Biochemical and Physiological Studies of *Arabidopsis thaliana* Diacylglycerol Kinase 7 (AtDGK7)

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By

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Eidesstattliche Erklärung

Diese Dissertation ist das Ergebnis experimenteller Arbeit, welche vom September 2002 bis November 2005 im Institut für Biochemie und Biologie der Universität Potsdam in Kollaboration mit dem Max-Planck-Institut für Molekulare Pflanzenphysiologie durchgeführt wurde. Ich erkläre, dass ich die vorliegende Arbeit an keiner anderen Hochschule eingereicht sowie selbständig und nur mit den angegebenen Mitteln angefertigt habe.

Potsdam, Dezember 2006 _____

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PREFACE

Plants are fantastic factories to convert simple molecules into products charged with high energy. Today the biotechnology has provided a diverse set of tools used to design and optimize the capture of solar energy through crops. Moreover, the development of biotechnological and genomics tools have allowed and still allowing the development of crops with specific characteristics, optimized for example to produce biofuels and bioenergy. However, every year a long discussion about the use of resources and the application of politics for food security define the agenda of diverse institutions around the world. Biological research, legal issues and society rights are combinatory keys to put in perspective and finally, drive the direction and interest of stakeholders and decision makers, with the aim of contributing to the economic progress of the countries.

This manuscript introduces a JBC´s paper on *AtDGK7*, a catalytic active member of the AtDGK family, and a biochemical characterization in comparison with *AtDGK2*, the first member of the family able to phosphorylate the DAG substrate *in vitro*. It follows a chapter about the molecular, functional and physiological analysis of *AtDGK7*. A hypothesis about the activity and involvement in aspects of cell proliferation and growth of lateral roots is discussed.

I have to say thank you initially to my parents for all their efforts to build a family with a nice values like e.g. freedom and respect.

I would like to gratefully acknowledge Prof. Dr. Bernd Müller-Röber for the opportunity to join his research group. For his continuous support, and his excellent supervision. I appreciate his knowledge and skills, and his assistance in writing… and especially his patience with me.

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Special thanks to the Captain Pollito for his big energy and tenderness pushing me to bring my thesis to an end.

"The most beautiful thing we can experience is the mysterious.

It is the source of all true art and science"

A. Einstein

ABSTRACT

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A family of diacylglycerol kinases (DGK) phosphorylates the substrate diacylglycerol (DAG) to generate phosphatidic acid (PA) . Both molecules, DAG and PA, are involved in signal transduction pathways. In the model plant *Arabidopsis thaliana*, seven candidate genes (named *AtDGK1* to *AtDGK7*) code for putative DGK isoforms. Here I report the molecular cloning and characterization of *AtDGK7*. Biochemical, molecular and physiological experiments of *AtDGK7* and their corresponding enzyme are analyzed.

Information from Genevestigator says that *AtDGK7* gene is expressed in seedlings and adult *Arabidopsis* plants, especially in flowers. The *AtDGK7* gene encodes the smallest functional DGK predicted in higher plants; but also, has an alternative coding sequence containing an extended *AtDGK7* open reading frame, confirmed by PCR and submitted to the GenBank database (under the accession number DQ350135). The new cDNA has an extension of 439 nucleotides coding for 118 additional amino acids The former AtDGK7 enzyme has a predicted molecular mass of ~41 kDa and its activity is affected by pH and detergents. The DGK inhibitor R59022 also affects AtDGK7 activity, although at higher concentrations (i.e. IC_{50} ~380 µM). The AtDGK7 enzyme also shows a Michaelis-Menten type saturation curve for 1,2-DOG. Calculated K_m and V_{max} were 36 µM 1,2-DOG and 0.18 pmol PA min^{-1} µg of $protein^{-1}$, respectively, under the assay conditions. Former protein AtDGK7 are able to phosphorylate different DAG analogs that are typically found in plants.

The new deduced AtDGK7 protein harbors the catalytic DGKc and accessory domains DGKa, instead the truncated one as the former AtDGK7 protein (Gomez-Merino *et al*., 2005).

SUMMARY

Arana-Ceballos, F. 2006 Biochemical and Physiological Studies of *Arabidopsis thaliana* Diacylglycerol Kinase 7 (AtDGK7). Dissertation. Institute of Biochemistry and Biology. University of Potsdam. Golm, Germany.

Diacylglycerol kinase (DGK) regulates the level of the second messenger diacylglycerol (DAG) and produces phosphatidic acid (PA), another signalling molecule. The *Arabidopsis thaliana* genome encodes seven putative diacylglycerol kinase isozymes (named AtDGK1 to 7), structurally falling into three major clusters. So far, the biological function of these enzymes has only marginally been analysed. In my PhD work I specifically concentrated on the analysis of AtDGK7, which belongs to the cluster II of the plant DGKs. In the following I am shortly summarizing the major discoveries of this work:

1. *AtDGK7*, encoded by gene locus At4g30340, was initially reported to encode a protein of 374 amino acids with an apparent molecular weight of 41.2 kDa. This conclusion was drawn from the report of a full-length *AtDGK7* cDNA deposited in the public databases (NCBI). AtDGK7 harbours an N-terminal catalytic domain, but in contrast to various of the characterized DGKs (including AtDGK2), lacks a cysteine-rich domain at its N-terminus.
2. Heterologous expression of the AtDGK7 protein in *Escherichia coli* proved it to be a genuine diacylglycerol kinase. The biochemical properties of the recombinant enzyme were determined. AtDGK7 activity was affected by pH, various detergents, and the DGK inhibitor R59022.
3. R59022 inhibits root elongation and lateral root formation and reduces plant growth, suggesting that DGKs play an important role in plant development.
4. Together with Fernando Gomez I was able to demonstrate that both, AtDGK2 and AtDGK7, phosphorylate various DAG molecular species that are typically found in plants, indicating that both enzymes convert physiologically relevant substrates.
5. Quantitative real-time reverse transcription qRT-PCR was used to measure *AtDGK7* transcript levels. *AtDGK7* was found to be expressed throughout the Arabidopsis plant, but expression is strongest in flowers and young seedlings.
6. The *AtDGK7* promoter was isolated and fused to the *Escherichia coli* β-glucuronidase (*uidA*) reporter gene. The promoter-reporter gene constructs was subsequently used to transform *Arabidopsis thaliana* using the floral dip method. Subsequently, the expression pattern of the *AtDGK7* gene was analysed in detail.

7. *AtDGK7* was found to be expressed early at the root-hypocotyl transition zone and also at the root apex. In 4- and 5-day old seedlings GUS staining was evenly distributed in cotyledons and root tips of primary roots; in 9- and 10-day-old seedlings staining was detected at the base of the cotyledons and the apical meristem. Later, strong *AtDGK7* promoter activity was observed in stipules, in the tip of the primary root and also in secondary root tips, emerging lateral roots and in root hairs. Additionally, cells of the leaf edges as well as guard cells were stained in juvenile, early- and late-adult leaves and in cauline leaves; in flowers, GUS staining was detected in the junction that connects filaments and stamens in the anthers.
8. The expression pattern of the *AtDGK7* gene was also analysed in plants grown on different solid media with varying carbon and nitrogen concentrations. I also tested the effect of auxin and the auxin transport inhibitor TIBA on gene expression. *AtDGK7* expression was affected negatively at low nitrogen concentration and also in the presence of TIBA; primary root growth was reduced and lateral root formation was suppressed.
9. Transgenic Arabidopsis lines harbouring T-DNA insertions in the *AtDGK7* gene (mostly in the 3´ UTR) were identified. Mutant lines exhibited altered root growth on various media tested. Additionally, after external application of auxin, *AtDGK7* T-DNA insertion lines partially reversed the mutant root phenotype.
10. I was able to clone of a novel cDNA for *AtDGK7* which codes for a protein that is larger than the previously described AtDGK7 polypeptide (54.6 kDa). The new *AtDGK7* cDNA sequence obtained was submitted to the GenBank database under the accession number DQ350135.
11. Based on the physiological analysis and the results obtained it is hypothesized that *AtDGK7* is involved in a signalling process affecting growth and development of lateral roots, mediated via an interaction with auxin.

CHAPTER 1

INTRODUCTION

1. General aspects about phospholipid signaling

All biological organisms sense environmental and chemical signals via perception mechanisms or specific receptors. Once stimulated, such receptors induce an intracellular cascade of events leading to the regulation of specific genes or to modification of cellular activity, which in turn produces a biological response. In a particular case, plants sense and respond to endogenous signals and environmental cues to ensure optimal growth and development. Sensing and processing of stimuli are mediated by signal transduction cascades and circuits. These molecular circuits in cells are constructed from receptors, enzymes, channels and regulatory proteins. They detect, amplify, and integrate diverse external signals. Plant cells have developed finely tuned cellular mechanisms to response to a variety of internal and external stimuli with rapid and dramatic rearrangements of their cytoplasm (Staiger, 2000). To contend with environmental variability, plants often show considerable plasticity in their developmental and physiological behaviours. Some of their apparent choices include: when and where to forage for nutrients and where to allocate those nutrients and derived organic molecules within the organism; when and what organs to generate or senesce; when to reproduce and the number of progeny to create; how to mount a defense against attack and in what tissues or organs; and when and where to transmit chemical signals to surrounding organisms. All these responses must occur within the context of a changing environment, including periodic and meteorological variation regarding light, nutrients, water, wind, temperature and attack. They must be made within the multicellular confines of the complex biological unit of the plant body and, thus, require coordinated cell-to-cell signalling, which requires a sophisticated information storage and acquisition system (Brenner et al., 2006).

In eukaryotes many extracellular signals (first messengers) bind to cell surface receptors which causes changes in the concentration of intracellular signals or second messengers. The known or probably second messengers include cyclic AMP, cyclic GMP, inositol trisphosphate (IP_3), phosphatidylinositol 3,4,5-trisphosphate (PIP_3), cyclic ADP ribose (cADPR) (Lee and Aarhus, 1991), arachidonic acid and diacylglycerol (DAG). Ca^{2+} may also be regarded as a second messenger: Ca^{2+} concentration increases in the cytoplasm due to the opening of voltage-gated ion channels, which might be e.g. be due to binding of messengers to ligand-gated ion channels, which can either span the plasma membrane (e.g. the nicotinic acetylcholin receptor) or membranes of internal organelles (e.g., the IP_3 and cADPR receptors) (Berridge and Irvine, 1984). Many, but not all, second messengers exert

their effects through allosteric binding to second messenger-dependent protein kinases. These include the cyclic AMP- and cyclic GMP-dependent protein kinases (PKA and PKG) which, with the exception of certain cylic nucleotide-gated ion channels, are thought to mediate all of effects of cyclic AMP and cyclic GMP. The various isoforms of protein kinase C (PKC) are activated by lipid second messsengers, including diacylglycerol, fatty acids and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Nakanishi et al., 1993), with some also requiring Ca^{2+}. Most effects of Ca^{2+} are mediated by Ca^{2+}-binding proteins of the calmodulin family, which bind to calmodulin-activated-protein. An important subgroup within the latter are the calmodulin-dependent protein kinases (e.g. CaMKII, SmMLCK and Twn). Variations of this theme are seen both in vertebrates, where phosphorylase kinase (PhK) contains endogenous calmodulin as a tightly bound subunit, and in higher plants, where Ca^{2+}-dependent protein kinases (CDPK) have a calmodulin-like domain on the same polypeptide as the kinase domain.

1.1. An overview

Signalling agents that are common to many different pathways include Ca^{2+}, inositol phospholipids, G-proteins, cyclic nucleotides, protein kinases and protein phosphatases (Clark et al., 2001). Plant signal transduction studies have benefited greatly from models developed for mammals and yeast. Many transduction pathways are common to plants and mammals, but some pathways, pathway components, or functions of those components are unique to plants (Wilson et al., 1997); (Blumwald E. et al., 1998).

Phospholipids, as phosphatidic acid (PA), are emerging as novel second messengers in plant cells (Munnik and Musgrave, 2001). They are rapidly formed in response to a variety of stimuli via the activation of lipid kinases or phospholipases. These lipid signals can activate enzymes or recruit proteins to membranes via distinct lipid-binding domains, where the local increase in concentration promotes interactions and downstream signaling (Meijer and Munnik, 2003).

The more common phospholipids are components of membranes, naturally located with membrane receptors; a receptor activation is often translated directly or indirectly (e.g., via G-proteins) into effector enzyme activity that uses lipids as substrates to convert them into signaling molecules. Each effector enzyme therefore heads a lipid-signaling pathway and those present in plants are represented in **Figure 1**, together with the signals they produce.

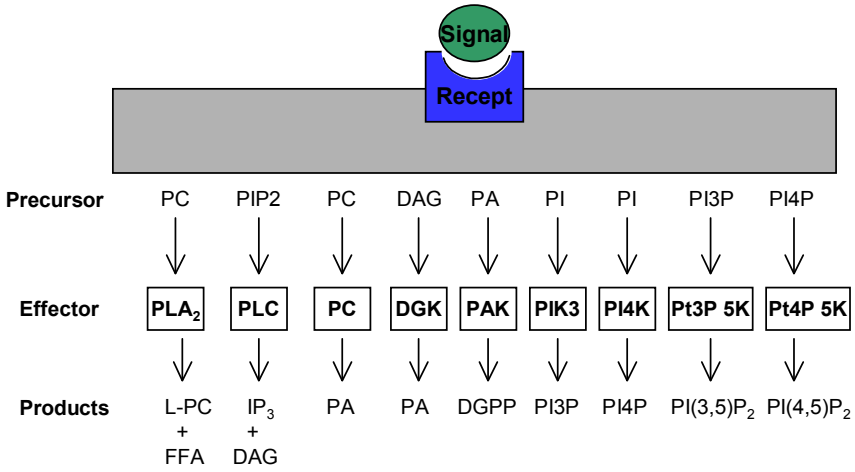

Figure 1. Schematic representation of lipid substrates (precursor) and messengers (product) produced by the action of some lipid enzymes and phospholipases (effector)

Similar lipid-signaling pathways exist in yeast and animal cells, but some of them are not yet clear for plant systems. Modified from Meijer and Munnik, 2003.

Phospholipases, enzymes hydrolyzing phospholipids (**Figure 2**), are divided into groups according to the site of substrate molecule cleavage (phospholipase A, C and D; PLA, PLC and PLD). PLD is the predominant family of phospholipases in plants and the biochemical properties, domain structure and genome organization of plant PLDs are much more diverse (McGee et al., 2003) and complex than those of other organisms: two PLD genes are present in *Arabidopsis* genome, whereas two PLD genes are found in mammals and one in baking yeast (*Saccharomyces cerevisiae*; (Wang, 2002; Wang, 2004). The twelve Arabidopsis PLDs can be classified into six types, i.e. PLDα (3 genes), β (2 genes), γ (3 genes), δ, ε, and ζ (2 genes).

Based on the overall protein domain structures, PLDs can be divided into two subfamilies, C2-PLD and PX/PH-PLDs. C2 is a Ca21 and phospholipid-binding domain, and the PX and PH domains refer to two distinct phosphoinositide-interacting structural folds, phox homology and pleckstrin homology, respectively. Ten of the twelve Arabidopsis PLDs (α, β, γ, δ and ε) contain the C2 domain. The PLDs contain the PX and PH domains, and this domain structure is present in mammalian PLDs (Elias et al., 2002); (Qin and Wang, 2002). The overall sequences of PLDζs are more similar to mammalian PLDs than to other *Arabidopsis* PLDs.

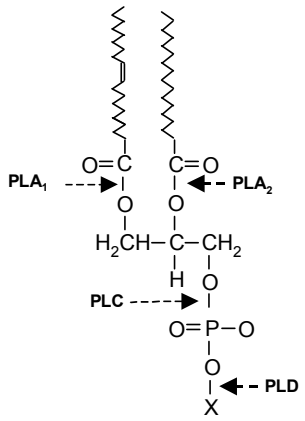

Figure 2. Phospholipid hydrolysis by different phospholipases

Schematic representation of the activity sites for phospholipases in a hypothetical phospholipid substrate; X could represent a diverse chemical species = Choline, Ethanolamine, Serine, Glycerol, Inositol, Inositol 4,5-bisphosphate

The PLD groups show different requirements for substrate, Ca^{2+} and phosphatidylinositol-4,5-bisphosphate (PIP_2) (Qin and Wang, 2002); (Wang, 2002). PLD hydrolyses various phospholipids, such as phosphatidylcoline, phosphatidylethanolamine, and phosphatidylglycerol, to PA and water-soluble free head groups. PLDγ from *Brassica oleracea* was found to be phosphorylated (Novotna et al., 2003). PI-PLC uses phosphoinositides as substrates to generate DAG and phosphorylated head groups such as inositol 1,4,5-trisphosphate (IP_3). PLA_2 cleaves phospholipids to lysophospholipids and free fatty acids. The products of individual phospholipases can be further metabolized in the cell. For example, PA, produced by PLD, can be dephosphorylated to DAG by phosphatidate phosphatase or phosphorylated to DAG pyrophosphate by PA kinase (Munnik et al., 2000). PLC-generated DAG can be phosphorylated to PA by DAG kinase. In addition, PA can be deacylated by PLAs to free fatty acids and lysoPA.

Regulation of the levels of various lipid messengers by phosphorylation and dephosphorylation can be thought of as analogous to the well-established regulation of protein functions by protein kinases and phosphatases. There are two major types of lipid phosphate phosphatases (Waggoner et al., 1999): Types I and II, which are structurally and catalytically unrelated; type I (PAP-1) is Mg^{2+}-dependent and is important in triacylglycerol synthesis, whereas type II (PAP-2) is Mg^{2+}-independent and is likely involved in signal transduction. PAP-2 is a family of phosphatases that hydrolyse a variety of lipid phosphates. Thus, the members of this family are renamed lipid phosphate phosphatases (LPPs). LPP-1, LPP-2, and LPP-3 can dephosphorylate PA, lysoPA, diacylglycerol pyrophosphate (DAGPP), ceramide 1-phosphate, and sphingosine 1-phosphate. The type-II-like PAPs have been recently cloned in plants (Marcel et al., 2000).

The multiple metabolic pathways by which a class of lipid messengers, such as PA, DAG, lysoPA, or a free fatty acid, is generated are likely to be important in regulating the amount of messenger produced, as well as the location, timing of production, and acyl composition of the messenger. Furthermore, not all PAs, DAGs, and lysoPLs are chemically identical; the acyl groups at the sn-1 and sn-2 positions are varied; thus there are numerous distinct molecular species. For example, PC and PtdIns $(4,5)P_2$ are distinct in acyl composition, thus, the DAGs released by PI-PLC are distinct from those produced by PLD, using PC as substrate. Different molecular species can have different effects on downstream targets and be acted upon differently by enzymes. Therefore, PA or DAG species produced by one pathway may differ in effect from those produced by another pathway. Analysis of molecular species in certain animal systems suggests that PLD activation results in the lipid messenger DAG, rather than PA, meaning that PA is dephosphorylated by LPP (Hodgkin et al., 1998).

The functional diversity of the polyphosphorylated inositol lipids is in part predicated by the stereospecificity of the phosphate groups on the inositol ring and by the subcellular localization of the phospholipids. In eukaryotic cells, the multiple phosphorylated isomers and the specific lipid kinases involved in their synthesis (phosphatidylinositol [PtdIns] 3-kinases, PtdIns 4-kinases and PtdInsP kinases), are located in various intracellular compartments, including the plasma membrane, endomembranes, the cytoskeleton, or the nucleus (Heilmann et al., 2000).

In animal cells the PtdIns 3-kinases are now known to be involved in a plethora of cellular processes, ranging from mitogenesis, membrane trafficking and ruffling to glucose uptake, oxidative burst responses, chemotaxis, and apoptosis. Whatever the precise roles of the PtdIns 3-kinases and 3-phosphorylated inositol lipids turn out to be in plant cells, their functions are almost certain to be considerably more diverse and multifaceted than is assumed currently (Bunney et al., 2000).

The classic example of a lipid-signaling pathway is that in which an activated receptor triggers phospholipase C (PLC) to hydrolyze the minor lipid phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] to produce the signals inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). However, it has become apparent that inositol phospholipids are an important complex group of signals or signal precursors, involved in a number of independent pathways (see below). They originate from the structural lipid phosphatidylinositol (PI) that is converted by PI- and PIP-kinases into different polyphosphoinositide (PPI) isomers. Plants contain three PIP (PI_3P, PI_4P, PI_5P) and three PIP_2 isomers ($PI(3,4)P_2$, $PI(3,5)P_2$, and $PI(4,5)P_2$) but no PIP_3. This is significant because $PI(3,4,5)P_3$ is a important signal in animal cells (Vanhaesebroeck et al., 2001).

Over the last few years, a number of lipid-binding domains in proteins have been identified and characterized and most of these data are summarized from the animal literature, but the same domains are found in plant proteins; even though their lipid-binding properties have seldom been characterized. Lipid-binding domains are of great importance not just because they epitomize the significance of lipid signals but also because they provide research tools for visualizing signaling. If green fluorescent protein (GFP) is coupled to a lipid-binding domain and the chimera is injected, or its gene construct transfected into a plant cell, the fluorophore can locate the signal and monitor changes in concentration (Balla and Varnai, 2002); (Kim et al., 2001).

There is accumulating evidence that individual phospholipids can have a profound effect on plant physiologies and one of the most important of these is the stress signalling lipid PA (Munnik, 2001); (Testerink and Munnik, 2005); (Wang, 2005). Phosphatidic acid induces an oxidative burst in tobacco cells (de Jong et al., 2004). In *Arabidopsis* leaves exogenous PA induces cell death, characterized by loss of turgor and chlorosis, whereas exogenous PC, PE, PI, and PS and related metabolites have no apparent effect (Park et al., 2004). Further analysis revealed that leaves of the *rop2* mutant of *Arabidopsis* exhibited earlier cell death in the presence of PA than the wild type indicating that PA impacts an additional factor to elicit ROP-regulated ROS production. Thus, exogenous PA can couple to intracellular signaling agents to initiate signaling systems. Exogenous application of dipalmitoyl-PA has by comparison been shown to stimulate tobacco pollen tube germination and elongation growth (Potocky et al., 2003). Similar results were obtained for egg yolk-derived PA containing oleoyl-, linoleoyl-, or stearoyl-acyl chains. Furthermore, using a fluorescent-labelled PA analogue these authors provided strong evidence that PA was incorporated into pollen tubes by an endocytotic mechanism. Based on these observations and the documented role of PLD-derived PA in cell swelling in animal cells it was concluded that PA plays a pivotal role in plant cell expansion.

1.2. Role of diacylglycerol, phosphatidic acid and diacylglycerol kinase

Diacylglycerol kinases (DGKs) phosphorylate DAG to PA. Whereas DAG is a second messenger in animals, this has not been formerly established for plants or fungi (Munnik et al., 1998); (Munnik, 2001), but evidence from (Wang, 2004) shows a possible signaling action. In contrast, PA is becoming accepted as a signaling molecule (Munnik, 2001), and therefore DGK could be an important signaling enzyme, especially since plant DGK rapidly converts the DAG produced by PLC into PA (Munnik et al., 1998); (van der Luit et al., 2000); (Munnik et al., 2000); (den et al., 2001); (Meijer et al., 2001); (de Jong et al., 2004). PA-kinase (PAK) is a lipid kinase originally discovered *in vitro* (Wissing and Behrbohm, 1993);

PAK phosphorylates PA to DAGPP, a new lipid that was later discovered to accumulate *in vivo* when PLC or PLD signaling is activated (Munnik et al., 1996).

1.2.1. Diacylglycerol (DAG)

Diacylglycerol, together with IP_3 (inositol 1,4,5-trisphosphate), is formed by the hydrolysis of PIP_2 mediated by PLC; also PA can be dephosphorylated by a phosphohydrolase to form DAG (**Figure 3**) and the dephosphorylation is believed to be coupled with PLD activation in some systems. The lipid product PA can be hydrolyzed by PA phosphatase to DAG and by acylhydrolase/PLA to lysoPA. Although these products may have effects, some studies suggest that DAG formed from PA may not be active because of the distinct acyl composition from the active DAG released by PLC (Hodgkin et al., 1998). DAG, can be an intermediate in the synthesis of PC, PE, and triacylglycerol, and also a potent activator of PKC (Drobak, 1993).

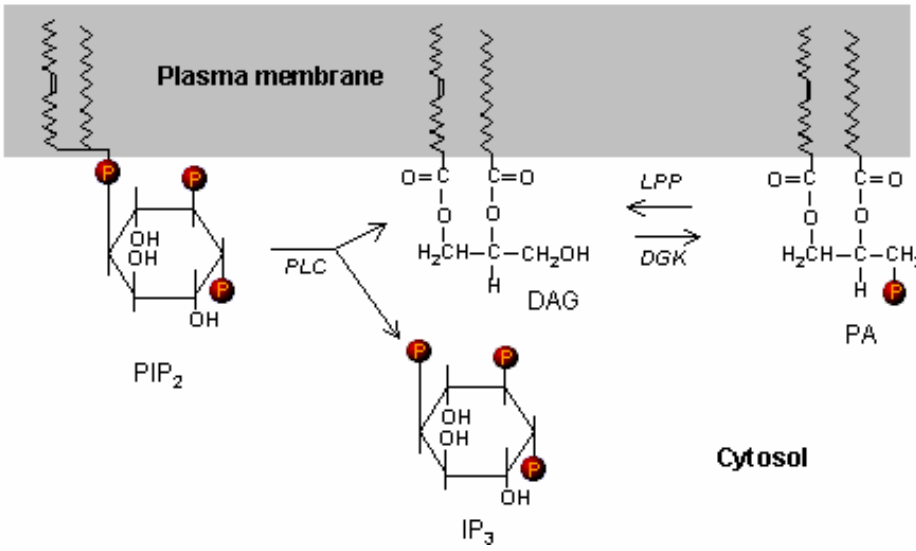

Figure 3. Synthesis of diacylglycerol (DAG)

DAG can be synthesized by hydrolysis or dephosphorylation of PIP_2 or PA, mediated by PLC or LPP, respectively. PLCs are cytosolic enzymes that act on membrane-inserted phosphoinositide substrates.

In animals, DAG, is an allosteric activator of classic and novel calcium-insensitive protein kinase Cs (PKCs α, β, γ, δ, ε, η, and θ), and DGK has been implicated as an attenuator of PKC activity, thereby inactivating downstream signaling pathways (Nishizuka, 1992). In addition to PKC, DAG is also a key player in other cellular processes, including activation of guanyl nucleotide-releasing protein, Ras-GRP (Ebinu et al., 1998). DAG is a biological alcohol that has been proposed to serve as a relevant transphosphatidylation substrate. The PLD-mediated condensation of PA and DAG to form bisphosphatidic acid is proposed as a

mechanism to rapidly attenuate the levels of these two signaling molecules in animal systems (van Blitterswijk and Hilkmann, 1993).

Additional information says that PA can be dephosphorylated by PA phosphatase to form DAG, which affects various aspects of plant cellular functions such as increasing proton-ATPase activity, stimulating stomatal opening, altering cell division, retarding movement via plasmodesmata, and increasing protein phosphorylation (Drobak, 1993).

The activation of PLC results in the initial rise of DAG, whereas PLD coupled with PA phosphatase provides the sustained supply of DAG required for cell proliferation. On the other hand, PA itself is a mitogen in animals and has been shown to stimulate PLC, PLA_2, and PKC. Analysis in certain animal systems further enforces the notion of networking of PLD activation with other lipid signaling pathways. Some researchers suggest that DAG rather than PA serves as the lipid messenger of PLD activation (Hodgkin et al., 1998). The network of PLD, PLC, and PLA_2 generates several potent lipid mediators, such as, PA, lysophospholipids, DAG, and free polyunsaturated fatty acids, which are involved in cellular regulation (Munnik et al., 1998); (Ryu and Wang, 1998).

DAG activates the multifunctional molecule, protein kinase C (PKC) and this one phosphorylates serine and threonine residues in many target proteins. Yasutomi Nishizuka (Nishizuka, 1992) found that PKC is enzymatically active only in the presence of Ca^{2+} and phosphatidylserine. DAG increases the affinity of PKC for Ca^{2+} and thereby renders it active at physiologic levels on this ion.

Many mammalian and *Drosophila* cDNAs for PKC have been cloned. They encode proteins with mass around 80 kd and all of them containing an N-terminal regulatory domain and a C-terminal catalytic domain. Proteolysis at the junction of these domains yields a persistently active catalytic fragment and a regulatory fragment that binds Ca^{2+} and DAG. The PKC's regulatory domain, similar to the R subunit of protein kinase A, contains a pseudosubstrate sequence that is rich in positively charged residues (-RFARKGALRKQNVHEVKN-): A competent substrate has a serine or threonine in place of the marked alanine. In the absence of DAG, the pseudosubstrate domain could access to the substrate binding site; however, the interaction is disrupted when DAG occupies the binding site and enables a protein substrate to enter.

DAG, as well IP_3, works transiently because it is rapidly metabolized. It can be phosphorylated to phosphatidate (PA), or it can be hydrolyzed to glycerol and its constituent fatty acids. Arachidonate is a C_{20}-polynsaturated fatty acid that often works in the 2-position on the glycerol moiety of PIP_2 and also, is the precursor of a series of C_{20}-carbon hormones such as the prostaglandins. Consequently, the phosphoinositide pathway generates a important kind of molecules that have signaling roles.

PKC has a recognized importance in controlling cell division and proliferations; the activity is revealed by the action of phorbol esters. These polycyclic alcohol derivatives from croton oil are carcinogenic and are known as tumor promoters. Phorbol esters activate PKC because they resemble DAG. The activation is persistent because phorbol esters, unlike DAG, are not readily degraded.

Among the components of the phosphoinositide cycle it is well known in animal cells that DAG is an established second messenger, whose best characterized function of receptor-stimulated signaling cascade is activation of PKCs (Nishizuka, 1992). In this respect, it should be noted that DAG is not a single entity but constitutes at least 50 structurally distinct molecular species, whose fatty-acyl groups can be polyunsaturated, diunsaturated, mono-unsaturated or saturated (Hodgkin et al., 1998); (Wakelam, 1998). Although it is very hard to predict the extent to which particular DAG species activate PKCs within stimulated cells, there is some preference for polyunsaturated DAG species: Saturated DAGs are generally poor activators; di-unsaturated DG is more active; and polyunsaturated DAGs, such as 1-stearoyl-2-arachidonoyl DAG are most potent (Marignani et al., 1996); (Schachter et al., 1996). Functional significance of DAG is not restricted to the PKC pathway. Recent studies have revealed that DAG may also activate several proteins including RasGRP, the chimaerins, Unc-13, protein kinase D, and some mammalian homologues of transient receptor potential (TRP) protein as hTRPC3 and hTRPC6 (Brose et al., 2004). To date, however, no data are available to show the extent to which various DAG species activate these molecules.

1.2.2. Phospatidic acid (PA)

At least, cellular PA could be synthesized using different enzymes (**Figure 4**):

- PLD, acting hydrolytically on membrane phospholipids;
- DGK, phosphorylating DAG;
- acyl transferase, adding a fatty acid to lysoPA; and
- *de novo* pathway enzymes from glyceradehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP).

Probably PLD and DGK are the two principal routes that produce signaling PA, and this activity is widespread in plants. Recent results indicate that PA and PLD play multiple regulatory roles in diverse plant processes, including abscisic acid (ABA) signaling, programmed cell death, root hair patterning, root growth, freezing tolerance, and other stress responses (**Figure 4**). In some cases, direct molecular targets of PA and PLD have been

identified, providing insights into the mechanism by which the phospholipase and lipid messenger mediate plant functions (Wang, 2005).

PLD hydrolyzes phospholipids into PA and a free head group (**Figure 4**); for example, PLD hydrolyzes phosphatidylcholine into PA and choline, or phosphatidylserine into PA and serine (Wang, 2002).A has emerged as a messenger involved in many cellular processes, such as ROS generation and protein kinase cascade (Munnik, 2001); (Wang, 2002). Elicitor N-acetylchitooligosaccharide induced phytoalexins in rice suspension cell cultures may be mediated by H_2O_2 and PLD (Yamaguchi et al., 2004). It is also suggested that H_2O_2-inducible PLD activation enhances signal transduction leading to phytoalexin biosynthesis in rice cells (Yamaguchi et al., 2004).

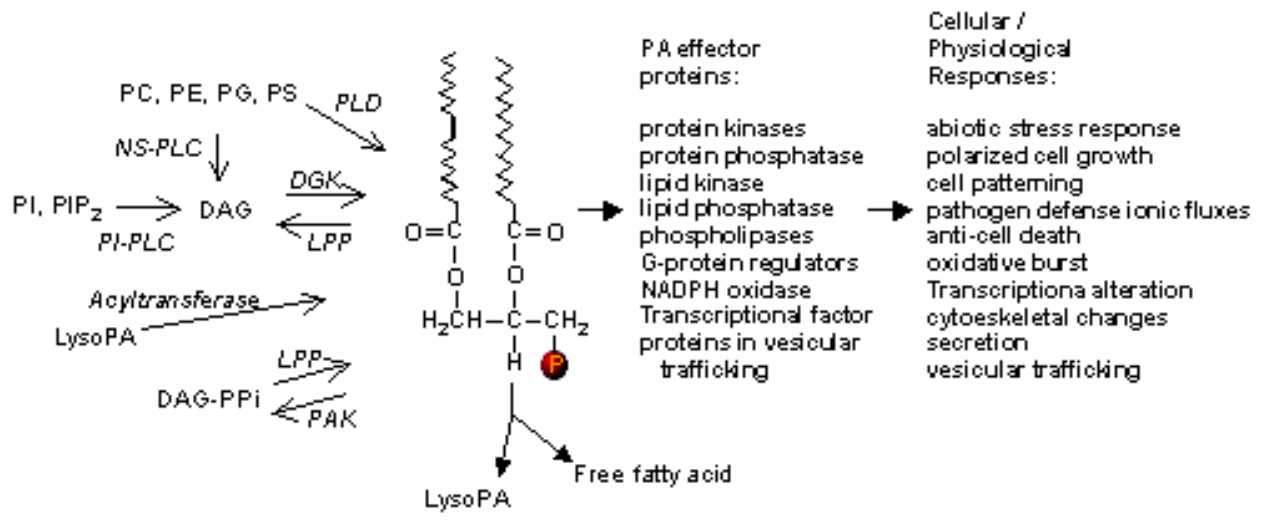

Figure 4. Enzymatic reactions lead to PA production, downstream targets, and cellular functions.

PA can be generated from DGK hydrolyzing DGA, coupled to the activation of PLC and/or potentially from acylation reactions and/or PLD hydrolyzing membrane lipids and/or from G3P and DHAP pathway. The same target proteins for PA were identified in animals, plants, and yeast. DGK, diacylglycerol kinases; DAG-PPi, diacylglycerol pyrophosphate; LPP, lipid phosphate phosphatase; LysoPA, lysophosphatidic acid; PAK, phosphatidic acid kinase; PE, phosphatidylethanolamine; PS, phosphatidylserine. Modified from (Wang, 2005; Wang et al., 2006).

Besides DGK, PLD is also an important PA generator. Interestingly, more and more proteins are found to bind PA (Munnik, 2001). So far the best characterized are the serine/threonine kinase Raf-1 and a cAMP-specific phosphodiesterase in animal cells (Rizzo et al., 1999; Rizzo et al., 2000); (Baillie et al., 2002). Others, including several plant proteins, range from ion channels to protein phosphatases and protein- and lipid-kinases (McPhail et al., 1999); (Manifava et al., 2001); (Cockcroft, 2001); (Munnik, 2001); (Munnik and Musgrave, 2001); (Baillie et al., 2002); (Jones and Hannun, 2002).

PA could also act as a specific inhibitor. A human protein phosphatase-1 catalytic subunit (PP1γ) is specifically inhibited by PA with an IC_{50} of 15 nM (Jones and Hannun, 2002). An

additional fact that shows to PA as an important signaling molecule in plants was the discovery that it is further phosphorylated to DGPP and obviously, attenuating the PA signal (Munnik et al., 1996; Munnik et al., 1998); (van Himbergen et al., 1999).

PA is an activator of some signaling enzymes such as Raf-1 kinase, PKC ζ, and the protein tyrosine phosphatase, PTP1C, and has been implicated in the stimulation of DNA synthesis (Zhao et al., 1993); (Limatola et al., 1994); (Ghosh et al., 1996). PA is the simplest biological membrane phospholipid present in small amounts and has a function as a central intermediate for the synthesis of membrane lipids and storage. In *Arabidopsis* leaves, the PA level is 0.5 to 1.5 nmol milligram^{-1} of dry weight, and the PA concentration is estimated to be 50–150 μM (Welti et al., 2002; Zhang et al., 2004). PA constitutes less than 1% of total phospholipids, or about 20-fold less than PC, the most abundant phospholipid in eukaryotic membranes (Welti et al., 2002). Various are the conditions to change in plants the cellular levels of PA:

(a) plant growth and developments during seed germination, in senescing leaves, aging seeds, and expanding pollen tubes;

(b) abiotic stresses, such as chilling, freezing, dehydration, drought, salts, nutrient starvation, wounding, and reactive oxygen assaults; and,

(c) biotic challenges, such as attacks by bacterial and fungal pathogens, elicitor treatments, and nodule induction.

Enzymes that metabolize PA play an important role in switching off the PA signal. The removal of PA can be accomplished by several enzymes (**Figure 4**): (1) lipid phosphate phosphatase (LPP) that dephosphorylates PA to produce DAG; (2) PA kinase that phosphorylates PA to form DAG pyrophosphate; (3) PA-selective A type phospholipases that deacylate PA to produce lysoPA and free fatty acids (FFAs). In addition to attenuating PA function, these enzyme activities can also generate new lipid messengers, such as DAG, FFAs, DAGPP and lysoPA. Mammalian LPPs are integral membrane glycoproteins that dephosphorylate several phosphorylated lipid mediators, including PA, lysoPA, and sphingosine 1-phosphate (S1P) (Sciorra and Morris, 2002). LPP-catalyzed dephosphorylation can disrupt the signaling actions of lipid mediators and generate new mediators. LysoPA and S1P function through actions at cell surface receptors while PA is an intracellular messenger. The *Arabidopsis* genome has 4 LPP genes (Pierrugues et al., 2001) (Katagiri et al., 2005); LPP1 and LPP2 differ in substrate preferences for PA and DAGPP, and their patterns of expression. LPP1, but not LPP2, is transiently induced by ionizing radiation (UV-B, X-ray) and elicitor, whereas the expression of LPP2 and LPP3 increases during seed germination. Both genetic and pharmacological approaches have been used to attenuate the degradation of PA. Genetic manipulation of LPP in Arabidopsis has resulted in

an increase in PA (Katagiri et al., 2005). Propanolol, which inhibits PA phosphohydrolases, has been used in animal cells to inhibit dephosphorylation of PA (Pierrugues et al., 2001). This treatment has been used to distinguish the cellular effects mediated by PA or DAG, particularly in the system where PA is dephosphorylated rapidly. A PA kinase activity is activated during responses that result in PA signaling (Laxalt and Munnik, 2002), suggesting that it may play a role in attenuating responses to PA. On the other hand, the production of DAG pyrophosphate from PA has been shown to play a role in ABA signaling (Zalejski et al., 2005). The gene for PA kinase has not yet been identified. There are multiple phospholipase As in mammals and plants, although few of these enzymes utilize PA as a major substrate in vivo (Ryu, 2004). (Hiramatsu et al., 2003) reported the identification of two human PA-specific PLA1s.

Root hair initiation and development is controlled by genetic and environmental factors, including the transcription factor GLABRA2 (*GL2*), Ca^{2+}, ROS, light, and rhizobium nodulation factors (Ohashi et al., 2003);(Charron et al., 2004). *GL2*, which encodes a homeodomain protein, is a negative regulator of root hair development. The PLDζ1 gene was identified as a direct target of GL2; GL2 suppresses the expression of PLDζ1 by binding to a 303-bp DNA fragment in its promoter. Increased PLDζ1 expression induces branched and swollen hair-roots, a phenocopy of the *gl2* mutant, suggesting that PLDζ1 is negatively regulated by *GL2* in root hair cells. Partial inhibition of PLDζ1 expression alters the position where the root hairs develop, while the diminishment of PA formation with 1-butanol eliminates root hair growth. These results suggest that PA from PLDs other than PLDζ1 is involved in root hair development. In *Medicago truncatula*, PA is necessary to Nod factor-induced *ENOD* (early nodulin) gene expression in root hairs (Charron et al., 2004).

How *PLDs* are involved in root hair growth and development is not clear. One potential way is through their roles in regulation of redox signals, such as ROS and NO (Prado et al., 2004);(Zhang et al., 2005). ROS are important mediators of root hair growth and development. Mutation of the catalytic subunit of an NADPH oxidase gene, *RHD2* (*root hair defective 2*), results in the formation of only root hair bulges, but not elongated root hairs (Foreman et al., 2003). While ROS accumulates in the growing wild-type root hairs, ROS levels are markedly reduced in the *rhd2* mutant. The ROS produced by RHD2 activity are required to stimulate Ca^{2+} influx and maintain a tip-focused Ca^{2+} gradient during hair elongation and cell extension. More recently, a serine/threonine kinase, OX1, has been identified as an essential component of the signal transduction pathway linking ROS to the MAPK system during root hair growth (Rentel et al., 2004). OX1 is identical to AGC2-1, which belongs to the AGC kinase family. AGC2-1 is a downstream target of phosphoinositide-dependent protein kinase 1 (PDK1) and it is activated in a PDK1-

dependent manner (Anthony et al., 2004). PA binds to PDK1 and activates the PDK1 and AGC2-1 kinases. These studies indicate that PA and ROS converge in the PDK1 → AGC2-1/OX1 signaling pathway.

NO has been found to be involved in the regulation root tip growth (Hu *et al*., 2005) and re-orientation of pollen tubes (Prado et al., 2004). In animal cells, PA induces iNOS expression and NO production in systemic inflammatory responses (Lim et al., 2003). However, a direct interaction between PLD/PA and the NO pathway has not been revealed in plants. In addition, PLDs and PA may affect root growth and development through their effects on membrane trafficking, exocytosis, and cytoskeletal rearrangements. PLDζ1 fused to GFP is found mainly in vesicles both in bulges and root-hair apices; these locations suggest that PLDζ1 may play a role in vesicle trafficking during root hair initiation and growth (Ohashi et al., 2003)*et al*., 2003). It has been proposed that vesicles are associated with microtubules for transport and uncoupled from microtubules at their sites of use (Sedbrook, 2004).

1.2.3. Diacylglycerol kinase (DGK)

In animals DAG plays an important role in cell regulation; it can be a direct product of PI–phospholipase C (PLC) and serves as a potent activator of protein kinase C. The downstream target of DAG in plants is unclear. One suggested role for DAG is as a substrate for the formation of PA in response to stresses, such as microbial elicitation, salt, and hyperosmotic conditions (Meijer et al., 2002; Meijer and Munnik, 2003; den Hartog et al., 2003). This reaction is catalyzed by DAG kinases (Gomez-Merino et al., 2004; Gomez-Merino et al., 2005); **Figures 1** and **4**) and leading to the transfer of the γ-phosphate of ATP to the hydroxyl group of DAG to generate phosphatidic acid (PA). Multiple DAG kinase genes are present in the *Arabidopsis thaliana* genome. This conversion of DAG to PA is important for phosphoinositide (PI) resynthesis within the PI cycle (Quest *et al*., 1996). Additionally, however, these kinases have the task of balancing the intracellular levels of two lipid molecules, diacylglycerol (DAG) and phosphatidic acid (PA).

DGK homologues have been identified in various organisms (**Figure 5**), including mammals, *Drosophila melanogaster* (Masai et al., 1993), *Caenorhabditis elegans* (Nurrish et al., 1999), *Arabidopsis thaliana* (Beisson et al., 2003);(Gomez-Merino et al., 2004), and *Dictyostelium discoideum* (Thanos and Bowie, 1996);(De la Roche et al., 2002). There are nine known mammalian isoforms of DGKs, which are subdivided into five different groups based on their domain organization; all of them contain either two or three cysteine-rich domains (CRDs) (Topham and Prescott, 1999; Jose and Koelle, 2005). The three Type I mammalian DGKs contain calcium-binding EF-hands motifs in their N-termini, while Type II have pleckstrin homology (PH) domains at the N-termini (Klauck et al., 1996);(Sakane et al., 1996). The

Type III group, with only a single member, has the simplest structure with no regulatory subunits. The two members of the Type IV group are characterized by C-terminal ankyrin repeats and a region homologous to the myristoylated alanine-rich C kinase substrate (MARCKS) protein's phosphorylation site on the upstream side of the catalytic domain. The single member of Type V has a PH domain, but, differing from Type II, it is located after the CRD domains, just upstream of the catalytic domain. DGK activity can be involved both in generating signaling PA and in removing signaling DAG. DAG is a class of potent lipid messengers that activate PKCs and other effectors in animal systems. In many examples reported in animals, DGK acts to attenuate the effect of DAG by converting it to PA (Regier et al., 2005). Many of these domains are shared between different isoforms.

Seven DGK genes are present in the Arabidopsis genome; however, no DGK gene has yet been identified in yeast. Plant DGKs fall into three distinct clusters (Gomez-Merino et al., 2004; Gomez-Merino et al., 2005). Cluster I DGKs typically contain two DAG-binding domains, which are flanked by an N-terminal basic region and CRD-like sequence, following the second DAG-binding domain 2 (**Figure 5**). The catalytic region and an accessory domain follow the CRD domain. Clusters II and III are simpler in organization, lacking the basic upstream region, the DAG-binding domain, and the CRD domain. Two DGK splicing variants, *LeDGK1* and *LeCBDGK*, have been cloned from tomato, and the latter has a 29 C-terminal amino acid extension harboring a calmodulin-binding domain (Snedden and Blumwald, 2000). The two tomato DGKs also lack the CRD found in other eukaryotic DGKs and have been shown to be active *in vitro*. Active DGK from the cluster I AtDGK2 and the cluster II AtDGK7 have been expressed and both of the DGKs can phosphorylate DAG species found typically in plants. The two DGKs differ in sensitivity to the DGK inhibitor R59022; the inhibitor at 50–100 μM inhibited the activity of AtDGK2, but not AtDGK7. Treatments of *Arabidopsis* with the DGK inhibitor R59022 at 50–100 μM inhibited root elongation and lateral root formation. AtDGK2 expression is upregulated in chilling and wounding (Gomez-Merino et al., 2005).

A study identified a single gene in *D. discoideum* that appears to encode a protein that is structurally similar to the θ isoform of mammalian DGK (DGK-θ) and was designated DGKA (**Figure 5**; (Thanos and Bowie, 1996);(De la Roche et al., 2002). (Ostroski et al., 2005) showed the DGK activity of DGKA using medium-chain and long-chain DAGs, catalyzing the phosphorylation for both DAGs with pH optima of 7.4 and 7.0, respectively.

The contributions of the DAG kinase and PLD reactions to signal-induced PA production have been assessed using a differential labelling technique and primary alcohol treatments. These assessments revealed the differential activation of the two reactions in response to different stimuli (Arisz et al., 2003);(den Hartog et al., 2003). The production of the DAG

substrate of DAG kinases has been suggested to result from the activation of PI-PLC (Arisz et al., 2003). DAG may also come from the activation of other reactions. For example, 18:3/16:3-PA increases during freezing (Welti et al., 2002) and pathogenesis. An 18:3/16:3 species is abundant in galactolipids but virtually undetectable in phospholipids. Thus, 18:3/16:3-PA is likely to be derived from galactolipids via 18:3/16:3 (DAG) and a DAG kinase. In addition, it has been reported that PC-PLC is activated by a glycoprotein elicitor (Scherer et al., 2002). Six putative non-specific PLC genes are present in *Arabidopsis* (Beisson et al., 2003).

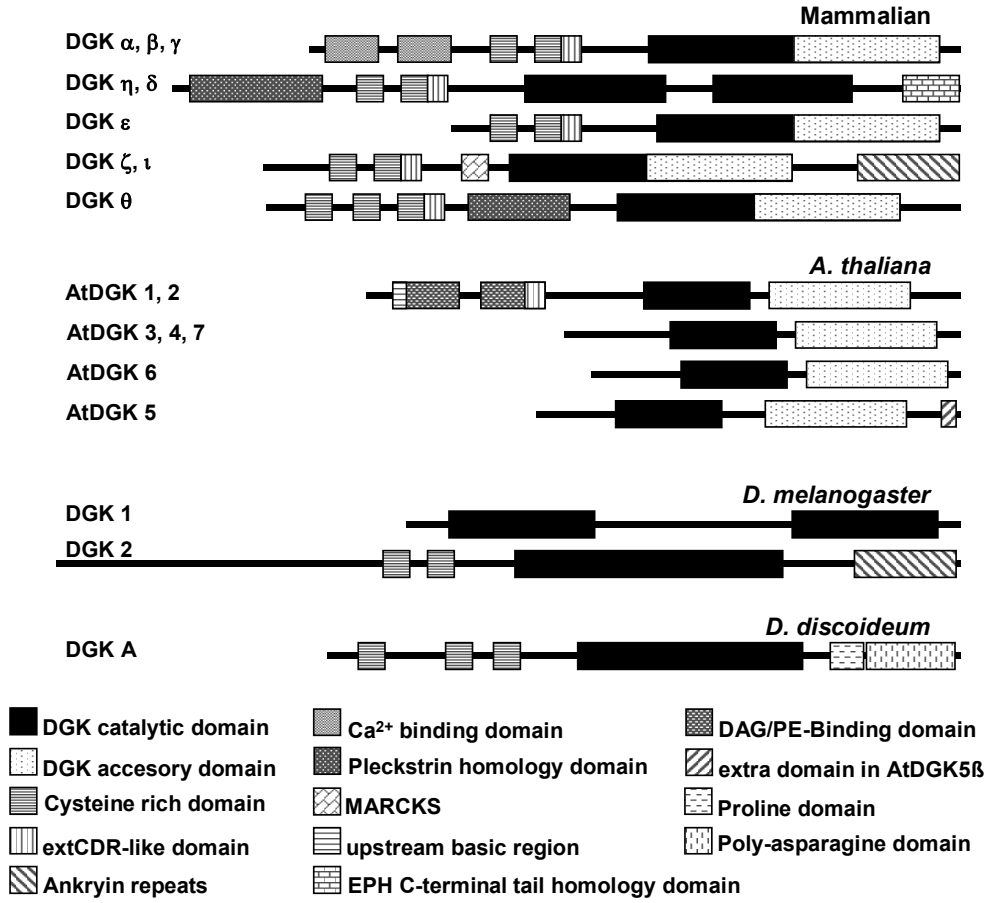

Figure 5. Summary of the DGK family in mammals, *Arabidopsis*, *Droshophila* and the unique representant from *D. discoideum*. EF-hands, Ca^{2+} binding domains; C1A, C1B and C1C, cysteine rich domains; EPH, EPH C-terminal tail homology domain; MARCKS, sequence homologous to the myristoylated alanine-rich C kinase substrate phosphorylation site domain. Other motifs shown in this figure regulate DGK activity, subcellular localization, or interaction with other proteins or lipids; other structural motifs of unknown significance are not included in this figure. Representation modified from (van Blitterswijk and Houssa, 2000);(De la Roche et al., 2002);(Luo et al., 2004);(Gomez-Merino et al., 2004);(Gomez-Merino et al., 2005);(Topham and Prescott, 1999);(Wang et al., 2006).

The cellular function of the PA produced by DAG kinases in plants remains to be established whereas, in animals, this reaction is often thought to remove signaling DAG produced by PI–PLC. The signalling function of PI–PLC has been well documented in animals. Animal PI–PLC consists of five types, PLCβ, PLCγ, PLCδ, PLCε, and PLCζ. *Arabidopsis* has nine PI–PLCs, and their domain structures all resemble that of the newly discovered animal PLCζ. Plant PI–PLCs were previously regarded as PLCδ-like (Mueller-Roeber and Pical, 2002), but they lack the PH domain present in animal PLDδ. Functional studies of plant PI–PLCs have mostly been concerned with the production of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which is a potent Ca^{2+} mobilizer (Mueller-Roeber and Pical, 2002). Recently, an IP_3-mediated transient increase in cytosolic Ca^{2+} was implicated in mediating the Phot1- and Phot2-mediated perception of blue light (Harada et al., 2003). PI–PLC has been suggested to be an effector protein for the G-protein coupled receptor GCR1, and PI-PLC's product, IP_3, may mediate DNA synthesis (Apone et al., 2003). Suppression of a recombinant NrPLC1 reduces the abscisic acid (ABA)-promoted closure of stomata, consistent with a role for PI–PLC, IP_3, and Ca^{2+} flux in stomatal movement (Hunt *et al*., 2003). The significance of inositol polyphosphates (IPs) to cellular signaling has also been investigated by overexpressing or ablating specific IP phosphatases (Perera et al., 2002);(Xiong et al., 2002). Perturbation of these phosphatase activities affects the expression of stress-responsive genes under salt, drought, cold, and ABA treatments (Xiong et al., 2002).

The *Arabidopsis* genome encodes seven putative DGKs named AtDGK1 to 7, enclosed in three clusters (Gomez-Merino et al., 2004). Isolated and mainly expressed in roots, shoots and leaves *AtDGK1* has not yet shown to encode an active enzyme (Katagiri et al., 1996). (Gomez-Merino et al., 2004) cloned the *AtDGK2* gene. Cloning and biochemical analysis of *AtDGK7* is reported in this thesis. Both proteins were able to phosphorylate DAG producing PA *in vitro* (Gomez-Merino et al., 2004; Gomez-Merino et al., 2005).

2. Chemical treatments affect phospholipid signaling in plants

A successful seedling yield after germination requires efficient utilization of endogenous storage reserves and resources from the environment. The development of a plant from a newly germinated seedling represents a special and unique transformation. Nearly all the structures that comprise the plant body are added at a postembryonic step. To get this transformation, seedlings must adapt both developmental and metabolic programs to the prevailing environmental conditions (Holdsworth et al., 1999);(Eastmond and Graham, 2001).

Because the number and location of organs is not predetermined in plant development; each plant can integrate information from its environment into the decisions it makes about root and shoot formation. This dynamic developmental strategy provides a clear advantage for a nonmotile organism. Plants are completely dependent on the resources that are available in their immediate neighbourhood. Unfortunately or not, nutrient availability and distribution are in constant flux in the environment (Malamy and Ryan, 2001).

The availability of macronutrients such as nitrogen is an important environmental parameter influencing seedling growth and development. For example, growth of tobacco (*Nicotiana tabacum*) seedlings under nitrogen-limiting conditions results in a dramatic redirection of biomass allocation to roots versus shoot and an accumulation of soluble carbohydrates (Paul and Stitt, 1993).

In addition to their metabolic function, soluble sugars play an important role in the regulation of many genes involved in physiological and developmental processes including photosynthesis, nitrate assimilation, assimilate storage, and the mobilization of starch and lipids (Graham et al., 1996);(Koch, 1996);(Jang and Sheen, 1997a);(Smeekens and Rook, 1997). Among various genes induced by sugars are those associated with nitrate assimilation such as nitrate reductase and genes encoding the high (*NRT*2) and low (*NRT*1) affinity nitrate uptake systems (Cheng et al., 1992);(Lejay et al., 1999). On the other hand, exogenous sugars repress other nitrate metabolism-associated genes such as the Gln-dependent Asn synthetase gene (*ASN*1) of *Arabidopsis* (Lam et al., 1994). Sugars are also known to repress many of the genes involved in photosynthesis related processes (Sheen, 1990);(von et al., 1990);(Krapp et al., 1993);(Krapp and Stitt, 1995).

Together, the increase in endogenous sugars and the break in lipid mobilization suggest a limited use of carbon resources under nitrogen restricted growth conditions. Previous studies agreed with these results reporting carbohydrates accumulation in leaves and roots of adult plants after nitrogen withdrawal (Thorsteinsson and Tillberg, 1990);(Henry and Raper, 1991);(Paul and Driscoll, 1997). Then, carbohydrate to nitrogen ratios play a basic and interactive role in regulating the processes supporting seedling organization (Martin et al., 2002).

Metabolic conditions, such as the nutritional state of a plant, may modulate its hormonal budget and thus become a signal at the hormonal level. Crosstalk of signals other than phytohormones is less common but not so far has been addressed in the case of sugar signals, which are linked in a signalling network to plant stress hormones. Close linkages between different types of signals was concluded from the fact that *Arabidopsis* mutants exhibiting reduced sugar sensitivity showed concomitant mutations in the biosynthesis of ABA or in the expression of the ethylene receptor (Leon and Sheen, 2003). Similarly,

(Richard et al., 2002) demonstrated combined effects of auxin (NAA), cytokinin (kinetin), and sucrose on cell cycle gene expression in *Arabidopsis* cell cultures. Crosstalk of various signals requires a platform which may be found at several regulatory levels. A superior level is a signal generation at which cytokinins control extracellular invertase and thereby the formation of the sugar signal in a tissue-specific manner. Also, at this level, environmental factors could affect on the cell cycle by modifying the external phytohormone setting of the cells. (Daan Kuiper, 1988) have shown that the endogenous cytokinin budget of *Plantago major* ssp. *pleiosperma* responds quickly to the supply of an artificial cytokinin; (Hartig and Beck, 2006) reporting to (Valdes, 2005) who have demonstrated a similar correspondence of the artificial auxin 2,4-D and the endogenous IAA level in *Chenopodium rubrum* suspension-cultured cells. These and other findings demonstrate that external signals from the phytohormone can be readily converted into cell internal auxin patterns.

2.1. Sugars and nitrogen availability

Specifically lateral root initiation is drastically repressed by high sucrose to nitrogen ratios under lab conditions. This response is not due to nitrogen starvation alone because lowering the sucrose concentration restored lateral root initiation even under low nitrogen conditions (Malamy and Ryan, 2001);(Martin et al., 2002). The idea that sugars and nitrogen salts can affect plant morphology is not unprecedented. Sugars and nitrate ions have been shown to act as signaling molecules (Gibson, 2001);(Zhang and Forde, 2000);(Coruzzi and Bush, 2001), inducing gene transcription and morphological changes. Furthermore, both photosynthetic activity and nitrogen availability have been implicated in the control of lateral root initiation (Drew and Saker, 1975);(Reed et al., 1998). High carbon-to-nitrogen ratios have also been reported to induce a specific set of responses in plants, including induction of metabolic genes (Coruzzi and Bush, 2001) and accumulation of anthocyanins (Boxall SF, 1996). Plants are able to control lateral root initiation in response to external nutritional conditions either by sensing nutrients directly or monitoring their internal metabolic status. Therefore, lateral root initiation is the target of a signal transduction pathway that interprets and integrates information about nutrient availability (Malamy and Ryan, 2001).

2.1.1. Sugar

Sugars such as sucrose, glucose, and fructose have an essential function in the metabolism of the plant. These sugars are important for intermediary and respiratory metabolism and are the substrate for synthesizing complex carbohydrates such as starch and cellulose. Moreover, sugars provide the building blocks for amino acid and fatty acid biosynthesis and essentially all other compounds present in plants (Smeekens, 2000).

Together to their essential roles as substrates in carbon and energy metabolism and in polymer biosynthesis, sugars have important hormone-like functions as primary messengers in signal transduction (Koch, 1996). The very important role of sugars as signaling molecules is well illustrated by the variety of sugar sensing and signaling mechanisms discovered in free-living microorganisms such as bacteria and yeast (Stulke and Hillen, 1999);(Rolland et al., 2001). In plants, sugar production through photosynthesis is a life basic process, and sugar modulates and coordinates internal regulators and environmental cues that govern growth and development (Koch, 1996);(Sheen et al., 1999);(Smeekens, 2000). Some research revealed the molecular mechanisms underlying sugar sensing and signaling in plants, including the demonstration of hexokinase (HXK) is a glucose sensor that modulates gene expression and multiple plant hormone-signaling pathways (Sheen et al., 1999);(Smeekens, 2000). In addition, sucrose, trehalose, and other HXK-independent sugar sensing and signaling pathways add more complexity in plants (Goddijn and Smeekens, 1998);(Lalonde et al., 1999);(Smeekens, 2000).

Multiple assays that involve biochemical, molecular, and genetic experiments have supported a central role of sugars in the control of plant metabolism, growth, and development; results have revealed interactions that integrate light, stress, and hormone signaling (Roitsch, 1999);(Sheen et al., 1999);(Smeekens, 2000);(Gazzarrini and McCourt, 2001);(Finkelstein and Gibson, 2002) and coordinate carbon and nitrogen metabolism (Stitt and Krapp, 1999);(Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001).

Although hexoses are potent signals sensed in plants, sucrose-specific (Chiou and Bush, 1998);(Rook et al., 1998) and trehalose-mediated (Goddijn and Smeekens, 1998) signaling pathways also play important roles in regulating development and gene expression. In developing seeds, it has been suggested that sucrose regulates differentiation and storage, whereas hexoses control growth and metabolism (Weber et al., 1997);(Wobus and Weber, 1999a). The ability of both 3-*O*-methylglucose and 6-deoxyglucose to regulate gene expression indicates the presence of HXK-independent pathways through novel sensors in plants (Martin et al., 1997);(Roitsch, 1999).

The effect of carbon allocation on organ and whole plant architecture is illustrated most dramatically by carbohydrate storage and the concomitant cell expansion in reserve organs such as roots, fruit, seed, and tubers. However, cell division and differentiation can be ascribed to both changes in metabolic activity and sophisticated developmental switches (Jackson, 1999);(Wobus and Weber, 1999b);(Hajirezaei et al., 2000);(White et al., 2000);(Giovannoni, 2001). In *Vicia faba* embryos, gradients of sugars have been reported to correlate spatially with mitotic activity (Borisjuk et al., 1998). Consistently, Arabidopsis D-type cyclin gene expression is regulated differentially by sugars (Riou-Khamlichi et al., 2000). Therefore, sugars also could act as morphogens, providing positional information to the cell

cycle machinery and different developmental programs. Remarkably, differential display analysis using portions of tomato meristems destined to form leaves revealed spatially regulated carbohydrate metabolism within the meristem and suggested the involvement of carbohydrate metabolism in organogenesis (Pien et al., 2001).

Sugar sensing and signaling are involved in the control of growth and development during the entire plant life cycle. During germination and early seedling development, sugars can repress nutrient mobilization, hypocotyl elongation, cotyledon greening and expansion, and shoot development (Yu et al., 1996);(Dijkwel et al., 1997);(Jang and Sheen, 1997b);(Peralta et al., 1997);(Kurata and Yamamoto, 1998);(Arenas-Huertero et al., 2000);(Gibson, 2001);(Smeekens, 2000);(Eastmond and Graham, 2001);(Gazzarrini and McCourt, 2001). High sugar accumulation during early seedling development may reflect undesirable growth conditions at a crucial developmental period (Lopez-Molina et al., 2001), resulting in a reversible developmental arrest that acts as a protection mechanism.

First, sugars have to be sensed to activate signal transduction pathways. The sugar's dual function as a nutrient and a signaling molecule, however, significantly complicates analysis of the mechanisms involved (Rolland et al., 2001). Even in yeast in which downstream components of sugar signaling pathways have been characterized in detail, elucidation of the initial glucose sensing and activation mechanisms has been difficult, but at least one involves in part a hexokinase metabolizing D-glucose transported into cells (Sheen et al., 1999);(Rolland et al., 2001);(Moore et al., 2003). There is emerging evidence that other sugar-signaling mechanisms exist in plants and yeast (Rolland et al., 2001);(Eastmond and Graham, 2001);(Tiessen et al., 2003);(Kolbe et al., 2005), including a hexokinase-independent mechanism involving key components of the G-protein heterotrimer (Ullah et al., 2002);(Chen et al., 2003);(Chen and Jones, 2004). This is not without precedent, as a G-protein–coupled D-glucose signaling mechanism was recently identified in the yeast *Saccharomyces cerevisiae*, in which it has been shown that sugar agonists and antagonists bind with low affinity to Gpr1, a G-protein–coupled receptor (GPCR) (Lemaire et al., 2004).

A regulatory role for HXK in plant hexose sensing was suggested by testing the effects of a variety of sugars, glucose analogs, and metabolic intermediates on photosynthesis and glyoxylate cycle gene repression in *Chenopodium* (Krapp et al., 1993) and cucumber (Graham et al., 1994) cell cultures and in a maize protoplast transient expression system (Jang and Sheen, 1994). Sugars that are substrates of HXK, including mannitol and 2-deoxyglucose, which are phosphorylated but inhibit Glc-6-phosphate and ATP production (Klein and Stitt, 1998), cause repression of photosynthetic gene expression at low physiological levels (1 to 10 mM in maize mesophyll protoplasts) (Jang and Sheen, 1994); he repression is blocked by the HXK-specific competitive inhibitor mannoheptulose.

In addition to germination and seedling development, sugars have a broad influence on other processes such as internode elongation, root formation, and mature leaf development (von et al., 1990);(Dickinson et al., 1991);(Jiang et al., 1993);(Weber et al., 1998), embryogenesis and organ differentiation (Tang et al., 1999), as well as leaf senescence (Ding et al., 1993);(Wingler et al., 2006). However, it has been shown that over-expression of *AtHXK1* can promote senescence in transgenic tomato but not in *Arabidopsis* under normal growth conditions; the differential response was presumably due to different plant species with different sugar sensitivity (Dai et al., 1999). Alternatively, it may be possible that the manifestation of sugar responses is dependent on other signaling pathways triggered by hormones, light, and environmental stimuli that crosstalk with the sugar signaling pathways (Zhou and Solomos, 1998);(Nemeth et al., 1998);(Sheen et al., 1999). It remains unclear whether HXK plays a role in plant senescence under different developmental and environmental conditions (Xiao et al., 2000).

(Moore and Sheen, 1999) showed that HXK also functions as a sugar sensor in plants. These results agreed with the information suggested from (Jang et al., 1997) working with *AtHXK1* and *AtHXK2* overexpression plants; they showed that hexokinases could work as sugar sensors in the inhibition of hypocotyl elongation, and in light-induced cotyledon opening.

D-glucose acts as a hormone-like signal, although a physiological concentration range of extracellular D-glucose in signaling has yet to be defined. High applications of D-glucose (Arenas-Huertero et al., 2000) cause physiologically appropriate responses, and genetic screens for mutants resistant to high D-glucose have revealed known elements in sugar signaling (Xiao et al., 2000);(Moore et al., 2003). Because cells develop in a wide range of D-glucose concentrations, a sugar-signaling mechanism operating from presumably 30 to 300 mM or higher D-glucose is expected. Molar levels of D-glucose are found in tissues such as fruit, but the levels of extracellular D-glucose in vegetative tissues such as root have yet to be successfully evaluated. Indirect measurements have been applied to address this problem, and estimates of apoplastic levels of D-glucose at 150 mM (3%) or higher have been proposed (McLaughlin and Boyer, 2004);(Makela et al., 2005). For example, sorghum (*Sorghum bicolor*) embryos develop in as high as 6% apoplastic D-glucose (Maness and Mcbee, 1986).

Externally supplied sugar has different effects on various stages of early growth in *Arabidopsis*. Whereas low concentrations can stimulate wild-type germination, higher concentrations repress both cotyledon, early seedling development and photosynthetic gene expression (Smeekens and Rook, 1997; Smeekens, 1998);(Sheen et al., 1999). Numerous experiments have now suggested a close interaction between the germination and growth of young seedlings on exogenous sugar and ABA/ethylene action. Young seedlings deficient in

ABA biosynthesis (i.e., *aba1*, *aba2* and *aba3*) or ABA sensitivity (i.e., *abi4* and *abi5*) are insensitive to high levels of sugar and, unlike wild-type plants, these mutants do not show sugar-dependent repression of photosynthetic gene expression. In addition, germination on high sugar increases ABA levels in wildtype plants (Arenas-Huertero et al., 2000). Conversely, low concentrations of exogenous sugar relieve the inhibitory effects of ABA on wild-type seed germination, although these seedlings fail to green or develop true leaves (Garciarrubio et al., 1997);(Finkelstein and Lynch, 2000). Therefore, low sugar levels interfere with the inhibitory effects of ABA on germination, whereas inhibition of seedling development post-germination by high sugar concentrations is dependent on ABA synthesis.

2.1.2. Nitrogen

The growth and development of a root system is highly sensitive to modification by both intrinsic and extrinsic factors (Forde and Lorenzo, 2001);(Bloom et al., 2002);(Porterfield, 2002). Intrinsic factors that influence root growth and development include the supply of photosynthesis products from the shoot and the nutrient status of the plant; in other hand, extrinsic factors include the supply and distribution of nutrients in the soil, soil compaction and gradients of water potential. One important aspect of root plasticity is the proliferation of lateral roots that occurs within soil patches enriched in certain nutrients, including NH^{+}_{4}, NO^{-}_{3}, Pi (Robinson, 1994);(Forde and Lorenzo, 2001) and even Zn^{2+} (Haines, 2002).

Increased branching (wherever is possible: shoots or roots) in resource-rich conditions serves to enhance the precision with which the leaves or roots are placed within the environment (Sutherland and Stillman, 1988). Thus, an understanding of the mechanisms underlying root foraging is very much dependent on understanding how intrinsic and extrinsic nutritional factors influence root branching.

The ability to respond to localized nitrate supplies by proliferating lateral roots within the nitrate-rich zone is a common characteristic to a spread number of plant species (Robinson, 1994);(Hodge, 2004). In barley, this ability is due to a combination of increased numbers of lateral roots and increased rates of lateral root elongation (Drew and Saker, 1975). In *Arabidopsis*, the primary effect of a localized nitrate treatment stimulated lateral root elongation (Zhang and Forde, 1998);(Linkohr et al., 2002), with one report indicating a small localized increase in lateral root numbers (Linkohr et al., 2002). This stimulation of lateral root elongation appears to be attributable to a signalling effect from the NO^{-}_{3} ion itself rather than to a downstream metabolite (Zhang and Forde, 1998);(Zhang et al., 1999). Nitrate stimulates lateral root elongation by increasing rates of cell production in the root tips directly exposed to the signal (rather than through any effect on cell elongation) (Zhang et al., 1999). How the nitrate signal is converted into an increase in meristematic activity in the root tip is

an intriguing question that as yet has no clear answer. One component of the NO^-_3 signalling pathway has been identified in the form of the product of the *ANR1* gene, which is a member of the MADS box family of transcription factors (Zhang and Forde, 1998). Using a reverse genetic approach it was shown that lateral roots of *Arabidopsis* lines in which *ANR1* was down-regulated were defective in their response to a localized supply of NO^-_3. (Walch-Liu et al., 2006) have obtained additional evidence that *ANR1* is a positive regulator of lateral root growth using transgenic *Arabidopsis* lines in which *ANR1* can be rapidly post-translationally activated by a treatment with the synthetic steroid dexamethasone (DEX). These transgenic lines carry a construct constitutively expressing a translational fusion between ANR1 and the ligand-binding domain of the rat glucocorticoid receptor (rGR). The ANR1-rGR fusion protein is held inactive in a cytoplasmic complex with the HSP90 protein until addition of DEX, when it is released and is able to enter the nucleus where it can activate or repress its target genes (Picard et al., 1988). When ANR1-rGR seedlings were treated with 1 μM DEX, lateral root growth was strongly stimulated (Filleur et al., 2005). By surprise, even though the expression of *ANR1-rGR* gene was under the control of a strong constitutive promoter (CaMV 35S), the result of the treatment with DEX was specific to lateral root growth; the result suggest that one or more components of the regulatory pathway of which ANR1 is a part should be absent in the primary root tip.

Well known information for widespread interactions between MADS box proteins at both the post-translational and the transcriptional levels. MADS box factors attach to DNA as dimers, which can be either homodimers or heterodimers with additional MADS box proteins (Theissen et al., 2000). Also are reported numerous cases in which one MADS box protein has been found to regulate, either directly or indirectly, the transcription of another MADS box gene (Riechmann and Meyerowitz, 1997);(Jack, 2004). Exist information that around half of the >100 members of the MADS box gene family in Arabidopsis are transcribed in roots (Rounsley et al., 1995);(Alvarez-Buylla et al., 2000);(Burgeff et al., 2002);(Parenicova et al., 2003); this data gives sufficient scope for possible regulatory interactions between *ANR1* and other MADS box genes. Therefore, is possible to realize about functional redundancy between different members of the gene family, i.e. a case among some MADS box genes involved in flower development (Jack, 2004). It has been suggested that the *AGL21* gene, which belongs to the same group as *ANR1* and performs a similar spatial pattern of expression in roots, probably is functionally redundant with *ANR1* (Burgeff et al., 2002).

However, recent evidence suggesting the possibility of regulatory interactions at the protein level, between some of the root-expressed MADS box genes. A close map of protein–protein interactions between the Arabidopsis MADS box family has been composed employing a matrix-based yeast two-hybrid screen (de Folter et al., 2005). An ‘interactome’ map was designed and the complexity of the network of possible interactions is indisputable high, with

each of the 11 proteins interacting with 2–10 other MADS box proteins (Walch-Liu et al., 2006). Nevertheless, these results must be interpreted with caution because is convenient to confirm the potential interactions *in planta* and in any case can only be biologically meaningful if the relevant proteins are expressed in the same cells at the same time. However, it is remarkable that was found ANR1 interacting just with three other proteins (AGL16, AGL21 and SOC1) and all of them are regulated by the provision of N similarly to ANR1 (Gan et al., 2005).

About inhibitory effects using nitrogen, *Arabidopsis* seedlings were grown at external NO^-_3 concentrations ≥10 mM which influenced the development of early lateral roots, resulting in the accumulation of short laterals roots that were blocked just after emergence from the primary root. The finding that this effect was enhanced in a nitrate reductase-deficient mutant suggested that the internal NO^-_3 concentration rather than accumulation of the products of NO^-_3 assimilation was the key factor triggering the developmental response (Zhang et al., 1999). It has been proposed that the accumulation of high tissue concentrations of NO^-_3 in the leaf are responsible for generating a long-distance signal that regulates lateral root development (Scheible et al., 1997);(Zhang et al., 1999). The nature of this long-distance signal is unknown, but an obvious candidate is auxin because it has been shown that shoot-derived auxin is important for stimulating lateral root emergence (but not initiation) in *Arabidopsis* seedlings (Bhalerao et al., 2002). An auxin-requiring checkpoint in lateral root development was hypothesized by (Forde, 2002); he proposes that NO^-_3 accumulation in the shoot may inhibit the pass of auxin to the root and this auxin inhibition drives to fail in the development of the lateral roots.

(Walch-Liu et al., 2006) propose that changes in root architecture in response to the presence of significant accumulations of L-glutamate in the soil may represent a second (morphological) adaptation that enhances a plant's ability to compete for organic N. It has been suggested that plants are most likely to be able to compete effectively with micro-organisms for soil amino acids within organic N-rich soil patches where the concentrations of these amino acids are highest (Raab et al., 1996);(Jones et al., 2005). The slowing of primary root growth, the increased root branching behind the root tip and the developmentally delayed inhibition of lateral root elongation, which are the responses observed when an *Arabidopsis* root system encounters a source of L-glutamate, can be seen as a potential foraging mechanism because they would serve to increase the precision of root placement within the soil (Sutherland and Stillman, 1988). Although the concentrations of L-glutamate normally found in the bulk soil solution may be too low to affect root growth (Jones et al., 2005), within regions of decomposing organic matter its concentration can be expected to frequently exceed that needed to elicit a growth response in roots of sensitive genotypes. Plant and animal tissues contain free glutamate at millimolar concentrations (Joy

et al., 1992);(Young and Ajami, 2000) and an even larger pool of glutamate is available for proteolytic release in the protein fraction (Tapiero et al., 2002).

2.2. Auxin

Julius von Sachs first was the first discussing the concept of a phytohormone in 1887 (Sachs, 1887). Phytohormones are chemicals that have specific effects on plant growth, and are active at low concentrations. Plants use a large variety of hormones, as steroids and peptides, including also the five classical classes of phytohormones (auxins, abscisic acid, cytokinins, ethylene and gibberellins), which are all relatively small molecules. However, the extent and significance of the mechanism of the phytohormone transport is not well understood for all of these classes (**Figure 6**).

The effect of auxin was first documented when Charles and Francis Darwin (Darwin, 1880) published *The Power of Movement in Plants*. After the perception of light in one area of a grass coleoptile, they realised that an "influence is transported" that causes bending towards the light in another. Forty-five years later, in 1926, this messenger was separated from plant tissues simply by being allowed to diffuse into agar blocks, which then retained a growth promoting activity (Cholodny, 1926; Went, 1926). Initially, three kinds of auxin were found in plants, of which one was also found in human urine. Afterwards, the first published reports began to appear on the crystallization and structural characteristics of auxin but for today is clear that only one of the structures, that of indole-3-acetic acid (IAA), was correctly identified.

The word 'auxin' or 'αυξανω' in Greek, or 'to grow' in English, is the name of these small class of molecules with a big capacity to induce growth responses in plants. In plants, growth is defined as an irreversible increase in size, and is achieved by the enlargement of individual cells driven by the uptake of water. Auxin refers to an important group of phytohormones that has been implicated in most of the quantitative growth changes that occur during a plant's life cycle: changes in growth direction, in shoot and root branching, and in vascular differentiation (Laskowski et al., 1995);,(Leyser, 2001);(Paquette and Benfey, 2001);(Reinhardt et al., 2000);(Teale et al., 2005). Chemically, auxins are a group of naturally occurring tryptophan derived indole-related compounds, of which indoleacetic acid (IAA) is the most common bioactive form. They are probably synthesized to some level in all plant cells and can be redistributed within the plant through the vasculature. However, early studies using radiolabelled IAA revealed that there is, in addition, a polar flux of auxin parallel to the shoot–root axis of the plant. Based on such transport studies, two decisive documents (Rubery and Sheldrake, 1974);(Raven, 1975) proposed that the observed directional flux of auxin along the shoot could be due to a molecular mechanism in which specific efflux

carriers for auxin were localized to one face of the component cells of a tissue, forming a column of cells along which auxin would move. Data from the last few years have put flesh on this model of polar auxin transport (PAT) and revealed a central molecular mechanism determining plant polarity, patterning and growth. These advances have come from the characterization of auxin transporters, in particular a family of efflux carriers encoded by the PIN genes.

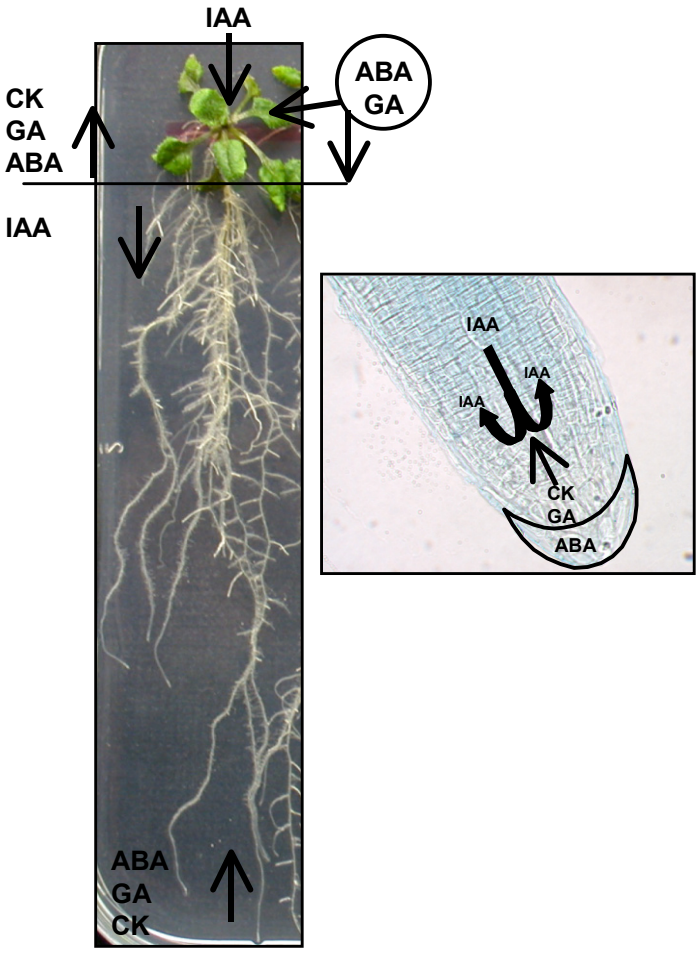

FIG. 6. Classical view of phytohormone movements.IAA: indole-3-acetic acid, a major auxin, GA: gibberellin, CK: cytokinin, ABA: abscisic acid. Transport of other hormones, brassinosteroid and jasmonate, are not well known, but these play a role in stress-response and injury-response in every part of the plants. Modified from (Tanimoto and Watanabe, 1986).Auxin is thought to be synthesized in young apical tissues and then transported downward to the maturing stem and the roots by a polar transport system (Sitbon et al., 2000).The most studied auxin with respect to the regulation of root growth is indole-3-acetic acid (IAA) among natural auxin including 4-chloroindole-3-acetic acid, indole-3-acrylic acid and indole-3-butyric acid (Marumo, 1986; Epstein and Ludwigmuller, 1993). The classical view of concentration dependency of IAA action on plant growth (Thimann, 1969)is a key characteristic to

understanding the regulatory function of auxin in root growth. In contrast to the accelerating effect on stem elongation, IAA strongly decelerates root elongation in a wide range of concentrations (Scott, 1972);(Feldman, 1984);(Porterfield, 2002);(Pilet, 2002).

Externally applied IAA rapidly inhibit the elongation growth of roots at very low concentrations; from 1 nM level in lettuce (Tanimoto and Watanabe, 1986) and in wild type *Arabidopsis*, down to 1 pM level in the hypersensitive transgenic *Arabidopsis* (Kim et al., 2001). The deceleration of root growth occurs within 10 minutes after IAA application (Tanimoto and Watanabe, 1986)and Watanabe, 1986). The inverse relationship between the endogenous IAA level and growth rate was confirmed in the elongation zone (2.5 to 5 mm from root tip) of maize roots (Pilet and Saugy, 1987; Pilet, 2002). They also presented a similar inverse relationship between ABA content and growth rate of the same maize roots. However, concentration dependent decrease in growth rate was much greater in IAA than that in ABA, indicating that the growth rate of maize roots is more sensitive to changes in IAA concentration than ABA concentration. The development of GC/MS method has greatly contributed to such quantifications of IAA and ABA, particularly in being able to take measurements in a tiny root tip (Edlund et al., 1995);(Koshiba et al., 1995);(Ribnicky et al., 1998). This technique has enabled measurement of the axial gradient of IAA levels even in *Arabidopsis* roots (King et al., 1995);(Koshiba et al., 1995);(Ljung et al., 2001);(Marchant et al., 2002);(Muller et al., 2002). The endogenous IAA level was the highest in the apical young zone of stems and in the apical and basal part of roots. Recent studies on the meristem of *Arabidopsis* suggest that such endogenous gradient of IAA plays an indispensable role for morphogenesis (Swarup et al., 2001);(Nakajima and Benfey, 2002);(Jiang and Feldman, 2002).

In addition to axial gradient of IAA level, radial gradient is also important. Much higher content of IAA was measured in stele tissues than in cortical cells of maize roots (Saugy and Pilet, 1987). They also detected more IAA in lower side of cortex than in upper side of the horizontally placed roots. Endogenous IAA concentration falls to the order of 0.1 micromole per liter (=17.5 ng/g FW) and these levels are compatible with the inhibitory concentration range of externally applied IAA. On the other hand, the amount of IAA conjugate is always several fold greater than free IAA (Saugy and Pilet, 1987);(Jensen and Bandurski, 1994). Since some IAA conjugates such as IAA-glucoside are reversibly convertible to free IAA by enzymatic hydrolysis, the interconversion of IAA to conjugates are also important reactions to regulate IAA level in roots (Kowalczyk and Sandberg, 2001);(Ljung et al., 2001). Results of dose response experiments and measurements of endogenous IAA levels indicate that IAA functions as a growth decelerator rather than an accelerator in roots by the concentration-dependent effect. Thus, the endogenous concentration of IAA plays a critical role for root growth. The inhibition mechanism of root growth by such low levels of IAA is not sufficiently

explained to date. However it is ascribed, at least partially, to the growth inhibitory effect of ethylene produced by IAA action, since the growth inhibition by IAA is partially eliminated by the treatment of roots by the inhibitor of ethylene biosynthesis, aminoethoxyvinylglycine (AVG) and cobalt ion (Mulkey et al., 1982).

The molecular mechanism of polar IAA transport has been elucidated by the discovery of membrane-located carrier proteins, named AUX1, PIN and MDR1. AUX1 is an influx protein and PIN is efflux facilitator of IAA. MDR1 belongs to multidrug-resistance-related protein and to ABC transporter family which carries ATP-binding cassette, and transport IAA and/or IAA-conjugate. Among these transporters, AUX1 and PIN proteins play a central role for polar auxin transport. Discovery of PIN proteins was initiated by the finding of pinformed mutant, pin-1 of *Arabidopsis* (Goto, 1987; Haughn and Somerville, 1988). The abnormal morphogenesis of the pinformed mutant was ascribed to the decreased IAA transport (Okada et al., 1991; Oka et al., 1998; Oka et al., 1999). These findings were followed by the identification of *pin* genes and proteins (Estelle, 1998). Eight kinds of PINs have been found in *Arabidopsis* to date and Four of them (PIN1-PIN4) are supposed to play roles in the root (PIN1: (Galweiler et al., 1998); PIN2: (Muller et al., 1998); PIN3: (Friml et al., 2002b); PIN4: (Friml et al., 2002a). PIN6 and PIN7 are also reported in roots (Benkova et al., 2003);(Blilou et al., 2005). Localization and gene expression of these carrier proteins are gravity-sensitive. In addition to the growth and tropic regulation of roots, movement and gradient of IAA in the root tip participate in the maintenance of root meristem (Friml et al., 2002a);(Jiang and Feldman, 2002);(Benkova et al., 2003);(Blilou et al., 2005);(Teale et al., 2005). An asymmetric auxin flux from columella cells to the lateral root cap cells is also observed by Green Fluorescence Protein(GFP)-based monitoring of auxin-responsive gene expression under gravity stimulation (Ottenschlager et al., 2003). Such auxin flow back to the elongation zone from columella cells is compatible with the localization of PIN proteins in these tissues. PIN family proteins were also cloned in cucumber and pea, and the gene expression of PIN2 and AUX1 are also gravity responsive (Kamada et al., 2003);(Miyamoto et al., 2003).

Quantitative measurement is necessary for the evaluation of these carrier proteins in the regulation of root growth, because root growth itself is strictly controlled by IAA concentration in the meristem and in the elongation zone. A regulatory mechanism of carrier proteins by signal transduction system may exist, since protein phosphorylation was suggested to play a role in basipetal auxin transport (Rashotte et al., 2001). Speed of IAA transport by these carriers remains to be investigated (Muday and DeLong, 2001; Muday and Murphy, 2002). In addition to the regulation of elongation growth, auxin plays an important function to produce lateral roots (Reed et al., 1998; Bhalerao et al., 2002; Benkova et al., 2003; Casimiro et al., 2003). Despite the accumulation of research on auxin responsive gene expression and its regulation by the proteasome dependent degradation of suppressor proteins (Ramos et al.,

2001; Dharmasiri and Estelle, 2002; Aspuria et al., 2002; Ueda et al., 2004; Dharmasiri and Estelle, 2004), little information is available to answer how auxin decelerate or accelerate root growth, because physiological and biochemical functions of these genes are not clear enough.

The reason why auxins have attracted so much attention for almost a century is not only that they have the capacity to influence growth, but that they have additional farther-reaching effects on the life cycle of plants. Recent evidence shows that, through a unique mechanism of perception and elicitation, the physiological responses that auxin governs are central to a plant's structure and functioning.

3. Aim & Scope

3.1. Aim

The more important aim of this research study consisted in the identification, cloning and characterization of a novel gene coding the *Arabidopsis thaliana* diacylglycerol kinase 7 (AtDGK7). This study groups the collection of experiments to analyze the molecular, biochemical and physiological aspects of *AtDGK7*.

The experimental activities followed the successful cloning and characterization of the gene *AtDGK2* (Gomez-Merino et al., 2004), the first published assay of an AtDGK enzyme that was catalytically active. From the AtDGK family the member *AtDGK7* was selected, its cDNA was cloned and biochemical assays showed the catalytic properties of the encoded protein.

3.2.Scope

Here, in this document, I will focus mainly on the *AtDGK7* gene, its biological roles as well as the biochemical and physiological properties of the encoded protein. In my PhD thesis I tested the hypothesis whether AtDGK7 plays are role in growth and differentiation in lateral root development. Therefore, several nutritional conditions and the effects of hormones were investigated.

In **Chapter 1**, the general introduction, I make an overview about phospholipid signaling and show the roles of dyacylglycerol and phosphatidic acid emerged as second messengers In this chapter I also present the signaling function by molecules as sugar, nitrates and auxin, and how they are affecting directly or indirectly biological processes.

Chapter 2 presents a paper with documented information about the molecular cloning and characterization of *AtDGK7*. The recombinant AtDGK7 enzyme is catalytically active and

phosphorylates DAG molecular species as their natural substrate. R59022 inhibits root elongation and lateral root formation and reduces plant growth, suggesting that DGKs play an important role in plant development.

In **Chapter 3**, I report the cloning of a cDNA for *AtDGK7* from *Arabidopsis thaliana* which codes for a protein that is longer than the previously described AtDGK7 polypeptide. Physiological analysis under abiotic stresses were performed with *AtDGK7* T-DNA insertion lines and also with plants expressing the GUS gene under the control of the native *AtDGK7* promoter (*AtDGK7prom::GUS*). It is hypothesized that *AtDGK7* is involved in a metabolic process affecting growth and development of lateral roots, mediated via an interaction with auxin.

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CHAPTER 2

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Arabidopsis AtDGK7, the Smallest Member of Plant Diacylglycerol Kinases (DGKs), Displays Unique Biochemical Features and Saturates at Low Substrate Concentration

*THE DGK INHIBITOR R59022 DIFFERENTIALLY AFFECTS AtDGK2 AND AtDGK7 ACTIVITY IN VITRO AND ALTERS PLANT GROWTH AND DEVELOPMENT**

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Diacylglycerol kinase (DGK) regulates the level of the second messenger diacylglycerol and produces phosphatidic acid (PA), another signaling molecule. The *Arabidopsis thaliana* genome encodes seven putative diacylglycerol kinase isozymes (named AtDGK1 to -7), structurally falling into three major clusters. So far, enzymatic activity has not been reported for any plant Cluster II DGK. Here, we demonstrate that a representative of this cluster, AtDGK7, is biochemically active when expressed as a recombinant protein in *Escherichia coli*. AtDGK7, encoded by gene locus At4g30340, contains 374 amino acids with an apparent molecular mass of 41.2 kDa. AtDGK7 harbors an N-terminal catalytic domain, but in contrast to various characterized DGKs (including AtDGK2), it lacks a cysteine-rich domain at its N terminus, and, importantly, its C-terminal DGK accessory domain is incomplete. Recombinant AtDGK7 expressed in *E. coli* exhibits Michaelis-Menten type kinetics with 1,2-dioleoyl-*sn*-glycerol as substrate. AtDGK7 activity was affected by pH, detergents, and the DGK inhibitor R59022. We demonstrate that both AtDGK2 and AtDGK7 phosphorylate diacylglycerol molecular species that are typically found in plants, indicating that both enzymes convert physiologically relevant substrates. *AtDGK7* is expressed throughout the *Arabidopsis* plant, but expression is strongest in flowers and young seedlings. Expression of *AtDGK2* is transiently induced by wounding. R59022 at ~80 μM inhibits root elongation and lateral root formation and reduces plant growth, indicating that DGKs play an important role in plant development.

Lipid second messengers, generated in response to diverse stimuli through the activity of lipid kinases and phospholipases, are involved in a variety of biological responses in plant cells (1, 2). Diacylglycerol kinase (EC 2.7.1.107) is a lipid kinase that phosphorylates diacylglycerol (DAG)[5] to yield phosphatidic acid (PA) in a reaction that uses ATP as phosphate donor. In plants, both DAG and PA may have signaling functions. DAG has been demonstrated to activate both ion pumping in patch-clamped guard cell protoplasts and opening of stomata (3), whereas PA accumulates in response to different kinds of stresses and regulates the activity of several enzymes (4).

Various PA targets have been identified in plants, including, for example, *Arabidopsis* phosphoinositide-dependent protein kinase 1 (5) and a calcium-dependent protein kinase from carrot (6). Recently, using PA affinity chromatography coupled to mass spectrometry, PA-binding proteins were identified in plants, including phosphoenolpyruvate carboxylase, Hsp90, 14-3-3 proteins, and others (7), providing new entries into the field of plant PA research. Furthermore, Zhang *et al.* (8) have demonstrated that PA interacts with ABI1 phosphatase 2C, thereby regulating abscisic acid signaling in *Arabidopsis thaliana*.

Animal DGKs have been extensively studied, and models for their function have been elaborated (9, 10). DGK activity has also been reported in several plant species, including *Catharanthus roseus*, tobacco, wheat, tomato, and *Arabidopsis* (11–16), and molecular data bases reveal that they are present in a number of other crop plants such as rice, maize, grape, sweet orange, and cotton. However, functional analysis of *DGK* genes is still fragmentary. Two *DGK* cDNAs, *LeDGK1* and *LeCBDGK*, respectively, have been cloned from tomato (15). The two enzymes are derived from the same gene via alternative splicing and are identical except for a 29-amino acid-long C-terminal extension in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY686593.

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[5] The abbreviations and trivial names used are: DAG, diacylglycerol; EST, expressed sequence tag; Bis-Tris, bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Na-DC, sodium deoxycholate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 1,2-SAG, 1-stearoyl-2-arachidonoyl-*sn*-glycerol; 1,2-DOG, 1,2-dioleoyl-*sn*-glycerol; 1,2-POG, 1-palmitoyl, 2-oleoyl-*sn*-glycerol; 1,2-SLG, 1-stearoyl, 2-linoleoyl-*sn*-glycerol; 1,2-OPG, 1,2–1-oleoyl, 2-palmitoyl-*sn*-glycerol; PA, phosphatidic acid; CaM, calmodulin; RT, reverse transcription; R59022, 6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo(3,2-α)pyrimidine-5-one; R59949, 3-{2-{4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl}ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone; MPSS, massively parallel signature sequencing; TPM, transcripts/million; HPLC, high pressure liquid chromatography.

the case of LeCBDGK, which represents a calmodulin (CaM)-binding domain. The two proteins lack the cysteine-rich domain (CRD) present in other eukaryotic DGKs, but are active *in vitro*. LeCBDGK is found both in association with membranes and in soluble cell extracts. The Ca^{2+}/CaM-dependent translocation of LeCBDGK to its membrane-associated substrate DAG might represent a means of activation *in vivo*, analogous to the Ca^{2+}-dependent translocation of certain mammalian DGKs. In contrast, LeDGK1 only associates with the membrane fraction, via a Ca^{2+}/CaM-independent mechanism, which might represent a means of encoding specificity in cellular responses by alternative splicing (15). In *A. thaliana*, seven candidate genes (named *AtDGK1* to *-7*) encode putative DGK isoforms. The *AtDGK1* cDNA has been isolated and reported to be mainly expressed in roots, shoots, and leaves, but its enzyme product was not active *in vitro* (17). We have previously cloned the *AtDGK2* cDNA and demonstrated that its encoded enzyme is catalytically active. *AtDGK2* transcripts are found in the whole plant except in stems and are induced by exposure to 4 °C, pointing to a role in cold signal transduction (18). AtDGK1 and -2 share a similar domain organization and fall into the Cluster I of plant DGKs.

Here we report the molecular cloning and characterization of *AtDGK7*, which encodes an enzyme structurally belonging to the Cluster II of plant DGKs. The *AtDGK7* gene is transcribed throughout the plant and most prominently in flowers and young tissues. Recombinant AtDGK7 enzyme is catalytically active and, importantly, accepts DAG molecular species containing at least one saturated fatty acid as the preferred substrate. We also demonstrate that both AtDGK2 and AtDGK7 are able to phosphorylate DAG molecular species that are typically found in plants, indicating that both enzymes convert physiologically relevant substrates. The DGK inhibitor 6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo(3,2-α)pyrimidine-5-one (R59022) at around 50–100 μM inhibits recombinant AtDGK2 *in vitro*. It also modifies root growth within the same concentration range, indicating the involvement of this enzyme in developmental processes. In contrast, AtDGK7 was found to be affected by R59022 only at concentrations above 100 μM.

EXPERIMENTAL PROCEDURES

General—Manipulation and analysis of nucleic acids were performed according to standard molecular-biological techniques described (19). Restriction enzymes were purchased from Roche Applied Science (Mannheim, Germany) and New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were obtained from TibMolbiol (Berlin, Germany) or Eurogentec (Cologne, Germany). DNA sequencing was performed by SeqLab (Göttingen, Germany). Unless otherwise indicated, other chemicals were purchased from Roche Applied Science, Merck, or Sigma. *Escherichia coli* strain XL-1 Blue (Stratagene, Heidelberg, Germany) was employed for general DNA work. For sequence analyses, the tools provided by the National Center for Biotechnology Information (available on the World Wide Web at www.ncbi.nlm.nih.gov/), the ExPASy Molecular Biology Server (available on the World Wide Web at us.expasy.org/), and The Arabidopsis Information Resource (available on the World Wide Web at www.arabidopsis.org/) were used. Gene expression data obtained through Affymetrix GeneChip hybridizations were retrieved using the software tools of the Genevestigator package (available on the World Wide Web at www.genevestigator.ethz.ch), version of July 2004. Massively parallel signature sequencing data were retrieved from the *Arabidopsis* massively parallel signature sequencing (MPSS) data base (available on the World Wide Web at mpss.udel.edu/at/java.html), version of August 2004.

Cloning of AtDGK7 cDNA—PCR amplification of the *AtDGK7* cDNA was carried out using the Advantage-HF2 PCR kit (Clontech, Heidelberg, Germany) according to the manufacturer's protocol. *A. thaliana* (L.) Heynh. Col-0 flower cDNA was used as template. Primer sequences were as follows: AtDGK7-F, 5′-GCGGATCCTGATGGAGGAGACGCCGAGATC-3′; AtDGK7-R, 5′-GCGCTCGAGTTATATGAACCTCTTAGGAAC-3′. Reaction details were as follows: 95 °C for 3 min; 36 cycles of 95 °C for 45 s, 62 °C for 45 s, and 72 °C for 72 s; 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis. Individual fragments were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subcloned into pGEM-T Easy (Promega, Mannheim, Germany), generating the plasmid pG7. The entire *AtDGK7* cDNA, present in plasmid pG7, had a length of 1125 bp (deposited under GenBank™ accession number AY686593).

Quantitative Reverse Transcription-PCR Analysis—Total RNA was digested with DNase I, RNase Free (Roche Applied Sciences, Mannheim, Germany). The absence of genomic DNA contamination was subsequently confirmed by PCR, using primers annealing to an intron of actin 2 (At3g18780) as a control gene, or alternatively, with primers LEH-fw (5′-AACAGCAACAACAATGCAACTACTGATT-3′) and LEH-rev (5′-ACAAACAGAGACAAGAGACAAGACATGG-3′), that span an intron of the *Arabidopsis* late elongated hypocotyl 1 gene encoding a Myb transcription factor. In some cases, genomic PCR products appeared after 35 amplification cycles, and such samples were discarded. RNA integrity was confirmed on 1.5% (w/v) agarose gel prior to and after DNase I digestion. Reverse transcriptase reactions were performed with avian myeloblastosis virus reverse transcriptase (Promega) or Superscript II reverse transcriptase (Invitrogen). Real time RT-PCR was performed with 1 μl of a 1:2 dilution of the first strand cDNA reaction and SYBR Green reagent (Applied Biosystems, Foster City, CA) in a 20-μl volume, on a PerkinElmer Life Sciences Geneamp 7300 machine, with the following primer pairs: *ACTIN2*, actin-fw (5′-ATGGCTGAGGCTGATGATATTCAAC-3′) and actin-rev (5′-TACAAGGAGAGAACAGCTTGGATG-3′); polyubiquitin *UBQ10*, UBQ-fw (5′-ATGCAGATCTTTGTTAAGACTCTCAC-3′) and UBQ-rev (5′-ATAGTCTTTCCGGTGAGAGTCTTC-3′); *AtDGK2*, AtDGK2-fw (5′-AAGCAAGTCTCGGACATGCCT-3′) and AtDGK2-rev (5′-TTCGTTTGTGCCCGCCTAT-3′); *AtDGK7*, AtDGK7F-RT (5′-TGTGGACTTAGCATCACAGG-3′) and AtDGK7R-RT (5′-AGCTGAGAGTCTGTCAAGG-3′).

Data were normalized to actin 2 or ubiquitin (*UBQ10*) as follows: $nCT = CT_{gene} - CT_{actin}$ or $CT_{ubiquitin}$, where *CT* refers to the number of cycles at which SYBR green fluorescence (ΔR_n) in a PCR reaches an arbitrary value during the exponential phase of DNA amplification, set at 0.3 in all experiments, and then compared according to the formula, *Cr* (change in signal log ratio) = $nCT_{control} - nC_{sample}$.

Wounding Experiments—Arabidopsis wild-type and transgenic plants harboring the promoter region of the *AtDGK2* gene fused to the *Escherichia coli GUS* reporter gene (18) were used to test the effect of wounding on *AtDGK2* expression. Wounding of 4-week-old soil-grown plants was performed by thoroughly crushing whole shoots. Wounded and nonwounded plants were harvested at different time points and frozen in liquid nitrogen for RNA and protein extraction.

Expression of Recombinant AtDGK7 in Bacteria—The coding region of the *AtDGK7* cDNA, flanked by XhoI and SpeI sites, was transferred from plasmid pG7 to plasmid pET43c (Novagen, Darmstadt, Germany), yielding plasmid pET7. The pET43c vector allows the generation of fusion protein containing a NusA tag and a His_6 tag (for detection and purification of target proteins; see Novagen, on the World Wide Web at www.novagen.com). Plasmid pET7 was transformed into *E. coli* BL21

(DE3) (Novagen). Cells were grown at 37 °C. At an A_{600} of 0.6, expression of NusA-His-AtDGK7 fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM final concentration). Cells were harvested 4 h later by centrifugation, resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, and Complete Mini Protease Inhibitor Mixture (Roche Applied Science), and lysed by sonification. Recombinant AtDGK7 was purified using Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer's protocol. All homogenates were frozen and stored at −80 °C until assayed. Protein concentration was determined according to Bradford (20), using bovine serum albumin as a standard.

Western Blot Analysis—Purified proteins (20 μg) produced by the pET7 plasmid or the pET43c empty vector were separated by SDS-PAGE electrophoresis (21) using a 4% spacer and a 8% (w/v) separating polyacrylamide gel and transferred onto nitrocellulose membranes (Amersham Biosciences) by semidry electroblotting. For detection, nitrocellulose membranes were blocked overnight in TBS (Tris-buffered saline) containing 0.150 M NaCl, 0.025 M Tris/HCl, pH 7, and 5% (w/v) nonfat dry milk (Bio-Rad) prior to a 1-h incubation in anti-His tag antibody (Pierce). After three 15-min washing steps using TBST (TBS plus 0.05% Tween 20 (v/v)) to remove residual antibody, membranes were developed with an enhanced chemiluminescence Western blot detection kit (Pierce SuperSignal) and exposed to x-ray films (Kodak X-Omat AR) for 5 s to 5 min.

DGK Enzymatic Assays—Diacylglycerol kinase activity was determined under standard conditions by measuring the incorporation of [γ-^{32}P]ATP into phosphatidic acid at 25 °C as described (18). To assess Michaelis-Menten kinetics, enzymatic activity of DGK was measured as a function of the sum of molar concentrations of CHAPS, Triton X-100, and DAG at a given mol fraction of DAG. Four stock solutions (A, B, C, and D) were prepared. Stock solution A contained 69.5 mM CHAPS, 30 mM Triton, 0.5 mM DAG (0.005 mM DAG); stock solution B contained 69 mM CHAPS, 30 mM Triton, 1 mM DAG (0.01 mM DAG); stock solution C contained 68 mM CHAPS, 30 mM Triton, 2 mM DAG (0.02 mM DAG); stock solution D contained 65 mM CHAPS, 30 mM Triton, 5 mM DAG (0.05 mM DAG). Stock solutions were prepared as follows: DAG, dissolved in chloroform/methanol (1:1), was placed in 7-ml Schott glass disposable reaction tubes (with screw cap; Schott, Mainz, Germany), dried under a stream of nitrogen vapor, resuspended in a solution of CHAPS and Triton X-100 dissolved in water, and sonified for 5 min in a Sonorex RK 100 sonifier (Bandelin, Berlin, Germany). Increasing volumes (from 3.6 to 35.0 μl) of the stock solution were pipetted into the glass disposable reaction tubes. The final volume of the reaction mix was 250 μl. Diacylglycerol kinase activity was determined by measuring the incorporation of [γ-^{32}P]ATP into PA as described previously (18). Extraction and separation of phospholipids were performed as follows: 1 ml of chloroform/methanol (1:1) and 500 μl of 1 M KCl plus 0.2 M H_3PO_4 were added, and the mixture was mixed thoroughly; samples were centrifuged at 2500 rpm for 5 min in a Labofuge 200 centrifuge (Heraeus Sepatech, Osterode, Germany). The lower phase (lipids) was transferred to a new glass reaction tube and washed once with chloroform/methanol and KCl to discard the remaining radiolabeled ATP. The amount of phosphate incorporated was determined by counting the radioactivity in a liquid scintillation counter.

Enzymatic activity of AtDGK7 was measured as a function of ATP concentration in mixed micelles. A solution containing 50 mM nonlabeled ATP was prepared as stock. Under standard conditions, each enzymatic reaction contained a final concentration of 1 mM nonlabeled ATP and ~5 mCi of ^{32}P-labeled ATP (18). To assess the effect of varying ATP on the enzymatic reaction, four different ATP final concentrations were tested: 0.10, 1.0, 2.0, and 5.0 mM, respectively. The same amount of radioactive ATP (5 mCi) was added to the assays, and the difference in specific radioactivities obtained were taken into account for calculating the enzyme activities in the individual reactions. For this experiment, the sum of the molar concentrations of CHAPS, Triton X-100, and DAG was 7.20 mM (containing 4.90 mM CHAPS, 2.20 mM Triton, and 0.10 mM DAG).

The effect of the DGK inhibitor R59022 or 3-{2-{4-[bis-(4-fluorophenyl)methylene]-1-piperidinyl}ethyl}-2,3-dihydro-2-thioxo-4(1*H*)-quinazolinone (R59949) on *Arabidopsis* AtDGK2 and AtDGK7 enzymes was tested at different concentrations of DAG and CHAPS as indicated above. The reaction mix was preincubated for 10 min with DGK inhibitor before the reaction was started by the addition of ATP.

PA standard was obtained from Sigma (product code P 9511). Hartmann Analytic (Braunschweig, Germany) provided [γ-^{32}P]ATP (15 TBq/mmol). Lipids (1,2-SAG, 1,2-DOG, and cardiolipin), salts ($MgCl_2$, LiCl, and NaCl) and detergents (Na-DC and CHAPS) were purchased from Sigma. Serva (Heidelberg, Germany) provided Triton X-100. DAG molecular species 1,2-POG, 1,2-SLG, and 1,2-OPG were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). DGK inhibitors R59022 and R59949 were purchased from Calbiochem.

HPLC Analysis of the Reaction Products of DGK Assay—The products of diacylglycerol kinase assays were obtained by two-phase partitioning. The organic phase was reduced to dryness under a stream of nitrogen gas, and the glycerolipids were deacylated (22). An aliquot of the water-soluble products of deacylation was mixed with L-[U-^{14}C]glycerol 3-phosphate (Amersham Biosciences; specific activity >100 mCi/mmol) and resolved by anion exchange high performance liquid chromatography with a gradient of $(NH_4)_2HPO_4$ (23). The column eluate was collected in 1-ml fractions, and 4 ml of Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA) was added. Radioactivity was estimated by dual label scintillation counting in a Wallac 1409 DSA (Turku, Finland) scintillation counter.

Effect of R59022 on Plant Growth and Development—A. *thaliana* (L.) Heynh. seeds (ecotype C24) were surface-sterilized with 70% ethanol for 5 min and sodium hypochlorite solution (5.0% $NaClO_4$ plus 0.05% Tween 20) for 5 min, followed by several washes with sterile water. Subsequently, seeds were sown on MS (Murashige Skoog) plates containing 0.8% agar (square Petri dishes, 100 × 100 × 14 mm; NUNC, Wiesbaden, Germany). The DGK inhibitor R59022 was dissolved in dimethyl formamide. Dimethyl formamide (without R59022) was used as control. The seeds were imbibed at 4 °C in the dark for 1 day. Plates were then placed vertically in a growth chamber under long day conditions (16 h of light, 8 h of dark). Lengths of primary roots from the shoot/root transition zone to the root tip were measured. To analyze the effects of the inhibitor R59022 on plant growth and development, 35–100 μM R59022 was added to MS medium containing 0.8% agar.

RESULTS

AtDGK7 cDNA and the Deduced Primary Structure of Its Encoded Protein—In the *Arabidopsis* genome, seven genes encode putative DGK isoforms that fall into three major clusters. AtDGK2, a member of Cluster I, is a catalytically active enzyme. The *AtDGK2* gene is located on chromosome V, and its expression is detected in various tissues of the *Arabidopsis* plant, including leaves and roots, as previously shown (18).

To investigate whether members of other clusters also encode functional DGKs, we concentrated here on AtDGK7 (encoded by gene locus At4g30340), representing a potential DGK of Cluster II. The *AtDGK7* gene is located on chromosome IV, and data base analysis revealed that *AtDGK7* is transcribed. In total, four *AtDGK7* expressed sequence tags

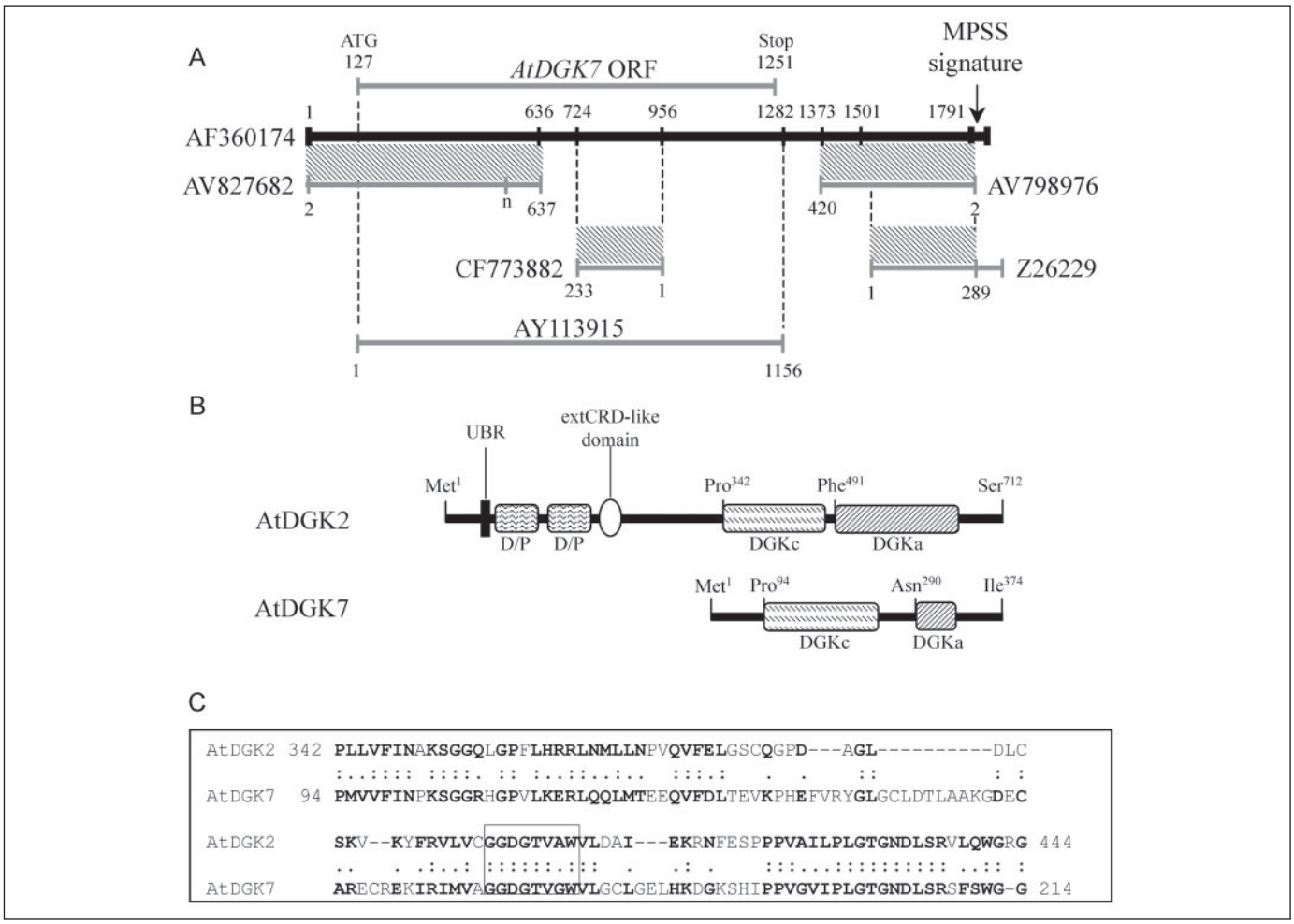

FIGURE 1. **Structural characteristics of AtDGK2 and AtDGK7 sequences.** *A*, arrangement of ESTs and cDNAs (GenBank™ accession numbers are given) corresponding to the *AtDGK7* gene. The longest cDNA (accession number AF360174) has a length of 1807 bp. The *numbers* indicate nucleotide positions of the overlapping ESTs/cDNAs. The 17-bp MPSS signature sequence (GATCTATGTTGAGCTTT) is located at the 3′-end of the *AtDGK7* transcript. The position of the predicted *AtDGK7* open reading frame (*ORF*) is indicated. *B*, structural alignment of the enzymes AtDGK2 and AtDGK7. Both enzymes share a conserved diacylgycerol kinase catalytic domain (DGKc; Pfam accession number PF00781). However, the diacylglycerol kinase accessory domain (Pfam accession number PF00609), which is full-length in AtDGK2, is incomplete in AtDGK7. Furthermore, the two diacylglycerol/phorbol esther binding domains (*D/P*; InterPro accession number IPR002219) as well as the upstream basic region (UBR) and the extended cysteine-rich domain (extCRD-like domain) are absent from the N-terminal region of AtDGK7. *C*, amino acid sequence comparison of the DGKc domains of AtDGK2 and AtDGK7. Amino acid residues identical or similar in the two proteins are highlighted in *boldface type*. The presumed ATP-binding site localized in the DGKc domain (consensus G*X*G*XX*G) is depicted in an *open box*.

(ESTs; GenBank™ accession numbers AV798976, AV827682, CF773882, and Z26229) and two cDNA sequences (AF360174 and AY113915) obtained through the full-length cDNA cloning projects at RIKEN and SALK can be retrieved from the NCBI data base (available on the World Wide Web at www.ncbi.nlm.nih.gov). Because of previous data base annotation ambiguities (reported in Ref. 18), we reanalyzed the *AtDGK7* sequence in detail here (see Fig. 1*A*). RIKEN ESTs AV827682 and AV798976 were both derived from clone RAFL 09-18-D16 and represent the 5′ and 3′ regions, respectively, of the AF360174 sequence, which in total encompasses 1807 base pairs. The EST Z26229 sequence was obtained from the Versailles EST collection and, like EST AV798976, corresponds to the 3′ part of the AF360174 full-length cDNA. The EST CF773882 covers the central part of the transcript. Finally, cDNA AY113915, generated at SALK, has a length of 1156 base pairs. The available data can be summarized as follows. (i) The AF360174 and AY113915 cDNAs code for the same protein, AtDGK7, which has a deduced length of 374 amino acids, a predicted molecular mass of 41.2 kDa, and an isoelectric point of 7.73. This prediction fully corresponds to the most recent TIGR annotation (version 5.0) for *AtDGK7* (available on the World Wide Web at www.tigr.org). (ii) The AF360174 cDNA contains a 5′-untranslated region at positions 1–126, the *AtDGK7* open reading frame at positions 127–1251, and an unusually long 3′-untranslated region at positions 1252–1807. The presence of this long untranslated region in the *AtDGK7* transcript was confirmed by the independently generated EST Z26229. The generation of the long transcript is further in full accordance with data from recent *Arabidopsis* MPSS analyses (24) (available on the World Wide Web at mpss.udel.edu/at/java.html). A 17-bp MPSS signature sequence corresponding to the very 3′-end of the *AtDGK7* gene was found (GATCTATGTTGAGCTTT on the Crick DNA strand). (iii) The nucleotide sequences of the two cDNAs are completely identical in the overlapping region (which encompassed the complete *AtDGK7* open reading frame and part of the 3′-untranslated region).

Protein sequence analysis reveals that AtDGK7 has a conserved catalytic domain (DGKc; Pfam accession number PF00781) (Fig. 1, *B* and *C*), located at amino acid residues 94–240, which contains a presumed ATP-binding site with a G*X*G*XX*G consensus sequence. Although the DGKc of AtDGK7 is ~40% identical to that of AtDGK2, the DGK accessory domain (DGKa; Pfam accession number PF00609) is incomplete in AtDGK7 (Fig. 1*B*). In most AtDGK isoforms, the DGKa domain encom-

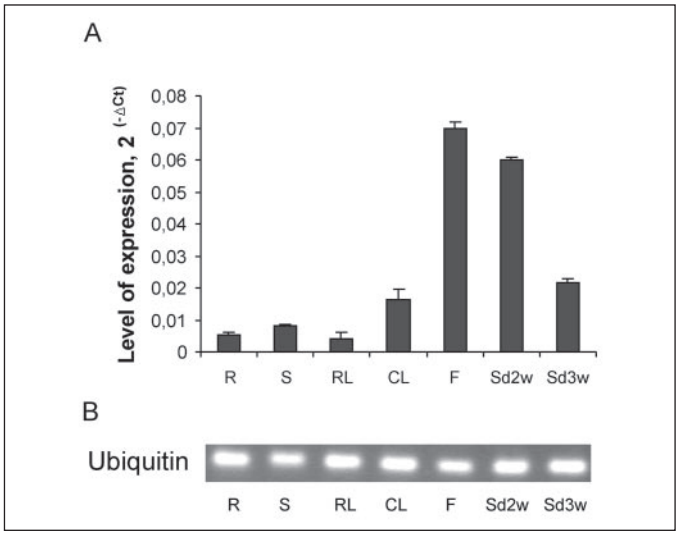

FIGURE 2. **Real time RT-PCR analysis of *AtDGK7* gene expression.** Quantitative RT-PCR was carried out using specific primers designed for the *AtDGK7* gene (locus At4g30340) and for the *UBQ10* gene (ubiquitin; locus At4g05320) used as control. *A*, relative abundance of *AtDGK7* transcripts in *Arabidopsis*. The expression levels were normalized against *UBQ10* mRNA in each sample. *B*, gel electrophoresis of *UBI10* amplicons derived from different *Arabidopsis* RNA pools. Total RNA was extracted from different organs of flowering *Arabidopsis* plants: roots (*R*), stems (*S*), rosette leaves (*RL*), cauline leaves (*CL*) from the inflorescence stem, and flowers (*F*) or from 2-week-old (*Sd2w*) or 3-week-old seedlings (*Sd3w*), respectively. Data are means of three independent experiments. *Bars*, ±S.D.

passes ~160 amino acid residues, but in AtDGK7, it includes only 52 residues (Asn^{290}–Asn^{341}). Furthermore, AtDGK7, like all other Cluster II and also Cluster III enzymes, lacks the N-terminal diacylglycerol/phorbol ester-binding domains (InterPro accession number IPR002219) as well as the upstream basic region and the extended cysteine-rich domain (extCRD-like domain) present in AtDGK2 (18).

AtDGK7 Transcripts Are Detectable throughout the Arabidopsis Plant, Especially in Flowers and Seedlings—We could not easily detect *AtDGK7* mRNA by standard Northern blot analysis (not shown), indicating weak expression. Therefore, we used real time RT-PCR for the quantitative measurement of *AtDGK7* transcripts. To ensure maximum specificity and efficiency during PCR amplification of *AtDGK7* cDNA under a standard set of reaction conditions, primer design was done as described by Czechowski *et al.* (25). Accordingly, the predicted melting temperatures (T_m) were 60 ± 2 °C, and primer lengths were around 20 nucleotides, containing a guanine-cytosine (GC) content of ~50%. The PCR amplicon length was ~100 base pairs. The resulting pair of primer sequences was compared with the *A. thaliana* genome by performing BLAST searches (available on the World Wide Web at www.ncbi.nlm.nih.gov/blast/) to ensure *AtDGK7* specificity. Real time RT-PCR on cDNA of different *Arabidopsis* tissues resulted in a single band of the expected size (~100 base pairs). As shown in Fig. 2*A*, *AtDGK7* transcripts were detected in all tissues analyzed. Expression levels were elevated in flowers and seedlings. Elevated expression of *AtDGK7* in seedlings was also evident from Affymetrix GeneChip hybridization data that were retrieved using the Gene Chronologer tool of the Genevestigator package (available on the World Wide Web at www.genevestigator.ethz.ch). The Digital Northern tool of this package indicated that *AtDGK7* may at some stages of flower development be highly expressed in petals, consistent with the elevated expression that we observed in whole flowers using RT-PCR. *AtDGK7* transcript level was not strongly affected by a number of applied biotic or abiotic stresses; however, transcript levels appear to be slightly (~2-fold) induced by sucrose, and perhaps by ozone treatment, as indicated by the Response Viewer tool of Genevestigator.

MPSS data indicate that *AtDGK7* is transcribed at a low to moderate level, with transcripts per million (TPM) values ranging from 0 to 116. For a weakly expressed gene, this value lies in the range of 1–10 TPM, whereas a very strongly expressed gene may be represented by a value of more than 1000 TPM (for detailed information, see, on the World Wide Web, mpss.udel.edu/at/java.html).

ESTs closely related to *AtDGK7* were also reported from other plant species, including *Brassica napus* (GenBank™ accession number CD826929), *Solanum tuberosum* (CK256071), and cotton (*Gossypium raimondii*; CO113886), indicating that *AtDGK7*-like genes are widely expressed in the plant kingdom.

AtDGK2 Expression Is Rapidly Induced by Wounding—We have previously described the generation of transgenic Arabidopsis plants harboring the promoter region of the *AtDGK2* gene (At5g63770) fused to the *E. coli* β-glucuronidase (*GUS*) reporter gene (*promAtDGK2*:*GUS* reporter lines) (18). Analysis of the *promAtDGK2*:*GUS* lines revealed that the *AtDGK2* gene is expressed throughout the plant and exhibits cold-inducible gene expression. To search for other environmental factors that could potentially alter *AtDGK2* expression, we screened the Genevestigator *Arabidopsis* microarray data base that provides Affymetrix GeneChip-based transcript profiles from a large number of independent experiments (26). *AtDGK2* transcript level appeared to be slightly induced (~2.3-fold) by nematode attack, as indicated by the Genevestigator Response Viewer tool. Microarray data also indicated that *Arabidopsis* plants exhibit a strong, but transient, increase of *AtDGK2* transcript level in leaves 15 min to 1 h after wounding (viewed with the Digital Northern software package of Genevestigator). At 3 h after wounding, *AtDGK2* transcript abundance had already returned back to background level that was observed before induction (Fig. 3*A*). No changes in gene expression were measured in roots.

In the case of the *promAtDGK2*:*GUS* reporter lines, we reproducibly observed strong GUS staining at sites where sections were cut before the incubation in staining solution. Fig. 3*B* shows an example of wound-induced GUS staining in leaves that had been cut. This result suggested that the activity of the *AtDGK2* promoter driving this *GUS* expression is enhanced upon wounding. Therefore, we quantitatively tested the induction of *GUS* activity in shoots of *promAtDGK2*:*GUS* plants.

To exclude the possibility that the increased GUS activity at the sites of wounding simply results from a better diffusion of substrate into the plant tissue, protein extracts were prepared from control plants and from two representative *promAtDGK2*:*GUS* plants harvested at different periods of time after wounding. The two lines were obtained from two independent transformations that employed different binary vectors (*i.e.* pCAMBIA-1303 and pGPTV-HPT, respectively), both carrying the *AtDGK2*:*GUS* fusion gene (for details, see Ref. 18). Total protein was extracted, and GUS activities were determined using 4-methylumbelliferyl-β-D-glucuronide as substrate, which yields a fluorescent product after hydrolysis by GUS. Quantification of the relative fluorescence data demonstrated elevated GUS activities in wounded transgenic plants (Fig. 3*C*), with an about 50% increase already 30 min after wounding. In both transgenic lines, GUS activity decreased progressively 4 h after wounding and returned to its original level ~24 after wounding. Northern blot experiments confirmed that *AtDGK2* expression is rapidly (within 30–40 min) induced in above ground wounded *Arabidopsis* tissues (Fig. 3*D*).

To further demonstrate transient, wound-triggered *AtDGK2* expression, we performed quantitative real time RT-PCR experiments, using RNA isolated from 4-week-old plants raised on soil. We observed that

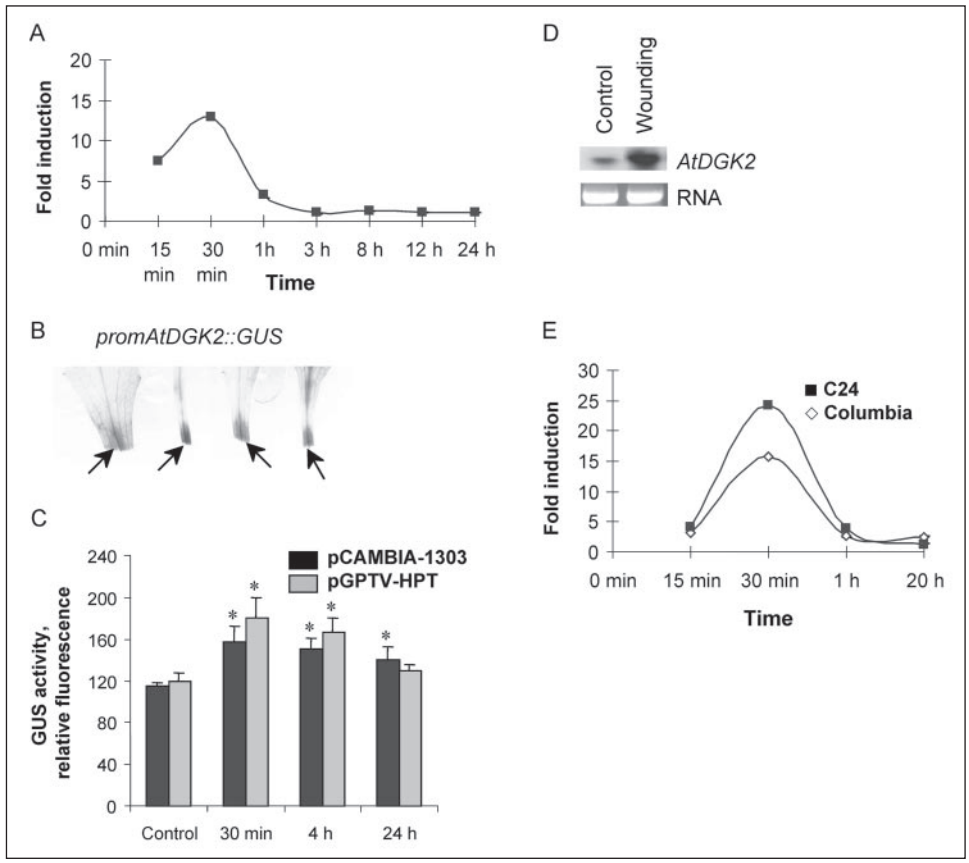

FIGURE 3. **Induction of *AtDGK2* expression upon wounding of *Arabidopsis* tissues.** Wild-type and transgenic *Arabidopsis* plants were used to test the transcriptional regulation of *AtDGK2* by wounding. *A*, Affymetrix expression values for *AtDGK2* (probe set 247346_at), retrieved from the Genevestigator *Arabidopsis* Microarray Database (using the Digital Northern software package) (26), indicate transient, wound-induced expression (shown as -fold induction in comparison with the corresponding controls). Data represent averages of two hybridization experiments. *B*, elevated GUS activity at the cut petiole (*gray shading image*, *arrows*). *C*, quantification of GUS activity. Methylumbelliferone fluorescence was determined in extracts of unwounded (control) and wounded *promAtDGK2*:*GUS* reporter lines obtained from two different transformation series. *Black bars*, data obtained from a *promAtDGK2*:*GUS* line that was transformed with a pCAMBIA-1303 construct; *gray bars*, data obtained from a reporter line that was transformed with a pGPTV-HPT construct (18). Plants were wounded, and samples were taken at different time points after the treatment. Data are means of four replicates ± S.D. The *asterisks* indicate significant differences ($p < 0.05$). GUS assays were performed as described (47). *D*, Northern blot analysis of *AtDGK2* transcript level in wild-type *Arabidopsis* above ground tissues. Wounding (see "Experimental Procedures") was applied for 40 min to 4-week-old plants. *Upper panel*, autoradiogram; *lower panel*, ethidium bromide-stained gel to demonstrate equal loading of RNA (25 μg/lane). The blot was hybridized with radiolabeled *AtDGK2* cDNA. *E*, real time RT-PCR. Leaves of the two *Arabidopsis* ecotypes C24 and Columbia-0 were wounded, and *AtDGK2* transcript levels were determined by quantitative RT-PCR. In both ecotypes, a slight increase in *AtDGK2* expression was observed already 10 min after wounding. Transcript level reached its maximum after 30 min and returned to the prewounding level after ~2–3 h. Expression values, normalized to actin 2, are expressed as a -fold change in comparison with the 0-h time point. Each data point represents the average of two technical repetitions.

the *AtDGK2* transcript level rose more than 16-fold in two different *Arabidopsis* accessions tested (*i.e.* C24 and Col-0) within 30 min after wounding and at later stages went down to the pretreatment level (Fig. 3*E*). Time course experiments indicated that *AtDGK2* expression started to increase as early as 10 min after wounding.

AtDGK7 Encodes a Functional Lipid Kinase—We have previously characterized the enzymatic activity of AtDGK2, representing one of the two Cluster I DGK enzymes from *A. thaliana* (18). So far, no enzyme activity has been demonstrated for any member of the Cluster II. We cloned the *AtDGK7* cDNA and tested recombinant NusA/polyhistidine-tagged AtDGK7, expressed in *E. coli* (see "Experimental Procedures" for details) for its potential to phosphorylate DAG. After isopropyl-β-D-thiogalactopyranoside induction, protein was extracted for both immunoassays and enzymatic analyses. Recombinant fusion protein of an apparent molecular mass of ~100 kDa was detected by an anti-His tag antibody in extracts of the transformed cells (Fig. 4), which compares favorably with the predicted size of the fusion protein (AtDGK7, 41 kDa; NusA tag/His_6 tag, ~60 kDa). Extracts from cells transformed with the pET43c control vector (lacking the *AtDGK7* cDNA) reacted with the antibody, revealing a protein of ~60 kDa that

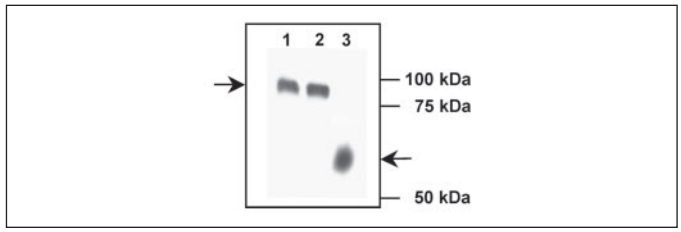

FIGURE 4. **Western blot analysis of recombinant AtDGK7.** *E. coli* BL21 (DE3) cells transformed with *AtDGK7* cDNA (plasmid pET7) express a ~100-kDa fusion protein (*left arrow*; *lanes 1* and *2*). *E. coli* cells transformed with the empty vector pET43c (lacking the *AtDGK7* cDNA) produce the ~60-kDa NusA-His_6 fusion protein (*lane 3*; *right arrow*). Cells were harvested 4 h after isopropyl-β-D-thiogalactopyranoside induction, and protein extracts were purified using Ni^{2+}-nitroacetic acid-agarose under native conditions. For protein detection, an anti-His tag antibody was used.

corresponds to the NusA tag/His_6 tag fusion expressed from the empty vector itself.

Proteins purified from cells transformed with the *AtDGK7* cDNA or the empty vector (pET43c) were assayed for DGK enzymatic activity. Cells transformed with *AtDGK7* showed strong DAG kinase activity against 1,2-DOG and 1,2-SAG, both of which contain unsaturated fatty

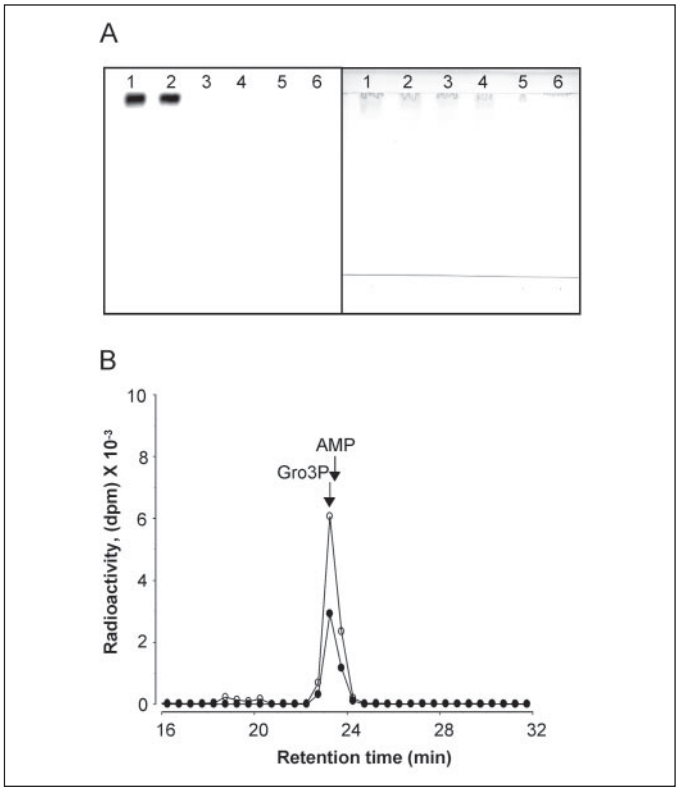

FIGURE 5. **Enzyme activity of AtDGK7.** Purified recombinant AtDGK7 catalyzes the *in vitro* phosphorylation of DAG to yield PA, using [γ-^{32}P]ATP as phosphate donor, according to the protocol described elsewhere (18). *A*, products of the enzymatic reaction were solubilized in choroform/methanol (2:1) and subsequently separated by thin layer chromatography and detected by autoradiography (*left panel*) and iodine vapor (*right panel*). The generated radiolabeled PA detected by autoradiography was identified by comparison with standard PA. Retention factor value for PA standard, radiolabeled PA, and cardiolipin is 0.95. Both the *left* and *right panels* contain the following. *Lane 1* and *2*, labeled PA produced by the activity of purified AtDGK7 enzyme, obtained from independent bacterial cultures transformed with the *AtDGK7* cDNA (plasmid pET7), using 1,2-DOG (*lane 1*) or 1,2-SAG (*lane 2*) as substrate; *lane 3*, purified NusA-His$_6$ fusion protein expressed from the cloning vector pET43c alone; *lane 4*, no enzyme added to the reaction mix; *lane 5*, PA standard; *lane 6*, cardiolipin. *B*, diacylglycerol substrate was incubated with affinity-purified AtDGK7 enzyme. The lipid products of assay were deacylated and mixed with L-[U-^{14}C]glycerol 3-phosphate, and the water-soluble products of deacylation were resolved on a Partisphere SAX HPLC column; *open symbols*, ^{14}C; *closed symbols*, ^{32}P. The position of elution of the internal standard is indicated with an *arrow*, as is the position of elution of an internal standard of AMP, monitored on-line at 258 nm.

acid moieties, whereas the transformants harboring the empty vector exhibited no activity after autoradiography of TLC plates (Fig. 5*A*), demonstrating that *AtDGK7* encodes a functional lipid kinase. This result was confirmed by HPLC analysis (Fig. 5*B*). Deacylation and subsequent HPLC of the ^{32}P-labeled products obtained by incubation of enzyme with 1,2-DOG yielded a ^{32}P-labeled peak that co-eluted with an internal standard of authentic [^{14}C]glycerol 3-phosphate.

Using standard experimental conditions (described in Ref. 18), incorporation of radioactive label was linear with time for at least 30 min in the activity assay. Therefore, in order to measure DGK activity, PA formation was determined in this linear kinetic range. Enzyme assays of purified recombinant AtDGK7 showed that the reaction was linear for up to ~2 μg of protein in the assay, using 1,2-DOG as substrate (Fig. 6*A*). Therefore, 1 μg of protein was used in all further experiments.

The AtDGK7 enzyme has an activity optimum at pH 6.8 and exhibits a fairly rapid decrease of activity below pH 6.8 and above pH 7.8 (Fig. 6*B*). NaCl (5–50 mM) increased the activity of the enzyme by up to 20% as compared with the standard assay. In the presence of LiCl, the highest enzyme activity was observed at a concentration of 50 mM. When the concentration of both salts was raised to 500 mM, the activity of the protein began to decrease (Fig. 6*C*). AtDGK7 activity was tested in the presence of different types of detergents. Sodium deoxycholate (Na-DC) at 1–5 mM concentration proved to be an efficient detergent for solubilization of lipids. Enzyme activity strongly decreased at higher Na-DC concentrations (10–100 mM). In contrast, Triton X-100 (0.07–0.52%) only marginally affected AtDGK7 activity in the presence of 1 mM Na-DC (Fig. 6*D*). Low concentrations of CHAPS (1–5 mM) replacing Na-DC were inefficient to activate the enzyme, whereas higher concentrations of this detergent (10–50 mM) strongly improved AtDGK7 activity. An inhibitory effect was observed at 100 mM CHAPS. At 1 mM CHAPS, 0.12–2.02% Triton X-100 strongly increased (around 8-fold) AtDGK7 activity. At the highest Triton X-100 concentration tested (2.02%), enzyme activity exceeded by almost 40% the activity determined under control conditions (Fig. 6*E*).

Because both the substrate and product of the DKG reaction are amphiphilic and not freely diffusible in aqueous solution, simple Michaelis-Menten kinetics might not be suitable to describe the enzymatic characteristics of this enzyme. The surface dilution kinetic model was developed for enzymatic reactions occurring at surfaces of mixed micelles in aqueous environment (27). This model is based on an enzyme converting amphiphilic substrates into reaction products in detergent-containing mixed micelles. Because the reaction partners localize to the surface of the micelles, they can only diffuse in two dimensions, and different kinetics apply in contrast to normal Michaelis-Menten kinetics, which are based on three-dimensional diffusion of enzymes, substrates, and products.

The surface dilution model predicts that enzyme activity should depend on the molar and micellar surface concentrations of the substrate when the molar substrate concentration relative to the other micellar components is low but be predominantly dependent on the surface concentration and be independent of the molar substrate concentration when the molar substrate concentration relative to detergent is high (27, 28). To test whether the surface dilution model is suitable to describe AtDGK kinetics, we measured DGK activity as a function of the total molar concentration of the three amphiphiles: CHAPS, Triton X-100, and diacylglycerol. CHAPS and Triton X-100 were considered as a single complex detergent. Fig. 7 demonstrates that within a range of total ([CHAPS] + [Triton X-100] + [diacylglycerol]) concentration between 5 and 8 mM, DGK activity was largely independent of the diacylglycerol molar concentration but dependent on the diacylglycerol surface concentration (given in mol fraction of diacylglycerol relative to total amphiphiles).

This result demonstrates that under our experimental conditions, DGK activity of both enzymes follows the surface dilution kinetic model. Fig. 7 shows that the specific activities for AtDGK2 and AtDGK7 were in the range of 0.5–3 pmol of PA min^{-1} μg^{-1} protein. Biochemical studies characterizing wheat (14) and tomato (15) DGK isoforms have reported specific activity values of up to 20 pmol of PA min^{-1} μg^{-1} protein, which are significantly higher than that observed here for the *Arabidopsis* enzymes.

Kinetic data for AtDGK2 and AtDGK7 were obtained from Michaelis-Menten kinetics using the surface dilution model. Calculated K_m and V_{max} values were obtained for 1,2-DOG using an ATP concentration of 1 mM and different total amphiphile concentrations of 1.44, 7.2, or 14 mM (Fig. 8, *A* and *B*). The K_m values for AtDGK2 and AtDGK7 at the three different total amphiphile concentrations were very similar, around 0.1 mol fractions of 1,2-DOG. The V_{max} values were in the same range for AtDKG2 and AtDGK7 but were dependent on the total amphiphile concentration (Fig. 8). This means that with increasing amounts of the total ([CHAPS] + [Triton X-100] + [diacylglycerol])

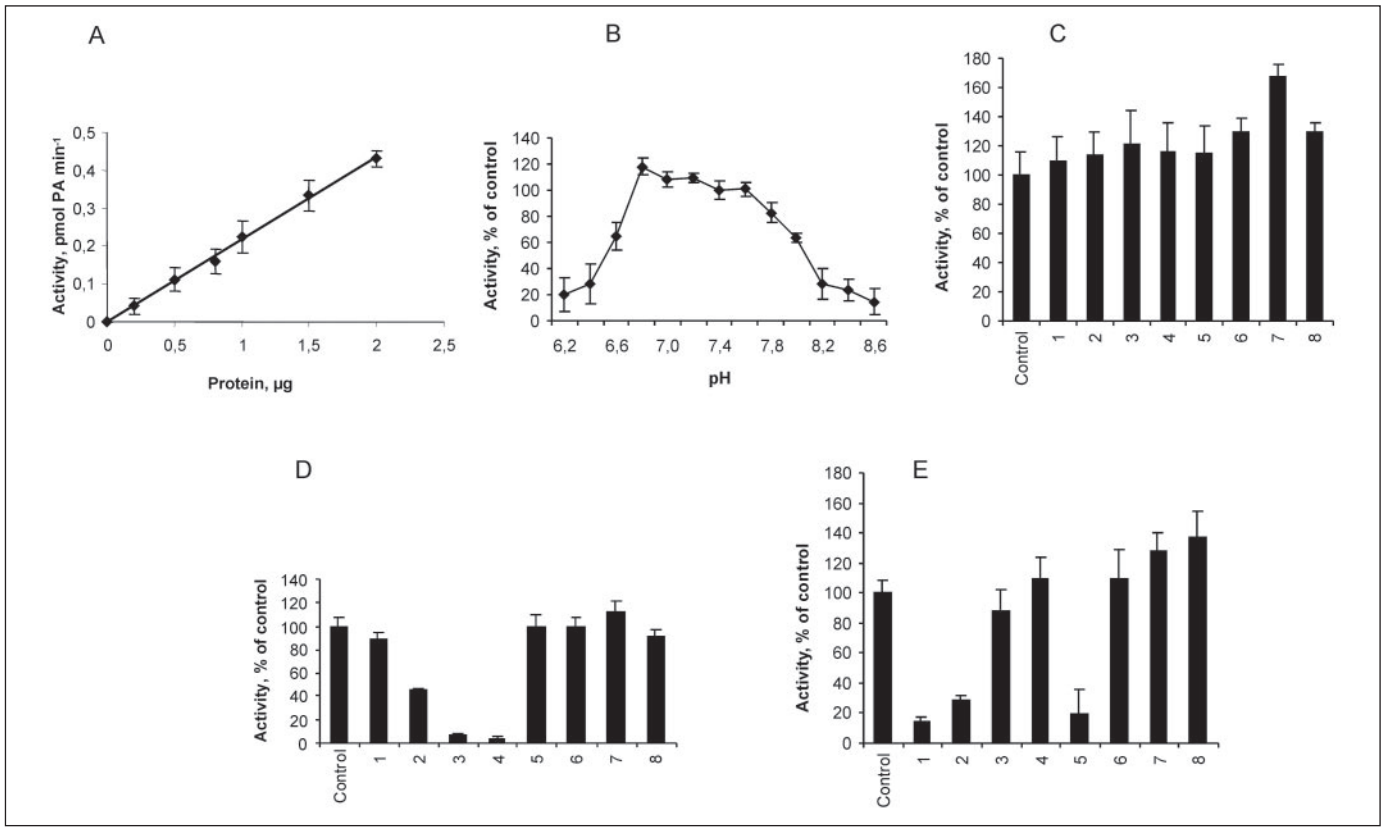

FIGURE 6. **Effect of different amounts of protein, pH values, salts, and detergents on AtDGK7 activity.** The standard assay is described under "Experimental Procedures" (18). *A*, diacylglycerol kinase activity assayed with different amounts of purified protein extracted from *E. coli* cells expressing AtDGK7 (*error bars* indicate mean ± S.D., three independent experiments). *B*, effect of pH on AtDGK7 activity. The enzymatic assay was performed in 40 mM Bis-Tris-HCl (pH 6.2, 6.4, and 6,6), 40 mM Bis-Tris-propane-HCl (pH 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0), and 40 mM Tricine-KOH (pH 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, and 8.6). The activity observed under standard assay conditions in Bis-Tris-propane, pH 7.4, was set to 100%. *C*, effect of extra salts on AtDGK7 activity. Enzyme activity determined under standard assay conditions containing no extra salt was set to 100% (*i.e. Control*). *1*, 5 mM NaCl; *2*, 10 mM NaCl; *3*, 50 mM NaCl; *4*, 500 mM NaCl; *5*, 5 mM LiCl; *6*, 10 mM LiCl; *7*, 50 mM LiCl; *8*, 500 mM LiCl. *D*, effect of Na-DC and Triton X-100 on AtDGK7 activity. Activity determined in the standard assay (*Control*) was set to 100%. The enzyme activities are presented as percentages of control activity. *Control*, 1 mM Na-DC, 0.02% Triton X-100; *1*, 5 mM Na-DC; *2*, 10 mM Na-DC; *3*, 50 mM Na-DC; *4*, 100 mM Na-DC; *5*, 1 mM Na-DC, 0.07% Triton X-100; *6*, 1 mM Na-DC, 0.12% Triton X-100; *7*, 1 mM Na-DC, 0.52% Triton X-100; *8*, 1 mM Na-DC, 2.02% Triton X-100. *E*, effect of CHAPS (in the absence of Na-DC) and Triton X-100 on AtDGK7 activity. *Control*, same as in *C*; *1*, 1 mM CHAPS; *2*, 5 mM CHAPS; *3*, 10 mM CHAPS; *4*, 50 mM CHAPS; *5*, 100 mM CHAPS; *6*, 1 mM CHAPS, 0.12% Triton X-100; *7*, 1 mM CHAPS, 0.52% Triton X-100; *8*, 1 mM CHAPS, 2.02% Triton X-100. Data are the means of three independent measurements. *Bars*, ±S.D.

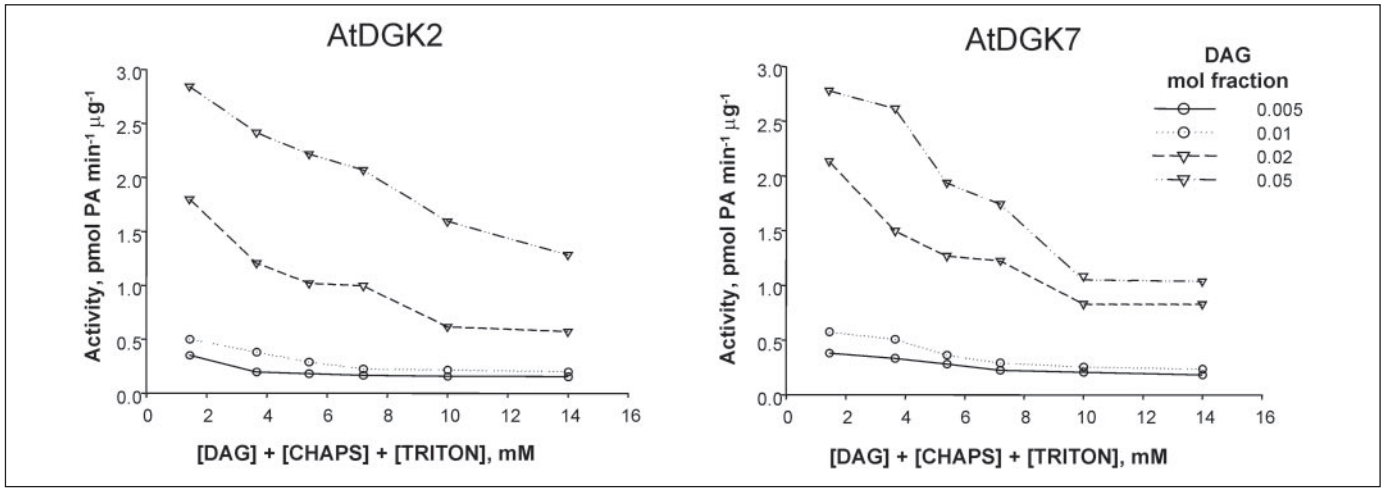

FIGURE 7. **Activity of two *Arabidopsis* DGK isoforms in mixed micelles, according to the surface dilution kinetic model.** Enzymatic activity of AtDGK2 and AtDGK7 was determined as described under "Experimental Procedures" and expressed as pmol of PA formed/min/µg of protein. DGK activity was measured as a function of the sum of the molar concentrations of CHAPS, Triton X-100, and DAG at given mol fractions of DAG to total amphiphiles (in mol/mol as indicated).

mixture, the V_{max} for AtDGK2 and AtDGK7 decreased. This can be explained by the surface dilution model, because by adding more and more detergent (and substrate), new and larger micelles are formed, whereas the amount of the enzyme remains the same. Enzyme is diluted out, because the reaction volume (which in this case is only two-dimensional) is enlarged. The inhibition of AtDGK2 activity by high concentrations of CHAPS was already found by Goméz-Merino *et al.* (18). Furthermore, V_{max} measured for AtDGK2 in the presence of CHAPS

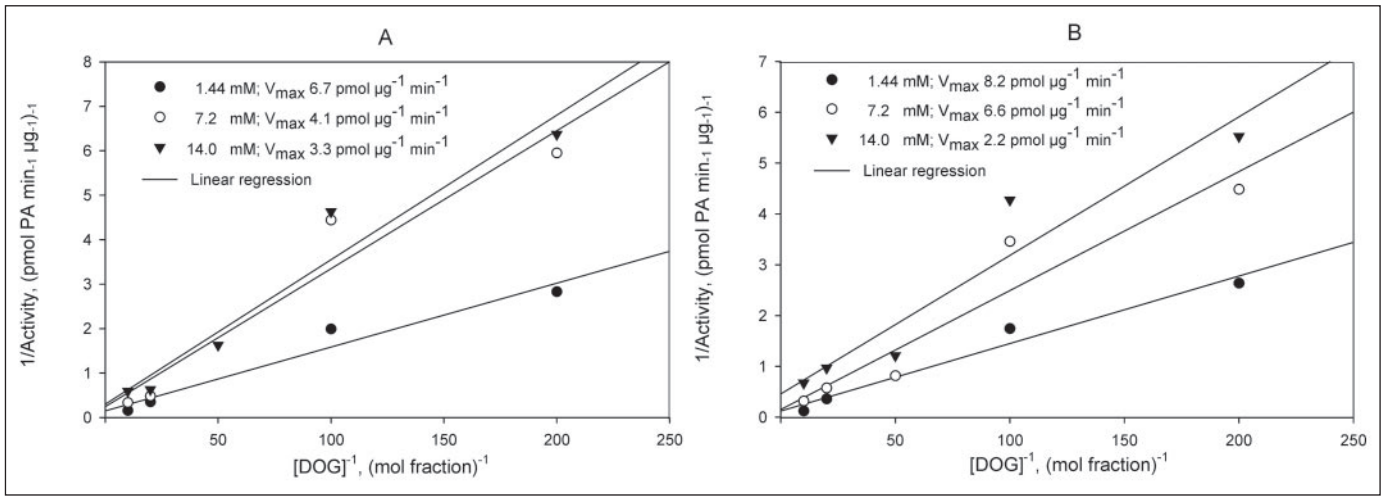

FIGURE 8. **Kinetics of recombinant AtDGD2 and AtDGK7 as a function of diacylglycerol concentration.** Lipid kinase activity of recombinant AtDGK2 and AtDGK7 was determined with different mol fractions of 1,2-DOG at three different concentrations of total amphiphiles (1.44, 7.2, and 14 mM) following the surface dilution model. Double reciprocal plots (Lineweaver-Burk) are shown to estimate K_m and V_{max}. *A*, AtDGK2. *B*, AtDGK7.

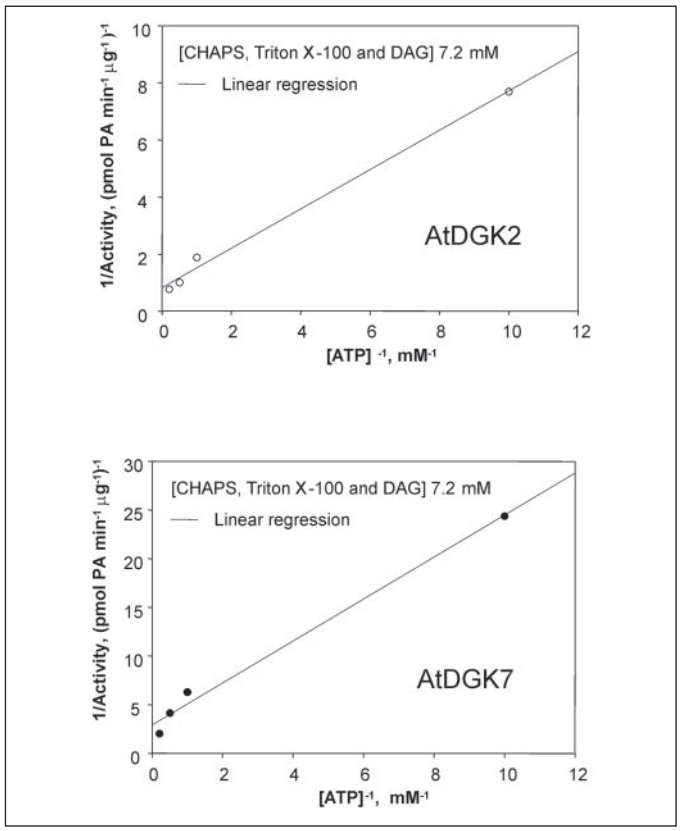

FIGURE 9. **Enzymatic activity of AtDGK2 and AtDGK7 in mixed micelles, as a function of ATP concentration.** DGK activity was determined with 1,2-DOG as substrate in the presence of increasing ATP concentrations (0.1–5.0 mM) and a fixed amount of CHAPS, Triton X-100, and DAG concentration (7.2 mM). For both enzymes that exhibited a Michaelis-Menten type saturation curve for the substrate ATP, Lineweaver-Burk plots are presented. The calculated K_m and V_{max} values were 1.20 mM for ATP and 1.20 pmol of PA min^{-1} μg of $protein^{-1}$ for AtDGK2 and 0.74 mM for ATP and 0.34 pmol of PA min^{-1} μg of $protein^{-1}$ for AtDGK7, respectively.

was much higher than previously reported in experiments done in the absence of CHAPS (0.76 pmol of PA min^{-1} μg^{-1}; see Ref. 18), indicating that low amounts of CHAPS stimulate AtDGK2 activity.

The effect of increasing concentrations of ATP on the enzymatic activities of AtDGK2 and AtDGK7 was determined using a sum of the molar concentrations of CHAPS, Triton X-100, and DAG of 7.2 mM at 0.02 mol fractions of 1,2-DOG (Fig. 9). AtDGK7 activity was compared with that of AtDGK2 under these experimental conditions. We observed that AtDGK2 was about 3.5-fold more active than AtDGK7, as its V_{max} reached 1.20 pmol of PA min^{-1} μg^{-1} protein, as compared with 0.34 pmol PA min^{-1} μg^{-1} protein for AtDGK7. The V_{max} values obtained in this experiment were lower than in the assays done with different amounts of 1,2-DOG (see above), indicating that the amount of 1,2-DOG used in this experiment (0.02 mol fractions) was not saturating. For AtDGK2 the K_m for ATP was 1.20 mM. The K_m of 0.74 mM for ATP in the case of AtDGK7 is somewhat lower and might indicate the high affinity of this enzyme for this substrate.

R59022 Differentially Affects Enzymatic Activity of AtDGK2 and AtDGK7 and Alters Root and Plant Growth—The DGK inhibitor R59022 modulates a number of cellular processes in animals that are regulated by DAG/PA signaling (e.g. see Refs. 29–31). In plants, R59022 has been demonstrated to reduce the formation of cold-induced PA in *A. thaliana* suspension cells at a concentration of around 50–100 μM of the inhibitor (32). It also enhanced phytoalexin accumulation in elicitor-treated (but not untreated) epicotyl tissues of pea, most likely involving an activation of phenylalanine ammonia lyase gene expression (33). Membrane-bound DGK activity in pea was inhibited ~50% at an inhibitor concentration of around 100 μM (33). We have previously reported that R59022 also reduces the activity of recombinant AtDGK2 at relatively low concentrations (IC_{50} = 50 μM) (18). To investigate whether AtDGK7 is inhibited in a similar manner, the reaction mix was preincubated with this inhibitor. Since the standard conditions for these experiments were different from those previously reported (dimethylformamide used as a solvent for R59022, instead of dimethyl sulfoxide) (18) and three different molar fractions were tested herein, we assayed both AtDGK2 and AtDGK7. Our data indicate that the decrease in PA formation and, hence, the degree of inhibition of DGK activity were indeed dependent on the concentration of the inhibitor (Fig. 10). R59022 had a stronger inhibitory effect on AtDGK2 (IC_{50} = 250 μM) in comparison with AtDGK7 (IC_{50} = 500 μM). Notably, at 50 μM R59022, no inhibition was detected for AtDGK7, whereas AtDGK2 was inhibited by 20%. At the highest R59022 concentration tested (1000 μM), AtDGK2 was still 20% active, whereas AtDGK7 was ~30% active. Additionally, we tested the effect of a second DGK inhibitor, R59949 (up to 1 mM in the reaction mix), but no inhibitory effect was observed, neither for AtDGK2 nor for AtDGK7 (data not shown). In animals, R59949

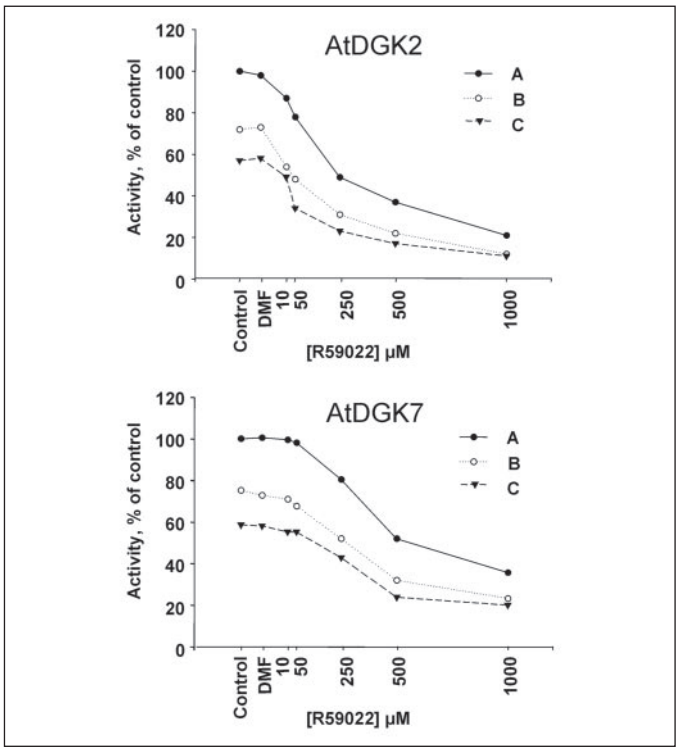

FIGURE 10. **Effect of the DGK inhibitor R59022 on AtDGK2 and AtDGK7 activities using different lipid mol fractions**. The DGK inhibitor R59022, dissolved in dimethyl formamide (*DMF*), was added prior to the start of the reaction containing AtDGK2 (*upper panel*) or AtDGK7 (*lower panel*) protein. Enzymatic activity was determined as described under "Experimental Procedures." Values are expressed as percentages of enzyme activity determined under control assay conditions (*i.e.* in the absence of R59022) showing the highest activity. Measurements were done as a function of the sum of molar concentrations of CHAPS, Triton X-100, and DAG at a given mol fraction. A, 1.44 mM (1.00 mM CHAPS, 0.43 mM Triton, 0.01 mM DAG); *B*, 3.60 mM (2.50 mM CHAPS, 1.08 mM Triton, 0.02 mM DAG); *C*, 7.20 mM (4.90 mM CHAPS, 2.20 mM Triton, 0.10 mM DAG). Data are means of three independent determinations ± S.D.

inhibits Ca^{2+}-activated DGKs, but Ca^{2+}-insensitive isoforms are only weakly affected (34). We have previously reported that AtDGK2 activity is not modified by Ca^{2+} in the reaction assay (18).

Evidence indicates that DGKs may regulate diverse cellular processes such as growth and differentiation in animals (10). In plants, studies involving the DGK inhibitor R59022 demonstrated an involvement of DGK in stress-related physiological processes (cold, elicitor treatment) (32, 33). We have shown that *AtDGK2* is prominently expressed under non-stress conditions in root tips of seedlings a few days after germination (18), indicating that it plays a developmental role in this tissue. Only weak expression in roots was observed for *AtDGK7* (this report; Fig. 2*A*). We grew plants in the presence of the DGK inhibitor R59022 to assess whether it affects root development. To this end, *Arabidopsis* seedlings were grown on agar plates (kept vertically) in the absence and presence of the inhibitor. Primary root elongation rate was analyzed and found to be strongly reduced at an inhibitor concentration above 50 μM (Fig. 11, *A* and *B*). At a concentration of 75 μM R59022, root elongation was inhibited by ~50%. R59022 also inhibited lateral root growth (not shown). Treatment with the inhibitor also affected overall plant development; biomass production as well as petiole lengths and sizes of leaf blades were reduced (not shown). Notably, leaves became chlorotic at inhibitor concentrations above 50 μM (Fig. 11*C*). At the highest R59022 concentration tested (*i.e.* 250 μM), seeds were able to germinate, but seedlings died 3–5 days after germination (data not shown), demonstrating that this inhibitor does not affect the process of germination but rather root elongation and plant growth.

AtDGK2 and AtDGK7 Phosphorylate 1,2-DAG Molecular Species Physiologically Important in Plants—Higher plants contain a variety of diacylglycerol molecular species that differ by the length and degree of unsaturation of the acyl groups linked in the *sn*-1- and *sn*-2-position of the glycerol backbone. Two major sites contribute to diacylglycerol formation in many plants (*e.g. Arabidopsis*) (*i.e.* the chloroplast and the ER). Due to differences in the specificity of the acyltransferases involved, two major types of diacylglycerol can be distinguished: a prokaryotic type derived from the plastid (C18 at *sn*-1, mostly C16 at *sn*-2), or a eukaryotic type (C16 or C18 at *sn*-1, C18 at *sn*-2) (35). We previously have proved that AtDGK2 has high specificity for 1,2-SAG and 1,2-DOG (18). To further investigate the ability of AtDGK2 and AtDGK7 to phosphorylate 1,2-*sn*-diacylglycerides found in plants, we tested 1,2-POG/1-palmitoyl, 2-oleoyl-*sn*-glycerol (1–16:0–2-18:1); 1,2-SLG/1-stearoyl, 2-linoleoyl-*sn*-glycerol (1–18:0–2-18:2); and 1,2-OPG/1,2–1-oleoyl, 2-palmitoyl-*sn*-glycerol (1–18:1–2-16:0) (Fig. 12). Interestingly, AtDGK2 activity increases by 46% with 1,2-SLG as substrate in comparison with the control (1,2-DOG). With 1,2-POG and 1,2-OPG used as substrates, AtDGK2 activity is more than 30% higher than the control. Regarding AtDGK7, our data indicate that this enzyme exhibits elevated activity in the presence of 1,2-POG and 1,2-OPG (nearly 20% higher than with 1,2-DOG). With 1,2-SLG, AtDGK7 activity is similar to that observed with the control substrate.

DISCUSSION

The experiments reported in this paper were initiated as an attempt to demonstrate that a member of Cluster II of plant DGKs is a functional enzyme, since no protein of such a cluster has been characterized so far from any species. AtDGK7 and previously characterized AtDGK2 share a well conserved catalytic domain, although the primary structure of the former is far simpler than the latter. The complex domain organization present at the N-terminal region of AtDGK2, which contains two copies of a DAG/PE-binding domain flanked by an upstream basic region preceding the DAG/PE-binding domain 1, and an extCRD-like sequence following the DAG/PE-binding domain 2, is absent in AtDGK7 (Fig. 1*B*). Furthermore, the DGK accessory domain is incomplete in AtDGK7.

In contrast to *AtDGK2*, *AtDGK7* transcripts were not detected under conventional Northern blot conditions. Quantitative RT-PCR revealed that *AtDGK7* is expressed throughout the plant, most prominently in flowers and seedlings. Affymetrix GeneChip hybridization studies so far have not revealed any profound affect of biotic or abiotic stresses on *AtDGK7* transcript levels, suggesting that *AtDGK7* may be more involved in regulating developmental or growth processes than in stress responses. In contrast, we have previously reported that expression of *AtDGK2* is induced by cold treatment, indicating an involvement in cold-related signal transduction (18). Here, we observed that wounding also triggers *AtDGK2* expression. Wounding is known to rapidly trigger the accumulation of PA in plants (36). Molecular-physiological evidence indicates that this rise in PA level to a large extent results from an involvement of phospholipase D activity. However, other biochemical pathways contributing to PA production under stress, such as cold (18, 32) and wounding (36), are likely. Our finding that *AtDGK2* expression rapidly reacts to a wound stress is consistent with this interpretation and conforms to the model that genes that code for signal pathway components are themselves often triggered by the stimulus that is transmitted by the signaling pathway (37).

The *AtDGK7* gene encodes a functional diacylglycerol kinase with unique biochemical properties. AtDGK7 saturates at lower substrate concentrations, and its activity appears to be approximately 3–4 times

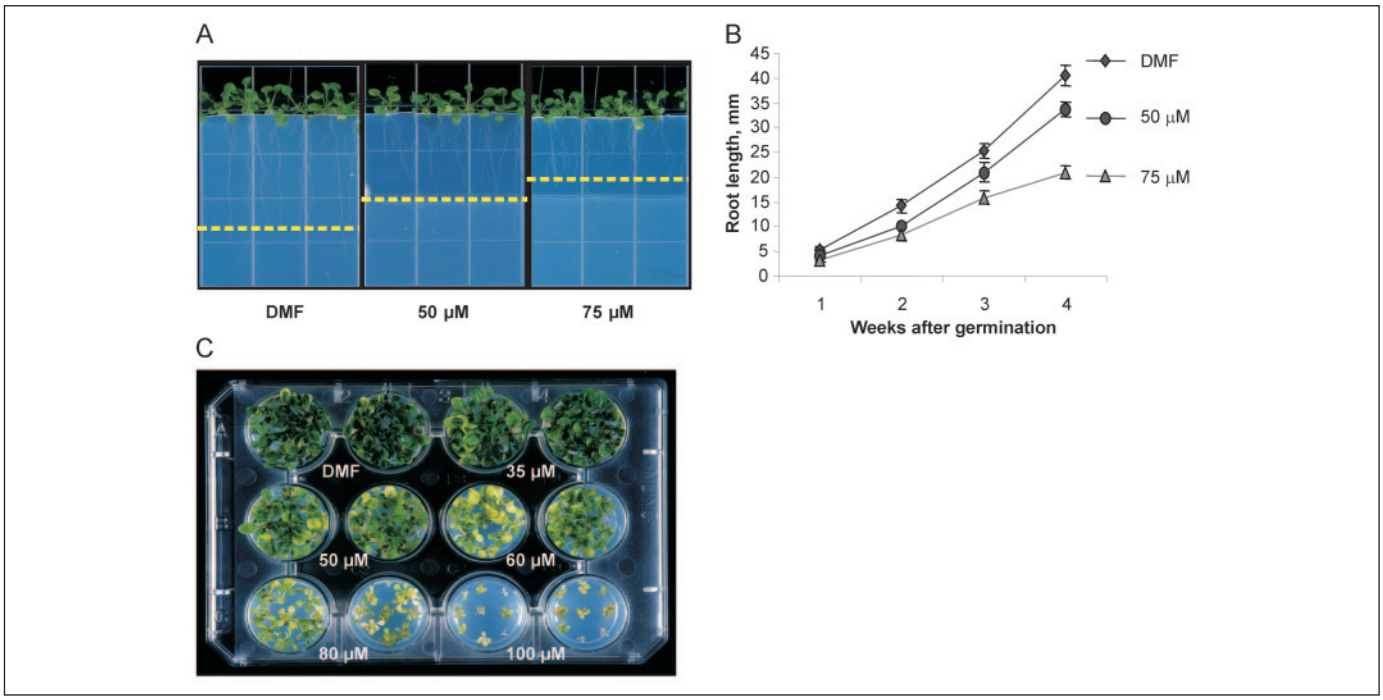

FIGURE 11. **Effect of the DGK inhibitor R59022 on root elongation and plant growth.** Surface-sterilized *Arabidopsis* seeds were sown on MS medium (48) containing 0.8% agar and different concentrations of the DGK inhibitor R59022. The inhibitor R59022, dissolved in dimethyl formamide (*DMF*), was added. *A*, root length of *Arabidopsis* seedlings is affected by increasing amounts of R59022. The *yellow line* marks the average root length of 25-day-old seedlings. *B*, length of the primary root (expressed in mm) was measured weekly with the help of a scaled magnifying glass for a period of 4 weeks. Values are means of 12 individual seedlings ± S.D. *C*, effect of R59022 on plant growth and development. Seedlings are 3 weeks old. Note that seedlings develop chlorosis when the concentration of the inhibitor in the medium is above 50 μM.

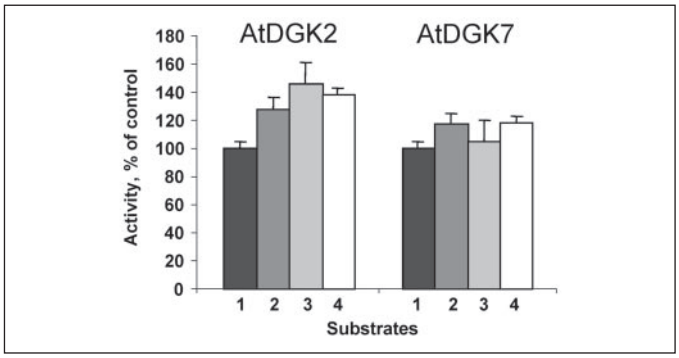

FIGURE 12. **Substrate specificity of the enzymes AtDGK2 and AtDGK7.** AtDGK2 (*left*) and AtDGK7 (*right*) were assayed with various DAG molecular species containing fatty acid moieties found in plants. For each substrate, the concentration used in the reaction mix was 500 μM. For both enzymes, 1,2-DOG control substrate (*1*); 1,2-POG (*2*), 1,2-SLG (*3*), and 1,2-OPG (*4*) are shown. Values are expressed as a percentage of enzyme activity determined under control assay conditions using 500 μM 1,2-DOG. Values are means of three independent determinations ± S.D.

lower than that of AtDGK2, possibly due to its partial DGK accessory domain. AtDGK7 is the smallest of the plant DGKs known (containing 374 amino acids) and apparently is also smaller than all other known eukaryotic DGKs described so far. Because of this, we can assume that all other DGKs encoded by the *Arabidopsis* genome are *bona fide* diacylglycerol kinases. Although an *Arabidopsis* Cluster III enzyme has not been analyzed biochemically so far, two isoforms of a Cluster III enzyme from tomato have been shown to be catalytically active (15).

AtDGK2 and AtDGK7 are differentially affected by pH, detergents, and the inhibitor R59022. The optimal pH found at 6.8 for recombinant AtDGK7 is 0.4 pH units lower than that of AtDGK2 (*i.e.* 7.2). Inhibitory effects of salts (800 mM NaCl) have been observed in a DGK isoform from *Rattus norvegicus* (38). Interestingly, at concentrations below 500 mM, LiCl and NaCl positively affected AtDGK7 activity. Instead, AtDGK2 activity is reduced when those salts are used at concentrations above 200 mM in the reaction mix. AtDGK7 activity is dependent on the presence of detergents. Increasing concentrations of the anionic detergent Na-DC decrease PA formation, whereas the nonionic detergent Triton X-100 does not. An opposite effect is observed with CHAPS, since enzymatic activity of AtDGK7 in buffers containing such zwitterionic detergent at low concentrations (*i.e.* 5 and 10 mM) is reduced.

Diacylglycerol found in plant membranes can be derived from PA phosphatase action on PA originating from *de novo* synthesis in the ER or the plastid. However, alternative pathways exist for diacylglycerol production from membrane lipids via phospholipase C or phospholipase D/PA phosphatase reaction. Therefore, the molecular species distribution of diacylglycerol is complex and depends on the subcellular membrane analyzed. Not much is known about the molecular species specificity of enzymes involved in diacylglycerol metabolism, and its analysis is hampered by the fact that only a few of the diacylglycerol molecular species presumed to be important in plant lipid metabolism are commercially available. We therefore focused our analysis on the specificity for diacylglycerols having a eukaryotic (1,2-DOG, 1,2-POG) or prokaryotic (1,2-OPG) structure or diacylglycerols with different degrees of unsaturation (1,2-SLG). Both enzymes, AtDGK2 and AtDGK7, were about 20–30% more active with 1,2-POG and 1,2-OPG as compared with 1,2-DOG. Therefore, diacylglycerol molecular species containing at least one saturated fatty acid seem to be the preferred substrates for both enzymes. In accordance with this finding, AtDGK2 showed higher activity with 1,2-SAG (18), and 1,2-SLG (this report), two substrates carrying stearate at the *sn*-1-position. However, no increase in AtDKG7 enzyme activity was observed when comparing 1,2-DOG (two monounsaturated acyl groups) and 1,2-SLG (stearate in *sn*-1, diunsaturated fatty acid in *sn*-2). AtDGK2 and AtDGK7 display different specificities for diacylglycerol substrates of different subcellular origin (eukaryotic: 1,2-POG, 1,2-DOG; prokaryotic: 1,2-OPG).

However, the significance of this finding is unclear, because the subcellular location for none of the respective enzymes, AtDGK2 or AtDGK7, is currently known.

The DGK inhibitor R59022 differentially affects the enzymatic activity of AtDGK2 and AtDGK7. In AtDGK7 enzymatic assays, half-maximal inhibition (IC_{50}) is reached at an R59022 concentration almost 2-fold higher than that observed for AtDGK2, which possibly could be explained in light of the complex domain organization of AtDGK2 that may result in a higher affinity of the inhibitor for the enzyme. Recently, R59022 has been shown to minimize cold-induced PA formation in *A. thaliana* suspension cells (32) and to stimulate phytoalexin accumulation in elicitor-treated pea epicotyl tissues (33), indicating an involvement of DGK in both stress-related physiological responses. In the present report, we further demonstrate that R59022 also affects growth and development. Root elongation was drastically reduced in the presence of 50–100 μM R59022, a concentration range that was found to partially inhibit recombinant AtDGK2 *in vitro*. We therefore suggest that DGKs play an important role not only in stress responses but also in developmental processes in plants, consistent with the observation that *AtDGK2* transcriptional activity undergoes a developmental shift during root growth (18). Although we have demonstrated here that R59022 inhibits plant DGK activity *in vitro*, the possibility cannot be excluded at present that other cellular proteins interact with the inhibitor, thereby triggering the developmental and physiological effects that we and others have observed.

In animals, DGKs play a pivotal role in many biological processes, such as cell proliferation, differentiation, survival, and apoptosis (10). Nuclear DGKs are associated with other regulatory enzymes of the phosphoinositide cycle and have an effect on cell cycle progression (39). For instance, some animal DGKs are found associated with and/or regulated by small GTPases of the Rho family, which participate in the organization of the cytoskeleton, stress-induced signal transduction, cell death, cell growth, and differentiation (40). Plants possess a large family of genes encoding Rop (Rho-related GTPase from plant) (41), implicated in pollen tube elongation (42), root hair initiation and root tip growth (43). As most of the eukaryotic DGKs contain cysteine-rich domains, which are thought to mediate protein-protein interactions as well as to bind acidic phospholipids in membranes (44–46), one might presume that R59022 annuls such interactions and that members of the Rop family may be potential candidate proteins that interact with *Arabidopsis* DGKs.

Both AtDGK2 and AtDGK7 phosphorylate 1,2-*sn*-DAG molecular species found in plants to generate the respective PA molecules. Therefore, both proteins might be implicated in regulating DAG and PA cellular levels in order to allow the plant to perform its proper function. In some cases, their actions may result in lowering the levels of DAG, thereby serving as an off signal for the activation of a number of potential enzymes, whereas at other times they may serve as a source of PA that then functions as a signaling molecule itself (9).

With the molecular cloning of *AtDGK7*, we add a new member to the growing family of plant DGKs. AtDGK7 is structurally different from previously characterized AtDGK2 and displays unique biochemical characteristics that point to a different biological function in plants.

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CHAPTER 3

The functional role of *AtDGK7* in lateral root growth and stress response in *Arabidopsis thaliana*

Running title: Role of *AtDGK7* for lateral root growth

No. of figures: 21

No. of tables: 6

Key words: *AtDGK* genes, AtDGK7, auxin, diacylglycerol, phosphatidic acid

Abbreviations:

The abbreviations used are: aa: amino acid; bp: base pairs; DAG: diacylglycerol; DGK: diacylglycerol kinase; DNA, deoxyribonucleic acid; EST: expressed sequence tag; GUS: β-glucuronidase; LR: lateral roots; NAA: α-naphtalene acetic acid; ORF: open reading frame; PA: phosphatidic acid; PCR: polymerase chain reaction; TIBA: 2,3,5-triiodobenzoic acid; UTR: untranslated region.

SUMMARY

In mammals diacylglycerol (DAG) can be metabolized in three ways but, under most circumstances, the phosphorylation of the free hydroxyl group to produce phosphatidic acid (PA) is considered the major route for metabolism of signalling DAG; the responsible molecules for this reaction are the members of the diacylglycerol kinase (DGK) family. No members are still reported in yeast but molecular and biochemical data have shown their presence in *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana* and, recently, in *Dictyostelium discoideum*. Here, I report the cloning of a cDNA for *AtDGK7* from *Arabidopsis thaliana* which codes for a protein that is longer than the previously described AtDGK7 polypeptide. Expression analysis using a promoter *ß-glucuronidase* reporter gene revealed *AtDGK7* activity in many tissues, including guard cells, the junction that connects filaments and stamens in the anthers and, strongly, in lateral root tips. Physiological analysis under abiotic stresses (changing concentrations of nitrogen, sucrose, auxin and auxin transport inhibitors) were performed with *AtDGK7* T-DNA insertion lines and also with the promoter-reporter plants. *AtDGK7* T-DNA insertion lines exhibited a strong inhibition of lateral root growth under limiting nitrogen and/or sucrose concentrations. Auxin also in some cases affected the T-DNA insertion lines different from the wild-type. Lateral root formation was totally absent and root size was reduced in the presence of the auxin transport inhibitor TIBA (2,3,5-triiodobenzoic acid). Lateral root formation recovered partially when mutated lines were grown at 50nM NAA (α-naphtalene acetic acid). It is hypothesized that *AtDGK7* is involved in a signalling process affecting growth and development of lateral roots, mediated via an interaction with auxin.

3.1. INTRODUCTION

Plants, because of their non-motile living conditions, are completely dependent on the resources existing around them. However, the availability of nutrients and their distribution are not fixed conditions in the environment; they are generally in constant flux. The flexible short-term adaptation of plants to diverse environments allows them to grow and develop in effective and opportune ways. A good example of developmental plasticity is lateral root formation in the root system. The number and placement of lateral roots are dramatically influenced by external cues or environmental signals (Leyser and Fitter, 1998; Malamy and Ryan, 2001). The development of an optimal root system is a key factor in a plant's ability to survive under adverse conditions (Hooker and Atkinson, 1992). Especially the available nutrients affect the number and location of lateral root initiation sites (Drew and Goss, 1973; Drew, 1975; Drew and Saker, 1978). It is of basic importance for plants to sense the level of nutrients in the soil directly via external sensors; having this information, plants must decide whether or not to start lateral root initiation.

The plant hormone auxin is known to play a critical role in lateral root initiation. Exogenous application of auxin stimulates lateral root formation (Evans et al., 1994) and some auxin-resistant mutants have reduced numbers of lateral roots (Malamy and Benfey, 1997). Furthermore, exogenous application of the plant hormone auxin to mature regions of the root can stimulate excess lateral root formation, suggesting that even cells that were not preselected are capable of being recruited to the lateral root program. Additionally, the accumulation of auxin to high levels in some mutants leads to excess lateral roots (King et al., 1995; Boerjan et al., 1995; Delarue et al., 1998). It is assumed that auxin is produced in young aerial tissues and transported in a polar way from the shoot system to the root to induce the formation of lateral root. An important support for this model comes from chemical inhibitors of polar auxin transport that completely block lateral root initiation (Reed et al., 1998). However, until present, it is mostly unknown how auxin movement or activity interacts with environmental signals to appropriately modulate lateral root initiation (Malamy and Ryan, 2001).

AtDGK7 encodes a member of the AtDGK enzyme family in *Arabidopsis thaliana*. We have previously reported the *AtDGK7* cDNA sequence and the biochemical characterization of the encoded protein (Gomez-Merino et al., 2005) – Chapter 2). Promoter-reporter (GUS) studies demonstrate that *AtDGK7* is expressed in a number of cell types and tissues including guard cells, the distal part of anther filaments, leaf margins, lateral root initiation sites and the root tips.

Here, the cloning of a novel *AtDGK7* cDNA is reported. This cDNA encodes a DGK protein that is, compared to the originally report AtDGK7 protein, extended at the C-terminus by 118 amino acids.

We employed T-DNA insertion lines to analyze the biological function of the *AtDGK7* gene. It appeared that lateral root formation was affected in these lines.

3.2. MATERIAL AND METHODS

General

Standard molecular-biological techniques were performed as described (Sambrook, 2001). Restriction enzymes were purchased from Roche Applied Science (Mannheim, Germany) and New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were obtained from TibMolbiol (Berlin, Germany). DNA sequencing was performed by AGOWA (Berlin, Germany). Unless otherwise indicated, other chemicals were purchased from Roche Applied Science, Merck, or Sigma. *E. coli* strain XL-1 Blue (Stratagene, Heidelberg, Germany) was employed for general DNA work. For sequence analyses, the tools provided by the National Center for Biotechnology Information (available on the World Wide Web at www.ncbi.nlm.nih.gov/), the ExPASy Molecular Biology Server (available on the World Wide Web at us.expasy.org/), and The *Arabidopsis* Information Resource (TAIR; available on the World Wide Web at www.arabidopsis.org/) were used. Cloning strategies were developed using Lasergene software (DNASTAR, Madison, MI). The NCBI Gene Expression Omnibus data repository was accessed via the World Wide Web at www.ncbi.nlm.nih.gov/geo/.

Plant growth conditions

Seeds were surface sterilized in 2 mL Eppendorf tubes (proportional ~500 µL of seeds) adding the same volume of 70% EtOH. After vortexing, seeds were centrifuged, the liquid was discarded and bleach solution containing ~1.5 mL NaOCl + three drops of Tween 20 was added. Followed by three washes in ~1.5 mL sterile water contains three drops of Tween 20, the seeds were collected by centrifugation and were distributed on sterile (autoclaved) filter paper kept in Petri dishes. Seeds were allowed to dry and then stored at 4°C for further use. To grow *Arabidopsis* plants, 14-day old seedlings from standard nutrient agar media containing antibiotics were sown in a 1:1 (v/v) mixture of soil (GS90; Gebr. Patzer, Sinntal Jossa, Germany) and vermiculite (Deutsche Vermiculite Dämmstoff, Sprockhövel, Germany) and cultured under controlled environmental conditions in growth chambers with an irradiance of 150 microeinsteins at a 20/18°C (day/night) temperature regime, a photoperiod of 16 h, and a relative humidity of 75%. At day 7 after germination, seedlings were transferred to a growth chamber under the same conditions except that the photoperiod was reduced to 8 h. Twenty-eight days after germination, plants were kept under 250 microeinsteins, 20/18 °C, 80/50% relative humidity (day/night).

Promoter-GUS fusions

A 1.5-kb 5´ genomic fragment upstream of the ATG start codon of the *AtDGK7* gene was amplified by PCR using primers pDGK-7F (5'-CCCAAGCTTCATCTGTACTAAATGTGATTAAACTTG-3'; added *Hin*dIII site underlined) and pDGK-7R (5'-CATGCCATGGCTTGTAAATCTAATATGTCATTCC-3'; added *Xba*I site underlined). *Arabidopsis thaliana* (L.) Heynh. Columbia-0 genomic DNA served as template. Cycling conditions were as follows: 95°C for 50 sec; 35 cycles of 95°C for 30 sec, 60°C for 1 min, 72°C for 2 min; 72°C for 10 min. The amplified promoter fragment was inserted into plasmid pCR2.1 (Invitrogen). Subsequently, the promoter was fused via *Hin*dIII/*Xba*I to the β-glucuronidase (*GUS*) reporter gene in the binary vector pGPTV-HPT (http://www.biotech.unl.edu/transgenic/vectors.html). The final constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) and transgenic *Arabidopsis* plants were obtained using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on sterile medium containing hygromycin. GUS activity was determined in vacuum-infiltrated plants or tissues using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate. Infiltrated plants were incubated in GUS staining solution overnight at 37°C. GUS staining patterns were viewed with a Leica ATC 2000 binocular microscope.

Cloning of AtDGK7 cDNA and generation of transgenic AtDGK7 overexpression and antisense plants

Polymerase chain reaction (PCR) amplification of the *AtDGK7* cDNA was carried out using the Advantage-HF2 PCR kit (Clontech, Heidelberg, Germany) according to the manufacturer's protocol. *A. thaliana* (L.) Heynh. Col-0 flower cDNA was used as template. Primer sequences were as follows: AtDGK7-F 5'-GCGGATCCTGATGGAGGAGACGCCGAGATC-3' and AtDGK7-R 5'-GCGCTCGAGTTATATGAACCTCTTAGGAAC-3'. Reaction details were as follows: 95 °C for 3 min; 36 cycles of 95 °C for 45 s, 62 °C for 45 s, and 72 °C for 72 s; 72 °C for 10 min. PCR products were analysed by agarose gel electrophoresis. Individual fragments were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subcloned into pGEM-T Easy (Promega, Mannheim, Germany), generating the plasmid pG7. The entire *AtDGK7* cDNA, present in plasmid pG7, had a length of 1125 bp (deposited under GenBank™ accession number AY686593). The coding region of the *AtDGK7* cDNA, flanked by *Eco*RI sites, was transferred from plasmid pG7 to vector pGreen0229-35S (provided by Isabel Witt, MPI of Molecular Plant Physiology, Golm, Germany), yielding the plasmid pAtDGK7. *Arabidopsis* ecotype Col-0 plants carrying the *AtDGK7* cDNA in sense (pOx-AtDGK7) and antisense (pAs-AtDGK7) orientation were generated, so that the transcription

of the corresponding *AtDGK7* strand was driven by the CaMV 35S constitutive promoter present in the vector pGreen0229-35S, which also harbors the *bar* gene that allows *in terra* selection with glufosinate ammonium (BASTA). *Agrobacterium tumefaciens* strain GV3101 (pMP90) was transformed by electroporation. *Arabidopsis* plants were transformed using the infiltration method (Clough and Bent, 1998b).

RNA isolation and northern blot analysis

Total RNA was isolated from leaves and seedlings, separated, and analysed by northern blot technique following standard procedures (Sambrook and Russell, 2001). The *AtDGK7* cDNA was radioactively labelled with [α-^{32}P]dCTP (Hartmann Analytic, Braunschweig, Germany) using the Rediprime II DNA labelling kit (Amersham Biosciences, Freiburg, Germany). Hybridisation was performed overnight at 65°C in sodium phosphate buffer. The membranes were washed twice for 10 min at 65°C (first wash: 5x SSC, 0.5% SDS; second wash: 1x SSC, 0.5% SDS). Membranes were exposed to x-ray films (Kodak X-OMAT AR) at -80°C for 1 day.

Cloning a full-length of AtDGK7 cDNA

For cloning of *AtDGK7* full-length cDNA, DNAse-treated RNA isolated from flowers served as template in a PCR using the Advantage-HF2 PCR kit (Clontech, Heidelberg, Germany). The *AtDGK7 cDNA* was amplified with gene-specific primers: AtDGK7-F 5'-GCGGATCCTGATGGAGGAGACGCCGAGATC-3' and AtDGK7-R L 5'-GCTCGAGATTTTGACTAATTGATGGAACAG-3'. Reaction details were as follows: 95 °C for 3 min; 36 cycles of 95 °C for 45 s, 62 °C for 45 s, and 72 °C for 72 s; 72 °C for 10 min. The amplified product was resolved by electrophoresis, gel purified, and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) generating the construct pGAtDGK7L. The 1.6 kb cDNA PCR fragment flanked by the pUC/M13 Forward sequencing primer binding site and SP6 RNA polymerase promoter site (from the polylinker region of the mentioned cloning vector) was sequenced and compared with the *AtDGK7* gene.

Analysis of root growth – variation of sucrose, nitrogen, auxin and TIBA concentrations

For these experiments, Arabidopsis wild-type plants (ecotype Columbia-0) and promAtDGK7::GUS seedlings were germinated on standard nutrient medium (commercial Murashige & Skoog medium, no. M 0221.0050, Duchefa, Haarlem, The Netherlands; supplemented with 0.5 g L^{-1} MES [2-(N-morpholino) ethanesulfonic acid]). Ten-day old seedlings were transferred to various nutrient media. Nutrient concentrations were as

follows: 4.5% (w/v) sucrose (Carl Roth, cat. no. 4621) and 0.02mM nitrogen (i.e. [0.01mM] NH_4NO_3); 0.5% (w/v) sucrose and 0.02mM nitrogen (i.e. 0.01mM NH_4NO_3); standard Murashige & Skoog medium is designated 4.5/60 because it contains 60mM nitrogen (supplied as 20mM NH_4NO_3 + 20mM KNO_3). Sterilized seeds were sown on standard media (pH was adjusted to 5.7 using 1 N KOH) containing 0.7% (w/v) Select Agar (no. 30391-049, Invitrogen). The 4.5/0.02 and 0.5/0.02 media were prepared using nitrogen-free Murashige & Skoog salts and additional components were added: boric acid, 0.10 [µM]; calcium chloride (anhydryde), 2.99 [µM]; cobalt chloride, 0.11 [µM]; copper sulfate, 0.10 [µM]; EDTA, 0.10 [µM]; glycine, 26.64 [µM]; myo-inositol, 0.56 [µM]; magnesium sulfate (anhydryde), 1.50 [µM]; manganese sulfate H_2O, 0.10 [µM]; nicotinic acid, 4.06 [µM]; potassium iodide, 5.00 [µM]; potassium phosphate monobasic, 1.25 [µM]; pyridoxine HCl, 2.43 [µM]; sodium molybdate, 1.03 [µM]; thiamine HCl, 0.30 [µM]; zinc sulfate, 29.91 [µM] (Murashige and Skoog, 1962). The number of lateral roots (LR) and lateral root primordia were examined using a stereoscopic microscope. Ten-day old seedlings were transferred from standard medium to 4.5/0.02, 0.5/0.02 and standard 4.5/60 media, respectively, in square vertical plates. Root growth and lateral roots were examined three and nine days later.

Further experiments were performed by transferring 10 –day-old seedlings from standard Murashige & Skoog media to plates with different sucrose concentrations (containing 4.5%, 10%, or 12% [w/v] sucrose, respectively, as indicated, and 60mM nitrogen [20mM KNO_3 and 20mM NH_4NO_3]) followed by a subsequent microscopic analysis of the seedlings.

The effect of auxin on lateral root initiation in primary roots was tested by growing seedlings for up to 10 days on standard medium and subsequently transferring them to 4.5/0.02 medium containing 100nM NAA (α-naphtalene acetic acid, no. N 0903 Duchefa). After three and nine days the seedlings were analysed by counting all lateral roots. NAA was dissolved in 0.1N NaOH and adjusted to a final concentration of 100nM. In control plates, an equivalent volume of 0.1N NaOH was added without NAA. Additionally, the same experiment was performed changing the concentration of NAA (1.0, 50.0 and 200nM) but using the in standard sucrose/nitrogen media, having a 4.5/60 + 0.1N NaOH as a control.

To determine the effect of the auxin transport inhibitor TIBA on lateral root initiation the seedlings were grown ten days in standard medium and transferred to standard medium containing + DMSO with and without 20µM of the auxin transport inhibitor TIBA (2,3,5-triiodobenzoic acid, no. T 0929 Duchefa) dissolved in DMSO. The concentration of DMSO in the growth media never exceded 0.1% (v/v).

At days three and nine, the lateral roots were analysed. The same experiment was done but using the sucrose standard medium with reduced nitrogen concentration, 4.5/0.02 medium + DMSO, with and without the auxin transport inhibitor TIBA (20 µM).

Plates were oriented vertically to allow roots to grow on the surface of the medium in a growth chamber set at 22°C for 16 h in the light and 8 h in the dark.

Isolation of AtDGK7 T-DNA insertion lines

Mutants from the SIGnAL (Salk Institute Genomic Analysis Laboratory) database collection were screened using the "T-DNA Express" Arabidopsis Gene Mapping Tool and five putative *AtDGK7* insertional mutants (Salk 110797, Salk 139504, Salk 139607, Salk 139760 and Salk 507896) were identified. Transgenic plants were selected on sterile medium containing kanamycin. For PCR screening of the mutant lines, the following combinations of primers were used: Left border primer (LBb1) 5'-GCGTGGACCGCTTGCTGCAACT-3' and *AtDGK7* specific forward primer (Fw-AtDGK7-T) 5'-GCATAGGTTTGGAATTTGGATACATGAAGT-3' or an *AtDGK7* reverse primer (Rv-ATDGK7-T) 5'-CGTCCCTCAATCCTCAAATCTTTCTGCTCA-3'. The left border primer in combination with the gene-specific primers flanking the T-DNA insertion sites were used to distinguish between heterozygous and homozygous T-DNA insertion lines.

3.3. RESULTS

3.3.1. Sequence characterization of AtDGK7

Until today the biological processes influenced by DGK activity in plants are still largely unknown. A step forward towards the understanding of the molecular mechanisms of DGK function was the isolation and characterization of the family of *DGK* genes from Arabidopsis. First results were published by (Gomez-Merino et al., 2004). This investigation presented data about *AtDGK2* of the *DGK* gene family in *Arabidopsis thaliana*. Subsequently, a cDNA encoding a polypeptide similar in its basic structure to other *AtDGK* members from *A. thaliana* and similar to DGKs from other plants species (rice, maize, tomato) was selected and compared with the database entries. It was found that the novel AtDGK belongs to the cluster II of plant DGKs as indicated by (Gomez-Merino et al., 2004). The coding region was represented by a cDNA reported to be full length (see GenBank acc. AY686593), coding for a novel DGK that we designated AtDGK7 (for **A**rabidopsis **t**haliana **d**iacyl**g**lycerol **k**inase **7**). The amino acid sequence of the AtDGK7 protein is available from GenBank under accession number NM_119180. The AtDGK7 protein deduced from the cDNA sequence has an isoelectric point of 7.73 and a molecular mass of 41.2 kDa (374 amino acid residues). The predicted AtDGK7 protein was reported to represent the smallest member of plant diacylglycerol kinases known so far (Gomez-Merino et al., 2005). The annotated *AtDGK7* gene is located on chromosome number 4 of the *A. thaliana* genome (gene locus number At4g30340, www.tigr.org) between positions 14.841.073 (5´ end) and 14.838.315 (3´ end). The predicted genomic sequence is 2759 bp long and includes a 126 bp 5´ UTR and a very long (758 bp) 3´ UTR. The annotated ORF contains 6 exons with a length of 1125 bp. AtDGK7 harbours an N-terminal catalytic domain, but in contrast to various of the characterized DGKs (including AtDGK2) it lacks a cysteine-rich domain at its N-terminus, and, importantly, its C-terminal DGK accessory domain was found to be incomplete (Gomez-Merino et al., 2005). The AtDGK7 protein shares 42.4% identical amino acids (187 aa) with AtDGK4 (GenBank accession number NP_200577), 28.4 % (165 aa) with Dgk-1 from *Caenorhabditis elegans* (NP_508190), 33.1% (232 aa) with KDGE_DROME eye-specific diacylglycerol kinase from *D. melanogaster* (GenBank Q09103), 27.3% (316 aa) with KDGG_RAT diacylglycerol kinase gamma from *R. norvegicus* (GenBank P49620), 34.9 % (106 aa) with diacylglycerol kinase 4 from *Homo sapiens* (NP_001338) and 28.1% (162 aa) with diacylglycerol kinase epsilon from *Mus musculus* (NP_062378). We demonstrated that the 41.2 kDa AtDGK7 protein is enzymatically active when expressed in *Escherichia coli* (Gomez-Merino et al., 2005); Chapter 2 of this thesis).

Five T-DNA insertion lines were obtained from the Salk stock center for further characterisation of *AtDGK7* function. The T-DNA insertion sites of the five *AtDGK7* Salk lines were identified (see chapter 3.3) and compared with the annotated At4g30340 gene (AY686593, 1125bp CDS size http://www.ncbi.nlm.nih.gov/). Despite phenotypic changes observed in the Salk lines when compared to wild-type plants (more shoots, altered root formation, differences in the response to abiotic stresses and hormone treatments; see Chapter 3) all T-DNA insertions were located in the predicted 3' UTR region of the *AtDGK7* gene. The genomic sequence of At4g30340 (http://www.tigr.org/ - The TIGR *Arabidopsis thaliana* Database) was therefore again subjected to a detailed sequence analysis. This analysis resulted in a possible alternative coding sequence that was elongated by part of the formerly predicted 3' UTR. To confirm the presence of a mRNA containing an extended *AtDGK7* open reading frame, a PCR experiment was performed employing a reverse primer annealing to the 3' UTR predicted. Using cDNA from flowers as template a ~1.6 kb long PCR fragment was amplified and cloned into vector pGEM-T Easy (Promega, Mannheim, Germany) yielding plasmid pGAtDGK7L. Sequence analysis revealed a full-length cDNA of 1564 bp encoding a polypeptide of 492 amino acids. Compared to the original *AtDGK7* sequence this new cDNA has an extension of 439 nucleotides coding for 118 additional amino acids. There are five differences in the nucleotide sequence compared to the previously database published *AtDGK7* cDNA (BAC locus name: F17I23A / acc. AF160182; AY686593, At4g30340. http://www.tigr.org; http://www.ncbi.nlm.nih.gov): the discrepancies, when comparing the newly determined sequence against the former clone for *AtDGK7* (AY686593), correspond to 3 exons and 2 introns located in the former 3' UTR. The protein encoded by the *AtDGK7* cDNA cloned here harbors the complete DGKa domain instead of a truncated one as reported in (Gomez-Merino et al., 2005). The new *AtDGK7* cDNA sequence obtained here was submitted to the GenBank database under the accession number DQ350135. **Figure 1** shows a schematic diagram of the *AtDGK7* gene and of the deduced AtDGK7 protein indicating the positions of the catalytic (DGKc) and accessory (DGKa) domains.

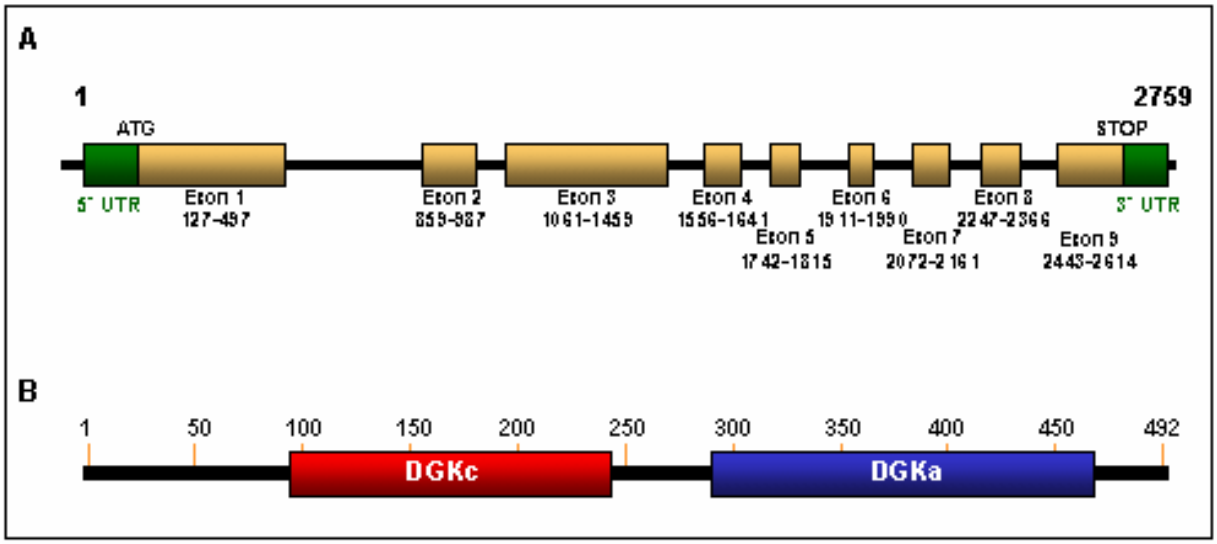


Figure 1. Schematic diagram of the *AtDGK7* gene (locus At4g30340) and domain composition of isozyme AtDGK7.

A. The *AtDGK7* gene is composed of nine exons (in brown color boxes) and eight introns (indicated with black wide line); the 5` UTR and 3` UTR are indicated by green color boxes). **B.** Schematic representation of the domain composition and order encountered in the large AtDGK7 protein. Catalytic and accessory domains are represented as a red box and blue box, respectively; DGKc; Diacylglycerol kinase catalytic domain (presumed, accession number: PF00781 at http://www.sanger.ac.uk/Software/Pfam/)), located at amino acid positions 94–240; and, DGKa; Diacylglycerol kinase accessory domain (presumed, accession number: PF00609), located at amino acid positions 287-464.

3.3.2. Analysis of GUS activity in transgenic Arabidopsis plants transformed with the promAtDGK7::GUS construct

3.3.2.1. Cloning of the AtDGK7 promoter into a GUS reporter vector

AtDGK7 transcript level was originally assessed by real-time RT-PCR (see Chapter 2). To define the transcriptional activity of the *AtDGK7* gene further, a 1.5-kb-long 5' upstream region of the *AtDGK7* gene was isolated, fused to the *gusA* reporter gene encoding *Escherichia coli* β-glucuronidase in vector pGPTV-HPT, and transformed into *A. thaliana* (Columbia-0) wild-type using the floral dip method (Clough and Bent, 1998a). Transgenic seeds from the plants transformed were selected on axenic medium containing hygromycin; twelve selected transgenic *promAtDGK7*::GUS seedlings were tested and all of them exhibited GUS activity at the seedling stage. After this test, ten seedlings were selected for further studies. Plants were also transferred to soil and grown in a phytotron. The seeds were kept at room temperature for future experiments. Additional GUS activity tests were performed (see below). Analysis of the *AtDGK7* promoter for the presence of potential *cis* regulatory elements using the PLACE database (available on the World Wide Web at www.dna.affrc.go.jp/htdocs/PLACE/fasta.html) revealed *cis* elements (**Figure 2**) that may

contribute to: tissue-specific expression and auxin-inducibility (Baumann et al., 1999), gene expression during root, shoot and meristem formation, sugar repressibility (Morita et al., 1998; Mena et al., 2002), and control of guard cell-specific gene expression (Plesch et al., 2001).

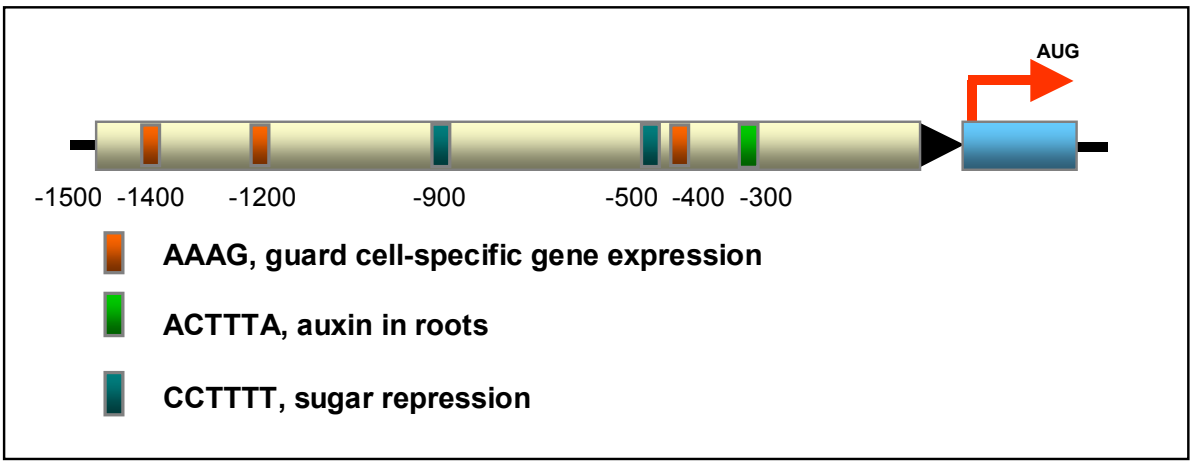


Figure 2. Schematic localization of putative *cis*-acting elements in the promoter region of the *AtDGK7* gene.

Analysis of the *AtDGK7* promoter for the presence of potential *cis* regulatory elements was done using the PLACE database (available on the World Wide Web at www.dna.affrc.go.jp/htdocs/PLACE/fasta.html). The position of the translational start codon (AUG) is indicated.

3.3.2.2. Analysis of AtDGK7 expression

3.3.2.2.1. Analysis of AtDGK7 expression in A. thaliana

The *promAtDGK7*::GUS lines were tested for the distribution of GUS activity throughout various stages of seedling development. Initially, seeds from *promAtDGK7*::*GUS* lines were germinated and selected on agar plates containing hygromycin. GUS activity was not observed in one-day-old seedlings (**Figure 3A**).

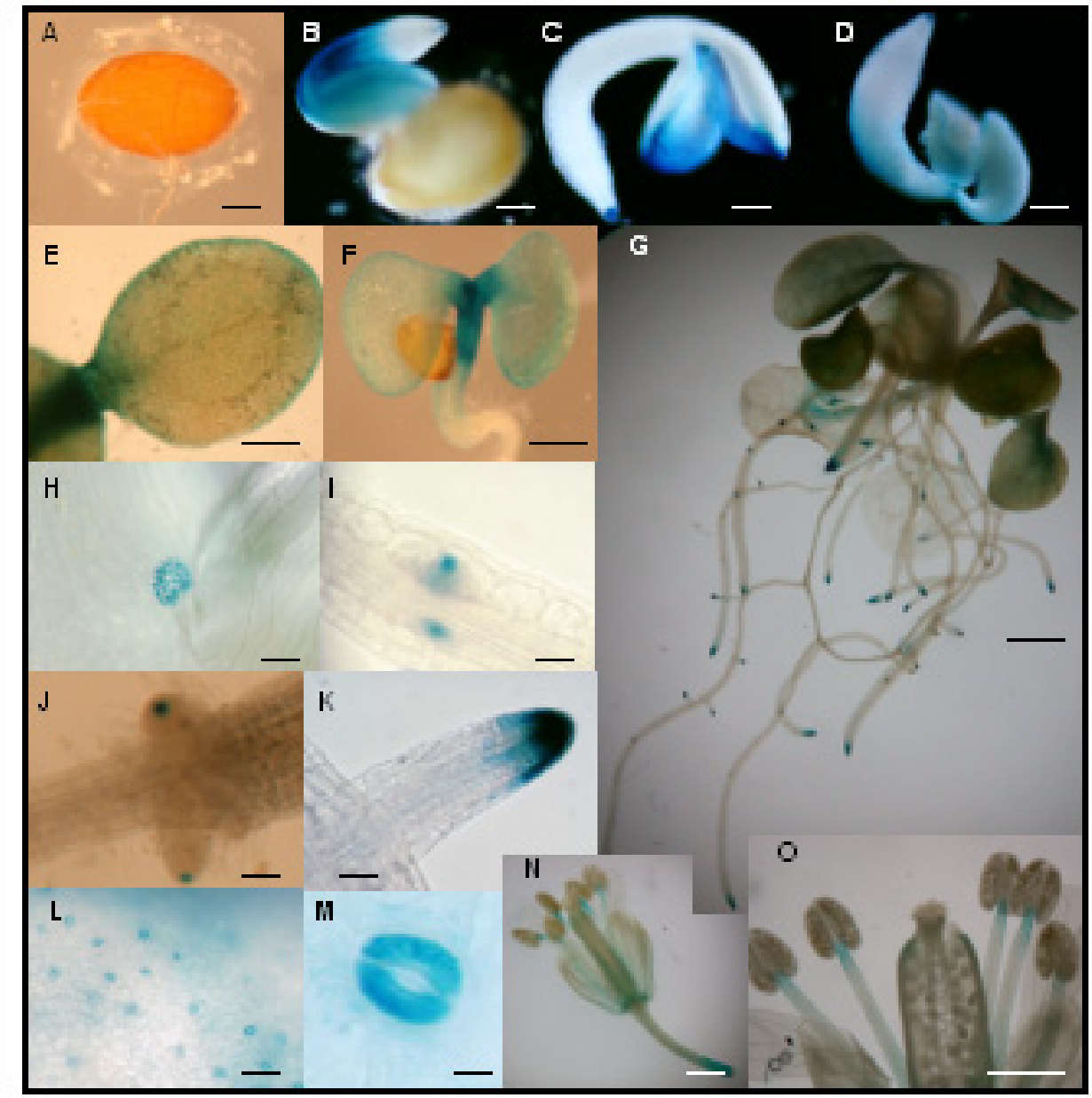


Figure 3. Analysis of GUS activity in transgenic *Arabidopsis* plants transformed with the *promAtDGK7::GUS* fusion construct.

(A), Seed kept for 1 day on agar medium; absence of GUS staining of the embryo inside the seed. (B), 3-day-old seedling showing GUS staining in the root-hypocotyl transition zone and the root apex. (C and D) 4-and 5-day-old seedlings; GUS staining in cotyledons, shoot apex and the root tip. (E and F) 9- and 10-day-old seedlings showing stained cells at the leaf margin and cotyledons. Also shown are roots (G) and tips of secondary roots (I, J and K) of a 14-, 15- and 17-day-old seedling. Note strong GUS staining at stipules (H) and guard cells in the leaves (L and M). Mature flower with intense GUS staining present in the junction connecting filaments and stamens in the anthers (N and O). Scale bars represent 5.0µm in M, 20µm in K, 50µm in L, J and O, 100µm in A, B, C, D and H, 250µm in E, 1.0mm in F and N, and finally 3.0mm in G. *Arabidopsis* tissues and seedlings were stained for 3 hours in X-Gluc buffer.

Three days after germination (DAG) strong GUS staining was observed at the root-hypocotyl transition zone and also at the root apex (**Figure 3B**). In 4- and 5-day old seedlings GUS

staining was evenly distributed in cotyledons; in addition, GUS staining was observed in root tips of primary roots (**Figure 3C and D**). In 9- and 10-day-old seedlings staining was detected at the base of the cotyledons and the apical meristem (**Figure 3F**). In the following days, strong *AtDGK7* promoter activity was observed in stipules, in the tip of the primary root and also in secondary root tips, emerging lateral roots and in root hairs (**Figure 3G, H, I, J and K**). Additionally, cells of the leaf edges as well as guard cells were stained in juvenile, early- and late-adult leaves (**Figure 3E, L and M**). The same staining pattern was present in cauline leaves. In flowers, prominent GUS staining was detected in the junction that connects filaments and stamens in the anthers (**Figure 3N and O**).

3.3.2.2.2. Analysis of AtDGK7 expression in Oryza sativa

We were interested to know whether *AtDGK7* promoter-driven reporter gene activity is retained in monocotyledonous species. The *promAtDGK7::GUS* construct was therefore transformed into rice. The vector was sent to the lab of Prof. Hong-Wei Xue (Shanghai Institute of Plant Physiology and Ecology, Shanghai, China) and transformed into *Agrobacterium tumefaciens* via electroporation. A positive clone was selected and used for transformation of immature rice embryos. Several independent rice transformants were obtained. GUS staining was observed in the tips of rice lateral roots. Strong reporter gene expression was detected in primordia of lateral roots; no expression was observed in primary root tips (**Figure 4**). The expression pattern of *AtDGK7* in rice plants was, therefore, similar to the one observed in *Arabidopsis thaliana*.

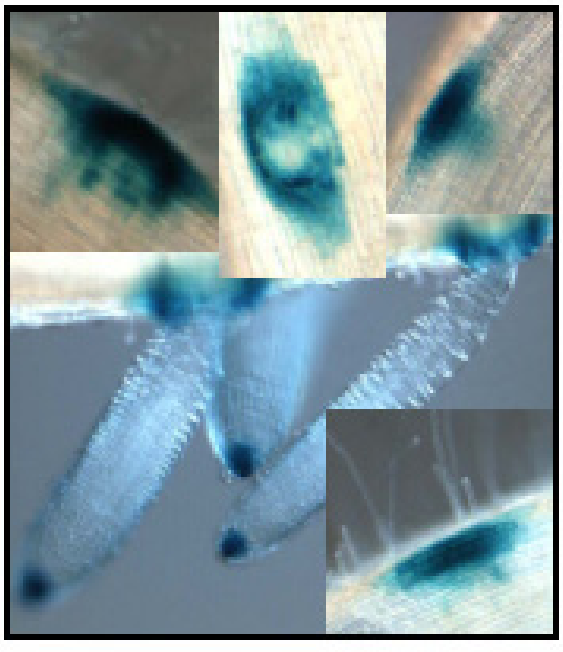


Figure 4. Histochemical localization of GUS activity in a rice plant transformed with the *promAtDGK7::GUS* fusion vector.

GUS activity was absent from primary roots (Prof. Hong-Wei Xue, personal communication), but *AtDGK7* promoter-triggered *GUS* expression was strong in lateral root primordia (photographies provided by Prof. Xue, Shanghai Institute of Plant Physiology and Ecology, Shanghai, China).

3.3.2.3. Analysis of promAtDGK7::GUS seedlings under various physiological conditions

The literature was screened to search for specific physiological tests to find growth conditions where lateral root initiation processes were evident and could be reliably analyzed. Subsequently, *A. thaliana* (Col-0) wild-type and transgenic seedlings expressing the *E. coli* ß-glucuronidase (*gusA*) reporter gene under the control of the *AtDGK7* promoter were grown on different media containing standard or altered sucrose and/or nitrogen concentrations. Additionally, the effects of auxin and the auxin transport inhibitor TIBA (2,3,5-triiodobenzoic acid) were tested. These parameters were altered as indicated in **Table 1**.

Table 1. Composition of media used for seedling growth assays.

	Sucrose (%)	Nitrogen (mM)	NAA (nM)	TIBA (µM)
Standard	4.5	60	-	-
Reduced nitrogen	4.5	0.02	-	-
Reduced sucrose and nitrogen	0.5	0.02	-	-
Reduced nitrogen + solvent	4.5	0.02	100mM NaOH[1]	-
Reduced nitrogen + high NAA	4.5	0.02	100	-
Standard + solvent	4.5	60	100mM NaOH[1]	-
Standard + low NAA	4.5	60	1.0	-
Standard + high NAA	4.5	60	50.0	-
Standard + higher NAA	4.5	60	200.0	-
Reduced nitrogen + solvent	4.5	0.02	-	1.0% DMSO[2]
Reduced nitrogen + TIBA	4.5	0.02	-	20
Standard + solvent	4.5	60	-	1.0% DMSO[2]
Standard + TIBA	4.5	60	-	20

[1] Control for NAA experiment; solvent used was NaOH;

[2] Control for TIBA experiment; solvent used was DMSO.

Interesting evidence from (Reed et al., 1998) indicates that auxin from the aerial tissues of the plant is essential for lateral root initiation. Also, (Malamy and Ryan, 2001) showed that NAA was effective at increasing the initiation of lateral roots in plants under standard (4.5/60) or repressive (4.5/0.02) growth conditions. They demonstrated, working with mutants with repressed root formation, that NAA can induce lateral root initiation; also, this fact is consistent with the idea that auxin is limiting in these tissues.

Here, GUS expression patterns were determined in seedlings grown for three weeks on the indicated media. The following observations were made:

- **Standard medium** (4.5% sucrose, 60mM nitrogen): the seedlings were of normal size and had an abundant number of lateral roots (LR) (**Figure 5A**), as expected from literature data. Histochemical staining of transgenic *promAtDGK7::GUS* seedlings revealed that *AtDGK7* transcription is strong in apical meristem, hypocotyls, root tips and the tips of lateral roots (**Figure 5B and C**), but was absent in vascular tissue in general. Also, GUS expression was present in petioles and at the edges of the cotyledons and younger leaves (**Figure 5D**), as already indicated in the previous section.
- **Growth medium with reduced nitrogen concentration** (4.5% sucrose, 0.02mM nitrogen): under these conditions roots were shorter than on standard growth medium and they produced a reduced number of lateral roots (**Figure 5E**). Histochemical assays of four independent transgenic lines revealed that all of them had an identical GUS expression pattern in hypocotyls (**Figure 5F**) and the vascular tissue of roots, hypocotyls and leaves (**Figure 5G, H and J**). Weak expression was visible in root tips (**Figure 5I**). However, no staining was observed in the shoot meristem, cotyledons and leaves.
- **Growth medium with reduced sucrose and nitrogen concentration** (0.5% sucrose, 0.02mM nitrogen): seedlings produced short primary roots with very short lateral roots that were reduced in number (**Figure 5K**). The GUS expression pattern was similar to plants grown on normal sucrose (i.e., 4.5%) but low nitrogen concentration (i.e., 0.02mM) (**Figure 5L, M, N and O**).

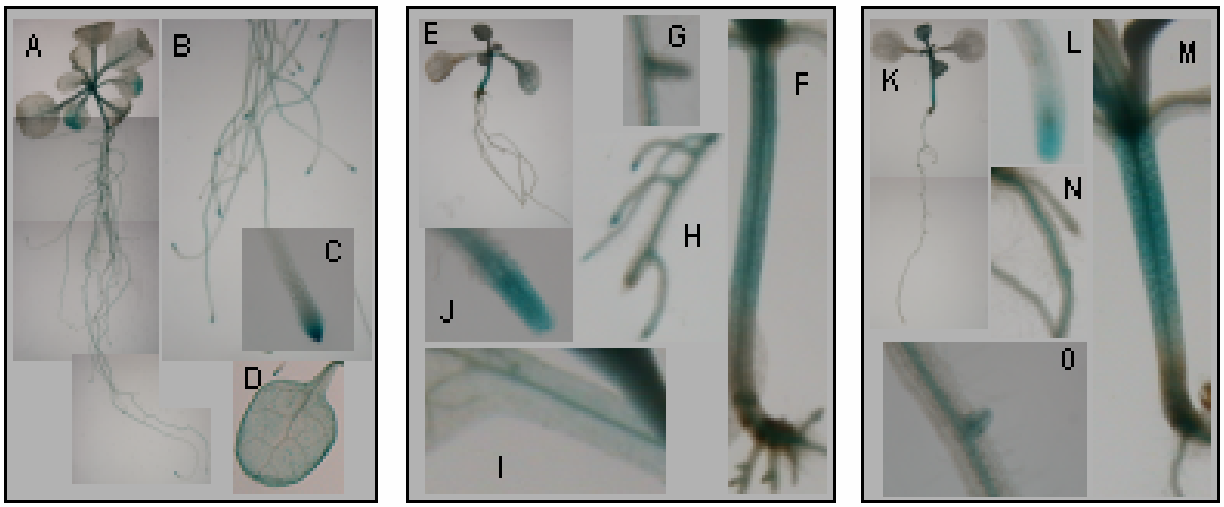


Figure 5. Expression pattern in 3-week-old wild-type and *promAtDGK7::GUS* seedlings grown on different media containing standard or altered sucrose and/or nitrogen concentrations.

(A-D), Standard growth medium, i.e., 4.5% sucrose, 60mM nitrogen. (E-J), Medium with reduced nitrogen concentration, i.e., 4.5% sucrose and 0.02mM nitrogen. (K-O), Medium with reduced sucrose and nitrogen concentrations, i.e., 0.5% sucrose, 0.02mM nitrogen. Seedlings grown on standard growth medium showed normal size and abundant lateral roots number (A), strong GUS staining in root tips (B) and lateral roots tips (C). Note the specific GUS signal at the leaf edges (D). Seedlings grown on reduced nitrogen concentration had a short size and a low number of leaves and lateral roots (E). All of them showed strong GUS expression in hypocotyls (F), vascular tissue of hypocotyls (F), roots (G and H) and leaves (I). Similar results we obtained with seedlings grown on medium with reduced sucrose and nitrogen concentrations. These plants produced few leaves (K) and short primary roots with very few and short lateral roots; also the GUS expression pattern was similar (L, M, N and O).

- **Growth medium with reduced nitrogen** (4.5% sucrose, 0.02mM nitrogen), **supplemented with high NAA concentration** (100nM): after transfer to this medium, (**Figure 6A, D**) seedlings did not grow further and the primary root (radicle) remained short. However, all plants produced lateral roots (**Figure 6A, C, D, E and F**). The expression of the *GUS* reporter gene was observed in the vascular tissue of the roots (**Figure 6E and F**), but not in the hypocotyls (**Figure 6H**), in the cotyledons (**Figure 6B**) or in the root tips (**Figure 6E and F**). In control experiments (i.e., without NAA, but with 100mM NaOH solvent), the seedlings did not grow further and their development stopped (**Figure 6H**). The primary roots were strongly shortened in length (**Figure 6H**) and lateral roots were virtually absent or only one lateral root was visible (**Figure 6K**). The *GUS* reporter gene was expressed in the vascular tissue of hypocotyls (**Figure 6J**) and roots (**Figure 6K and L**), in the shoot meristem and in the mesophyls cells of cotyledons (**Figure 6I**). GUS staining was absent from the root tips (**Figure 6K**).
- **Standard medium** (4.5% sucrose, 60mM nitrogen) **supplemented with low NAA concentration** (1.0nM): seedlings grown under these conditions showed a normal size (**Figure 6M**); however, the auxin treatment affected the number of lateral roots. Lateral root number was strongly reduced (**Figure 6N**) and only few of them developed further. **Figures 6O** and **P** show that the expression of the *GUS* reporter gene in lateral roots and leaves was similar to seedlings grown under standard conditions. In a control experiment without NAA, where only 100mM NaOH was supplemented, the presence of lateral roots was similar to plants grown on standard medium and the size of the seedlings was also similar (**Figure 6Q**). *GUS* expression was detected in the cotyledons (**Figure 6U**) and edges of leaves (**Figure 6T**). The GUS staining was generally strong (**Figure 6R and S**).
- **Standard medium** (4.5% sucrose, 60mM nitrogen) **supplemented with high NAA concentration** (50nM): Under these conditions the size of the seedlings was similar (**Figure 6V**) to seedlings that were grown in a medium with low concentration of auxin,

although the lateral root number was larger but their lengths were reduced (**Figure 6W**). GUS expression in the presence of auxin was detected in leaves (**Figure 6Y**) and cotyledons (**Figure 6AA**) and was strong in the tip of primary roots (**Figure 6Z**) and lateral roots tips (**Figure 6X and 6B**).

- **Standard medium** (4.5% sucrose, 60mM nitrogen) **supplemented with higher NAA concentration** (200nM): high auxin concentration resulted in strong *GUS* expression in all lateral roots (**Figure 6AD, AE, AH and AF**) and leaves (**Figure 6AG**); however, a strong reduction in the size of the seedlings (**Figure 6AC**) and lengths of the roots (**Figure 6F**) was observed.

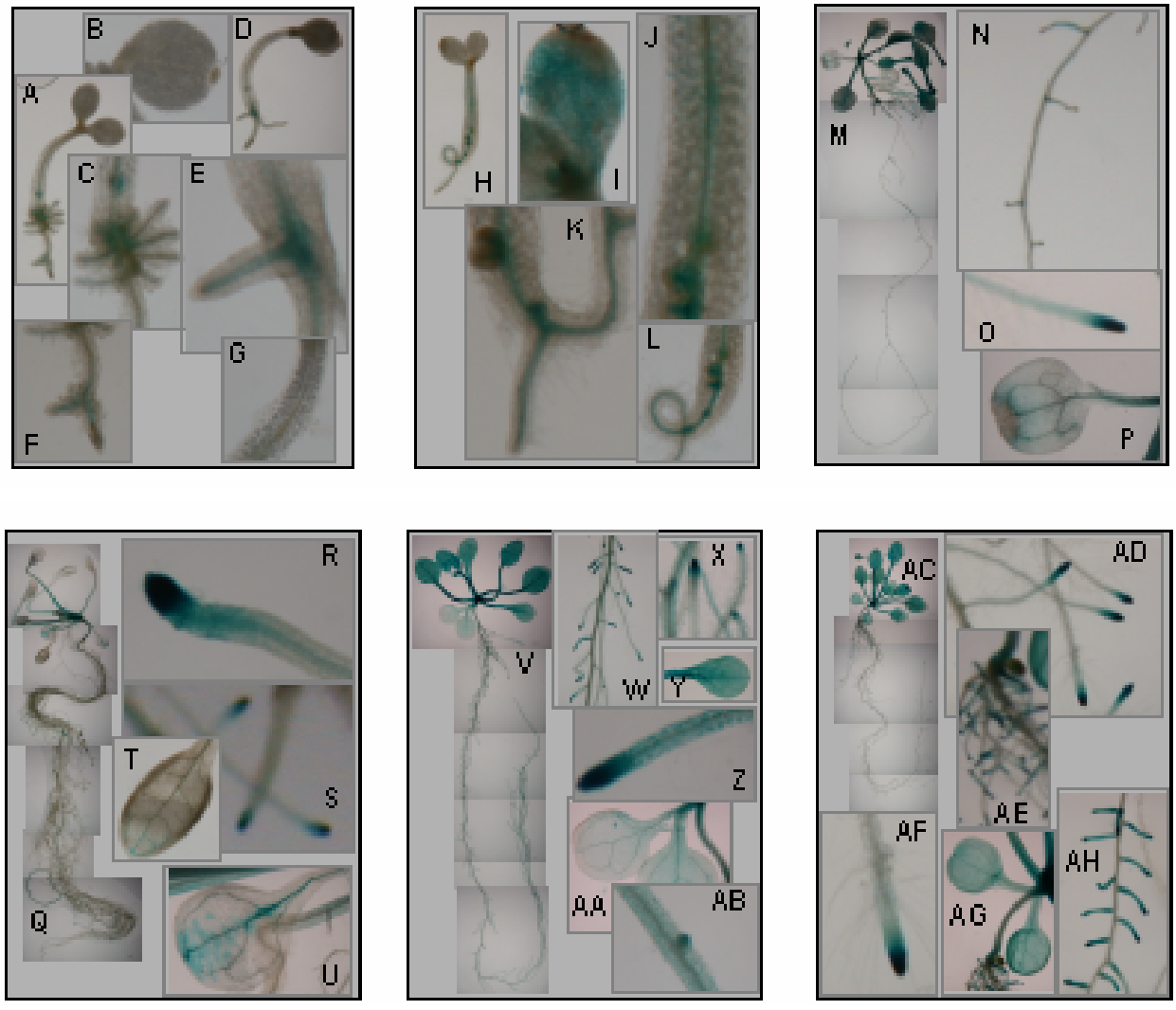


Figure 6. Expression pattern in a 3-week-old wild-type and *promAtDGK7::GUS* seedlings grown on different media containing standard or altered nitrogen concentrations, plus/minus auxin.

(A-G), Medium with reduced nitrogen concentration, i.e., 4.5% sucrose and 0.02mM nitrogen supplemented with high auxin concentration, 100nM. (H-L), Medium with reduced nitrogen concentration supplemented with auxin solvent NaOH, 100mM. (M-P), Standard growth medium, i.e.,

4.5% sucrose, 60mM nitrogen supplemented with low auxin concentration, 1.0nM. (Q-U) Standard growth medium, i.e., 4.5% sucrose, 60mM nitrogen supplemented with auxin solvent NaOH, 100mM. (V-AB), Standard growth medium, i.e., 4.5% sucrose, 60mM nitrogen supplemented with high auxin concentration, 50nM. (AC-AH), Standard growth medium, i.e., 4.5% sucrose, 60mM nitrogen supplemented with higher auxin concentration, 200nM. The seedlings grown under reduced nitrogen concentration and high auxin concentration showed a strongly reduced size (A) and few lateral roots and/or in some cases only one (C, D, E and F). A visible *GUS* signal was present in vascular tissue of roots (E and F) but was absent in cotyledons (B), hypocotyls and root tips (G); in control experiments (without auxin, only with NaOH) the seedlings showed reduced size (H) and only one (K) or a total absence of lateral roots (H). The *GUS* reporter gene was expressed in vascular tissue of hypocotyls (J) and root (K and L), in shoot meristem and cotyledons (I), but was absent in the root tips (K). Seedlings cultured on standard growth medium with low auxin concentration were normal in size and the number of leaves (M) but lateral roots number was substantially reduced (N). Hypocotyls (M), leaves (P) and root tips (O) showed GUS expression. The seedlings grown on standard growth medium, without auxin, plus the solvent NaOH, had a normal size and number of leaves, with normal production of lateral roots number (Q) and the GUS reporter gene showed strong expression in root tips (R and S) and edges of leaves (T and U). When the seedlings were grown on standard growth medium supplemented with high auxin concentration they were normal in size, and produced numerous lateral roots (V) which were reduced in length (W). The *GUS* reporter gene was expressed in cotyledons (AA), leaves (Y) and strongly in lateral roots tips (X, Z and AB). Finally, the seedlings grown under standard growth medium supplemented with higher auxin concentration were small in size and had short primary and lateral roots. The expression of the *GUS* reporter gene was strong in leaves (AG) and lateral root tips (AD, AE, AF and AH).

In the following we tested, whether or not the auxin transport inhibitor, TIBA (2,3,5-triiodobenzoic acid) has an effect on AtDGK7 expression.

- **Growth medium with reduced nitrogen** (4.5% sucrose, 0.02mM nitrogen), **supplemented with auxin transport inhibitor TIBA** (20µM): seedlings remained small and had an atrophied primary root with only a minimal number of lateral roots (**Figures 7A, B and C**). GUS staining was observed in cotyledons, stipules and was strong in hypocotyls (**Figures 7D, E and F**). No GUS activity was detectable in roots including the vascular tissue (**Figures 7B and A**). In a control experiment where TIBA was omitted (and only DMSO solvent was added) the seedlings remained small with a short primary and only one or two short lateral roots (**Figures 7H, G and I**). As shown in **Figure 7F** weak *GUS* expression was observed in the hypocotyl region and the vascular tissue of hypocotyls and roots (**Figure 7I**) but was totally absent from cotyledons or root tips (**Figures 7J, G, H and I**).
- **Standard medium** (4.5% sucrose, 60mM nitrogen) **supplemented with auxin transport inhibitor TIBA** (20µM): seedlings grown in the presence of TIBA had a reduced size of the hypocotyls (**Figure 7K and L**) and lateral roots were totally absent (**Figure 7O**). Additionally, weak GUS staining was present at the leaf margins (**Figure 7M**). and in the epidermis of the root (**Figure 7N and O**). As expected, seedlings grown under control conditions (i.e., in the absence of TIBA) were normal in size and lateral roots formation (**Figure 7Q**). A high level of GUS activity was detected in all leaves (**Figure 7Q, S and T**), the tips of lateral roots (**Figure 7P and R**) and, in shoot apex and

petioles (**Figure 7U**). The pattern of GUS expression is similar to that of transgenic *promAtDGK7::GUS* seedlings grown on standard medium.

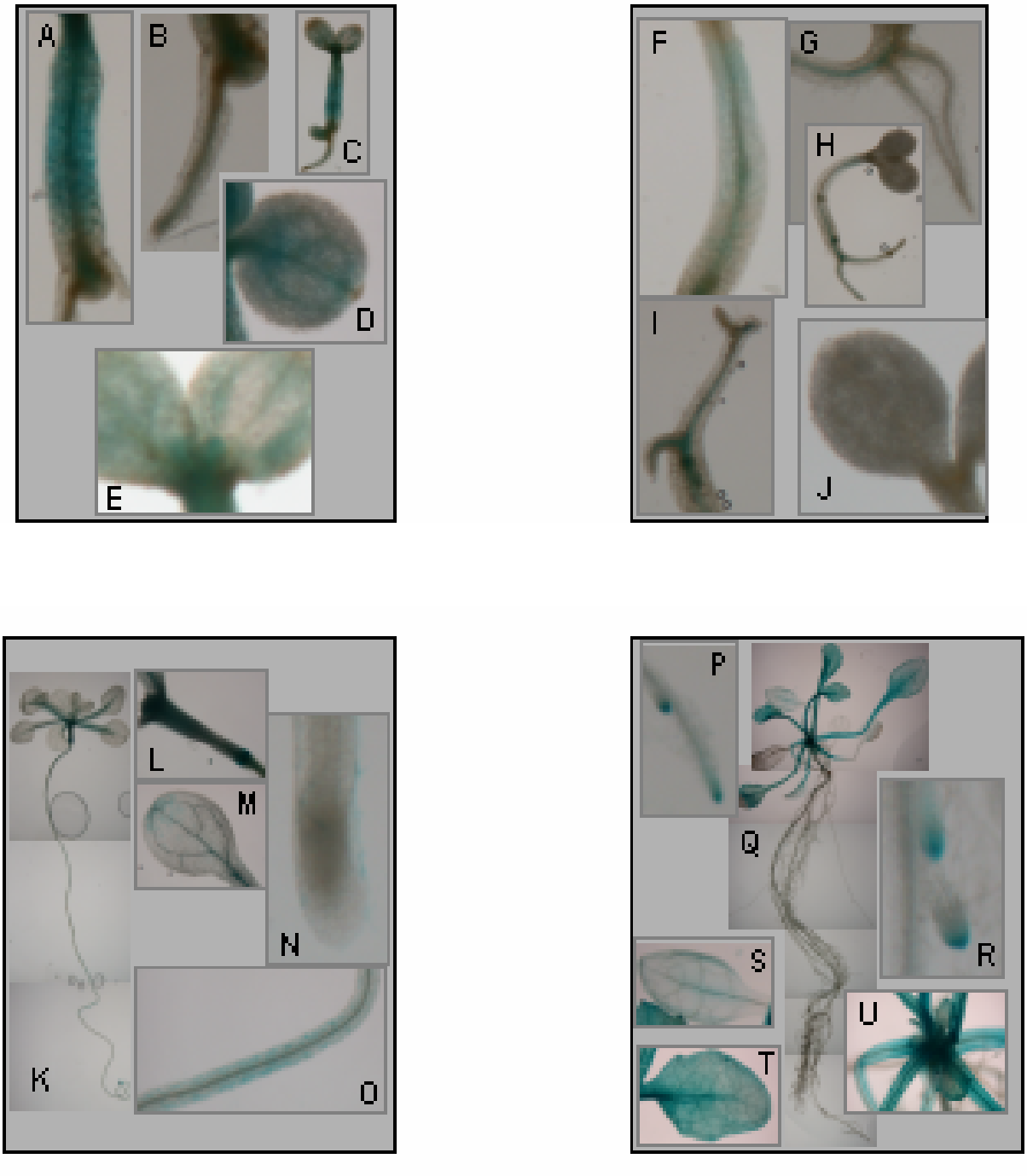


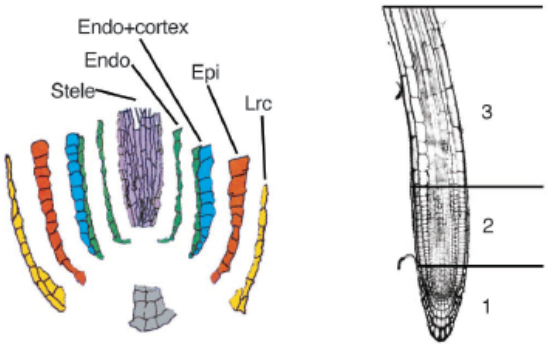
Figure 7. Expression pattern in a 3-week-old wild-type and *promAtDGK7::GUS* seedlings grown on different media containing standard or altered nitrogen concentrations plus/minus auxin transport inhibitor TIBA.

(A-E), Medium with reduced nitrogen concentration, i.e., 4.5% sucrose and 0.02mM nitrogen supplemented with 20µM TIBA. (F-J), Medium with reduced nitrogen concentration supplemented with 1.0% DMSO (solvent). (K-O), Standard growth medium, i.e., 4.5% sucrose, 60mM nitrogen

supplemented with 20µM TIBA. (P-U), Standard growth medium, i.e., 4.5% sucrose, 60mM nitrogen supplemented with 1.0% DMSO. A minimal number of lateral roots and short size was observed in the seedlings grown on medium with reduced nitrogen concentration plus the auxin transport inhibitor TIBA (A-C). The *GUS* reporter gene showed expression in cotyledons (D), stipules (E) and very strong in hypocotyls (A) but expression was totally absent in roots. In a control experiment with DMSO only (without TIBA) and medium with reduced nitrogen concentration the seedlings were also small in size and produced few lateral roots (H); the *GUS* expression was absent in stipules (J). In the seedlings grown on standard growth medium with TIBA ,the expression of the *GUS* reporter gene was absent in cotyledons (K) and also, in the root tips (N) and, weak in the hypocotyls (L), margins of leaves (M) and epidermis of roots (O). The size, presence and number of lateral roots and the GUS expression pattern of seedlings in control experiments was normal (Q) showing the expected signal in all leaves (S and T), petioles and apex meristem (U) and, tips of lateral roots (P and R).

3.3.2.4. AtDGK7 expression pattern as revealed from microarray experiments

Philip Benfey's group developed a set of techniques to generate high-resolution spatial and temporal expression profiles throughout the *Arabidopsis* root. The method measures gene expression among cell types and tissues and along a developmental gradient, resulting in a digital readout with resolution approaching that of *in situ* hybridization (Birnbaum et al., 2003). Gene expression was mapped to 15 different zones of the root that correspond to cell types and tissues at progressive developmental stages using stage-specific GFP marker lines; they got data with the localization of expression of more than 22,000 genes, covering about 90% of the genome. From the root tip until the elongation zone, the root was divided in three developmental stages and taking five specific cell types 15 separate subzones (protoplasted by enzymatic digestion) were established that were hybridized separately to collect microarray data. Finally the information that was could be obtained from their data shows that *AtDGK7* expression level is around 7 times higher at the tip of the root (stage 1 – **Figure 8**, graphic) compared with the others two stages; additionally, cells from the more outer areas of the root (subzone lateral root cap – **Figure 8**, graphic) exhibited two-three times highest expression than the stele or epidermis (**Figure 8** - table).



Cell types	Mean expression value of the transcripts stages		
	1	2	3
Stele	59,50	8,09	7,54
Endo	62,69	8,53	7,95
Endo+cortex	100,23	13,64	12,70
Epidermis	97,38	13,25	12,34
Lateral root cap	195,36	26,58	24,76

Figure 8. Expression profiling of cells and tissues of Arabidopsis roots at three developmental stages.

Abbreviations are: Endo, endodermis; Endo+cortex, endodermis and cortex; Epi, epidermal atrichoblasts; and Lrc, lateral root cap. For developmental stage profiles (right), numbers indicate developmental stages profiled, which were dissected with the use of the following landmarks as upper borders: stage 1, where the root tip reached its full diameter (about 0.15 mm from the root tip); stage 2, where cells transition from being optically dense to a more transparent appearance as they begin longitudinal expansion (about 0.30 mm from the root tip); and stage 3, where root hairs were fully elongated (about 0.45 to 2 mm from the root tip). In the table, the numbers correspond to the expression values of the transcript abundance of *AtDGK7* in every zone and stage of the microarray results. Figure modified from Birnbaum *et al*. (2003) A gene expression map of the Arabidopsis root. Science. 302: 1956 - 1960.

AtDGK7 appeared to be strong expressed (7-8 times) in stage 1 than in stage 2 and 3. Additional microarray data were analysed using a bioinformatic tool called “Genevestigator” (https://www.genevestigator.ethz.ch/); this online tool comprises a gene expression database and a number of querying and analysis functionalities developed to facilitate gene functional discovery (Zimmermann et al., 2004). The use of this instrument allows assigning contextual information to gene expression data and directing the design of new experiments. Using the *Gene Annotator* tool (https://www.genevestigator.ethz.ch/at/index.php) it was straight forward to identify biological processes that *AtDGK7* might be involved in: responses to abiotic or biotic stimuli and signal transduction. Here, the search was focused on two kinds of stress response experiments and each stress factor corresponded to a respective control allowing a direct comparison of gene expression un the conditions evaluated (**Figure 9**). For the first stress category (**Table 2**), I chose four experiments that tested anoxic conditions, the deprivation of nitrogen and sucrose, and the incubation in mannitol. I also analysed the effect of auxin and auxin transport inhibitors (**Table 3**) on *AtDGK7* gene expression.

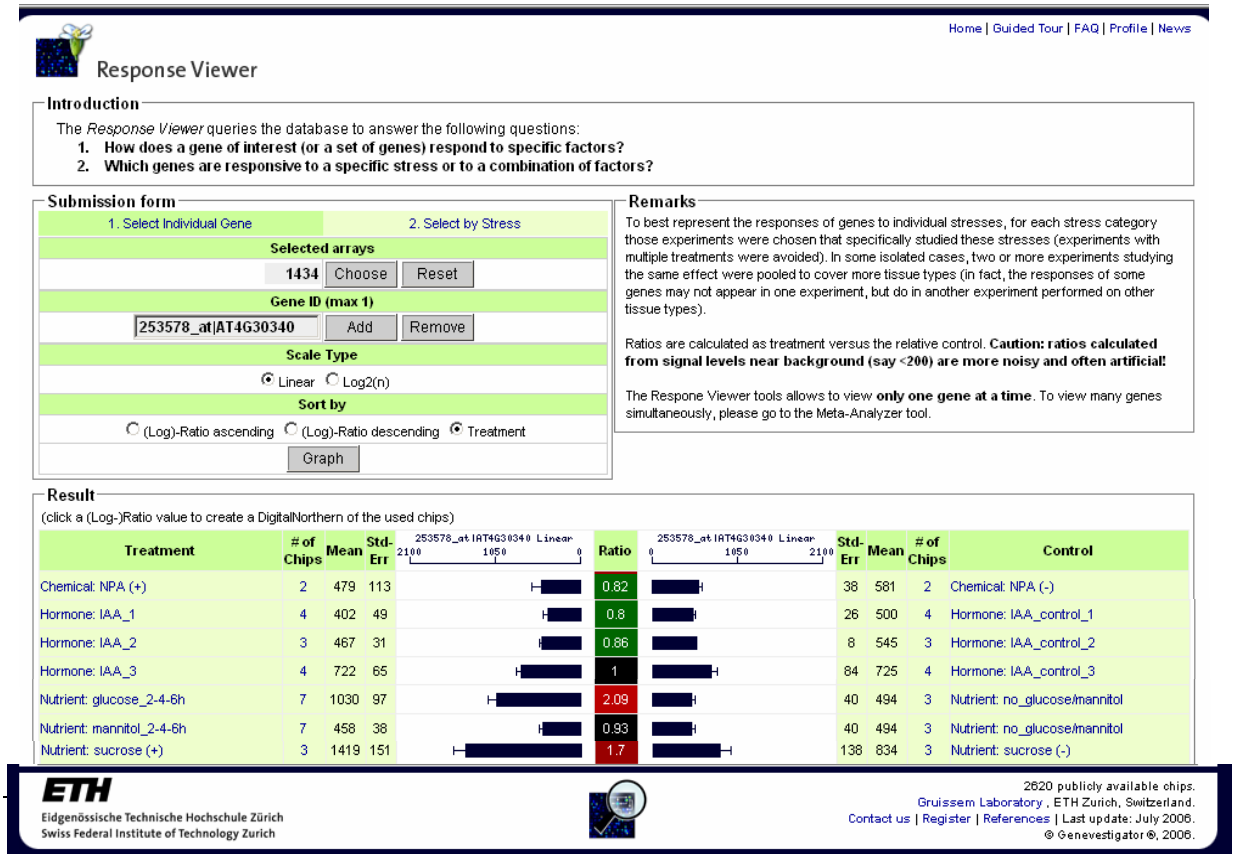


Figure 9. Representation of *AtDGK7* expression profile (Genevestigator)

Modified from the Genevestigator webpage (https://www.genevestigator.ethz.ch/at/index.php?page=5; Gruissem Laboratory, ETH Zurich, Switzerland.). Information regarding *AtDGK7* gene expression was extracted for two types of experiments, i.e. responses to sugar and to auxin/auxin transport inhibitors, respectively.

The following results were obtained for the first type of experiments (**Table 2**):

- A NASC Array Experiment (Reference number: NASCARRAYS-103) tested for genes that respond to N-deficiency and N-resupply. *AtDGK7* expression level was found to be repressed under N deprivation.
- *Arabidopsis thaliana* seedlings were grown for four days in the dark without added sucrose. Samples were subsequently kept for 6h either under aerobic conditions, under anoxia in the absence of sucrose, or under anoxia in the presence of sucrose (Gonzali et al., 2005). *AtDGK7* expression was reduced under both stresses; no reference to control samples grown in normal MS medium was provided.
- Experiments were undertaken to investigate some of the mechanisms that may function to regulate lignin biosynthesis (lignification) in *Arabidopsis thaliana*. In two independent experiments, six day-old, dark-grown seedlings were subjected to transcriptome analysis using Affymetrix GeneChip microarrays. The transcript abundance found in wild-type plants grown in the dark in the presence of sucrose was compared against that found in plants grown under identical conditions (including osmotic stress), but without sucrose (Rogers et al., 2005). The hybridized Affymetrix GeneChips showed that the *AtDGK7* expression was down-regulated.
- Data regarding the effect of osmotic stress on *AtDGK7* expression level were retrieved via accession number ME00327 (the study is part of the AtgenExpress project) from the TAIR (http://www.arabidopsis.org) project. Shoot and root tissue from 18 day-old *A. thaliana* wild-type plants were analyzed separately. The material was harvested at the following time points: 0,5h; 3h; 6h; 12h; 24h. For each time point two samples serving as replicates were collected. Control samples were collected from non-treated plants at the respective time points. The *AtDGK7* expression was down-regulated after 3h, 6h and 12h of stress treatment and after 6h in shoots.

Regarding the effect of auxin and auxin transport inhibitor the following results were obtained (**Table 3**):

- Seven day-old seedlings of wild-type Col-0 were treated with 1.0μM IAA for 30 min, 1h and 3h (TAIR accession number ME00336). *AtDGK7* expression was slightly down-regulated after 3h treatment but was not affected at the 0.5h and 1h time point.
- For the root auxin sensitivity assay *Arabidopsis thaliana* four day-old seedlings grown in light were transferred to vertically oriented agar plates containing 5μM IAA or EtOH for 2 h at 24 °C (Okushima et al., 2005). The *AtDGK7* transcription level did not change under these conditions.
- Wild-type Col-0 seven day-old seedlings grown in liquid culture were subjected to the treatments for 1h with 5μM IAA and DMSO in control experiments (Armstrong et al., 2004). *AtDGK7* transcription level did not change under these conditions.
- For auxin induction experiments from 6 week-old wild-type Col-0 plants, flower bunches containing flowers from stage 1 to stage 14 were kept (untreated control) or sprayed with 10 μM IAA (in 1% methanol, 0.05% Tween-20) or with buffer alone (mock) (Nagpal et al., 2005). *AtDGK7* expression was down-regulated in IAA and mock treated samples as compared to values obtained for the untreated controls.
- Arabidopsis seedlings (Col-0) were grown in suspension in MS medium; after 10-12 days the seedlings were treated by adding IAA (0.1 or 1.0μM) to each flask; plant material was harvested after 1 or 3h incubation. Controls were not treated and harvested at 0h (Redman et al., 2004). *AtDGK7* was slightly down-regulated at higher IAA concentration.
- Experiment ME00358 deposited at TAIR used seven day-old seedlings that were treated with auxin inhibitors for 3h; after this time whole plants were harvested. The four chemical treatments were 10μM TIBA, 10μM p-chlorophenoxyisobutyric acid, 10μM 2,4,6-trihydroxybenzamide and 10μM NPA; DMSO was used as a control. Gene expression for *AtDGK7* was only slightly affected in all treatments.

Table 2. *AtDGK7* transcript abundance: Nutritional stress conditions were tested.

Tissue	Age plants (weeks)	Treatment	Signal	P value	Regulation		Source
					up	down	
Whole root system	4-5	8h light/16h dark, MS normal	60,23			●	https://www.genevestigator.ethz.ch/
		8h light/16h dark, MS (-)N	24,75	n.s.			
Whole plant	4 days	Dark, MS (-) sugars, 6h aerobic conditions	154,45	n.s.			(Gonzali et al., 2005)
		Dark, 6h anoxia, MS (-) sugars	130,30			●	
		Dark, 6h anoxia, MS normal	111,50	n.s.		●	
Whole plant	1	Control, dark, MS normal	141, 86	n.s.		●	(Rogers et al., 2005)
		Dark, MS + no sucrose	83,43	n.s.			
Root	2	Control, 0.5h	71,00	n.s.	-	-	https://www.genevestigator.ethz.ch/
		Mannitol stress 0.5h	70,00				
		Control, 3h	71,15	n.s.		●	
		Mannitol stress 3h	50,20	n.s.			
		Control 6h	74,35	n.s.		●	
		Mannitol stress 6h	42,50				
		Control 12h	72,20			●	
		Mannitol stress 12h	48,05	n.s.			
		Control 24h	60,55	n.s.	-	-	
		Mannitol stress 24h	62,35	n.s.			
Shoot		Control, 0.5h	102,90	n.s.	-	-	
		Mannitol stress 0.5h	98,35	n.s.			
		Control, 3h	91,90	n.s.	●		
		Mannitol stress 3h	111,65	n.s.			
		Control 6h	93,25			●	
		Mannitol stress 6h	76,00	n.s.			
		Control 12h	108,60		-	-	
		Mannitol stress 12h	116,20	n.s.			
		Control 24h	101,80		-	-	
		Mannitol stress 24h	95,70				

Data were obtained through the Genevestigator database from transcript profiling experiments performed with Affymetrix GeneChip (8K and ATH1) arrays.

Table 3. *AtDGK7* transcript abundance data: Response to auxin and auxin transport inhibitors.

Tissue	Age plants (weeks)	Treatment	Signal	P value	Regulation		Source
					up	down	
Whole plant	1	Control, 0.5h	90,55	n.s.	●		https://www.genevestigator.ethz.ch/
		1.0µM IAA, 0.5h	99,95	n.s.			
		Control, 1h	100,95	n.s.	-	-	
		1.0µM IAA, 1h	95,50	n.s.			
		Control, 3h	99,15	n.s.		●	
		1.0µM IAA, 3h	65,20				
Whole plant	1	Control, EtOH 2h	6,97		-	-	(Okushima et al., 2005)d}
		5µM IAA + EtOH, 2h	6,63				
Whole plant	1	Control, DMSO 1h	8,01		-	-	(Armstrong et al., 2004)d}
		5µM IAA, 1h	8,07				
Flowers	6	control - untreated	523,90	*			(Nagpal et al., 2005)d}
		10µM IAA - Methanol 1% + 0.05% Tween, 0.5h	406,20	*		●	
		Methanol 1% + 0.05% Tween, 0.5h	433,50	*		●	
Whole plant	2	control - untreated	272,03	n.s.			(Redman et al., 2004)d}
		0.1µM IAA, 1h	292,80	n.s.	●		
		0.1µM IAA, 3h	290,90	n.s.	●		
		1.0µM IAA, 1h	220,60	n.s.		●	
		1.0µM IAA, 3h	168,50			●	
Whole plant	1	control - untreated	116,15	n.s.			https://www.genevestigator.ethz.ch/
		10µM TIBA, 3h	88,20			●	
		10µM NPA, 3h	95,85	n.s.		●	
		10µM 2,4,6-trihydroxybenzamide, 3h	83,95			●	
		10µM p-chlorophenoxyisobutyric acid, 3h	96,00			●	

Data were obtained through the Genevestigator database from transcript profiling experiments performed with Affymetrix GeneChip (8K and ATH1) arrays.

3.3.2.5. Summary

Summarizing the analysis of transgenic *A. thaliana* plants harboring an *AtDGK7* promoter-GUS fusion construct led to the following conclusions:

- The *AtDGK7* gene is highly regulated during plant development. The gene is preferentially expressed in stipules, petioles, edges of cotyledons and younger leaves; strong transcription is also visible in hypocotyls, root tips and tips of lateral roots when grown on standard media.
- When seedlings were grown on media with reduced nitrogen they developed a shorter primary root and a reduced number of lateral roots; under these conditions GUS activity was detected in hypocotyls, roots, the vascular tissue of leaves and in root tips, but it was absent from the shoot meristem, cotyledons and leaves.
- On low-nitrogen media supplemented with 100nM NAA seedlings exhibited reduced root development. GUS activity was detected in the vascular tissue of roots but was absent from hypocotyls, cotyledons and root tips. In control experiments with NaOH, the root phenotype was similar, but GUS activity was observed in the vascular tissue of hypocotyls and roots, shoot meristems and cotyledons.
- On standard medium supplemented with low NAA concentration [1.0nM], the GUS staining pattern was similar to the pattern observed on standard medium in the absence of NAA (activity in cotyledons, leaf edges and root tips), but lateral root number was substantially reduced in auxin treated seedlings.
- Higher NAA concentrations (50 and 200nM) did not markedly influence the GUS activity pattern in cotyledons, leaves and lateral roots tips; however, the number of lateral roots was increased, but root length was reduced at 50nM NAA. At 200nM NAA a strong reduction in the length of lateral roots and primary roots was observed.
- Seedlings grown on standard medium supplemented with 20µM TIBA generated short hypocotyls and lateral roots were almost totally absent. Weak GUS staining was observed in the root epidermis and leaf margins.
- Seedlings grown on nitrogen-limited medium supplemented with TIBA were strongly impaired in growth; GUS activity was strongest in hypocotyls, cotyledons and stipules.

3.3.3 AtDGK7 T-DNA insertion lines

3.3.3.1 Identification of AtDGK7 T-DNA insertion lines

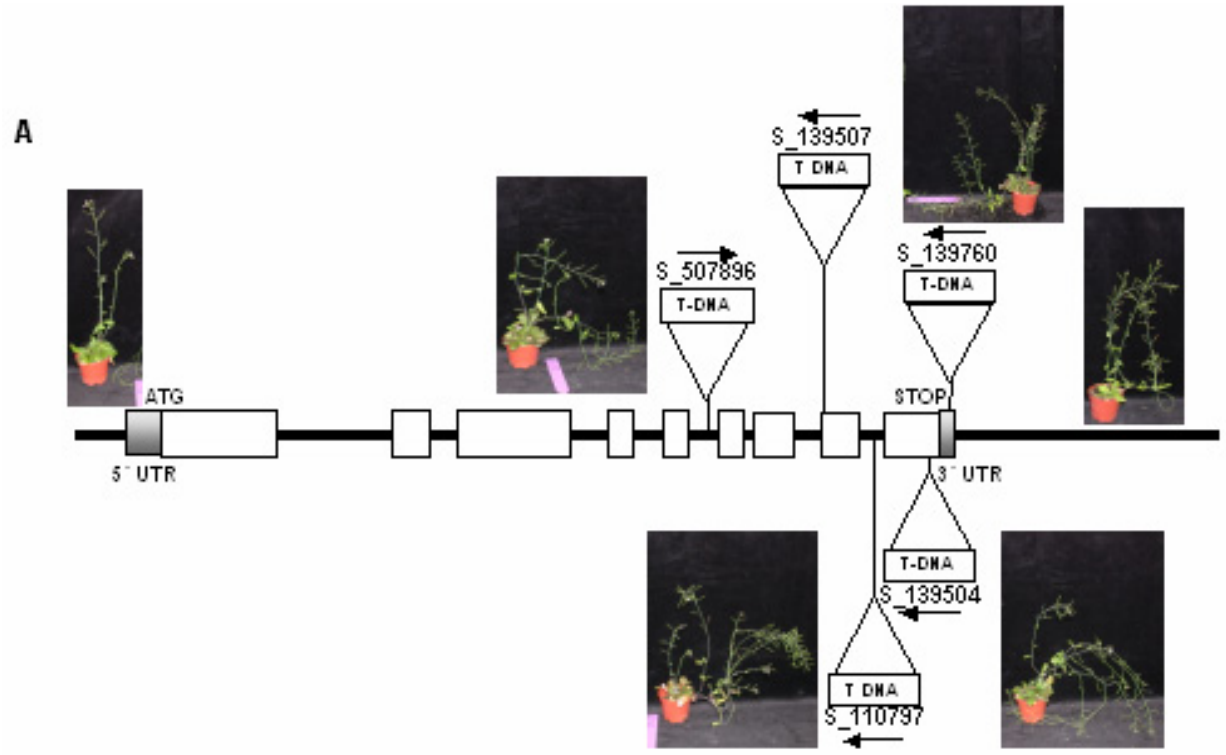
To elucidate the physiological function of *Arabidopsis* diacylglycerol kinase 7, T-DNA insertion mutants were identified by screening the database of the Salk Institute Genomic Analysis Laboratory using the *AtDGK7* cDNA sequence as a bait. Five mutant lines were available and ordered from the Salk Institute. The seedlings were grown on MS medium containing kanamycin for selection and subsequent propagation. Leaves were selected for the isolation of genomic DNA to confirm the presence of the T-DNA insertion in the *AtDGK7* gene in its homozygous or heterozygous state using PCR (for conditions see "Material and Methods - *Isolation of AtDGK7 T-DNA insertion lines*"). Using the information available from the database of the Salk Institute the five mutant lines selected showed the insertion of the T-DNA at the positions listed in **Table 4**. The sequences of the *AtDGK7* gene flanking the T-DNA insertions were used to exactly locate the insertions using bacterial artificial chromosome (BAC) clone **F17I23**. Additionally, a PCR experiment was performed to test whether the mutant lines were homozygous or heterozygous with respect to the T-DNA insertion.

Table 4. Location of T-DNA insertions in the *AtDGK7* gene.

Line	Coordinates (5' - 3')*	Orientation (T-DNA insertion)	PCR fragment size expected (kb)
At4g30340 (wild-type)	11504 - 11272	-	3.4
Salk 110797	11172 – 11007	Reverse	1.0
Salk 139504	11779 – 11044	Reverse	0.8
Salk 507896	11134 - 11374	Forward	1.2
Salk 139507	11123 - 11048	Reverse	1.0
Salk 139760	11274 - 11705	Reverse	0.7

* The bacterial artificial chromosome clone F17I23 (NCBI database accession number F17I23, http://www.ncbi.nlm.nih.gov/) has a size of 134.784bp and carries 37 loci, including the At4g30340 gene (http://www.tigr.org/tigr-scripts/euk_manatee/BacAnnotationPage.cgi).

Using the information of the Salk database the position of the T-DNA insertions were located in relation to the annotated sequence of the *AtDGK7* gene (At4g30340). **Figure 10** shows the exact position of each insertion.



B

Salk line	Whole T-DNA insertion (bp)	Sequence shared	Size sequence shared (bp)
507896	441	1037 AGGGGACTAA - - - - - -TTTAGAGAAG 1113 23 AGGGGACTAA - - - - - -TTTAGAGAAG 110	111
139507	227	1229 ATGACGGACT - - - - - -TGGCTGAAAT 1295 111 ATGACGGATT - - - - - -TGGCTGAAAT 102	77
110797	131	1111 TTGTGGAGGC - - - - - -CTAAGCACAT 1313 131 TTGTGAAGGC - - - - - -GTAAGCACAT 33	102
139504	375	1112 AGAGAGGATT - - - - - -ATTGCTCAGG 1311 142 AGGCGGCTGC - - - - - -TTCAATCGTT 1	141
139760	432	1434 GATAAAAAAG - - - - - -TAGTCAAAAT 1574 432 GATAAAAAAG - - - - - -TAGTCAAAAT 302	130

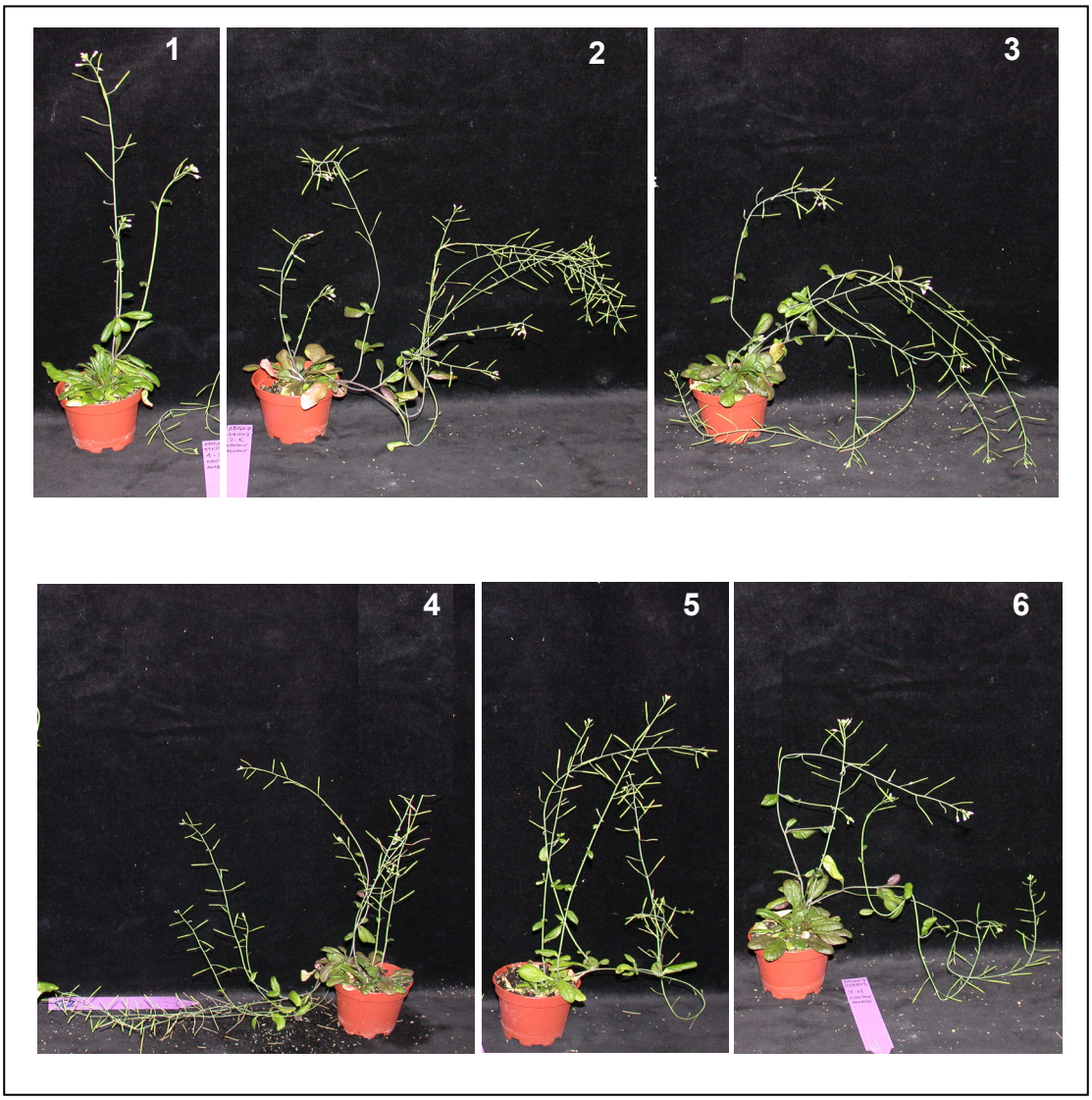
Figure 10. Schematic diagram of the *AtDGK7* gene (locus At4g30340) providing information about the positions of the T-DNA insertions.

A. The orientations of the T-DNA insertions are indicated and also the positions of T-DNA insertions in the genome (deduced from sequence comparisons done with BLAST searches). Only the Salk line 139760 carries a T-DNA insertion in the 3' UTR region; all other mutants have the insertions within the *AtDGK7*. All mutants were produced by the Salk Institute Genomic Analysis Laboratory (SIGnAL). B. The columns in the table summarizes: the size of the T-DNA insertion fragment for each mutant line, between the wild-type gene (first row) and the mutant lines (second row), the shared sequence and their size.

3.3.3.2 Phenotypic description of the AtDGK7 mutant lines

The seeds of the mutant lines were germinated on MS medium supplemented with kanamycin for selection and after four weeks transferred to soil. After 70 days of growth the mutants were analysed for phenotypic changes compared to the wild-type plants (Col-0). All mutants showed similar growth patterns with a large number of shoots that grew tall and exceeded the height of the wild-type plants (between 1.5 and 3 times) and also all of them had the tendency to bent downwards (**Figure 11A**). The number of siliques was increased by 2-4 times in the mutants compared to the wild-type, and at the time point of data acquisition the number of flowers present was also increased in the mutant lines by around 30% on average (**Figure 11B**). In contrast, the number of leaves was slightly greater in wild-type plants in comparison to the mutants.

A



B

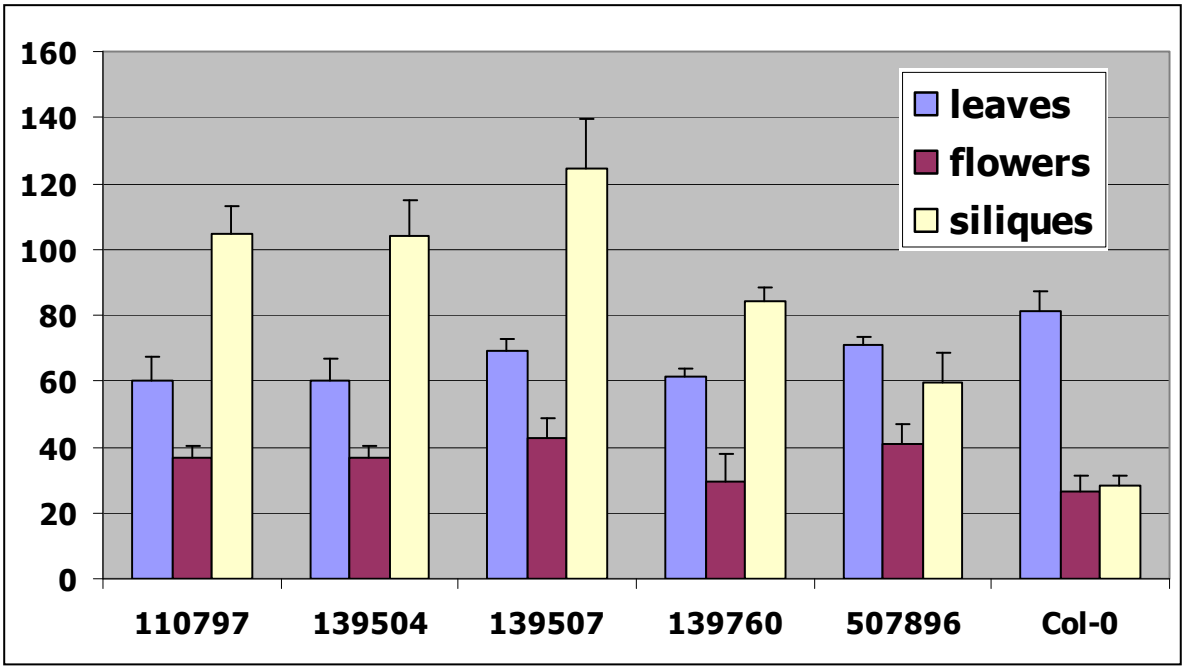


Figure 11. Phenotypic analysis of *AtDGK7* T-DNA insertion lines.

A. 1. Wild-type Col-0; 2. Salk 110797; 3. Salk 139504; . Salk 139507; 5. Salk 139760; 6. Salk 507896. Pictures were taken from mature homozygous plants (70 days old). An interesting characteristic of the mutants was their fall down phenotype because of stem branching. **B.** Wild-type plants produced slightly more leaves than all T-DNA insertion lines. In contrast, the number of flowers and siliques was increased in the mutant lines. Measurements were done using mature plants (70 days old). Plants were grown under controlled environmental conditions in growth chambers. Twenty-eight days after germination, plants were kept under 250 microeinsteins m^{-2} s^{-1}, 20/18 °C, 80/50% relative humidity (day/night). SD bars are shown; n = 15

3.3.4. Physiological characterisation of the AtDGK7 mutant lines

The *promAtDGK7::GUS* experiments showed a strong expression of the *AtDGK7* gene in the tip of lateral roots. Having this information and searching a potential physiological role of the *AtDGK7* gene, I screened the *Arabidopsis AtDGK7* T-DNA insertion lines for obvious root phenotypes using various growth media. I also employed growth conditions reported by (Malamy and Ryan, 2001) that allow lateral root formation to occur in a reliable and consistent manner; when *Arabidopsis* seedlings were grown on nutrient media with a high sucrose to nitrogen ratio, they showed that lateral root initiation and number were dramatically repressed.

For all growth experiments the seedlings were grown for 2 weeks on standard MS medium (4.5% sucrose, 60mM nitrogen) and subsequently transferred to media with different sucrose and/or nitrogen concentrations which in some studies were supplemented with auxin or the auxin transport inhibitor TIBA (2,3,5-triiodobenzoic acid). The plants were analysed for root length and lateral root formation after 3 and 9 days of growth on the various media.

3.3.4.1. Phenotypic effects of growth media on AtDGK7 mutant lines

3.3.4.1.1. Variation of the sucrose-to-nitrogen ratio

To investigate the influence of nitrogen and sucrose supply on the phenotype of *Arabidopsis thaliana* (Col-0) wild-type and *AtDGK7* T-DNA insertion lines, all plants were examined under standard growth conditions (i.e., 4.5% sucrose, 60mM nitrogen) as well as in the presence of altered sucrose and/or nitrogen concentrations. Seedlings were germinated and grown for two weeks on standard growth medium and were subsequently transferred to modified growth media to be evaluated after two additional weeks of growth.

Summarizing the results all *AtDGK7* mutant seedlings showed the same strong inhibition of growth, in comparison with the WT, on all media tested especially with respect to the number and formation of lateral roots (**Table 5 – Appendix D**). In general the Salk lines 110797 and 507896 exhibited the strongest effect compared to the other lines and 139760 showed a phenotype similar to the wild-type (**Figure 12**). The number of lateral roots was drastically reduced in all mutant lines and the strongest effect was observed in the Salk line 110797 (the number of lateral roots was 7% of that of the Col-0 wild-type; **figure 12**). Auxin application appears to be a key factor that overcomes the phenotype in the *AtDGK7* mutant lines.

3.3.4.1.2. Effect of auxin (naphthalene acetic acid, NAA) on growth of mutant Arabidopsis seedlings under standard and/or reduced nitrogen conditions

To determine the effect of NAA supply on lateral root (LR) initiation in primary roots in *Arabidopsis thaliana* (Col-0) wild-type and *AtDGK7* T-DNA insertion lines seedlings were germinated and grown for two weeks on standard growth media (i.e., 4.5% sucrose, 60mM nitrogen); subsequently all the seedlings were transferred on different modified Murashige and Skoog media containing 0.02mM nitrogen plus 100nM NAA, or 60mM nitrogen complemented with different auxin concentrations: 1.0, 50.0 and 200nM (**Table 6 - Appendix E**). I used 0.1N NaOH to dissolve NAA; NaOH was used in control experiments.

The addition of 1.0 and 200nM NAA led to a very strong growth reduction in all seedlings treated (**Figure 13F** and **G**); also the application of excess NAA under low nitrogen conditions led to a strong inhibition of seedling development (**Figure 13F**).

A more interesting result was observed in the presence of 50nM NAA which allowed normal growth of all mutant lines like wild-type under standard conditions (**Figure 13C**, all the lines).

Figure 12. Phenotypes of *Arabidopsis thaliana* Col-0 wild-type and *AtDGK7* mutant seedlings grown on standard growth media and media containing altered sucrose and/or nitrogen concentrations.

After two weeks on standard growth medium the seedlings were transferred and grown for two additional weeks on: A. Standard growth medium (i.e., 4.5% sucrose, 60mM nitrogen). B. Reduced nitrogen concentration (i.e., 4.5% sucrose, 0.02mM nitrogen). C. Reduced sucrose and nitrogen growth medium (i.e., 0.5% sucrose, 0.02mM nitrogen). D. High sucrose (i.e., 10% sucrose, 60mM nitrogen); and E. Higher sucrose (i.e., 12% sucrose, 60mM nitrogen). Scale bar, 10 mm.

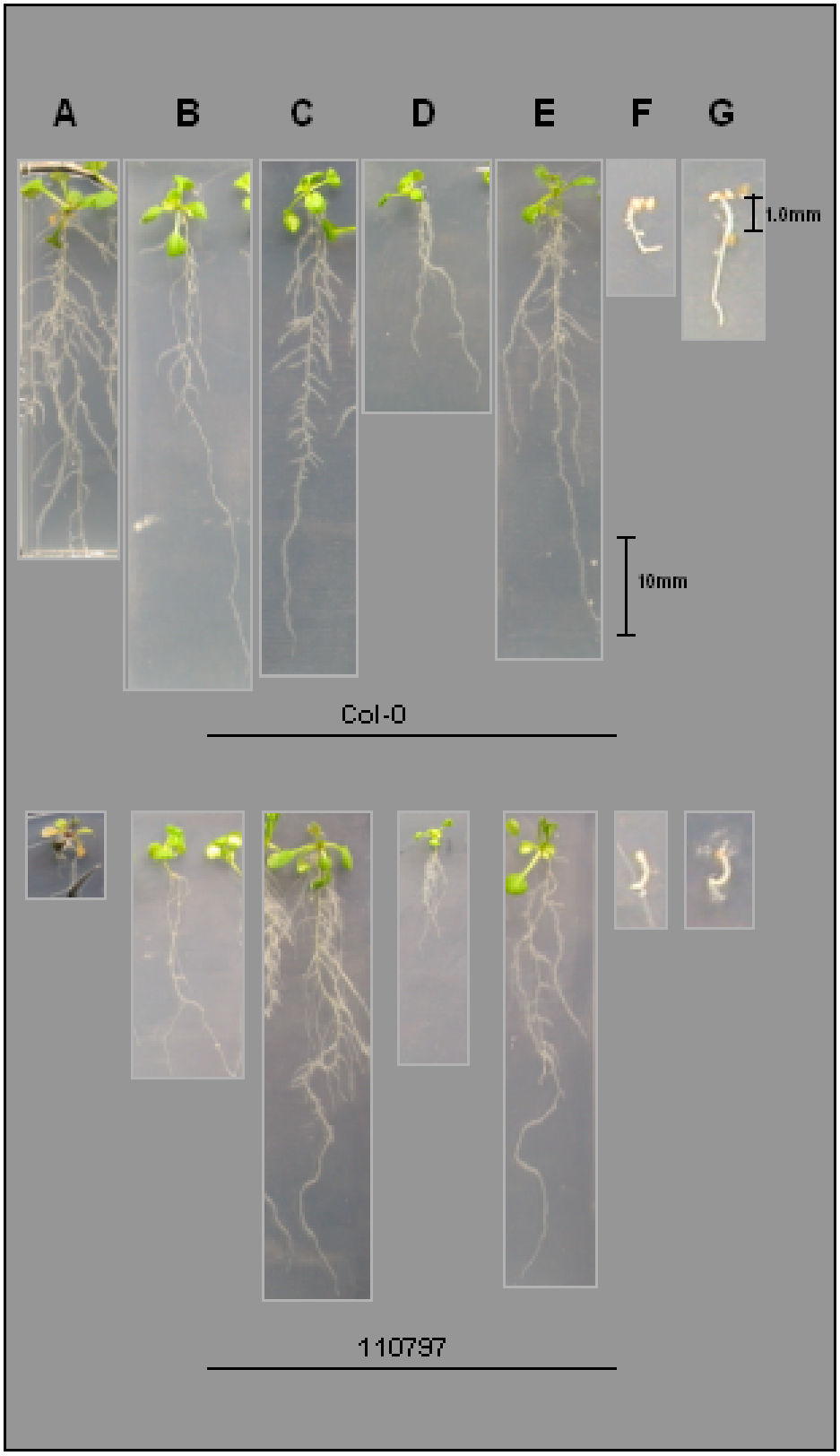


Figure 13. Continues on the following page.

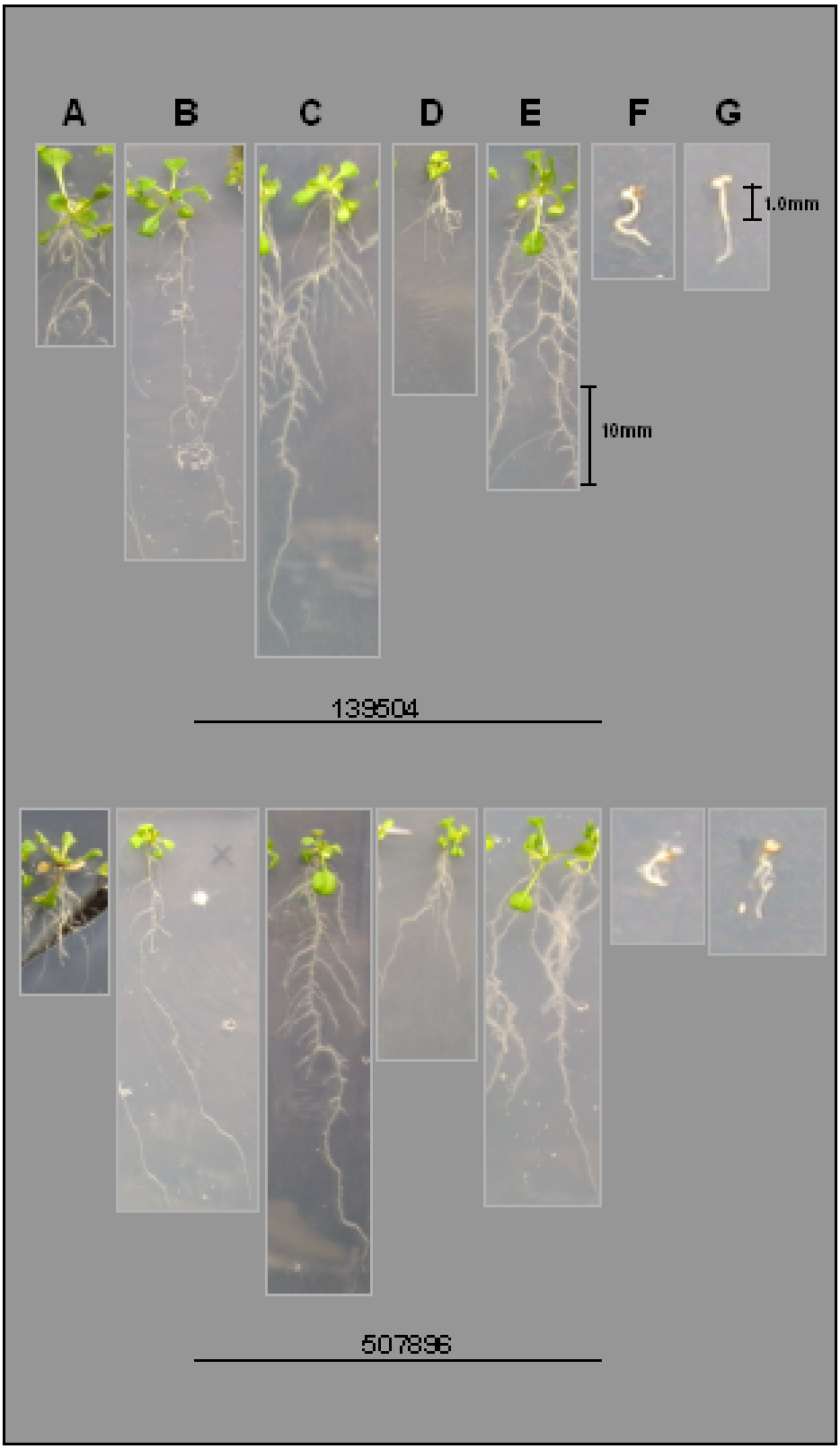


Figure 13. Continues on the following page.

Figure 13. Phenotypic analysis of *A. thaliana* (Col-0) wild-type and T-DNA insertion lines grown on different media containing standard or altered nitrogen concentrations plus auxin.

Seedlings were grown on vertical agar plates containing: A. Standard growth medium (i.e, 4.5% sucrose 60mM nitrogen); B. Standard growth medium, plus 1.0nM NAA; C. Standard growth medium, plus 50nM NAA; D. Standard growth medium, plus 200nM NAA; E. Standard growth medium, plus 100mM NaOH (NAA solvent);.F. Reduced nitrogen growth medium (0.02mM) with standard sucrose (4.5%) plus 100nM NAA; and, G. Reduced nitrogen and standard sucrose growth medium with 100mM NaOH, instead of NAA. Photographs of representative seedlings from each treatment are shown. The 10mm bar applies for A to E, and the 1.0mm bar applies to F and G.

3.3.4.1.3. The effect of the auxin transport inhibitor TIBA

To evaluate the consequences of the auxin transport inhibitor TIBA on lateral root initiation and lateral root growth, *Arabidopsis thaliana* (Col-0) wild-type and *AtDGK7* T-DNA insertion seedlings were grown for up to five days on standard media (i.e., 4.5% sucrose, 60mM nitrogen) and transferred to reduced nitrogen growth media (i.e., 0.02mM) contain DMSO alone or the auxin transport inhibitor TIBA (20μM) dissolved in DMSO; in parallel, experiments with standard growth media, the same inhibitory and control conditions were done. At day 12, the lateral roots and lateral root primordia produced in the new growth media were analyzed.

- After 5 DAG in standard growth medium *Arabidopsis* wild-type Col-0 and mutant *AtDGK7* seedlings were transferred and incubated in three different vertical growth media plates: standard growth medium (**Figure 14A**), medium supplemented with 20μM TIBA (**Figure 14B**), and medium containing 1.0% DMSO as TIBA's solvent (**Figure 14C**). The phenotype of seedlings grown on standard growth media was described already (see "*3.3.3.1.1. Variation of the sucrose-to-nitrogen ratio*" and "*3.3.3.1.2. Effect of the auxin (naphthalene acetic acid, NAA), on growth of mutant Arabidopsis seedlings under standard and/or reduced nitrogen media conditions*"). The inhibitory experiments (20μM TIBA) showed a slower growth of the wild-type Col-0 and mutant seedlings and their sizes were small in general (**Figure 14B**, all lines). In addition to the stunted roots, the cotyledons and leaves of the seedlings were tiny, but they remained green. Additionally, in many cases, their primary roots had a weak growth orientation often changing the way. Although the control experiments (with DMSO) exhibited a non-identical seedling phenotype to those grown on standard growth medium, their appearances were similar (**Figure 14A** and **C**, all lines).

Figure 14. The *A. thaliana* (Col-0) wild-type and *AtDGK7* mutant seedlings were sensitive to the auxin transport inhibitor TIBA.

For stress treatments on MS plates, seedlings were first grown on vertical plates on standard growth medium (4.5% sucrose, 60mM nitrogen) for 5 days before being transferred to the treatment plates: **A**. Standard growth medium; **B**. Standard growth medium supplemented with 20μM TIBA; and **C**. plus 1.0% DMSO as TIBA's solvent. The pictures were taken two weeks after the transfer. The 10mm bar applies to **A**, **B** and **C**.

- **Figure 15** shows the strong inhibition of growth induced by nutrient limitation (in this case, the nitrogen concentration was 0.02mM). The seedlings were transferred to inhibitory nitrogen growth media supplemented with auxin transport inhibitor TIBA

(**Figure 15A**) and the solvent, DMSO, as a control (**Figure 15B**). Note the stunted phenotype in all seedlings (including the wild-type Col-0) two weeks after of transfer to low-nitrogen medium, which led to a reduction of seedling and cotyledon size, the absence of visible primary leaves and root hairs. The seedlings were completely pale.

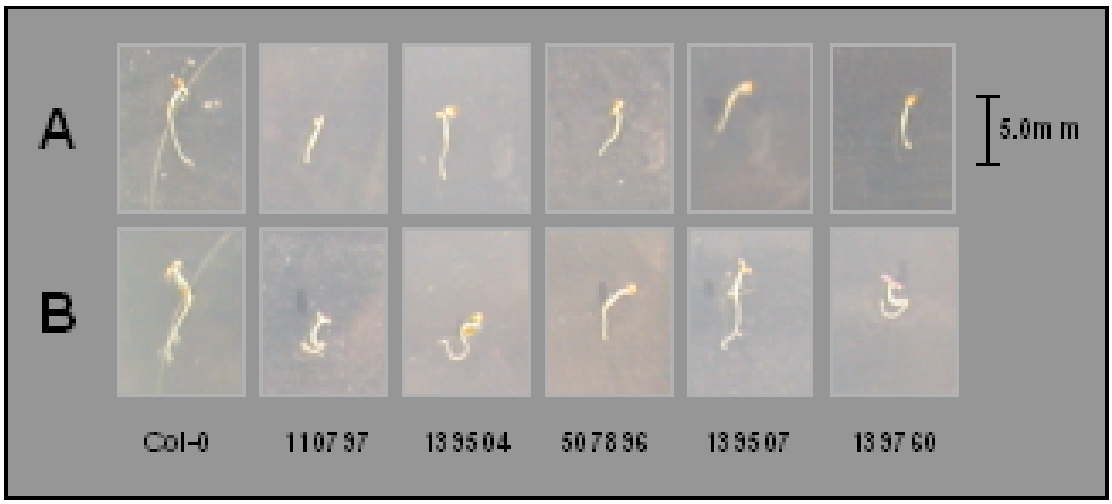


Figure 15. Phenotypic analysis of *A. thaliana* (Col-0) wild-type and *AtDGK7* mutant seedlings grown on altered nitrogen concentration plus the auxin transport inhibitor TIBA.

Effect of TIBA (A) and the solvent DMSO (B), in low nitrogen medium (i.e., 0.02mM), on *A. thaliana* (Col-0) wild-type and *AtDGK7* T-DNA insertion seedlings are displayed. Plants were grown for 5 d on agar medium containing standard concentrations of sucrose and nitrogen (i.e., 4.5% and 60mM, respectively) and subsequently they were transferred to low nitrogen medium, supplemented with auxin transport inhibitor TIBA (i.e., 20μM) and the solvent DMSO (i.e., 1.0%), as a control as indicated in the text above. In general, the response of the seedlings to the treatment was a strong inhibition of germination due to the low nitrogen concentration. The 10mm bar applies to A, B and C.

3.3.4.1.4. AtDGK7 T-DNA mutants exhibit increased sensitivity to the effect of nitrogen and carbon availability

For all growth experiments the seedlings were grown for two weeks on standard MS medium and were subsequently transferred to media with different sucrose and/or nitrogen concentrations. The plants were analysed for root length and lateral root (LR) formation after 3 and 9 days of growth on the various media. All wild-type plants reacted to low nitrogen and high sucrose concentration with the same change of the growth phenotype, i.e. root growth was almost completely inhibited, whereas under standard conditions root length increased by around 100% between days 3 to 9 (**Figure 16**). Under standard conditions the *AtDGK7* mutant lines exhibited a strong phenotype. Root growth was strongly inhibited in all T-DNA insertion lines with the exception of line Salk 139760 (**Figure 16,** Salk 139760) and the line Salk 507896 (**Figure 16,** Salk 507896). The strongest phenotype was observed in line Salk 110797 which exhibited only 10% of root length compared to wild-type measured 3 days after the transfer and did not continue to grow subsequently (**Figure 16,** Salk 110797). Under the stress conditions at low nitrogen and high sucrose concentration all mutant lines showed the same inhibition of root growth like the wild-type plants.

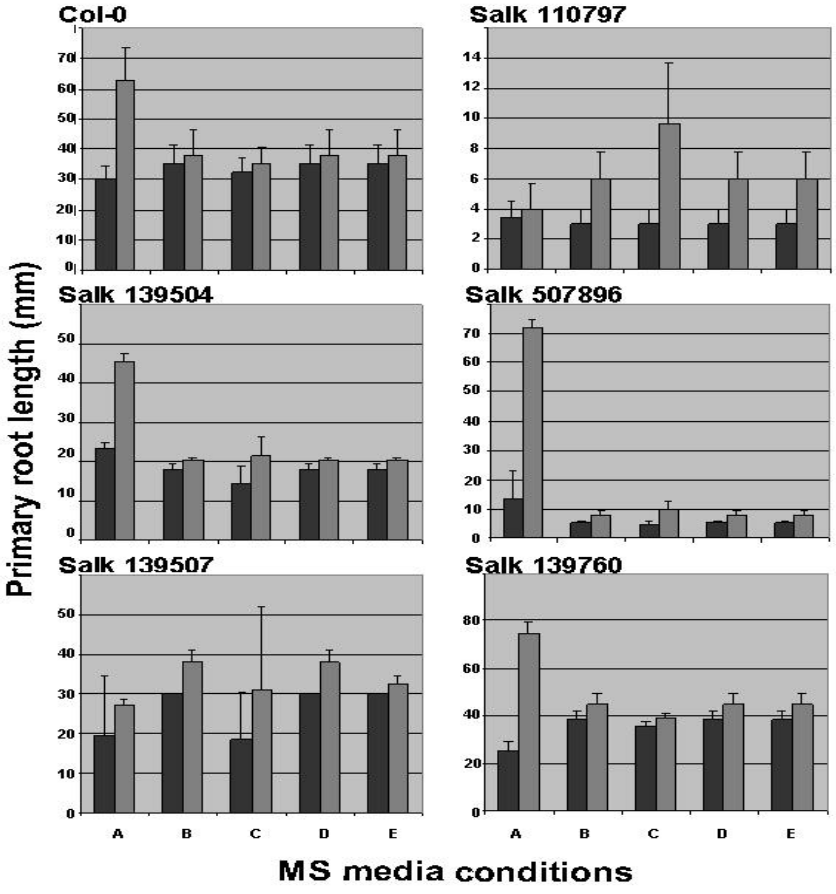


Figure 16. Quantitative analysis of the effect of standard or altered concentrations of nitrogen and sucrose on primary root length in seedlings.

Arabidopsis Col-0 and *AtDGK7* mutant seedlings were grown in the presence of various nitrogen and sucrose concentrations; the length of the primary root was measured 3 days (dark grey columns) and 9 days (light grey columns) after the transfer from standard growth medium (4.5% sucrose, 60mM nitrogen) to the other media. After growth for 15 days on standard growth medium, seedlings were transferred to the following media: **A.** 4.5% sucrose, 60mM nitrogen; **B.** 4.5% sucrose 0.02mM nitrogen; **C.** 0.5% sucrose 0.02mM nitrogen; **D.** 10% sucrose 60mM nitrogen; and, **E.** 12% sucrose 60mM nitrogen. Bars represent means +/-; *n* = 10.

The number of the lateral roots was also analysed under these growth conditions. Under standard conditions the wild-type plants had developed 30 times more lateral roots after nine days compared to the situation after three days of transfer. Stress conditions led to a dramatic decrease in the number of lateral roots. Plants grown at low nitrogen had only three times more lateral roots at day 9 compared to day 3 and a high sucrose concentration (12%) inhibited the formation of lateral roots almost completely (**Figure 17,** Col-0). The mutant lines also started to form lateral roots after transfer to the various media, but in contrast to the wild-type plants all mutant lines produced a dramatically reduced number of lateral roots (**Figure 17**).

Under low nitrogen all mutant lines showed a performance that was similar to that of to the wild-type. The formation of lateral roots started after transfer to the low-nitrogen medium, and the total number of lateral roots was similar to that of the wild-type plants. Only line Salk 110797 exhibited a strong reduction in the formation of lateral roots (**Figure 17,** Salk 110797). High sucrose (12%) led to a complete inhibition of lateral root formation in lines Salk 110797 and 507896, but in lines Salk 139504 and 139760 the number of lateral roots formed 9 days after the transfer was higher than in wild-type plants compared to the number of lateral roots present 3 days after transfer. The rate of lateral root formation in wild-type increased by 20%, whereas it increased by ~300% in line Salk 139504 and by ~200% in line Salk 139760, respectively (**Figure 17,** 12% sucrose + 60mM nitrogen growth medium).

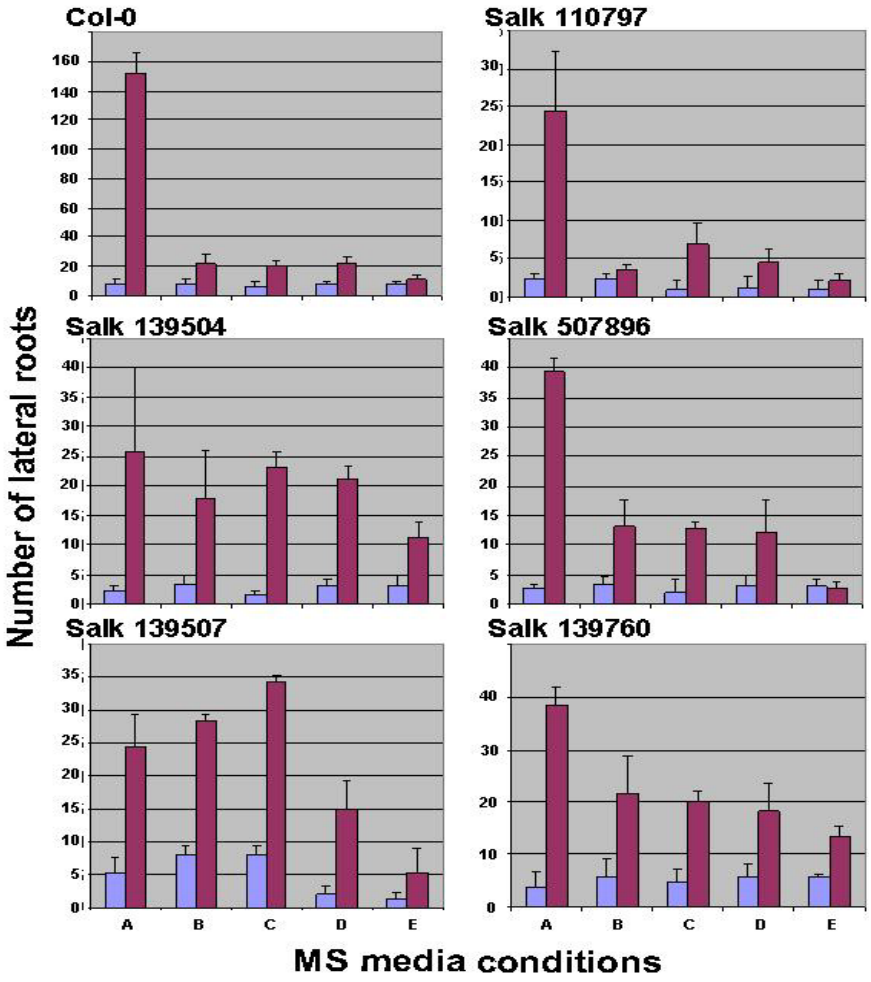


Figure 17. Quantitative analysis of the effect of standard or altered concentrations of nitrogen and sucrose on lateral root number in seedlings.

Arabidopsis Col-0 and *AtDGK7* mutant seedlings were grown in the presence of various nitrogen and sucrose concentrations; the number of lateral roots (LR) was measured 3 days (blue columns) and 9 days (red columns) after the transfer from standard growth medium (4.5% sucrose 60mM nitrogen) to the other media. After growth for 15 days on standard growth medium, seedlings were transferred to the following media: **A.** 4.5% sucrose 60mM nitrogen; **B.** 4.5% sucrose 0.02mM nitrogen; **C.** 0.5% sucrose 0.02mM nitrogen; **D.** 10% sucrose 60mM nitrogen; and, **E.** 12% sucrose 60mM nitrogen. Bars represent means +/-; *n* = 10.

3.3.4.1.5. AtDGK7 T-DNA mutants show positive lateral root response to the presence of 50nM auxin

The conditions and growth procedure for the analysis of length root and lateral roots (LR) number of wild-type and *AtDGK7* mutant seedlings under auxin treatment, NAA, were showed in M&M "*Analysis of root growth – variation of sucrose nitrogen, auxin and auxin transport inhibitor TIBA concentrations*". For all growth experiments the seedlings were grown for 2 weeks on standard MS medium (4.5% sucrose 60 mM nitrogen) and were subsequently transferred to standard media with different auxin concentrations or to medium depleted for nitrogen and containing NAA. All seedlings were analyzed for root length and lateral roots formation after 3 and 9 days of growth on the various media.

In the presence of auxin, the lenghts of the primary roots were similar in mutant and wild-type plants under normal growth conditions, excepting for Salk lines 507896 and 139760 (**Figure 18**); however, all mutant seedligs produced less lateral roots than the wild-type (**Figure 18**). At low nitrogen and high auxin, mutant lines and the wild-type showed the same inhibition of root growth; similar results were obtained in the control experiments, using the solvent NaOH instead of NAA (**Figure 18**). These results allow to assume that wild-type and mutant seedlings were more hypersensitive to very low nitrogen presence (0.02mM), than the presence of high NAA concentration. At 1.0nM NAA all mutant lines (with the excepction of Salk 110797 and 507896) and wild-type seedlings exhibited similar root lengths at days 3 and 9 when compared with seedlings kept on standard media. Coincidentally, the analysis with the seedlings grown under NaOH conditions (the NAA solvent) produced an identical result (**Figure 18,** Salk 110797 and 507896).

A further increase of the auxin concentration in the standard growth medium to 200nM NAA, between the 3rd day and 9th day, led to a significant decrease of the mean primary root length 1.5 to 3-times in all *AtDGK7* mutant seedlings and the wild-type; however, Salk 110797, showed a four-fold increase in the primary root length (**Figure 18,** Salk 110797).

The elongation of the primary root of *AtDGK7* mutants and wild-type in the 50nm NAA medium makes necessary a special comment. The rate of primary root growth for wild-type and Salk 507896 and 139760 lines, as was mentioned already, was very similar having a similar behaviour under standard media condtions. But in the case of Salk lines 139504 and 139507, the rate was 2 times and close to 3 times respectively; and significantly different, with around 20 times, for Salk 110797 seedlings.

In general terms, the availability for nitrogen affects root growth (Stitt and Krapp, 1999) and this was observed in *AtDGK7* mutant and wild-type seedlings. A slight increase in auxin concentration (1.0nM) did not markedly change the length of the primary roots, but high NAA concentration (200nM) strongly inhibited root growth. At 50nM NAA root growth was slightly induced in mutant and wild-type seedlings.

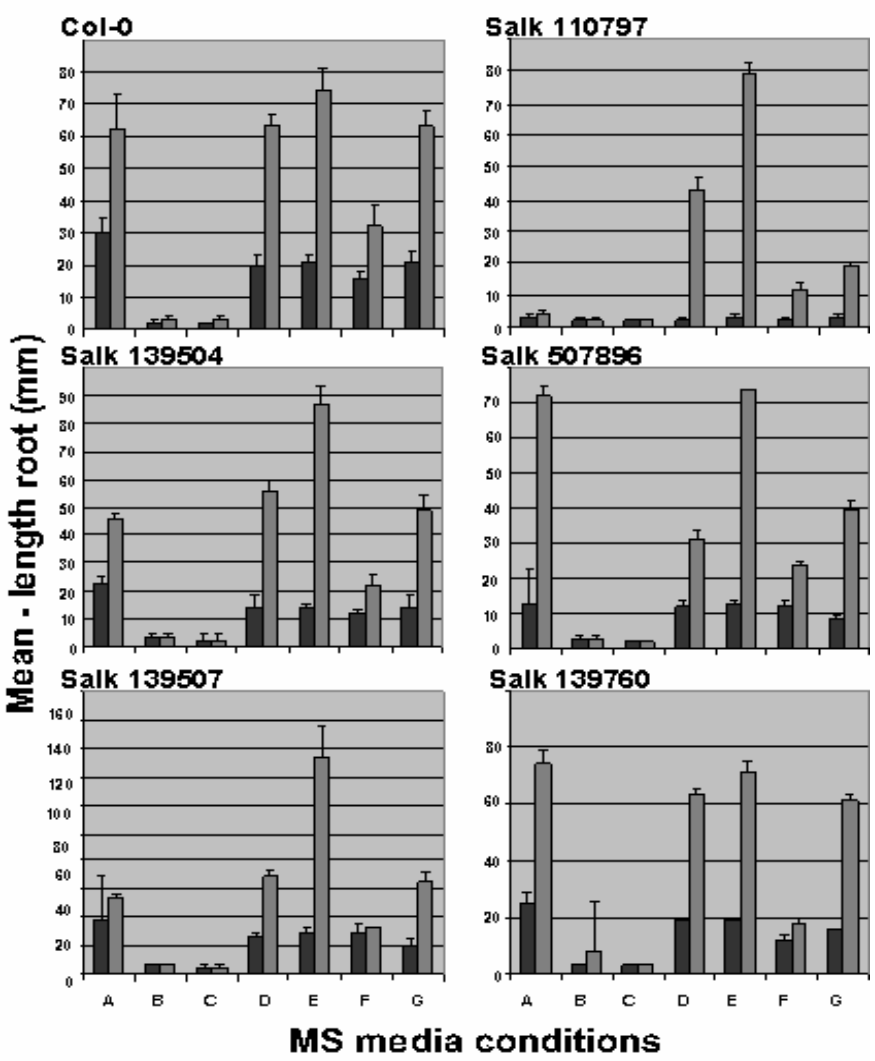


Figure 18. Primary root length of wild-type and *AtDGK7* mutant seedlings grown on media containing the auxin homologue NAA.

Arabidopsis Col-0 and *AtDGK7* mutant seedlings were grown in the presence of two nitrogen concentrations and supplemented with various NAA concentrations; the length of the primary root was measured after 3 days (dark grey columns) and 9 days (light grey columns) of transfer from standard growth medium (4.5% sucrose 60mM nitrogen). Grown during 15 days in standard growth media, seedlings were transferred to different standard or altered nitrogen concentrations and supplemented with different NAA concentrations: **A.** 4.5% sucrose 60mM nitrogen; **B.** 4.5% sucrose 0.02mM nitrogen + 100nM NAA; **C.** 4.5% sucrose 0.02mM nitrogen + 100mM NaOH; **D.** 4.5% sucrose 60mM nitrogen + 1.0nM NAA; **E.** 4.5% sucrose 60mM nitrogen + 50nM NAA; F. 4.5% sucrose 60mM nitrogen + 200nM NAA; and, G. 4.5% sucrose 60mM nitrogen + 100mM NaOH. Bars represent means +/-; *n* = 10.

In *AtDGK7* mutant lines grown on low nitrogen with 100nM NAA lateral root formation was almost absent. The same was observed in low nitrogen media without additional application of NAA, indicating the prominent effect of nitrogen limitation. A reduction of lateral formation was generally also seen at 200nM NAA, although the effect was not always strong in all the mutants analyzed (**Figure 19,** Salk lines 507896 and 139760). At 50nM all the *AtDGK7* T-DNA insertion lines and the wild-type produced an increased number of lateral roots compared to plants kept on standard growth medium. This result demonstrates that lateral

root formation is recovered in the mutant seedlings at intermediate NAA concentrations (**Figure 19,** treatment E in all lines).

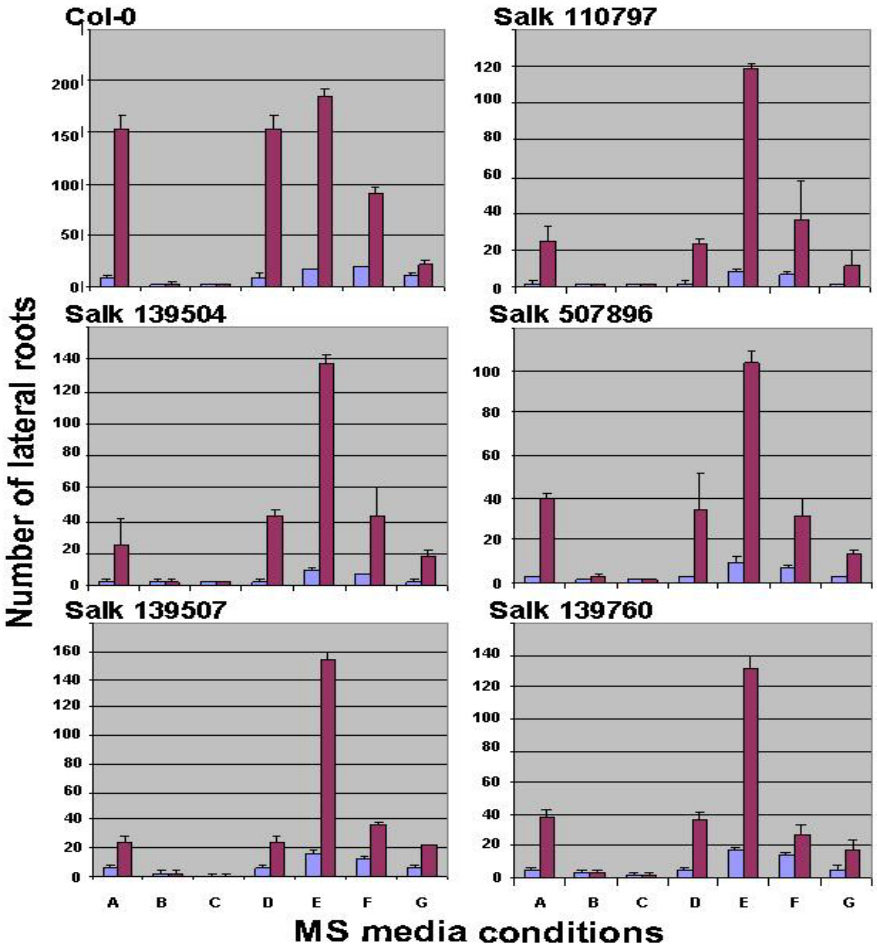


Figure 19. Quantitative analysis of LR phenotypes in wild-type (Col-0) and *AtDGK7* mutant seedlings at different auxin concentrations.

The number of lateral roots per primary root was determined. At least 10 measurements were made for each genotype and visible lateral roots were counted: after 3 days (purple columns) and 9 days (red columns) of transfer to auxin containing medium. Grown during 15 days in standard growth media, seedlings were transferred to different standard or altered nitrogen concentrations and supplemented with different NAA concentrations: **A.** 4.5% sucrose 60mM nitrogen; **B.** 4.5% sucrose 0.02mM nitrogen + 100nM NAA; **C.** 4.5% sucrose 0.02mM nitrogen + 100mM NaOH; **D.** 4.5% sucrose 60mM nitrogen + 1.0nM NAA; **E.** 4.5% sucrose 60mM nitrogen + 50nM NAA; F. 4.5% sucrose 60mM nitrogen + 200nM NAA; and, G. 4.5% sucrose 60mM nitrogen + 100mM NaOH. Bars represent means +/-; *n* = 10.

3.3.4.1.6. Reduced lateral root formation in the presence of auxin transport inhibitor TIBA

The TIBA concentrations used here was 20µM according to work performed by (Malamy and Ryan, 2001). Mutant and wild-type seedlings were grown on standard growth medium for 15 days and then transferred to medium containing TIBA, or solvent alone (dimethyl sulfoxide, DMSO). After 3 and 9 days the seedlings were examined for primary root length and laterel root number (**Figures 20 and 21**).

Severely reduced length of primary root and lateral root presence were observed under repressive growth media conditions for all seedlings. Except for Salk 110797, primary root growth and lateral root formation was stongly reduced in all seedlings when treated with TIBA (**Figure 20**; **Figure 21,** treatment B). Plants were also treated with the solvent DMSO, which in most lines (including wild-type, but excluding Salk lines 110797 and 139760) stimulated primary root growth (**Figure 20**). The situation appeared to be different with respect to the formation of lateral roots, where a decrease of lateral root number was observed (**Figure 21**) suggesting that the effect of DMSO on primary root growth might be independent of lateral root initiation.

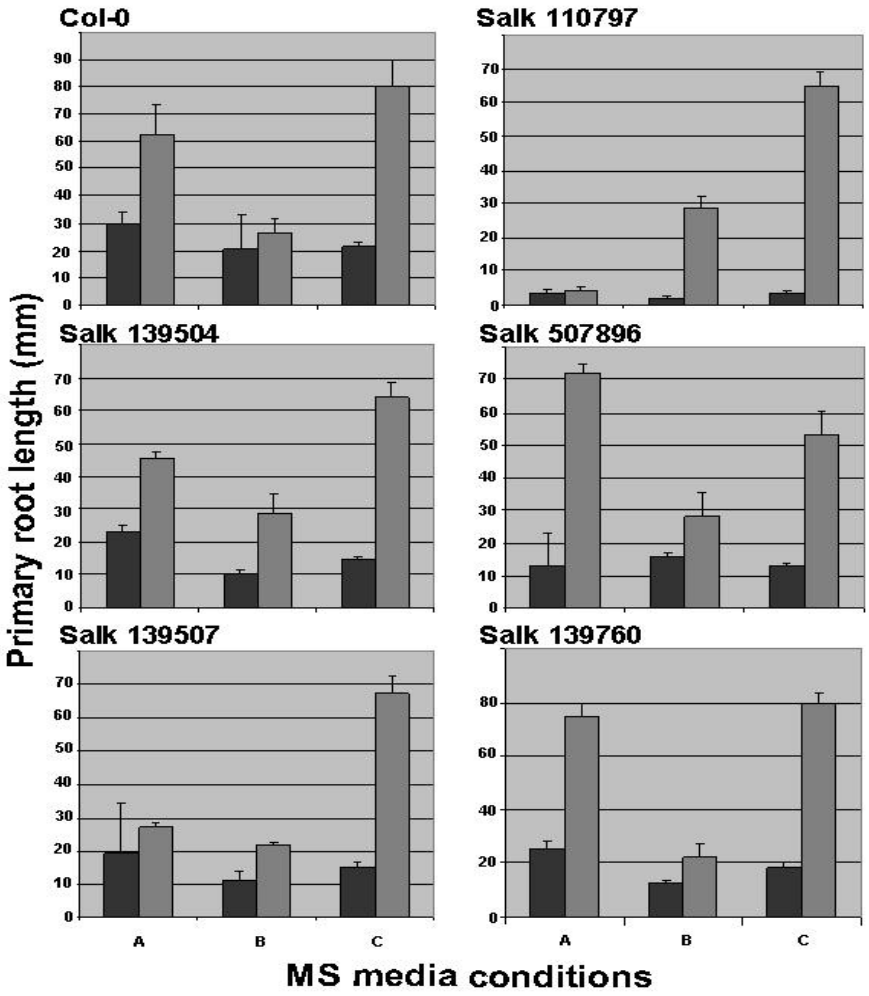


Figure 20. Effect of hormone treatment on primary root length in *Arabidopsis* Col-0 wild-type and *AtDGK7* mutant seedlings.

All seedlings were grown for 15 d on standard growth medium and transferred to new medium supplemented with transport inhibitor TIBA on vertically oriented agar plates. The length of primary roots was measured after 3 days (dark grey columns) and 9 days (light grey columns). MS media conditions were: **A.** 4.5% sucrose 60mM nitrogen; **B.** 4.5% sucrose 60mM nitrogen + 20µM TIBA; and C. 4.5% sucrose 60mM nitrogen + 1.0% DMSO. Bars represent means +/-; *n* = 10.

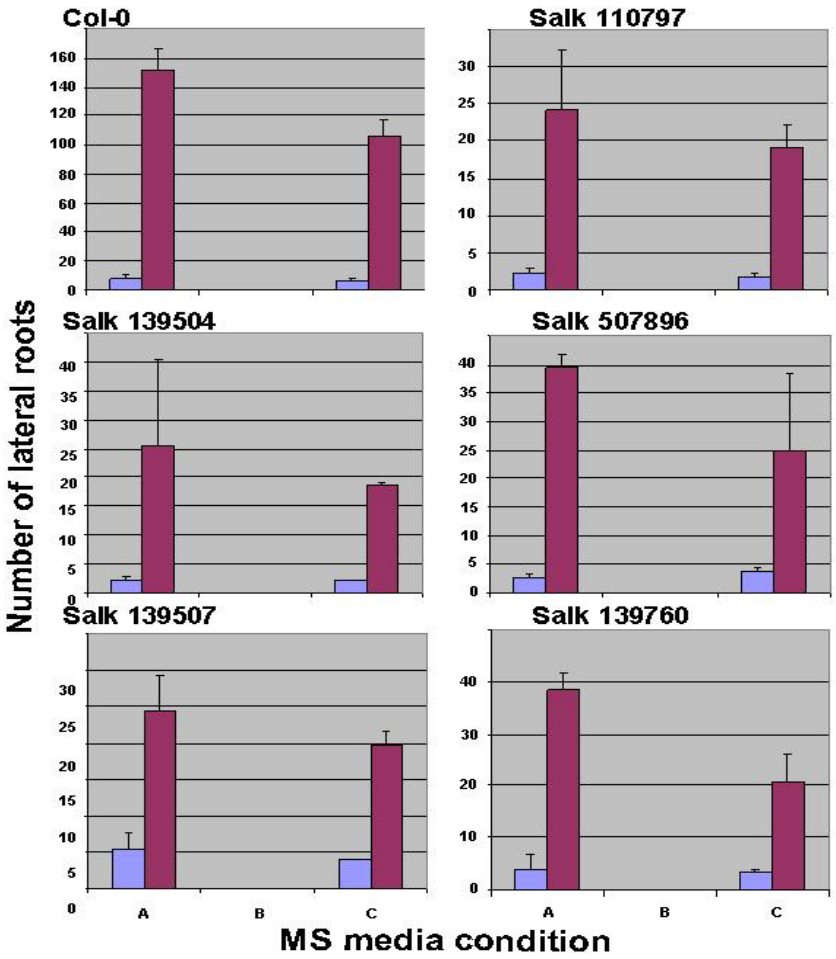


Figure 21. Effect of hormone treatment on lateral root formation in *Arabidopsis* Col-0 wild-type and *AtDGK7* mutant seedlings.

All seedlings were grown for 15 d on standard growth medium and transferred to new medium supplemented with transport inhibitor TIBA on vertically oriented agar plates. The number of lateral roots per primary root was determined after 3 days (purple columns) and 9 days (red columns). MS media conditions were: **A.** 4.5% sucrose 60mM nitrogen; **B.** 4.5% sucrose 60mM nitrogen + 20µM TIBA; and C. 4.5% sucrose 60mM nitrogen + 1.0& DMSO. Bars represent means +/-; *n* = 10.

3.4. DISCUSSION

3.4.1.A new annotation for the AtDGK7 gene

Two possible sequences were reported for the coding region of the *AtDGK7* gene that code for proteins of 374 and 492 amino acid residues having molecular masses of 41.2 and 54.6 kDa, respectively (Gomez-Merino, F. C. et al. 2004). The first cloning and characterisation started with the short version of *AtDGK7* (GenBank accession number NM_119180; (Gomez-Merino, F. C. et al. 2004); see Chapter 2). The obtained cDNA was previously reported to be full length (GenBank accession no. AY686593). The length of the underlying genomic

sequence was 2759 bp, in which the first 126 bp were assigned as the 5´ UTR (untranslated region) and the last 758 bp as the 3´ UTR. AtDGK7 harbored an N-terminal catalytic domain, and in contrast to various of the characterized (mostly animal) DGKs, it lacks a cysteine-rich domain at its N-terminus. Its C-terminal DGK accessory domain was found to be incomplete.

Checking the biochemical activity (Gomez-Merino, F. C. et al. 2004) (Chapter 2 of this thesis) we found that the AtDGK7 enzyme had a substrate saturation rate 3-4 times lower than that of AtDGK2, possibly due to its partial accessory domain. Also, AtDGK2 and AtDGK7 were differentially affected by pH, salts, detergents, prokaryotic and eukaryotic diacylglycerols, and the known DAG inhibitor R59022. Specifically, the R59022 half-maximal inhibition (IC_{50}) measured for AtDGK7 was around 2-fold higher than that observed for AtDGK2. These differences were proposed to be due to the more complex domain organization in AtDGK2 compared with AtDGK7.

Five *AtDGK7* T-DNA insertion lines were obtained from the Salk stock centre. All of them showed a defined phenotype (altered root formation, differences in the response to abiotic stresses and hormone treatments, early flowering and an increase in the amount of flowers and siliques) compared to the *Arabidopsis thaliana* wild-type (ecotype Col-0). The genetic analysis of the mutant lines showed that the T-DNA insertions in four of the mutant lines were in the predicted long 3' UTR. In accordance with the observed phenotype these findings were unexpected. Therefore, it was hypothesized that the long predicted 3' UTR was in fact still part of the coding region. This hypothesis led finally to the cloning of a longer version of *AtDGK7* (F17I23.320), encoding an extended accessory domain.

Sequence analysis showed that the new *AtDGK7* cDNA that was cloned during the course of my experiments encodes a protein harboring a complete DGKa domain instead of a truncated one of the previously reported full-length cDNA (see details in (Gomez-Merino, F. C. et al. 2005). Whether the biochemical characteristics of the 54.6 kDa AtDGK7 protein are different from that of the 41.2 kDa protein (Gomez-Merino, F. C. et al. 2004, Gomez-Merino, F. C. et al. 2005) remains to be tested.

The extended *AtDGK7* cloning sequence, submitted to the GenBank database under the accession number DQ350135, is disrupted by the T-DNA insertion in four of the Salk mutants obtained. Hence, the observed phenotype in these lines is probably due to the absence of full-length AtDGK7 protein.

3.4.2. Possible role of AtDGK7 in lateral root growth

In the present work I have shown that the *A. thaliana DGK7* promoter exhibits a specific pattern of expression in hypocotyls, root tips and the tips of lateral roots but, in general,

activity was not found in vascular tissue. Also, GUS expression was present in petioles and at the edges of the cotyledons and younger leaves. Temporally, lateral and primary root tips showed *AtDGK7* expression starting at day three of seedling growth; however, the vascular tissue of the primary and lateral roots did not show any *AtDGK7* transcriptional activity.

The most apical part (2 mm) of the root tip shows strong auxin biosynthesis activity (Ljung, K. et al. 2005), but the auxin synthesis rate and the auxin concentrations decrease drastically along the elongation zone. The most apical 2 mm of the root tip is the region of the root that includes a range of tissue types, such as the root cap, the root meristem, the elongation zone, and the differentiation zone where lateral root formation is initiated (Benfey, P. N. and Schiefelbein, J. W. 1994, Dolan, L. and Davies, J. 2004). *AtDGK7* showed the strongest transcriptional activity in the region of the most apical lateral root tip and coincidentally, their elongation zones did not show any *AtDGK7* promoter driven GUS activity.

In animal cells, several DGK isoforms are localized in the nucleus (Martelli, A. M. et al. 2002) where they regulate intranuclear DAG levels and, as a consequence, control cell proliferation (Martelli, A. M. et al. 2002, Topham, M. K. et al. 1998). Moreover, in mouse cells, a DGK isozyme interacted with phosphoinositide-specific phospholipase Cß1 (PLCß1) that is involved in inositide-dependent signaling pathways important for the regulation of cell proliferation and differentiation (Evangelisti, C. et al. 2006).

Ras-like proteins, a group of small guanosine triphosphatases (GTPases), constitute a super-family of over 150 members in mammals, subdivided into five main families, which each control particular ways of cell metabolism, such as cell proliferation (Hancock, J. F. and Parton, R. G. 2005, Wennerberg, K. et al. 2005), cell morphology (Wennerberg, K. and Der, C. J. 2004), vesicle trafficking (Donaldson, J. G. and Honda, A. 2005);(Bucci, C. and Chiariello, M. 2006) and nuclear trafficking (Pemberton, L. F. and Paschal, B. M. 2005). Proteins of the family are found in protists, fungi, plants and animals (Madaule, P. and Axel, R. 1985);(Yang, Z. and Watson, J. C. 1993);(Lohia, A. and Samuelson, J. 1996). A total of 93 small GTP-binding proteins were identified in *Arabidopsis* and classified into four of the five small GTPase families: 57 Rab GTPases, 21 Arf GTPases, 11 Rho GTPases, and 4 Ran GTPases (Vernoud, V. et al. 2003).

When activated by external (or internal) signals Rho family GTPases are converted to GTP-binding forms that interact with cellular target proteins or effectors and produces a variety of cellular responses (Hall, A. 1998), including cell growth and differentiation by mediating signals from membrane-bound receptors (Jones, M. A. et al. 2002);(Cheung, A. Y. et al. 2003);(Chen, C. Y. et al. 2003);(Brembu, T. et al. 2005). When lateral root start to grow, small GTP-binding proteins from the Rop family appear at the growth site (Molendijk, A. J. et

al. 2001). Rops are unique to plants, but are related to the small GTPases that control the morphogenesis of animal and yeast cells (Chant, J. 1999);(Eaton, S. et al. 1996).

A type I phosphatidylinositol-4-phosphate (PtdInsP) 5-kinase, independent of GTP, and DGK isozyme interacted with both GTP- and GDP-bound Rac1, a member of the Ras family (Tolias, K. F. et al. 1998). So far it is known that the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by phosphatidlyinositol 3-kinase (PI 3-kinase) activity is essential for receptor-mediated activation of Rac in mammalian cells (Lemmon, M. A. et al. 1997). Another target of Rac that may play a major part in actin polymerization is phosphatidylinositol-4-phosphate 5-kinase (PIP_5-kinase), the enzyme that converts PIP to phosphatidylinositol-4,5-bisphosphate (PIP_2; (Hartwig, J. H. et al. 1995).

Root hair tip growth requires calcium (Schiefelbein, J. W. et al. 1992) and the Ca^{2+} concentration at the tip of the swelling increases from about 200 nM to at least 1 µM, when *Arabidopsis* root hairs are 5-10 µm long (Wymer, C. L. et al. 1997);(Bibikova, T. N. et al. 1999); the Ca^{2+} concentration falls again when growth ceases, and the Rop protein (Molendijk *et al*., 2001) and calcium channel activity (Schiefelbein, J. W. et al. 1992);(Very, A. A. and Davies, J. M. 2000) are lost from the root tip.

Phosphatidic acid (PA) is known to function as a signaling molecule in various plant signal transduction pathways (Ritchie, S. et al. 2000, Ritchie, S. and Gilroy, S. 1998);(Lee, S. et al. 2001);(de Jong, C. F. et al. 2004);(Testerink, C. et al. 2004). It has a role in the activation of AtPDK1 signaling pathway, which is involved in root hair formation in *Arabidopsis thaliana* (Anthony, R. G. et al. 2004). Although it remains to be determined whether the binding of PA to AtPDK1 results from the activation of PLDs because signaling PA can alternatively be synthesised from DGK coupled to the activation of PLC (den Hartog, M. et al. 2003);(de Jong, C. F. et al. 2004) and potentially from acylation reactions.

It is important to note that PA may mediate cellular processes via different modes of action as a signaling messenger, e.g. as a lipid component of membranes. Also, PA may function via its structural effect on membranes to promote membrane fusion and trafficking. In addition, PA serves as a substrate and/or an activator for enzymes that promote the formation of other lipid regulators, such as lysoPA, free fatty acids, DAG, DAG-pyrophosphate, and oxylipins (Wang, X. M. 2002, Wang, X. M. 2004);(Testerink, C. and Munnik, T. 2005). Also, the binding of calcium ions by PA-rich sites providing sites for stimulation of enzyme activity by calcium binding appears possible (Wang, X. M. et al. 2006).

Lateral root initiation, differentiation and development is known to be controlled by many genetic and environmental factors, including transcription factors, light, calcium, pH, free radicals, nutrients, ethylene, auxin and phosphorylation events (Carol, R. J. and Dolan, L. 2002). The function of these different molecules and their interactions are rather complex.

Although the function of *AtDGK7* has not been established completely, its participation as a part of this interlinking metabolic machinery can perhaps be proposed. Here, I am providing information from published resources that might relate AtDGK7 activity to growth and cell proliferation:

- DAG, the substrate of AtDGK7 action, appears to have a role in root hair formation;
- PA, resulting form AtDGK7 activity, is related to root hair formation in *Arabidopsis thaliana*, involving the activation of the AtPDK1 signaling pathway;
- Auxin, functional in the most apical region of the lateral root tip, interacts functionally with Rob GTPases in the initiation of root hair formation and also controls the direction of growth, is directly or indirectly together with Ca^{2+} required for lateral root formation and growth;
- As mentioned before, a close interaction exists between Ca^{2+} and/or DAG and/or PA in some of the metabolic pathways.

Therefore, the working hypothesis that can be drawn is that AtDGK7 functions in the signaling pathway that links auxin to lateral root formation, perhaps via Rop GTPases, which might or might not involve the activation of Ca^{2+} mediated processes. AtDGK7 appears to represent a positive regulator mediating lateral root formation. In the future it will be interesting to see how the different signaling components interact during lateral root formation. Nevertheless, it can not be excluded at present that the enzyme AtDGK7 participates in other metabolic ways that might perhaps be involved in physiological processes not directly linked to lateral root development (note for example that the *AtDGK7* gene is also active in stipules and in leaves, including stomatal guard cells and the leaf margin).

3.4.3. Auxin transport inhibitor affects lateral root initiation

The auxin polar transport inhibitor TIBA strongly reduced the size of the main root and affected lateral root initiation in *AtDGK7* T-DNA insertion seedlings, as it did in wild-type plants. Similar data were previously reported for *Arabidopsis* wild-type plants (Reed, R. C. et al. 1998);(Malamy, J. E. and Ryan, K. S. 2001) and rice (Zhuang, X. et al. 2005) grown under standard conditions. In other publications it was reported that roots exhibited an aberrant gravitropism as a response to treatment with TIBA (Sieburth, L. E. 1999);(Ni, D. A. et al. 2001), as I have observed here in the case of the *AtDGK7* mutants. My data from *AtDGK7 promoter:GUS* seedlings grown on TIBA confirmed the absence of lateral roots; *GUS* activity was not observed in the tip of the main root. I relate the absence of GUS activity with the absence of auxin in the tip root. This result agrees with the fact that auxin transport in plants

is disrupted by TIBA (Li, H. et al. 1999);(Ni, D. A. et al. 2001);(Avsian-Kretchmer, O. et al. 2002). The author´s results also showed that auxin polar transport inhibitors cause an accumulation of auxin in the root apex of the seedlings, and also inhibit the initiation of lateral root and root elongation (Casimiro, I. et al. 2001).

3.4.4. Lateral root initiation is promoted in AtDGK7 T-DNA insertion lines by external auxin application

The experiments described here provide evidence that *AtDGK7* T-DNA seedlings recovered partially the wild-type (Col-0) phenotype after an external application of NAA. The mutated Salk lines showed a strong phenotype on standard growth medium, i.e. reduced plant sizes and primary roots were obvious at 9 –day-old seedlings; also, lateral root formation was strongly reduced (an exception was the size of the primary root in the Salk 139760 line that appeared to be similar to roots of the wild-type, which could be explained by the insertion in the 3' UTR after the stop codon). Without exceptions, all T-DNA insertion lines responded positively to a treatment with 50nM auxin supplied in standard medium, leading to an increased number of lateral roots and a longer primary root, thereby producing a phenotype that was similar to the root system of the Col-0 wild-type.

In *Arabidopsis*, lateral root primordia arise from asymmetric transverse divisions of pericycle founder cells, pericycle cells in cell files adjacent to the two xylem poles (Dubrovsky, J. G. et al. 2000) at some distance from the primary root meristem (Dolan, L. et al. 1993) and situated deep inside the parent root (Casimiro, I. et al. 2001). Although lateral roots display more variability in cell numbers and precise cellular organization, at later stages of lateral root formation the cellular organization becomes very similar to that of the primary root. However, the initial cell division patterns that give rise to new primordia are very different from those occurring during primary root formation (Casimiro, I. et al. 2001). This new primordial tissue grows through the epidermis of the primary root to form a functional lateral root meristem (Malamy, J. E. and Benfey, P. N. 1997).

Although plants tightly control lateral root initiation because lateral roots are initiated at a very well defined region in the main root, through which mechanism this happens is still not completely known. Auxin plays a dominant role in the process and its application (Himanen, K. et al. 2002, Torrey, J. G. 1950) and endogenous production (Boerjan, W. et al. 1995, Celenza, J. L., Jr. et al. 1995, King, J. J. et al. 1995) result in an increase formation of lateral roots. Three central aspects indicate why auxin is required for the initial cell divisions during lateral root initiation (De, Smet, I et al. 2006): first, experiments performed with an auxin-responsive promoter-reporter system, i.e. DR5::GUS, had shown the presence of auxin and/or an auxin response just prior to and during the asymmetric division (Benkova, E. et al.

2003); second, several genes related with auxin function have been reported to exhibit altered expression during lateral root initiation (Marchant, A. et al. 2002); (Tatematsu, K. et al. 2004); and, third, a repression of lateral root initiation occurs when auxin transport is inhibited (Casimiro, I. et al. 2001).

As mentioned before, PA specifically binds to AtPDK1 and stimulates AGC2-1; AGC2-1 is an AtPDK1-dependent molecule and its abundantly expressed in fast-growing organs and dividing cells. Evidence was provided that shows that AGC2-1 is activated during re-entry of cells into the cell cycle after sugar starvation-induced G_1-phase arrest (Anthony, R. G. et al. 2004); additionally, auxin and cytokinin synergistically activate the AtPDK1-regulated AGC2-1 kinase, one more reason to evidence its role in growth and cell division. GFP-fused DGKγ (GFP-DGKγ), expressed in CHO-K1 cells, became localized in the nucleus as well as the cytoplasm and also, expression of DGKθ and DGKζ in the nucleus has been already described (Matsubara, T. et al. 2006) (Bregoli, L. et al. 2001, Topham, M. K. et al. 1998). The authors have shown that the S phase of cells expressing the DGKγ-negative mutant was extended; an involvement of DGK in cell-cycle regulation was suggested by (Flores, I. et al. 1999) reporting that inhibition of DGK activity prevents the G_1 to S transition. About DGK effects on the cell cycle, (Topham, M. K. et al. 1998) by expressing DGKζ in COS-7 cells showed an increase in the population of G_1 cell and a decrease of cells that are in the G_2/M transition phase. The amount of nuclear phosphatidylinositols fluctuates during the cell cycle (York, J. D. and Majerus, P. W. 1994), and the percentage of DAG in the nucleus increases during the G_2/M phase (Deacon, E. M. et al. 2002). In human cells, DGKs are considered to be a one of the key players in lipid signaling, but their physiological functions remain to be solved in detail. Finally, it is suggest that the regulation of nuclear DAG activity is important for cell-cycle regulation, and apparently, DGK might be involved in the control of nuclear DAG (Matsubara, T. et al. 2006). Alternatively, PA produced by DGK may have a function in cell-cycle regulation (Bregoli, L. et al. 2002).

AtDGK7 phosphorylates DAG and the product of its action is PA. Substrate and product are recognized second messengers and their phospholipid signaling activity control multiple cellular signaling pathways among critical biological functions such as cell division and proliferation. As well auxin properties are related to cell division, cell growth and expansion; auxin stimulates positively or negatively the expression and activity of specific genes. Among others tissues, auxin promotes and is directly responsible in the initiation of lateral roots; also, PA via the AtPDK1 signaling pathway participates in the formation of lateral roots. In human cells, DGKs have an effect on cell cycle progression controlling the intracellular population levels of DAG and PA. Therefore, I propose the participation of the catalytic activity of AtDGK7 in lateral root formation. The fact that auxin is able to overcome the short root size and positively affect the number of lateral roots in *AtDGK7* mutant seedlings

supports the hypothesis. The regulation could be mediated by levels of PA and/or DAG, or through an interaction with small GTPases of the Rho family; also Ca^{2+} might be required for a proper AtDGK7-dependent cellular response.

Finally, I was able to demonstrate clear GUS activity in the margins of cotyledons and young leaves of *AtDGK7 promoter:GUS* seedlings grown on standard medium. There, available information about the role of polar auxin transport during leaf development in *Arabidopsis* and other observations suggest that within the leaf, the primary site of auxin synthesis is adjacent to the margin (Avsian-Kretchmer, O. et al. 2002); also studies on leaf vascular patterns indicate that the leaf margin is a source of auxin (Aloni, R. et al. 2003, Mattsson, J. et al. 1999, Mattsson, J. et al. 2003, Sieburth, L. E. 1999). Evidence suggests that the auxin produced by the leaf margin besides controlling leaf blade expansion also controls cell division (Zgurski, J. M. et al. 2005). This information partially supports the hypothesis developed here that tries to explain the relationship between the *AtDGK7* expression pattern and auxin activity (*AtDGK7* expression and high auxin levels are observed in the same regions of the leaf).

The data provided here provide a good starting point for further analyses. In the future it will be necessary to demonstrate in detail the involvement of AtDGK7 in aspects of cell proliferation and growth in general. The transgenic lines used here – as well as a recently obtained line that carries a T-DNA in the central region of the *AtDGK7* gene – could provide a useful tool to study the role of DAG and / or PA in more detail.

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Appendix A

Allgemeinverständliche Zusammenfassung zur Dissertation mit dem Thema

Biochemische Physiologische Studien an der *Arabidopsis thaliana* Diazylglyzerol Kinase 7 (AtDGK7)

vorgelegt von Fernando Alberto Arana-Ceballos
im Rahmen der Dissertation im Dezember 2006

Wachstum und Entwicklung sind die Kennzeichen lebender Systeme. Diese Prozesse unterliegen einer strengen Regulation im Organismus. Diacylglycerol (DAG) und Phosphatidsäure (PA) sind wesentliche Elemente in der Signalübertragung in Organismen. In Säugetieren kann DAG auf drei verschiedenen Wegen metabolisiert werden, die Entstehung von PA durch Phosphorylierung der freien Hydroxyl-Gruppe von DAG ist jedoch der am häufigsten vorkommende Stoffwechselweg. Die enzymatische Umsetzung dieser Reaktion wird von der Familie der Diacylglycerol-Kinasen (DGKs) katalysiert. Molekulare und biochemische Untersuchungen konnten die Anwesenheit von DGKs in *Drosophila melanogaster, Arabidopsis thaliana* und jüngst auch in *Dictyostelium discoideum* zeigen. In der vorliegenden Arbeit wird die Klonierung und Charakterisierung von *AtDGK7* aus *Arabidopsis thaliana* präsentiert, einem Vertreter des pflanzlichen DGK-Clusters II. Das Transkript von AtDGK7 findet sich in der gesamten Pflanze, jedoch sind die Transkriptmengen in Blüten und jungem Gewebe stark erhöht. Rekombinant hergestelltes AtDGK7 ist katalytisch aktiv und akzeptiert DAG-ähnliche Moleküle mit mindestens einer ungesättigten Fettsäure als bevorzugtes Substrat. AtDGK2, ein weiteres Mitglied der DGK-Familie, und AtDGK7 metabolisieren Substrate, welche in Pflanzen physiologisch relevant sind. Das als DGK-Inhibitor beschriebene Molekül 6-{2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl}-7-methyl-5*H*-thiazolo(3,2-α)pyrimidine-5-one (R59022) inhibiert bei Konzentrationen von 50-100 µM rekombinant hergestelltes AtDGK2 *in vitro.* In ähnlichen Konzentrationen eingesetzt modifiziert R59022 das Wurzelwachstum. Dies weist darauf hin, dass DGKs in Entwicklungsprozessen eine Rolle spielen. In *in vitro* Experimenten wurde AtDGK7 von R59022 allerdings erst in Konzentrationen über 100 µM inhibiert. Ferner wird in der vorliegenden Arbeit die erfolgreiche Klonierung einer cDNA beschrieben, die für AtDGK7 aus *A. thaliana* kodiert und welche im Vergleich zu der bereits bekannten cDNA um 439 bp länger ist. Expressionsanalysen mit Hilfe eines Promotor-*ß-glucuronidase* (GUS) Fusions-Produktes zeigten die Aktivität von AtDGK7 in vielen Geweben, vor allem aber in Schließzellen, im Konnektiv-Gewebe der Antheren, sowie besonders in den Spitzen der Seitenwurzeln. Physiologische Untersuchungen unter abiotischem Stress (Verwendung

verschiedener Konzentrationen von Stickstoff, Saccharose, Auxin und Inhibitoren von Auxin-Transportern) wurden mit *AtDGK7* T-DNA-Insertionslinien sowie mit den Promotor-GUS-Linien durchgeführt. *AtDGK7* T-DNA-Insertionslinien zeigten eine starke Inhibierung des Seitenwurzel-Wachstums unter limitierenden Stickstoff- und/oder Saccharose-Konzentrationen. In einigen der T-DNA-Insertionslinien inhibierte die Zugabe eines Inhibitors für Auxin-Transport (TIBA; 2,3,5-triiodobenzoic acid) die Bildung von Haupt- und Seitenwurzeln fast vollständig. Die Inhibition des Wurzelwachstums in den T-DNA-Insertionslinien konnte teilweise durch die Zugabe von 50nM NAA (α-naphtalene acetic acid) revertiert werden.

Aus den vorliegenden Ergebnissen wird die Hypothese abgeleitet, dass AtDGK7 im Zusammenspiel mit Auxin in Signaltransduktionsprozessen eine Rolle spielt, welche das Wachstum und die Entwicklung in Pflanzen regulieren.

Appendix B

Curriculum vitae

Name:	Fernando Alberto Arana-Ceballos
Date of Birth:	December, 17th, 1966
Place of Birth:	Buga
Nationality:	Colombian
Marital Status:	Single

1977 - 1983	Secondary School, Colegio Académico - Buga
07/1983	General certificate of education: High School (Abitur)
08/1982 - 06/1985	Studies in Music – Casa de la Cultura, Buga
08/1985 - 09/1996	Studies in biology at the Universidad del Valle, Cali, Colombia
09/1995 - 05/1996	Centro Internacional de Agricultura (CIAT), Colombia
	Diploma thesis under the supervision of Dr. Joe Tohme, Leader of Agrobiodiversity and Biotechnology Project, (CIAT)
	"Characterization of Mitochondria and Chloroplast genome by RFLP from wild American *Phaseolus vulgaris* Accessions"
10/1996	Degree in Biology, specialization in Genetics
10/1991 - 06/1993	Universidad del Valle, Cali, Colombia. Faculty of Sciences of the Life, Microbiology Department, Molecular Pathogenesis Project. Acute Respiratory. Infection (ARI). Diploma student

10/1991 - 06/1993	Molecular Biochemistry Lab, CIAT, Cali, Colombia. Application of Labelling, Hybridisation and Detection Non-Radioactive Techniques in Genome Mapping (CIAT-Gibco/BRL), Technical Assistant
06/1996 - 09/1996	Unemployment
11/1996 - 04/1999	AgrEvo S.A., Bogotá, D.C., Colombia. Molecular Biochemistry Lab, AgroBiologics Unit, Researcher
03/1999 - 06/2001	Universidad El Bosque Escuela Colombiana de Medicina, Bogotá, D.C., Colombia. Postgraduate studies in Bioethics, Student
05/1999 - 11/2000	Live Systems Technology, S.A., Bogotá, D.C., Colombia. Molecular Biochemistry Lab, Manager
02/2000 - 12/2000	Universidad El Bosque Escuela Colombiana de Medicina, Bogotá, D.C., Colombia. Specializations in Environmental Education and Bioethics, Teacher
01/2001 - 02/2001	Unemployment
03/2001 - 12/2001	Amazon Institute of Scientific Research (SINCHI), National Ministry of the Environment, Republic of Colombia, Bogotá, D.C., Colombia Genetic Resources Program, Coordinator
08/2001 - 06/2002	Presidential campaign of the national candidate Horacio Serpa 2002-2006, Social Democratic Party of Colombia, Bogotá, D.C., Colombia. National Program of Professional Youths, Co-director
07/2002 - 11/2005	Institut fuer Biochemie und Biologie, Universität Potsdam, Potsdam-Golm, Germany PhD thesis under supervision of Prof. Dr. Bernd Müller-Röber "Biochemical and Physiological Studies of *Arabidopsis thaliana* Diacylglycerol Kinase 7 (AtDGK7)"
12/2005 - 12/2006	Institut fuer Biochemie und Biologie, Universität Potsdam, Potsdam-Golm, Germany. Assistant to the Coordinator; EU MolConnect Project, Integration of Genomics and Biodiversity Research: Implementation of an International Platform.

List of Publications

Articles (press):

Yang, G., Zhang, Z-B, Chen, Z., **Arana-Ceballos, F.A.** and Xia, H-J. (2006). Inositol polyphosphate 6-/3-kinase (*AtIpk2ß*), an early auxin-responsive gene, positively regulates axillary shoot branching in *Arabidopsis thaliana*. Plant Physiol. (in press)

Gomez-Merino FC, **Arana-Ceballos FA**, Trejo-Tellez LI, Skirycz A, Brearley CA, Mueller-Roeber B (2005) *Arabidopsis* AtDGK7, the smallest member of plant diacylglycerol kinases (DGKs), displays unique biochemical features and saturates at low substrate concentration: The DGK inhibitor R59022 differentially affects AtDGK2 and AtDGK7 activity in vitro and alters plant growth and development. J Biol Chem **280:** 34888–34899

Arana-Ceballos F.A., Otero L. and Mayer J.E. (1993). Development of a uniform DNA extraction protocol across crops for molecular mapping. BRU. Annual Report. CIAT

Talks

22.10.2004 "Function analysis of the signalling enzymes Diacylglycerol Kinases (DGK) in *Arabidopsis thaliana*" In: XV. Berliner Graduiertenkolloquium der Pflanzenwissenschaften *„Havel-Spree-Kolloquium"* Berlin, Germany

Awards

30.06.1998 "ENVIRONMENTAL NATIONAL AWARD - BUSINESS MODALITY" of The National Ministry of the Environment in representation of the Environmental National System (SINA) to AgrEvo INC., Unit of Agrobiologics, by the effort of research and development in the area of Biological Control of species that affect the agriculture as different alternative to the culture of the agrochemicals

Appendix C

Fecha: Fri, 20 Jan 2006 10:47:44 -0500 (EST)
De: gb-admin@ncbi.nlm.nih.gov
Para: arana@uni-potsdam.de
Asunto: GenBank DQ350135

Dear GenBank Submitter:

Thank you for your submission.

Based on the data submitted to us, the scheduled release date
for your submission is:

Feb 1, 2008

However, if the accession number is published prior to that date, the sequence
will be released upon publication.

Note that the entire sequence will be released when the article citing this
accession number is published or on theabove release date, which ever comes first.
If thisis not what you intended, please notify us immediately so that we can
discuss your submission in more detail.

If this date is not correct, please get in touch with us as soon as possible (gb-
admin@ncbi.nlm.nih.gov, telephone at (301) 496-2475, orfax at (301) 480-2918),
otherwise this submission will be releasedon the date indicated above. The data
would then be available overthe network data servers which provide daily updates
of GenBank data.The data are simultaneously made available to EMBL in Europe and
the DNA Data Bank of Japan.

Minor changes may have been made in your original submission in order to conform
to database annotation conventions. You can greatly assist us in presenting your
data in as accurate a manner as possible by paying specific attention to the
following in your review:

- Spelling (particularly author names)
- Citation data (author order, page span, etc.)
- Nomenclature ('official' gene names, product labels, etc.)
- Taxonomic and source data
- Feature spans and descriptions (particularly non-coding regions)

Please send any revisions, including bibliographic information (e.g., conversion
from unpublished to published), biological data (e.g., new features), or sequence
data as text in the body of an email to:

gb-admin@ncbi.nlm.nih.gov

Since the flatfile record is a display format only and is not an editable format
of the data, do not make changes directly to a flatfile. For complete
information about different methods to update a sequence record, see:
http://www.ncbi.nlm.nih.gov/Genbank/update.html

An accession number has been assigned to each nucleotide sequence and was provided
to you at the time receipt of your submission was acknowledged. Note that during
the processing of your records, we have assigned protein identifiers to any
proteins that are in your records. This is fielded as /protein_id.

We strongly recommend that these numbers appear in any publication which reports
or discusses these data, as they give the community convenient labels with which
they may retrieve your data from our on-line servers.

Thank you once again for your submission.

Sincerely,

Kellie Kelm, PhD
GenBank Direct Submission Staff
gb-admin@ncbi.nlm.nih.gov

GenBank flat file:

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  TITLE     Arabidopsis AtDGK7 regulates lateral root formation
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1564)
  AUTHORS   Arana-Ceballos,F.A., Gomez-Merino,F. and Mueller-Roeber,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-JAN-2006) Dep. Molecular Biology, University of
            Potsdam, Karl-Liebknecht-Str. 24-25, Haus 20, Potsdam 14476,
            Germany
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Appendix D

Table 5. Growth media – Effect of the variation of sucrose-to-nitrogen ratio on *Arabidopsis* seedlings

	LINES	Col-0	Salk 110797	Salk 139504	Salk 507896	Salk 139507	Salk 139760
GROWTH MEDIA							
Standard (**Figure 12A**)	Cotyledons	Size WT standard (WT-S), green	Size reduced, chlorotic	Size WT-S, green	Size WT-S, chlorotic	Size WT-S, green	Size WT-S, green
4.5% sucrose, 0.02mM N (**Figure 12B**)		Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation
0.5% sucrose, 0.02mM N (**Figure 12C**)		Size reduced, chlorotic	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic	Size reduced, chlorotic with anthocyanin accumulation
10% sucrose, 60mM N (**Figure 12D**)		size WT-S, chlorotic	Size reduced, chlorotic	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic
12% sucrose, 60mM N (**Figure 12E**)		Size reduced, chlorotic with anthocyanin accumulation (edges)	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation	Size reduced, chlorotic with anthocyanin accumulation
Standard (**Figure 12A**)	Leaves	size WT standard (WT-S), green	Size reduced, pale green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green
4.5% sucrose, 0.02mM N (**Figure 12B**)		Size reduced, anthocyanin accumulation	Size reduced, anthocyanin accumulation	Size reduced, anthocyanin accumulation	Size reduced, chlorotic	Size reduced, anthocyanin accumulation	Size reduced, anthocyanin accumulation
0.5% sucrose, 0.02mM N (**Figure 12C**)		Size reduced, curly shape, anthocyanin accumulation	Size reduced, anthocyanin accumulation	Size reduced, pale green	Size reduced, anthocyanin accumulation	Size reduced, chlorotic	Size reduced, curly shape, anthocyanin accumulation
10% sucrose, 60mM N (**Figure 12D**)		Size WT-S, anthocyanin accumulation	Size reduced, curly shape, green	Size reduced, anthocyanin accumulation	Size reduced, curly shape, anthocyanin accumulation	Size reduced, high anthocyanin accumulation (necrosis)	Size reduced, curly shape, anthocyanin accumulation

12% sucrose, 60mM N (**Figure 12E**)		Size reduced, curly shape, pale green	Size reduced, chlorotic	Size reduced, curly shape, anthocyanin accumulation	Size reduced, chlorotic	Size reduced, anthocyanin accumulation, necrosis	Size reduced, anthocyanin accumulation
Standard (**Figure 12A**)	Primary root length	WT standard (WT-S)	7% of WT-S	70% of WT-S	+15% of WT-S	40% of WT-S	+20% of WT-S
4.5% sucrose, 0.02mM N (**Figure 12B**)		50% of WT-S	10% of WT-S	35% of WT-S	10% of WT-S	70% of WT-S	70% of WT-S
0.5% sucrose, 0.02mM N (**Figure 12C**)		50% of WT-S	15% of WT-S	35% of WT-S	15% of WT-S	50% of WT-S	70% of WT-S
10% sucrose, 60mM N (**Figure 10D**)		50% of WT-S	10% of WT-S	35% of WT-S	10% of WT	70% of WT-S	70% of WT-S
12% sucrose, 60mM N (**Figure 12E**)		50% of WT-S	10% of WT-S	35% of WT-S	10% of WT	50% of WT-S	70% of WT-S
Standard (**Figure 10A**)	Number of lateral roots	WT standard (WT-S)	15% of WT-S	15% of WT-S	25% of WT-S	15% of WT-S	25% of WT-S
4.5% sucrose, 0.02mM N (**Figure 12B**)		15% of WT-S	3% of WT-S	10% of WT-S	10% of WT-S	20% of WT-S	15% WT-S
0.5% sucrose, 0.02mM N (**Figure 12C**)		15% of WT-S	5% of WT-S	15% of WT-S	10% of WT-S	25% WT-S	15% WT-S
10% sucrose, 60mM N (**Figure 12D**)		15% of WT-S	3% of WT-S	15% of WT-S	10% of WT-S	10% of WT-S	15% WT-S
12% sucrose, 60mM N (**Figure 12E**)		7% of WT-S	1% of WT-S	7% of WT-S	2% of WT-S	5% of WT-S	15% WT-S

Appendix E

Table 6: Growth media – Effect of auxin (naphthalene acetic acid, NAA) on the growth of mutant *Arabidopsis* seedlings under standard and/or reduced nitrogen conditions.

	LINES	Col-0	Salk 110797	Salk 139504	Salk 507896	Salk 139507	Salk 139760
GROWTH MEDIA							
Standard (**Fig. 13A**)	Cotyledons	Size WT standard (WT-S), green	Size reduced, chlorotic	Size WT-S, green	Size WT-S, chlorotic	Size WT-S, green	Size WT-S, green
Standard + 1.0 nM NAA (**Fig. 13B**)		Size WT-S, green	Size reduced, green	Size reduced, green	Size reduced, pale green	Size reduced, curly shape, pale green	Size reduced, green
Standard + 50 nM NAA (**Fig. 13C**)		Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green
Standard + 200 nM NAA (**Fig. 13D**)		Size reduced, green	Size reduced, green	Size reduced, green	Size reduced, curly shape, green	Size reduced, green	Size reduced, green
Standard + 100 mM NaOH (**Fig. 13E**)		Size WT-S, green	Size WT-S	Size WT-S, green	Size WT-S, chlorotic	Size WT-S, green	Size WT-S, green
4.5% sucrose, 0.02 mM N + 100 nM NAA (**Fig. 13F**)		Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development
4.5% sucrose, 0.02 mM N + 100 mM NaOH (**Fig. 13G**)		Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development	Cotyledon formation, but no development
Standard (**Fig. 13A**)	Leaves	Size WT standard (WT-S), green	Size reduced, pale green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green
Standard + 1.0 nM NAA (**Fig. 13B**)		Size WT-S, green	Size reduced, green	Size WT-S, green	Size reduced, pale green	Size reduced, curly shape, pale green	Size reduced, green

Standard plus 50 nM NAA (**Figure 13C**)		Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green
Standard plus 200 nM NAA (**Figure 13D**)		Size reduced, green	Size reduced, green	Size reduced, green	Size reduced, green	Size reduced, green	Size reduced, green
Standard plus 100 mM NaOH (**Figure 13E**)		Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green	Size WT-S, green
4.5% sucrose, 0.02 mM N + 100 nM NAA (**Figure 13F**)		No leaf formation	No leaf formation	No leaf formation	No leaf formation	No leaf formation	No leaf formation
4.5% sucrose, 0.02 mM N + 100 mM NaOH (**Figure 13G**)		No leaf formation	No leaf formation	No leaf formation	No leaf formation	No leaf formation	No leaf formation
Standard (**Figure 13A**)	Primary root length	WT standard (WT-S)	7% of WT-S	70% of WT-S	+15% of WT-S	45% of WT-S	+20% of WT-S
Standard + 1.0 nM NAA (**Figure 13B**)		WT-S	70% of WT-S	90% of WT-S	50% of WT-S	55% of WT-S	WT-S
Standard plus 50 nM NAA (**Figure 13C**)		+20% of WT-S	+30% of WT-S	+40% of WT-S	+20% of WT-S	+25% of WT-S	+15% of WT-S
Standard plus 200 nM NAA (**Figure 13D**)		50% of WT-S	20% of WT-S	35% of WT-S	40% of WT-S	30% of WT-S	30% of WT-S
Standard plus 100 mM NaOH (**Figure 13E**)		WT-S	30% of WT-S	80% of WT-S	75% of WT-S	50% of WT-S	90% of WT-S
4.5% sucrose 0.02 mM N plus 100 nM NAA (**Figure 13F**)		5% of WT-S	3% of WT-S	7% of WT-S	5% of WT-S	5% of WT-S	15% of WT-S

4.5% sucrose 0.02 mM N plus 100 mM NaOH (**Figure 13G**)		7% of WT-S	3% of WT-S	5% of WT-S	3% of WT-S	3% of WT-S	5% of WT-S
Standard (**Figure 13A**)	Number of lateral roots	WT standard (WT-S)	15% of WT-S	15% of WT-S	25% of WT-S	15% of WT-S	25% of WT-S
Standard plus 1.0 nM NAA (**Figure 13B**)		WT-S	15% of WT-S	30% of WT-S	20% of WT-S	15% of WT-S	25% of WT-S
Standard plus 50 nM NAA (**Figure 13C**)		+20% of WT-S	80% of WT-S	90% of WT-S	70% of WT-S	WT-S	85% of WT-S
Standard plus 200 nM NAA (**Figure 13D**)		70% of WT-S	25% of WT-S	30% of WT-S	20% of WT-S	25% of WT-S	20% of WT-S
Standard plus 100 mM NaOH (**Figure 13E**)		15% of WT-S	8% of WT-S	15% of WT-S	10% of WT-S	15% of WT-S	10% of WT-S
4.5% sucrose 0.02 mM N plus 100 nM NAA (**Figure 13F**)		2% of WT-S	1% of WT-S	1% of WT-S	2% of WT-S	2% of WT-S	2% of WT-S
4.5% sucrose 0.02 mM N plus 100 mM NaOH (**Figure 13G**)		1% of WT-S	1% of WT-S	1% of WT-S	1% of WT-S	1% of WT-S	1% of WT-S