12* calluses (Fig. 5.1; 5.6). After 21 days on SIM, 87% of wild type calluses (139 out of 159), 98% of *dMP* calluses (157 out of 159), and 82% of *nph4-1* calluses (124 out of 143) had formed roots (Fig. 5.6 A). However, roots formed in only 24% of *mpG12* calluses (39 out of 150) (Fig. 5.6 A).

The mean number of roots per root regenerating callus also differed among the genotypes. In wild type, the mean number of roots per callus was 6.98±0.22, range 1 to 30 per callus in 139 calluses (Fig. 5.6 B). In *dMP*, the mean number was slightly higher than that in wild type (8.75±0.3, range 1 to 32 in 157 calluses, P<0.1) (Fig. 5.6 B). In *nph4-1*, the mean number of roots per callus (6.06±0.45, range 1 to 20 in 124 calluses) was similar to that in wild type (Fig. 5.6 B). In *mpG12*, the mean number of roots was the lowest compared to that in wild type (1.9±0.42, range 1 to 5 in 39 *mpG12* calluses, P<0.001, and to that in the other genotypes (P<0.001) (Fig. 5.6 B).

In summary, compared to root formation on RIM, fewer roots were formed in all the genotypes on SIM. However, root development was particularly suppressed in the *mpG12* calluses, which barely produced any roots on the cytokinin-rich SIM.
Figure 5.5 Calluses derived from adventitious root explants after 12 (A, C, E, G) and 21 (B, D, F, H) days on RIM (following 6 days on CIM to produce the calli). Genotypes, and days on RIM, bottom right.
Figure 5.6 Percentage of root regenerating calluses (A), and the mean numbers of roots per root regenerating callus (B) in calluses derived from adventitious root explants after 21 days on SIM (following 6 days on CIM to produce the calli)

In (A) “n” is the total number of explants used in the experiments; in (B) “n” is the number of root regenerating calluses. Bars represent SE.
Figure 5.7 Root outgrowth in calluses derived from adventitious root explants after 8 (A, C, E, G) and 13 (B, D, F, H) days on SIM (following 6 days on CIM to produce the calli). Genotypes, and days on SIM as indicated on bottom right.
5.4 Discussion and Conclusions

The results in this chapter show that root regeneration differed among the auxin signal transduction genotypes, and was also influenced by the origin of the explant. Quite unexpectedly, root regeneration responses were only partially correlated with the assumed level of auxin sensitivity in the explants.

Root regeneration on RIM in cotyledon and leaf derived calluses was elevated only in the dMP explants, yet undiminished in the arf mutant genotypes, nph4-1 and mpG12. Earlier Krogan (2006) showed that dissected dMP cotyledons were highly competent for adventitious root formation when they were cultured in liquid RIM containing IBA. By contrast, rooting of mp cotyledons in similar conditions was greatly decreased compared to that in wild type (Mattsson et al., 2003). The discrepancy between the results of this study and the previous data on root regeneration in mpG12 cotyledon explants can possibly be explained by a difference in the experimental methods of rooting. Culture on liquid RIM may trigger different developmental processes compared to those after pre-incubation on CIM, during which competence for organogenesis stimuli is acquired (Christian and Warnick, 1985; Che et al., 2007; see Chapter 2.8 for details).

Another possible explanation for the normal root regeneration here in the aerial explants of the arf mutant genotypes, particularly mpG12, may be related to the different molecular properties of the two auxins, 2,4-D in CIM, and IBA in RIM. IBA is one of the natural forms of auxin with physiological properties similar to IAA (Rashotte et al., 2003; Gaba, 2005). 2,4-D is a powerful synthetic auxin, used for callus initiation (Gaba, 2005), which is known to be a good influx and a bad efflux substrate - the qualities which allow 2,4-D to accumulate intracellularly in high concentrations (Delbarre et al., 1996; Estelle, 1998). Possibly, the high intracellular auxin (2,4-D) concentration during CIM incubation diminished differences in the relative auxin responses among the tested genotypes. Also, the genotype related sensitivity of the explants to these two auxins can differ (Estelle, 1998).

All the calluses derived from the adventitious root explants produced roots on RIM. The calluses of the arf mutant genotypes (nph4-1 and mpG12) had a lower mean number of roots per callus. Differences in the mean number of roots per callus were the only discriminative phenotypical
trait found in this category of explants. Also, lower root numbers in the arf mutants were only in partial agreement with the original levels of auxin sensitivity in root explants, where low auxin sensitivity is known for mpG12 (Mattsson et al., 2003), but not for nph4-1 roots (Watahiki and Yamamoto, 1997; Okushima et al., 2005).

Another explanation for the lower root formation on RIM in the arf root derived calluses may be their genotype related ability for lateral root formation. Root development in root derived calluses is initiated from the pericycle of the root explants, i.e., it is similar to lateral root formation (Casimiro et al., 2003; Atta et al., 2009; Sugimoto et al., 2010). Auxin stimulates the development of lateral roots (Casimiro et al., 2003; De Smet et al., 2010). Because of that, the lower root numbers on RIM found in nph4-1 and mpG12 could reflect their ability for lateral root formation in response to auxin. As shown recently, ARF7/NPH4 and ARF5/MP are components of a bimodular organogenesis system, and both ARFs are required for proper organogenesis and lateral root formation in response to auxin (De Smet et al., 2010). LOF of these ARFs can lead to reduced lateral root formation; and possibly, to lower number of roots in the mpG12 and nph4-1 adventitious root explants on RIM.

In this study, calluses derived from adventitious roots on SIM, had abundant root formation in most of the wild type, nph4-1, and dMP calluses—although in lesser numbers compared to that on RIM. However, root formation was dramatically reduced in mpG12. This was in broad correlation with known root auxin sensitivities, where mpG12, the only genotype with reduced auxin sensitivity in roots, also had greatly reduced root formation (Watahiki and Yamamoto, 1997; Mattsson et al., 2003; Krogan, 2006). Cytokinin inhibits root formation (Aloni et al., 2006). When roots appear on cytokinin-rich SIM after CIM, this occurs against the external hormonal signal. The latter is overcome by the inherent ability of roots to produce lateral roots, which is normally an auxin-dependent process (Casimiro et al., 2003; Hardtke, 2006). However, in the results here, the difference in genotype related auxin sensitivity comes out more clearly on SIM, rather than on RIM. The reasons for this are as yet unclear. To attempt to clarify them, further study with a wide range of mutants with altered auxin sensitivity would be required. Also in this study, cytokinin counter activity on SIM was inversely correlated to auxin sensitivity in the genotypes. In these results, low auxin sensitivity in mpG12 can also be paired with elevated cytokinin sensitivity. The latter was also indicated by early and intensive greening of mpG12 roots on SIM (discussed in Chapter 4.3.7). The lack of roots, or root primordia, and
the intensive greening of calluses on SIM are known as cytokinin responses (Sugiyama 1999; Kubo and Kakimoto, 2000; Gaba, 2005). As shown recently, ARF5/MP is implicated in crosstalk between auxin and cytokinin transduction pathways (Zhao et al., 2010; see for details Chapter 1.2.5). Thus, auxin/cytokinin balance and cytokinin sensitivity may be changed in the mpG12 explants due to the implication of MP in auxin/cytokinin crosstalk, and due to the interrelated and antagonistic activity of these hormones in organogenesis (Coenen and Lomax, 1997; Nordstrom et al., 2003; Zhao et al., 2010). These changes can result in visually noticeable elevated cytokinin responses, and a lack of roots in the mpG12 root derived calluses on SIM.
Chapter 6

General Discussion and Conclusions

6.1 Effects of altered MP/ARF5 and NPH4/ARF7 activities on callusogenesis and de novo generation of shoot and root

Plant cells have the ability to generate a whole plant from a wide variety of tissues and organs, or even from a single cell (Skoog and Miller, 1957; Vogel, 2005; Sugimoto et al., 2010). Several researchers have shown that auxin signaling genes may be involved in de novo shoot and root formation (Coenen and Lomax, 1998; Gordon et al., 2007; Chatfield and Raizada, 2008; Zhao et al., 2008; Kakani et al., 2009). Auxin signaling operates through auxin response factors (ARFs), a family of transcription factors that regulate the expression of auxin responsive genes (reviewed in Teale, 2006; Guilfoyle and Hagen, 2007). In this research, the LOF arf mutants, mpG12 and nph4-1, and the GOF transgene line dMP with truncated regulatory domains III and IV (Krogan, 2006; Fig. 1.2) were tested to determine the roles of altered MP and NPH4 activity on callus formation and de novo organogenesis in vitro. The results of the study indicated genotype, and organ related differences during developmental processes in plant regeneration.

Detailed discussion of the results related to each type of organogenesis is presented at the end of each result chapter (3.4; 4.4; 5.4). The main focus of this section is the discussion and general conclusions of the main results.

6.1.1 NPH4/ARF7 in developmental processes in tissue culture

The results of this study indicated that callus proliferation, root formation, and shoot regeneration were diminished in some of the nph4-1 explants, though relatively normal in others (Fig. 3.1; 4.1; 5.1). The observed variations in the organogenesis responses correlated with the level of auxin sensitivity in the tested genotypes only during callus proliferation, but not always during root and shoot formation.

Callusogenesis and root formation - developmental processes induced by auxin--have been considered to share similar molecular pathways (Gaba, 2005; Che et al., 2006; Atta et al., 2009;
Sugimoto et al., 2010). However, in this study, callusogenesis and root regeneration in nph4-1 mutants were affected differently. For example, only nph4-1 aerial organs were defective in callusogenesis, and only calluses derived from adventitious roots had fewer roots compared to those in wild type. Interestingly, only aerial parts of nph4-1 plants have been known to be auxin insensitive (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Okushima et al., 2005). The diminished level of auxin sensitivity in the nph4-1 aerial explants correlated with the diminished callus proliferation, but it did not correlate with the ability for root regeneration (Fig. 3.1).

Callus proliferation depends on the activation of cell cycle genes in response to auxin and cytokinin signals (Hemerly et al., 1993; Soni et al., 1995; Shaul et al., 1996; Riou-Khamlichi et al., 1999; Sugiyama, 1999; Richard et al., 2002; Che et al., 2007). AuxREs elements have been found in the promoters of various cyclin genes and some CDKs (Richard et al., 2002; Perrot-Rechenmann, 2010). In that light, NPH4/ARF7 function in aerial organs might be related to the auxin mediated activation of cell cycle genes.

The most straightforward interpretation of the apparently normal callusogenesis in nph4-1 adventitious roots is that auxin signal transduction is not defective in nph4-1 roots – because of the presence of other ARFs, particularly ARF19, which can compensate for NPH4 function specifically in this organ (Okushima et al., 2005). This interpretation is plausible, because it is consistent with the observed absence of root defects, and normal root auxin sensitivity in nph4 single mutants (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Okushima et al., 2005). This is contrasted by the appearance of root specific defects in the nph4 arf19 double mutants (Tatematsu et al., 2004; Okushima et al., 2005; Wilmoth et al, 2005; De Smet et al., 2010).

Similarly, apparently normal de novo root development in nph4-1 aerial explants (cotyledons and leaves) (Fig. 5.1) reflects the lack of an NPH4 requirement for de novo rooting in aerial organs. This may be due to ARF redundancy in these organs (Hardtke et al., 2004; Tatematsu et al., 2004; Wilmoth et al., 2005). By contrast, the results suggest that the molecular mechanisms of root initiation in adventitious root explants are different from those in aerial explants, as the latter seem to selectively require NPH4.
Shoot regeneration in *nph4-1* calluses also reflected the origin of the explants. Shoot regeneration was relatively mildly, or not at all, affected in explants from aerial organs. By contrast, shoot regeneration in *nph4-1* adventitious root explants was significantly less than that in wild type (Fig. 4.1). Thus, regarding the *NPH4* requirement in organogenesis, the organ origin of the *nph4-1* explants determines the efficiency of *de novo* shoot, and root formation.

These organ related similarities in the efficiency of root and shoot regeneration may indicate that these processes share a common developmental pathway. This statement accords with the model in which shoot or root organogenesis is preceded by a state of tissue competence for either type of organogenesis (Christianson and Warnick, 1985; Schwarz et al., 2005).

For example, tissue structures similar to lateral root meristems (LRMs) develop during callus proliferation on CIM (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010). Interestingly, similar structures were also found in aerial explants when they were cultured on CIM (Sugimoto et al., 2010). When transferred on RIM or SIM, LRM-like structures convert to either root or shoot meristems (Che et al., 2007; Atta et al., 2009). Taking into consideration that lateral root formation is slightly diminished in *nph4-1* roots (Okushima et al., 2005; Wilmoth et al., 2005), *nph4-1* adventitious root explants may have fewer organogenesis-competent cells that are the precursors of lateral root meristems. This may explain reduced shoot and root regeneration in *nph4-1* adventitious root explants. The organogenesis aspect of the *NPH4* function was almost irrelevant to the auxin sensitivity of *nph4-1* organs, as measured by auxin sensitivity assays (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Okushima et al., 2005).

The variation in organogenesis productivity among the different organs and types of explants of the same genotype is a well known fact in tissue culture research (Akama et al., 1992; Zhao et al., 2002; Gaba, 2005). The question remains, however, of how calluses are able to maintain their organ related identity, which causes the variations in organogenesis activity among calluses from different explants. One explanation is the variation in endogenous hormone levels among plant organs, which can affect their organogenesis responses *in vitro* (Davies, 2004; Gordon et al., 2007). Also, the difference in epigenetic regulation may be a factor contributing to the variation in organogenesis activity among plant organs (Zhao et al., 2008). Calluses generally comprise heterogeneous mixtures of mostly undefined cell types (Schwarz et al., 2005). A further analysis
of calluses derived from various organs is necessary to understand the causes of organ related differences in organogenesis responses.

### 6.1.2 MP/ARF5 in auxin and cytokinin mediated developmental processes

The MP gene together with NPH4 plays a key role in embryonic, vascular, and phyllotactic responses (Hardtke et al., 2004). However, the overexpression of NPH4 can not rescue the phenotype of mp mutants, which indicates only unilateral redundancy of their developmental roles (Hardtke et al., 2004; Wilmoth et al., 2005). The results in this study showed that callusogenesis and root regeneration had relatively similar features in mpG12 and nph4-1 mutants. The quantitative differences that distinguish callusogenesis and rooting responses in mpG12 from those in nph4-1 possibly reflect the levels of contribution of NPH4 and MP in a particular process (Fig. 3.1; 5.1). However, the ability for shoot regeneration strongly distinguished mpG12 from nph4-1 mutants (Fig. 4.1).

In particular, the impairment of callus proliferation in mpG12 aerial explants was less pronounced than that in nph4-1. However, dissimilar to nph4-1, the fresh weight of the mpG12 adventitious root derived calluses was below that of the wild type ones. This may indicate that some level of auxin insensitivity exists in all mpG12 organs. The generally milder phenotype of mpG12 aerial explants on CIM can possibly be explained by the presence of the functional NPH4 gene. NPH4 contribution to callusogenesis seems stronger than that of the MP gene. Based on the callusogenesis phenotype of mpG12 mutants, the possible contribution of the MP gene to the regulation of cell cycle genes may also be a consideration (Richard et al., 2002; Perrot-Rechenmann, 2010).

Root regeneration in mpG12 and nph4-1 explants had rather similar features. Based on normal root regeneration in the mpG12 aerial explants, two suggestions can be made. Either MP is not involved in de novo root formation in aerial explants; or, other ARFs substitute for the lack of MP function (Hardtke et al., 2004; Wilmoth et al., 2005). This might be surprising, given MP requirement for embryonic root formation, but it has previously been noted that this does not imply a similar role in other cell types (Berleth and Jurgens, 1993). Similarly to NPH4, MP can facilitate root formation in calluses derived from adventitious root explants, as indicated by the result that fewer roots were formed in the mpG12 adventitious root calluses. The low root
formation in *mpG12* root explants may reflect the regulation of lateral root formation by the *MP* gene, shown recently by De Smet et al. (2010).

Normally, the *MP* gene is not expressed in mature root tissue (Dr. W. Ckurshumova, personal communication), although *MP* expression is reactivated during callus proliferation (Dr. W. Ckurshumova, personal communication), and during lateral root initiation (De Smet et al., 2010). This concords with the data presented in this thesis, which indicate that the *MP* gene might be involved in callusogenesis and root formation in adventitious root explants based on the phenotype of *mpG12* mutants.

The major difference between *mpG12* and *nph4-1* mutants was in their ability for shoot regeneration (Fig. 4.1). Unlike in *nph4-1*, shoot regeneration was consistently very low in all the types of *mpG12* explants. Hardtke et al. (2004) demonstrated that the functions of *NPH4* and *MP* during SAM formation in embryos are only partially redundant, and the *NPH4* gene could not rescue the loss of *MP* functions in *mp* mutants. Likewise, the low rate of *de novo* shoot regeneration in *mpG12* may indicate one of the major differences between *MP* and *NPH4* functions in *de novo* SAM formation.

As previously reported (Przemeck et al., 1996), the overall morphology of the developing *mpG12* shoots was relatively normal during vegetative stages. Similarly, in this study, no major abnormalities were detected in regenerated shoots on SIM. Taking into consideration that the competence to form organs is acquired sequentially, first roots and then shoots (Ozawa et al., 1998), and that *de novo* root regeneration in *mpG12* explants was not impaired, it is possible to suggest that *MP* function is somehow related to the conversion of LRM-like structures to shoot meristems in response to cytokinin (Che et al., 2007; Atta et al., 2009; Sugimoto et al., 2010).

A growing body of research shows that *MP* activity is linked to regulation of shoot meristem size and formation (Vidaurre et al., 2007; Schuetz et al., 2008; Zhao et al., 2010), and to the activity of several key meristem genes (Aida et al., 2002; Cole et al., 2009; Chapter 1.2.5). Moreover, the *MP* gene facilitates auxin-cytokinin crosstalk via the auxin mediated regulation of two A-type ARRs (Zhao et al., 2010). The strong phenotype of *mpG12* mutants in shoot regeneration also demonstrates the key role of the *MP/ARF5* gene in *de novo* SAM initiation.
6.1.3 Enhancement of *de novo* organogenesis in the *dMP* genotype

*MP* function is elevated in the *dMP* genotype due to a constitutive *MP* activity in the truncated *dMP* transgene (Fig. 1.2; Krogan, 2006). Krogan (2006) demonstrated that auxin responses are strongly elevated in the *dMP* genotype. His results also showed a high capacity of cotyledon explants for rooting in liquid RIM (Krogan, 2006). The data of this study further indicated an enhancement of organogenesis responses in *dMP*, such as callusogenesis, root regeneration, and particularly strongly, shoot regeneration. This enhancement, however, was detected only in the aerial, but not in the adventitious root explants, indicating the organ dependent character of these responses in the *dMP* genotype.

The dependence of organogenesis responses on *MP* activity in the GOF and LOF genotypes (*dMP* and *mpG12*) turned out to be complex. Elevated organogenesis responses in *dMP* were not always paired with a reduction of the same responses in LOF *mpG12*, as was observed during shoot regeneration in aerial explants (Fig. 4.1). However, that was not always the case in other organogenesis responses and explant types. Moreover - though to a different degree - shoot organogenesis was diminished in the adventitious root explants of *dMP* and *mpG12* (Fig. 4.1). Thus, the data of this study indicated that the effect of *MP* on organogenesis processes depended on the level of gene activity only in particular processes, such as shoot regeneration in aerial explants. In other cases, the increase in organogenesis responses by *dMP* was not significant, or it may have been prevented by some unknown limiting factors. For example, low shoot regeneration in *dMP* adventitious roots was greatly elevated when the time of CIM preculture was increased, although the latter had no effect on *mpG12* explants (Fig. 4.5). This may indicate that some factors suppressing shoot regeneration in *dMP* were eliminated by the prolonged time on CIM. More experiments would need to be done to be able to characterize the factors and their molecular bases that possibly counteract *MP* activity in organogenesis responses.

### 6.2 General Conclusion

According to the results of this study, callusogenesis, and root and shoot regeneration showed genotype and organ related differences. Loss of *MP* and *NPH4* functions affected callus and root regeneration in *mpG12* and *nph4-1* mutants in a relatively similar manner. However, a major
difference in organogenesis responses between these *arf* mutants was indicated in their shoot regeneration. Putatively elevated *MP* activity levels in the *dMP* genotype seem to have resulted in the promotion of root regeneration, callus growth, and shoot regeneration in aerial, but not in adventitious root explants. The different levels of gene redundancy, and the joint functions of *MP* and *NPH4* (Hardtke et al., 2004) during *de novo* organogenesis can be suggested based on the results of this study. Overall, screening *arf* mutant genotypes in tissue culture assays can be a powerful tool to elucidate unknown functions of *ARF* genes, as demonstrated in this thesis. The possibly dominant effects of other *ARF* gene transgenes on organogenesis processes *in vitro* can be of significant interest for plant biotechnology, if they can be reproduced in suitable plant species - ones with normally poor regeneration properties.
References


