Putative phospholipase C (phosphatidylcholine-hydrolysing) genes in *Arabidopsis thaliana* with functions in hormone-dependent root development

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ABSTRACT

Phosphatidylcholine specific phospholipase C (PC-PLC) catalyzes the hydrolysis of phosphatidylcholine (PC) to generate phosphocholine and diacylglycerol (DAG). In animal cells DAG is known to be an important second messenger in signal transduction. Based on amino acid sequence similarity to bacterial PC-PLC, six putative *PC-PLC* genes (*NPC1* to *NPC6*) were identified in the *Arabidopsis* genome. The present study was aimed at investigating the potential function of *NPC* genes in signal transduction by studying the transcriptional regulation of the *NPC* genes during development and in response to various environmental stimuli and by characterization of T-DNA insertional knockouts of *NPC3* and *NPC4*.

Expression of all the *NPC* genes was investigated by semi-quantitative RT-PCR. All the *NPC* genes except *NPC5* were expressed in roots, stems, leaves, flowers and siliques but with differences in transcript abundance. The transcription response to a number of exogenous stress and hormonal stimuli was investigated. Transcriptional responses often indicate a functional relationship to these stimuli. *NPC4* transcription was up-regulated by phosphate deficiency, auxin, cytokinin and brassinolide whereas the transcription of the other genes was not differentially regulated. *NPC3* and *NPC4* were chosen for further analysis by promoter-GUS and knockout plants. Promoter-GUS fusion plants of *NPC3* (*PNPC3*) and *NPC4* (*PNPC4*) exhibited high degree of expression similarity across the entire developmental cycle. The overlapping transcription profiles suggest functional redundancy of these genes.

The expression of *PNPC3* and *PNPC4* was observed in primary and lateral root tips, in leaves prominently around the margins including hydathodes and in young pollen sac tissues which resembled the expression pattern of the auxin-regulated *DR5* promoter. Moreover, a strong increase of GUS activity was visible in roots, leaves and shoots of auxin-treated *PNPC3* and *PNPC4* seedlings and to a weaker extent, when brassinolide-treated. *PNPC4* plants responded also to cytokinin with an increase of GUS activity in young leaf tissues.

Two independent T-DNA insertional lines each of *NPC3* (*npc3-1* and *npc3-2*) and *NPC4* (*npc4-1* and *npc4-2*) were isolated. All lines were tested for potential phenotypes when grown on media either containing physiological amounts of phosphate or on phosphate deficient media each supplemented with or without 0.03 µM 1-naphthaleneacetic acid. Whereas both *npc3* and *npc4* knockouts were not impaired in lateral root formation in response to exogenous auxin and phosphate deficiency, they showed slightly impaired lateral root formation in normal growth media without exogenous auxin addition. As the response to auxin was not impaired, this may be explained by impairment in a function downstream of auxin signal transduction such as cell cycle activity. In response to 0.05 µM

brassinolide, both *npc3* and *npc4* knockout plants clearly differed from wild type plants in which lateral root density was higher.

The results are discussed with respect to potential roles of *NPC* genes in phospholipid and phosphate metabolism and/or in signal transduction providing DAG as a potential second messenger. Taken together, the results suggest a function of *NPC3* and *NPC4* genes in meristems or meristematic tissue, perhaps in cell cycle regulation.

Key words: *Arabidopsis,* phosphatidylcholine specific phospholipase C, signal transduction

ZUSAMMENFASSUNG

Die Phosphatidylcholin- spezifische Phospholipase C (PC-PLC) katalysiert die Hydrolyse von Phosphatidylcholin (PC) zu Phosphocholin und Diacylglycerol (DAG). In tierischen Zellen ist DAG als ein wichtiger sekundärer Botenstoff in der Signaltransduktion bekannt. Basierend auf einem Aminosäuresequenzvergleich mit bakteriellen PC-PLCs wurden im *Arabidopsis*- Genom sechs mutmaßliche *PC-PLC*- Gene (*NPC1* - *NPC6*) identifiziert. Die vorliegende Studie war auf Untersuchungen der potentiellen Funktion von *NPC*- Genen in der Signaltransduktion ausgerichtet, wobei die Regulation von *NPC*- Genen während der Entwicklung und in Reaktion auf verschiedenartige Umweltreize auf Transkriptebene analysiert sowie T-DNA- Insertionsknockouts von *NPC3* (*npc3*) und *NPC4* (*npc4*) charakterisiert wurden.

Die Expression aller *NPC*- Gene wurde mithilfe semi-quantitativer RT-PCR untersucht. Mit Ausnahme von *NPC5* waren alle *NPC*- Gene in Wurzeln, Stengeln, Blättern, Blüten und Schoten exprimiert, jedoch in unterschiedlicher Transkriptionsstärke. Als Antwort auf bestimmte Reize deuten Änderungen auf Transkriptebene häufig auf eine funktionelle Beziehung zu eben diesen Reizen hin. Hier wurde die Expression der sechs *Arabidopsis PC-PLC*- Gene nach Behandlung mit einer Reihe von exogenen Stressfaktoren und hormonellen Reizen analysiert. Phosphatmangel, Auxin, Cytokinin und Brassinolid lösten eine deutliche Zunahme der Transkription von *NPC4* aus, während die Transkription der anderen Gene nicht signifikant reguliert wurde. *NPC3* und *NPC4* wurden für die weitere Analyse durch Promotor-GUS-Pflanzen und Knockout-Pflanzen ausgewählt. Untersuchungen an Promotor::GUS- Pflanzen von *NPC3* (*PNPC3*) und *NPC4* (*PNPC4*) ergaben einen hohen Grad an Ähnlichkeit in der Gen- Expression durch den gesamten Wachstumszyklus hindurch. Diese Übereinstimmung in den Transkriptionsprofilen deutet auf eine funktionelle Redundanz zwischen beiden Genen hin.

Die Expression von *PNPC3* und *PNPC4* wurde in den Spitzen der Haupt- und Seitenwurzeln, in Blättern am markantesten an den Blatträndern einschließlich der Hydathoden und im jungen Pollensackgewebe beobachtet und glich damit dem Expressionsmuster des auxin- regulierten *DR5* Promoters. Eine starke GUS- Aktivität wurde außerdem in den Wurzeln und Sprossen von *PNPC3* und *PNPC4*- Keimlingen nach Behandlung mit Auxin und, in geringerem Umfang, auch nach Behandlung mit Brassinolid detektiert. Cytokinin löste in jungem Blattgewebe von *PNPC4*- Pflanzen ebenfalls einen Anstieg der GUS- Aktivität aus.

Für die Gene *NPC3* und *NPC4* wurden je zwei unabhängige T-DNA- Insertionslinien (*npc3-1, npc3-2* bzw. *npc4-1, npc4-2*) isoliert. Zur phänotypischen Analyse wurden alle Linien entweder auf Phosphatmangelmedium oder auf Medium mit physiologischen Mengen an Phosphat angezogen,

jeweils in Kombination mit oder ohne 0.03 µM 1- Naphthylessigsäure. Während beide T-DNA-Insertionslinien (*npc3* und *npc4*) in ihrer Seitenwurzelbildung durch exogenes Auxin und Phosphatmangel nicht beeinträchtigt wurden, zeigten sie in normalem Wachstumsmedium ohne Auxinbehandlung eine leicht verringerte Ausbildung von Seitenwurzeln. Dass die Reaktion auf Auxin nicht beeinflusst wurde, könnte mit einer Schädigung in einer Funktion, die der Auxin-Signaltransduktion nachgelagert ist, erklärt werden, wie zum Beispiel der Beeinträchtigung der Zellzyklus-Aktivität. Klare phänotypische Unterschiede zwischen Wildtyp *Arabidopsis* und *npc3* und *npc4-* Knockoutpflanzen wurden nach der Behandlung mit 0.05 µM Brassinolid sichtbar, letztere zeigten eine deutlich höhere Seitenwurzeldichte als der Wildtyp.

Zusammengefasst lassen die Ergebnisse der Untersuchungen den Schluss zu, dass *NPC-* Gene entweder eine Funktion im Phospholipid- und Phosphatstoffwechsel und/oder bei der Signaltransduktion übernehmen, wobei dann DAG als sekundärer Botenstoff dienen könnte. Insgesamt deuten die Resultate eher auf eine Funktion der *NPC3-* und *NPC4-* Gene in Meristemen oder meristematischen Geweben, vielleicht bei der Regulation des Zellzyklus hin.

Stichwörter: *Arabidopsis,* Phosphatidylcholin- spezifische Phospholipase C, Signaltransduktion

ABBREVIATIONS

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AM	Arabidopsis medium
bp	base pair
cDNA	complementary Deoxyribonucleic acid
DAG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
DGPP	Diaclylglycerol pyrophosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxy-ribonucleotide-5'-triphosphate
E. coli	*Escherichia coli*
FA	Fatty acids
GLB	Gel loading buffer
GUS	ß-glucuronidase
IAA	β-indole-3-acetic acid
IP$_3$	Inositol-1,4,5-triphosphate
JA	Jasmonic acid
kb	kilo basepair
kDa	kilo dalton
L	Liter
LB medium	Lauri Bertani medium
LPC	Lysophosphatidylcholine
LRR	Leucine-rich repeats
M	Molar
MAPK	Mitogen-activated protein kinases
μM	micromolar
mg	milligram
MGDG	Monogalactosyldiacylglycerol
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MS medium	Murashige and Skoog medium

OD	Optical density
OPDA	*cis*-12-oxophytodienoic acid
PA	Phosphatidic acid
PAF	Platelet-activating factor
PC	Phosphatidylcholine
PC-PLC	Phosphatidylcholine hydrolysing Phospholipase C
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIP$_2$	Phosphatidylinositol 4,5-bisphosphate
PIP$_3$	Phosphatidylinositol-3,4,5-trisphosphate
PI-PLC	PIP$_2$ hydrolysing Phospholipase C
PKC	Protein kinase C
PLA	Phospholipase A
PLA$_2$	Phospholipase A$_2$
PLC	Phospholipase C
PLD	Phospholipase D
PS	Phosphatidylserine
RLK	Receptor-like protein kinase
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	rotations per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic acid
TNF	Tumor necrosis factor
WT	Wild type
X-Gluc	5-bromo-4-chloro-3-indolyl- ß-glucuronidic acid

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1   INTRODUCTION

The main structural lipids in membranes are the polar glycerolipids. The two major categories of polar glycerolipids are glyceroglycolipids and glycerophospholipids. Plant membranes have additional structural lipids, including sphingolipids and sterols that can be considered as minor components (Stryer, 1995). Glycerolipid compositions differ in different cell types. Photosynthetic tissues are composed primarily of glyceroglycolipids (galactolipids) whereas nonphotosynthetic tissues are composed mainly of phospholipids (Stryer, 1995; Buchanan et al., 2000; Taiz and Zeiger, 2002).

1.1 Phospholipids

Phospholipids are a key component of all biological membranes. They are derived either from glycerol, a three-carbon alcohol or from spingosine, a more complex alcohol. Phospholipids derived from glycerol are called phosphoglycerides. In phosphoglycerides the hydroxyl groups at *sn-1* and *sn-2* carbons of glycerol are esterified to the carboxyl groups of two fatty acid chains. The *sn-3* hydroxyl group of the glycerol backbone is esterified to a phosphate group. Attached to this phosphate group is a variable alcohol component called the head group (Figure 1.1).
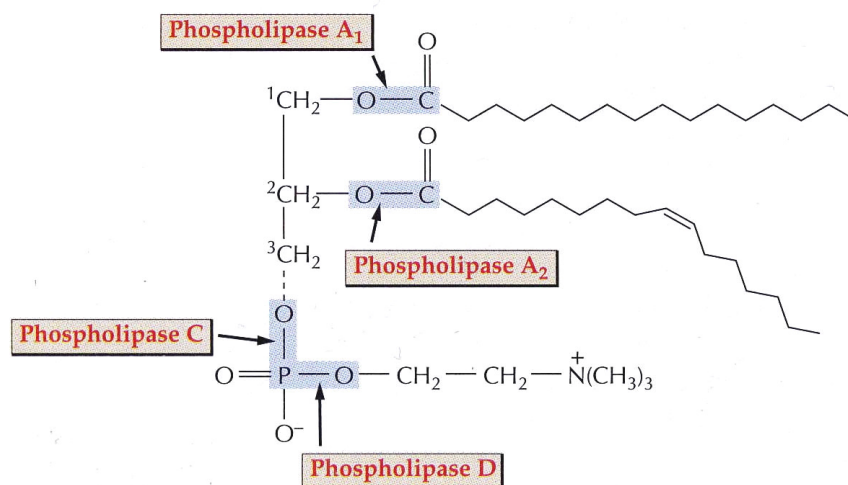
(Source: Buchanan et al., 2000)

Figure 1.1: Glycerophospholipid structure and the cleavage sites of the phospholipases A_1, A_2 C and D.

The simplest phosphoglyceride is called phosphatidate where the *sn-3* hydroxyl group of the glycerol backbone is esterified to phosphoric acid (Stryer, 1995; Munnik et al., 1998; Taiz and Zeiger, 2002; Meijer and Munnik, 2003). Only small amounts of phosphatidate are present in membranes but it is a key intermediate in the biosynthesis of other phosphoglycerides (Stryer, 1995). The other phosphoglycerides share the same basic phosphatidyl moiety with just different head groups. The common alcohol moieties of phosphoglycerides are serine, ethanolamine, choline, glycerol and inositol. Resulting phosphoglycerides are phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), diphosphatidylglycerol (PG) and phosphatidylinositol (PI) respectively (Stryer, 1995; Munnik et al., 1998; Taiz and Zeiger, 2002; Meijer and Munnik, 2003).

Phospholipid composition varies among the membranes of plant and animal systems. Each membrane system of a plant cell has a characteristic and distinct complement of lipid types. PC and PE are the major phospholipids in membranes of higher plant cells and are found in all non-plastidic membranes (Stryer, 1995; Taiz and Zeiger, 2002; Cowan, 2006). PC comprises 47% of endoplasmic reticulum and 43% of mitochondrion lipids as a percentage of total lipid composition. In contrast, only 4% of PC is found in chloroplasts (Taiz and Zeiger, 2002).

In addition to providing the major structural base of cell membranes, phospholipids play a key role in generating signalling substances, rearranging cytoskeleton and membrane trafficking (Wang et al., 2002; Cowan, 2006). Further, phospholipid metabolism is integral to embryo maturation, seed germination, auxin-stimulated cell division and growth, cell polarity, osmotic adjustment and stress tolerance and organ senescence (Chapman 1998; Testerink and Munnik, 2005). Phospholipids and related catabolites such as lysophospholipids can change the physical properties of membranes to increase or decrease ion flux and membrane transport, endo and exocytosis and vesicle formation (Cowan, 2006). Genetic studies using *Arabidopsis thaliana* confirm that changes in phospholipids homeostasis profoundly affect plant growth and development (Cowan, 2006).

In plant glycosylglycerides, the polar head group consists of monogalactose, digalactose or sulphated galactose without a phosphate group. The resulting most common glyceroglycolipids are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (sulfolipid) respectively. In bacteria it is well established that membrane phospholipids can function as a phosphate reserve and can be

replaced by glycolipids during phosphate starvation (Lopez-Lara et al., 2003). In *Arabidopsis thaliana* and *Avena sativa* an increase in the DGDG was observed as a compensation for decrease in tissue phospholipids when grown in phosphate limited conditions (Härtel et al., 2000; Härtel and Benning, 2000; Andersson et al., 2005; Nakamura et al., 2005).

1.2 Phospholipases

Phospholipases are a diverse series of enzymes that hydrolyze phospholipids. The functions of phospholipases can be described broadly in three categories namely:

1. Cell regulation, such as signal transduction, vesicular trafficking and cytoskeletal dynamics

2. Lipid catabolism, such as membrane lipid degradation during cell differentiation, senescence, aging, stress injuries and removal of undesirable lipids from membranes

3. Membrane remodelling, such as changing acyl and head group composition in membrane lipids during growth and development and in response to stresses (Wang, 2001).

Phospholipases are grouped into three main families designated as phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD) according to their sites of hydrolysis on phospholipids (Figure 1.1) (Stryer, 1995; Munnik et al., 1998; Buchanan et al., 2000; Wang, 2001; Wang et al., 2002). Each of these families is further divided into subfamilies based on sequences, biochemical properties or a combination of both (Wang et al., 2002).

An array of products such as phosphatidic acid, diacylglycerol, lysophospholipids and fatty acids are generated by an individual or coordinated action of these phospholipases on different classes of phospholipids (Stryer, 1995; Chapman, 1998; Munnik et al., 1998). The products of individual phospholipases can be further metabolised in the cell. Cross talks among different families and within the same family of phospholipases and the interconversions of hydrolytic products often mediate the cellular functions. Moreover, phospholipase signalling is linked to the mechanism of action of plant hormones elucidating the downstream functions of phospholipases more complex (Cowan, 2006).

1.3 Phospholipase A

Phospholipase A_1 and A_2 are acylhydrolases. Phospholipase A_1 (PLA_1) hydrolyses the *sn-1* acylester bond of phospholipids to free fatty acids (FA) and 2-acyl-1-lysophospholipids. PLA_1s have been identified and cloned from animals and yeast (Nagai et al., 1999; Wang, 2001). PLA_1 in plants is shown to liberate linolenic acid as a precursor to jasmonic acid (JA) synthesis (Ishiguro et al., 2001; Liechti and Farmer, 2002).

Phospholipase A_2 (PLA_2) hydrolyses the *sn-2* acylester bond of phospholipids to free fatty acids (FA) and 1-acyl-2-lysophospholipids. Both products have many downstream roles. PLA_2 is a diverse class of enzymes with regard to function, localization, regulation, mechanism, sequence and structure (Dennis, 1994; Exton, 1994; Kim et al., 1994; Stryer, 1995; Senda et al., 1996; Munnik et al., 1998; Ståhl et al., 1998; Dhondt et al., 2000; Jung and Kim, 2000; Wang, 2001; Holk et al., 2002). In animal systems the characteristics and functions of PLA_2 is extensively studied specially in relation to eicosanoid pathway. In plants the function of PLA_2 is described in many cellular processes. PLA_2 activity is involved in auxin stimulated growth (Scherer and Andrè, 1989, 1993; Andrè and Scherer, 1991; Paul et al., 1998; Scherer et al., 2000) probably by second messenger function of FA. Substantial evidence supports the role of PLA_2 products and their metabolites in elicitor induced plant defence responses (Kawakita et al., 1993; Roy et al., 1995; Chandra et al., 1996; Scherer et al., 2000, 2002; Viehweger et al., 2002). It was proposed that interaction of elicitors with receptors initiate the so-called octadecanoid pathway leading from linolenic acid to biosynthesis of jasmonic acid (JA) which is subsequently involved in activation of defence genes (Narváez-Vasquez et al., 1999; Dhondt et al., 2000). Up-regulation of PLA_2 was observed in wound signalling in plants (Farmer and Ryan, 1990; Ryan, 2000; León et al., 2001). However, overexpression of several patatin related cytosolic PLA_2 genes in petunia did not increase JA or *cis*-12-oxophytodienoic acid (OPDA) levels neither when challenged by pathogens nor when untreated (Zahn et al., 2005; Camera et al., 2005).

Lysophospholipids produced by PLA_2 may attenuate $PLD\alpha$ as a feedback mechanism and activation of $PLD\alpha$ may signal an increase in PLA activity indicating the potential network of signalling (Wang et al., 2002). Lysophospholipids may serve as second messengers in response to elicitors leading to opening of Na^+/H^+ exchange channel in the tonoplast, generating a pH signal to the cytosol (Viehweger et al., 2002, 2006).

1.4 Phospholipase D

Phospholipase D (PLD) is a phosphodiesterase that hydrolyses the terminal phosphodiesteric bond of phospholipids forming phosphatidic acid (PA) and free head groups. PLDs are grouped into five types, PLDα, β, γ, δ and ζ based on their sequences and biochemical properties (Qin and Wang, 2002). PLD is widely distributed in plants, fungi, animals and bacteria and thought to play an important role in an array of cellular functions (Stryer, 1995; Munnik et al., 1998; Buchanan et al., 2000; Wang, 2001). In plants, PLD activity has been implicated in processes like seed germination (Dyer et al., 1994; Ueki et al., 1995), abscisic acid signalling and ethylene promoted senescence (Fan et al., 1997; Ritchie and Gilroy, 1998), wounding (Lee et al., 1997) and pathogen attack (Young et al., 1996; Laxalt et al., 2001).

In animals it is well known that PA stimulates protein kinases such as protein kinase C, mitogen-activated protein kinases and Raf-kinases. Recent studies indicate the involvement of PA in signalling cascades in plants especially in abscisic acid signalling (Wang, 2001). Further, PA can be phosphorylated to diacylglycerol pyrophosphate (DGPP), dephosphorylated to DAG or deacylated to generate lysoPA and free fatty acids which are known to have second messenger function in many cellular processes (Munnik et al., 2000; Wang, 2001; Wang et al., 2002; Meijer and Munnik, 2003; Zalejski et al., 2005).

1.5 Phospholipase C

Phospholipase C (PLC) hydrolyses the glycerophosphate ester bond to generate diacylglycerol (DAG) and phosphorylated head groups. The products DAG and head group are known to play key roles in signal transduction. According to the substrate specificity two major types of PLC have been identified; the well characterised phosphoinositide specific phospholipase C (PI-PLC) and phosphatidylcholine specific phospholipase C (PC-PLC) (Stryer, 1995; Munnik et al., 1998, 2000; Wang et al., 2002). To detect PLC activity in plants caution is required because of the relatively high activity of PLC and the possible interconversions of PLD and PLC products by phosphatases and kinases (Wang, 2001).

1.5.1 Phosphoinositide specific phospholipase C (PI-PLC)

In higher eukaryotes PI-PLC catalyse the hydrolysis of the substrate phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Stryer, 1995; Munnik et al., 1998; Buchanan, 2000; Munnik et al., 2000). Phosphoinositide metabolism contributes an important signalling system that is involved in a variety of cellular functions both in prokaryotes and eukaryotes. IP_3 diffuses freely in the cytoplasm and binds to specific Ca^{2+} channels in the endoplasmic reticulum (ER) and tonoplast paving the way to open and release Ca^{2+} into cytoplasm. DAG remains membrane-bound and activates certain members of protein kinase C. Both of these are known to be of prime importance in signalling in animal systems. However, DAG-mediated protein kinase C activation in plants remains elusive because of lack of information about the presence of protein kinase in plants (Wang, 2001; Meijer and Munnik, 2003). PLC-generated DAG can be phosphorylated to PA by DAG kinase and PLC activation may influence PLD activities by modulating the levels of cellular Ca^{2+} making the PI-PLC signalling cascade more complex (Wang et al., 2002). PA kinase phosphorylates PA to DGPP which then may accumulate in PLC signalling.

PI-PLC have been identified and characterised in a wide variety of bacteria (Heinz et al., 1998), mammals (Nakahara et al., 2005) and plants (Kopka et al., 1998; Meijer and Munnik, 2003; Mueller-Roeber and Pical, 2003).

PI-PLC activity has been reported from a number of plant species (Chapman, 1998; Munnik et al., 1998) and plant PI-PLCs characterised so far are related more closely to mammalian PI-PLC δ (Kopka et al., 1998; Wang, 2001). PI-PLC mediated signalling has been proposed to play important roles in a number of plant responses to various stimuli including abscisic acid induced stomatal closure (Hunt et al., 2003; Mills et al., 2004), light regulated leaf movement (Chapman, 1998), drought and salinity (Hirayama et al., 1995, 1997; Hunt et al., 2004; Kim et al., 2004), pathogen attack (Legendre et al., 1993; Chapman, 1998), root nodulation (Den Hartog et al., 2001) and in regulating pollen tube growth (Monteiro et al., 2005; Pan et al., 2005; Dowd et al., 2006).

1.5.2 Phosphatidylcholine specific phospholipase (PC-PLC)

PC-PLC hydrolyses the common membrane phospholipid, phosphatidylcholine to phosphocholine, a soluble potential second messenger and DAG a second messenger that activates protein kinase C (Stryer, 1995; Wang, 2001). PC-PLC is able to hydrolyse other phospholipids in a lesser efficiency. Phosphatidylcholine is often referred to as lecithin and originally PC-PLC was also called lecithinase and also α-toxin in bacteria. Animal sphingomyelinases catalyse the hydrolysis of sphingomyelin (ceramide phosphorylcholine) into ceramide and phosphorylcholine. PC-PLC and sphingomyelinases sometimes collectively referred to as PC-PLC and has been identified and characterised from a wide variety of bacteria and to a limited extend in animal systems (Stryer, 1995; Argüello et al., 1998). Although phosphatidylcholine is abundant in plant membranes, information on plant PC-PLC is scarce.

1.5.2.1 Bacterial PC-PLC

Occurrence

PC-PLC has been isolated and characterized from a wide variety of bacterial species (Titball et al., 1989; Tso and Siebel, 1989; Titball, 1993, 1998). PC-PLC and sphingomyelinase (SPH) have been cloned, expressed, purified and characterised from *Bacillus cereus* (Gilmore et al., 1989; Argüello et al., 1998; Preuss et al., 2001; Pomerantsev et al., 2003). A phospholipase C gene from *Clostridium perfringens* coding for alpha toxin was cloned in *Escherichia coli* which shows considerable amino acid sequence homology to PC-PLC of *Bacillus cereus* (Titball et al., 1989). Several members of *Pseudomonas* family have been reported to produce PC-PLC. *Pseudomonas aeruginosa* produces haemolytic and nonhaemolytic PLCs (Ostroff et al., 1990). PC-PLC has been isolated from *Pseudomonas fluorescens* as a contaminant while investigating PC-PLC from bovine rat brain cytosol (Crevel et al., 1994) and from human placentae (Preuss et al., 2001). Korbsrisate et al. (1999) have cloned and characterized a *PC-PLC* gene from *Burkholderia pseudomallei* which exhibits 48% amino acid sequence similarity to that of PLC from *P. aeruginosa*.

Properties and regulation

Several evidences indicated that bacterial PC-PLCs are regulated under low-phosphate conditions (Ostroff et al., 1990; Shortridge et al., 1992; Titball, 1993). PC-PLC of *Bacillus cereus*, *Clostridium bifermentans* and *Listeria monocytogenes*, alpha-toxin of *Clostridium perfringens* and gamma-toxin of *Clostridium novyi* essentially require Zn^{2+} ions and are reversibly inactivated by EDTA or o-phenanthroline (Gilmore et al., 1989; Titball and Rubidge, 1990; Crevel et al., 1994). PC-PLC purified from *P. fluorescens*, *C. perfringens*, and *B. cereus* is reported to be Ca^{2+} regulated (Titball, 1993; Preuss et al., 2001). *Bacillus anthracis* and *B. cereus* PC-PLC and spingomyelinase enzymes have shown very similar catalytic properties (Pomerantsev et al., 2003). It has been reported that *C. perfringens* alpha toxin is inactivated when heated to 60°C. The expressed PC-PLC from *B. pseudomallei* is heat stable, nonhemolytic for sheep erythrocytes and active at pH 2-8 (Korbsrisate et al., 1999).

Cellular functions

Phospholipase activity of bacteria was mainly related to the pathogenicity in humans and animals by producing an array of toxic products (Titball, 1993). Alpha toxin plays a key role in the pathogenesis of *C. perfringens* mediated gas gangrene infection and necrotic enteritis of fowl (Titball et al., 1989). PC-PLC activities of *L. monocytogenes* and *P. aeruginosa* are involved in the pathogenesis of listeriosis and skin infections respectively (Titball et al., 1989). PC-PLC of *B. pseudomallei* is shown to be involved in the fatal disease called melioidosis (Korbsrisate et al., 1999) and PC-PLC activity is shown to be relevant to *Mycobacterium tuberculosis* pathogenesis (Johansen et al., 1996). Cooperative actions of *B. cereus* PC-PLC and spingomyelinases were needed to lyse human erythrocytes (Pomerantsev et al., 2003). Apart from the pathogenesis some of the research and therapeutic applications of bacterial PC-PLC are production of vaccines, membrane probes and generation of immunotoxins.

1.5.2.2 Animal PC-PLC

Experimental evidences regarding isolation and characterisation of PC-PLC is scarce. The understanding of PC-PLC function has been hindered by the lack of molecular information on this enzyme (Wang, 2001). The monoclonal antibody raised against *B. cereus* PC-PLC showed a reaction with human monocytic-cell (Clark et al., 1986) indicating that *B. cereus* enzyme may serve as a readily available model for investigating eukaryotic PC-PLC. A

number of studies with animal cells have demonstrated that PC-PLC appears to be critically involved in signal transduction mechanisms controlling cell growth and tumour transformation (Larrodera et al., 1990).

PLC which hydrolyses ^{14}C labelled PC but not ^{14}C labelled PI and PE was purified from bull seminal plasma (Sheikhnejad and Srivastava, 1986). This enzyme had an optimum pH 7.2 and inhibited by EDTA, Cd^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+} and Zn^{2+}. It has been observed that PC-PLC is localized in the acrosome and hence has significant role in fertilization. When isolated rat platelets were incubated with [^{3}H] PC, the formation of [^{3}H] DAG and [^{3}H] phosphorylcholine was observed suggesting the presence of PC-PLC (Randell et al., 1992). PC-PLC activity was increased when incubated with Ca^{2+}. The activity was inhibited by EDTA. A pH optimum was 7.2-7.6 as observed with bull seminal PC-PLC. Rat brain cytosol contains a PLC that preferentially hydrolyses the PC (Edgar and Freysz, 1982). Optimum pH for the enzymatic activity was around 7. The enzymatic activity was increased with Ca^{2+} and inhibited by EDTA as observed for PC-PLC activity of rat platelets and bull seminal plasma. Experimental evidences indicate that the PC-PLC activity is important in relatively late stages of mitogenic responses. PC-PLC activity as measured by intracellular increase of phosphocholine levels was shown to occur following stimulation of quiescent Swiss 3T3 fibroblasts by platelet-derived growth factor (Larrodera et al., 1990). Concomitant with the increase in intracellular phosphocholine a significant elevation of DAG levels were observed in Swiss 3T3 fibroblasts. Osada et al. (1992) reported that the prolonged DAG formation in rat hepatocytes during mitogenic signal transduction is induced by hepatocyte growth factor and that DAG is principally derived from PC-PLC action. In a study with oocytes of *Xenopus laevis* it has been shown that activation of PC-PLC is a target for transforming growth factor β in mitogenic signal transduction mechanism (Diaz-Meco et al., 1992). In another study, using the cell lines NIH (M17) it has been demonstrated that the Raf kinase is activated downstream of PC hydrolysis, implying PC-PLC plays a pivotal role in Ras-mediated mitogenic signalling (Cai et al., 1993). In agreement with the above studies Bjørkøy et al. (1995) investigated the involvement of Raf-1 and PKC as necessary components of PC-PLC mediated mitogenic signalling. Tumour Necrosis Factor (TNF) induces PC-PLC in osteoblast like cells of mouse producing phosphokinase C as a second messenger for TNF induced IL-6 synthesis (Kozawa et al., 1997). Presence and subcellular distribution of PC-PLC in human NK cells is demonstrated by Ramoni et al. (2001). Despite these numerous

implications in animal intercellular signalling the molecular identification of PC-PLC in animals did not emerge.

1.5.2.3 Plant PC-PLC

Experimental evidence on the presence, regulation and functions of the plant PC-PLC is lacking except for a few recent findings. The first evidence for the existence of plant PC-PLC is reported by Scherer et al. (2002). When glycoprotein was added to the parsley cells a rapid decrease in DAG pools was observed indicating a down-regulation of PC-PLC (Scherer et al., 2002). Similarly, when cryptogein was added to the tobacco cells decreased DAG pools were observed. In all these cases observed PA pool was very low. These observations suggest that DAG arises mainly due to PC-PLC but not largely due to consecutive action of PLD and PA phosphatase to produce DAG. Mastoparan also showed strong decrease in DAG suggesting involvement of the newly identified PC-PLC activity in plants is implicated in elicitor signalling in cell cultures and perhaps other signalling pathways.

Based on amino acid sequence similarity to bacterial PC-PLC, six putative *PC-PLC* genes have been identified in *Arabidopsis* genome (Scherer, unpublished work; Nakamura et al., 2005). One of these genes designated as *NPC4* has been cloned and expressed in *E. coli* and purified enzyme is shown to have a significant DAG-producing activity from PC (Nakamura et al., 2005). Evidence was presented that during phosphate starvation NPC4 was highly induced indicating involvement of this enzyme in the hydrolysis of plasma membrane phospholipids. It was concluded that PC hydrolysing NPC4 plays an important role in the supply of both inorganic phosphate and DAG from membrane localized phospholipids for galactolipid synthesis (Nakamura et al., 2005). Further, in phosphate starved *Arabidopsis,* a transient increase in PC content was followed by its rapid decrease and a concomitant increase of DAG and DGDG having similar composition of FA as of PC suggesting that it is derived mainly from PC and hence possible contribution of PC-PLC (Jouhet et al., 2003).

Despite the fact that PC is abundant in plant membranes and considerable amount of evidences regarding the presence and cellular functions of PC hydrolyzing PLC in bacteria and animals, information about plant PC-PLC is scarce. Accessibility to complete genomic sequence of *Arabidopsis* and availability of array of molecular techniques pave the pathway for searching and characterising PC-PLC in *Arabidopsis* as an initial step of gathering basic information about plant PC-PLC.

The objective of the present study is to identify putative PC-PLC genes in the *Arabidopsis* genome based on amino acid sequence similarities of known bacterial PC-PLC, to find the transcription regulation of the gene family during the plant growth and development, in response to various environmental stimuli and to elucidate the probable functions of the individual genes by reverse genetic approach.

2   MATERIALS AND METHODS

2.1   Sequence alignment and physical property prediction

Consensus multiple sequence alignments of amino acid sequences of PC-PLC of
Burkholderia thailandensis, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, putative
phosphoesterase proteins of *Oryza sativa* and *Arabidopsis thaliana* were determined. This
was done by using the database National Centre for Biotechnology Information (NCBI) Basic
Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST) and ClustalW
(http://www2.ebi.ac.uk/clustalw/). Afterwards a phylogenetic tree was created in ClustalW
and displayed with the programme TREEVIEW. This phylogenetic tree is based on amino
acid sequences of putative PC-PLC of *Arabidopsis*, PC-PLC of bacteria, putative
phosphoesterase family proteins of   *O. sativa*, a hypothetical protein of fungus *Aspergillus
nidulans* and a phosphoesterase protein of the protozoan *Tetrahymena thermophilia*. The
predicted physical property data of putative PC-PLC of *Arabidopsis* were obtained from
database search provided by TAIR (http://www.arabidopsis.org/), TIGR (http://tigr.org) and
ExPASy-SWISS-PROT (http://www.expasy.ch). Signal peptide predictions were obtained by
using SignalP program (www.cbs.dtu.dk/services/SignalP/output.html).

2.2   *Arabidopsis thaliana* as plant material

Arabidopsis thaliana (L.) Heynh. Columbia 0 stock, originally obtained from Arabidopsis
Stock Centre Nottingham (N1092), was used as wild type line for gene expression studies and
for *promoter::uid*A transformation. All the T-DNA insertional mutants investigated were
from *Arabidopsis thaliana* Columbia 0 background.

2.3   Plant growth, maintenance and seed harvest

Arabidopsis seeds were sown by sprinkling them onto the soil mix Einheitserde type P and
fine sand (2:1 mix). They were germinated in the greenhouse at 23°C-25°C in short day
conditions (8 h light/16 h dark) with regular watering. After 2-3 weeks of sowing, seedlings
were transplanted in pots containing the same substrate. Plants were further grown in short
day conditions as above, to favour vegetative growth. They were then transferred to long day
conditions (16 h light/8 h dark) to accelerate flowering. At the initial flowering stage, plants
used for seed collection were covered by transparent Aracon plastic sleeves (Arasystem, Beta

Tech. Belgium) to prevent cross pollination. When the plants have completed flowering and approximately 50% of the siliques were mature, watering was stopped in order to fully dry the plants before harvest (Detlef and Glazebrook, 2002). Mature dry siliques were harvested and seeds were threshed with a tea strainer to remove chaff. Seeds were collected in microfuge tubes (with perforated lids) and dried for 24 h at 37°C followed by 2-3 days of air drying at room temperature. Seeds were stored at 4°C.

2.4   Surface sterilization of seeds

Seeds were washed in 70% ethanol for 10 min under vigorous shaking. Ethanol was discarded. Seeds were washed further for 10 min in sterilization solution (Appendix III) under vigorous shaking. Then the seeds were rinsed for 5-6 times with sterile distilled water to ensure that bleach had been removed. Seeds were incubated in sterile distilled water for 24 h at 4°C. Seeds were washed again with sterile distil water before proceeding with the experiments.

2.5   *In planta* transformation of *Arabidopsis* with *promoter::uidA* construct

Arabidopsis can be stably transformed with *Agrobacterium tumefaciens* mediated transfer of T-DNA by vacuum-infiltration. The plant transformation procedure is a modification of the procedure described by Bechtold et al. (1993).

2.5.1 Plant growth

Plant health and vigour are important factors in the success of the transformation. Four *Arabidopsis* seedlings were transferred to each pot (8 cm, 20-30 pots) and grown as described in section 2.3 in short day conditions for 4-5 weeks to facilitate thicker rosettes development. Then the plants were transferred to long day conditions to induce flowering. As soon as the primary inflorescence shoots emerge they were clipped off to encourage growth of multiple inflorescence shoots. After 8-10 days of clipping the inflorescence, plants developed a number of flower shoots and at this stage they were transformed.

2.5.2 *Agrobacteria* culture preparation

A single colony of *Agrobacterium tumefaciens* strain GV3101 having the recombinant expression vector pKGWSF7 was selected from LB agar plate (Appendix VIII) and was

inoculated into 2.5 ml LB medium supplemented with 100 µg/ml rifampicin and 100 µg/ml spectinomycin and was incubated overnight at 28°C shaking at 180 rpm. From this culture, 100 µl were re-inoculated into 25 ml LB medium supplemented with the same antibiotics and grown for about 20 h at 28°C shaking at 180 rpm. Again 20 ml of pre-culture were inoculated into 250 ml LB and incubated as above until the optical density (OD_{600}) was 1.0-1.2. Cells were harvested by centrifugation of 250 ml culture at 4000 rpm for 13 min at 4°C (Sorvaal SLA 1500 rotor). The sediment was suspended in infiltration medium (Appendix IV) until the OD_{600} was approximately 0.8.

2.5.3 Vacuum infiltration

Agrobacteria suspension (in infiltration medium) was transferred to a 600 ml beaker. Inflorescence shoots were dipped into the suspension and the beaker was transferred into a desiccator connected to a water vacuum pump. Vacuum was applied for 2 min and released very rapidly forcing the bacterial cells into the plant tissue. Infiltration was visibly apparent as uniform darkened, water-soaked tissues. Plants were removed from the beaker and were placed on their sides on a plastic tray and closed with a transparent cover for 24 h to maintain high humidity. Then plants were set upright and placed back in long day conditions at 25°C. Vacuum infiltration was repeated twice unless otherwise the transformation efficiency is low. Plants were allowed to grow for approximately four weeks and when the siliques started to get dried, the seeds were collected as described in section 2.3.

2.5.4 Selection of putative transformants

For the selection of T_1-generation of the transformants, the seeds were sown on selection medium (Appendix V) supplemented with kanamycin (50 µg/ml). First, the seeds were sterilized as described in section 2.4, then the sterilized seeds were mixed with 1 ml of sterile water and approximately 1000 seeds were spread on each petri dish containing selection medium. This assures a good separation of the seeds. Higher seed density can make antibiotic selection less effective. The plates were first dried under the laminar flow and then they were incubated at 4°C for 48 h for stratification and transferred to a growth chamber (23°C, 16 h light/8 h dark, 35% RH). In 7-10 days, transformants were clearly identifiable as seedlings with green leaves and roots that extend over and into the selective growth medium. The untransformed seedlings were with yellow leaves and roots were poorly developed.

2.5.5 Transplantation of T$_1$ seedlings to soil

When the transformed seedlings developed 5-6 leaves they were carefully transferred to soil and grown in greenhouse until seed harvest. Before transplanting, all the adhering chunks of agar from roots were removed carefully by washing the roots several times with lukewarm water. The survival of the plants was improved by saturating the soil with water soon after transplanting and growing plants under high humidity for 2-3 days. When the plants start flowering they were covered with Aracon tubes (Arasystem, Beta Tech. Belgium) to prevent cross pollination. The T$_2$-generation seeds were collected, dried and stored until use (section 2.3).

2.6 Induction tests *promoterNPC3::uidA* (*PNPC3*) and *promoterNPC4::uidA* (*PNPC4*) transgenic plants

Histochemical GUS expression analysis was done for three independent lines each of *PNPC3* and *PNPC4* transformed plants with respect to plant developmental stages and a range of chemical tests for *in vitro* grown seedlings or greenhouse-grown plants.

2.6.1 Induction during development

After sterilization and stratification (section 2.4) T$_2$ seeds were sown on Murashige and Skoog medium (MS, Murashige and Skoog, 1962) (Appendix I) or Arabidopsis medium (AM, (Estelle and Somerville, 1987) (Appendix II-A) supplemented with 2% saccharose and 1% agar. The plants were grown for 30 days (23°C, 16 h light/8 h dark). Seedlings were tested for the expression of the promoter by histochemical GUS assay (section 2.7) after 3, 5, 8, 10, 12, 14, 18, 21 and 30 days of growth. Documentation was done by Zeiss Axioskop MR-5 and by scanning at 800 dpi (Fa. Epson, Perception 1650).

2.6.2 Induction with hormones, other biologically active substances, nutrient deficiency and physical stresses

In vitro grown or greenhouse-grown seedlings/plants were subjected to various chemical treatments, nutrient deficient conditions or physical stresses. Afterwards a histochemical GUS assay was performed (section 2.7). Plants were photographed using Zeiss Axioscop MR-5 and Axioskop-MR mot plus.

2.6.2.1 Hormones and other biologically active signal substances

The T_2 seeds were sterilized and grown on MS (Appendix I) or AM (Appendix II-A) supplemented with 1% agar and 2% saccharose for 12 days (23°C, 16 h light/8 h dark). Five seedlings per line were transferred from solid medium to 2.5 ml of liquid MS or AM in 12-well culture plates (Nunc™ Denmark). A number of different treatments were given (Table 2.1) and incubated at the same conditions as above for 48 h. In addition to 48 h treatment, 6 h and 24 h incubation times were tested for 2,4-D, IAA and 1-NAA. As controls for the various treatments, 5 seedlings per line were treated with the respective solvent in appropriate volume. Histochemical GUS assay was done (section 2.7).

Table 2.1: Chemical compounds used for induction of *promoter::uidA*

Chemical treatment	Treatment concentration	
2,4 Dichlorophenoxyacetic acid (2,4-D) dissolved in 70% EtOH (stock 10 mM)	1.0 μM	10 μM
Indole-3-acetic acid (IAA) dissolved in 70% EtOH (stock 10 mM)	1.0 μM	10 μM
1-Naphthaleneacetic acid (1-NAA) dissolved in 70% EtOH (stock 10 mM)	1.0 μM	10 μM
24-epi-brassinolide dissolved in 70% EtOH (stock 1 mM)	0.1 μM	1 μM
1-Aminocyclopropane-carboxylic acid (ACC) dissolved in H_2O (stock 50 mM)	5.0 μM	50 μM
Jasmonic acid (JA) dissolved in 70% EtOH (stock 50 mM)	10 μM	50 μM
Salicylic acid (SA) dissolved in 70% EtOH (stock 50 mM)	10 μM	50 μM
Zeatin-trans dissolved in 1N NaOH (stock 5 mM)	2.5 μM	5 μM
Gibberellic acid (GA_3) dissolved in 70% EtOH (stock 50 mM)	10 μM	50 μM
Abscisic acid (ABA) dissolved in 70% EtOH (stock 50 mM)	10 μM	50 μM
Sodiumnitropruside (SNP) dissolved in H_2O (stock 10 mM)	1.0 mM	
Spermine dissolved in H_2O (stock 100 mM)	1.0 mM	
Spermidine dissolved in H_2O (stock 100 mM)	1.0 mM	

2.6.2.2   Nutrient deficiency

2.6.2.2.1 Phosphate (P_i) deficiency

PNPC3 and *PNPC4* transformed T_2 seedlings were first grown on 1% MS agar medium for 10 days in a growth chamber (23°C, 16 h light/8 h dark). After 10 days the seedlings were transferred to half strength 1% AM agar (Appendix II-B) either containing 1.25 mM KH_2PO_4 (+Pi) or lacking KH_2PO_4 (-Pi) (Kobayashi et al., 2004). In –Pi medium KH_2PO_4 was replaced

with the same concentration of KCl. Seedlings were grown further 10 days and subjected to histochemical GUS assay.

2.6.2.2.2 Iron (Fe) deficiency

PNPC3 and *PNPC4* transformed T_2 seedlings were grown on 1% MS agar medium for 10 days in a growth chamber (23°C, 16 h light/8 h dark). After 10 days, plants were transferred and grown for an additional 6 days on half concentrated 1% AM agar either containing 40 µM FeEDTA (+Fe) or lacking Fe (-Fe) but with 100 µM 3-(2-pyridyl)-5,6-diphenyl-1,2,4 triazine sulfonate (FerroZine) to capture free Fe ions (Schmidt et al., 2000). Seedlings were subjected to histochemical GUS assay.

2.6.2.2.3 Sulfur (S) deficiency

PNPC3 and *PNPC4* transformed T_2 seedlings were grown on 1% MS agar for 10 days in a growth chamber (23°C, 16 h light/8 h dark). After 10 days the seedlings were transferred to S containing (+S) or S lacking (-S) half strength 1% MS agar and grown for further 6 days. In -S medium $MgSO_4$ was replaced with same concentration of $MgCl_2$. Seedlings were subjected to histochemical GUS assay.

2.6.2.3 Physical stresses

2.6.2.3.1 Cold stress

The seedlings were grown on 1% AM agar for 12 days (23°C and 16 h light/ 8 h dark). Then they were transferred to 4°C. Control seedlings were incubated at 23°C. After 48 h the seedlings were subjected to histochemical GUS assay.

2.6.2.3.2 Drying test

The seedlings grown on 1% AM agar for 12 days were transferred to petri dishes lined with 50 µl of water containing Whatman paper. The control seedlings were lined with 2 ml of water soaked Whatman paper. Seedlings were subjected to GUS assay after 24 h of incubation.

2.6.2.3.3 Wounding

Wounding of the leaves was done for 4-5 week old T$_2$ *PNPC3* and *PNPC4* plants grown in greenhouse, by scratching a leaf along the blade with a needle. Wounded leaves were detached after 24 h and histochemical GUS staining was performed. Unwounded detached leaves were subjected to GUS assay as the control.

2.7 Histochemical GUS assay

The gene coding for ß-glucuronidase (*uidA* or *gusA*) originate from *gus*-Operon of *E. coli* is widely used as a reporter gene (Jefferson et al., 1987). GUS activity was assayed qualitatively by staining with the 2 mM X-Gluc buffer (Appendix VI). The plants were incubated at 37°C for 30 min to 16 h depending upon the treatment. After the incubation the plants were treated with 70% ethanol until complete removal of chlorophyll. Destained plants were stored in 70% ethanol.

2.8 Treatments given for RT-PCR expression analysis

2.8.1 Chemical treatments

Arabidopsis thaliana (L.) Heynh. Columbia 0 wild type (WT) seeds were sterilized and sown either on 1% MS agar or 1% AM agar. Stratification was done for 48 h at 4°C. The plants were incubated in a climatic chamber (16 h light/8 h dark, 23°C, 65% RH, 9600 lx/m^2 light intensity) for 12-20 days depending on the experiment. Ten seedlings were transferred to 2.5 ml liquid MS or AM in sterile 12-well culture plates (Nunc™ Denmark) and pre-incubated under the same conditions for 24 h before applying the chemical treatments. Seedlings were treated with different hormones and other biologically active substances for indicated time periods as depicted in Table 2.2. Then they were frozen in liquid nitrogen and stored at -80°C until used for RNA extraction. For the controls 10 plants were mock treated with the respective solvent.

Table 2.2: Chemical compounds used for RT-PCR

Chemical treatment	concentration and treatment duration	
2,4 Dichlorophenoxyacetic acid (2,4-D) dissolved in 70% EtOH (stock 10 mM)	10 μM	for 1 h, 3 h, 6 h, 24 h
Indole-3-acetic acid (IAA) dissolved in 70% EtOH (stock 10 mM)	10 μM	for 1 h, 3 h, 6 h, 24 h
1-Naphthaleneacetic acid (1-NAA) dissolved in 70% EtOH (stock 10 mM)	10 μM	for 1 h, 3 h, 6 h, 24 h
1-Aminocyclopropane-carboxylic acid (ACC) dissolved in H_2O (stock 50 mM)	50 μM	for 6 h, 24 h
Jasmonic acid (JA) dissolved in 70% EtOH (stock 50 mM)	50 μM	for 6 h, 24 h
Salicylic acid (SA) dissolved in 70% EtOH (stock 50 mM)	50 μM	for 6 h, 24 h
Zeatin-trans dissolved in 1N NaOH (stock 5 mM)	5 μM	for 3 h, 6 h, 14 d
24-epi-brassinolide dissolved in 70% EtOH (stock 1 mM)	0.1 μM	for 24 h

2.8.2 Aluminium treatment

Sterilized *Arabidopsis thaliana* Columbia 0 WT seeds were plated on modified MS medium containing MS salts, B5 vitamins, 1% saccharose and 1% agar pH 5.8 (Ezaki et al., 2001). Seedlings were incubated in a chamber (16 h light/8 h dark, 23°C, 65% RH, 9600 lx/m^2 light intensity) for 10 days. Then the seedlings were transferred to one-sixth concentrated modified MS pH 4.0 containing 0.5 μM $AlCl_3$ (+Al) or lacking 0.5 μM $AlCl_3$ (-Al). Seedlings were incubated further 4 days and snap frozen in liquid nitrogen until RNA was extracted.

2.8.3   Nutrient deficiency

2.8.3.1 Phosphate (P$_i$) deficiency

Arabidopsis thaliana (L.) Heynh. Columbia 0 WT seedlings were grown as described in section 2.6.2.2.1. RNA was extracted from the seedlings grown on +Pi and –Pi AM after 10 days of growth.

2.8.3.2 Iron (Fe) deficiency

Arabidopsis thaliana (L.) Heynh. Columbia 0 WT seedlings were grown as described in 2.6.2.2.2. RNA was extracted from the seedlings grown on +Fe and –Fe AM after 6 days of growth.

2.8.3.3 Sulfur (S) deficiency

Arabidopsis thaliana (L.) Heynh. Columbia 0 WT seedlings were grown as described in 2.6.2.2.3. RNA was extracted from the seedlings grown on +S and −S MS media after 6 days of growth.

2.8.4 Wounding

Leaves of 5 to 6 week old greenhouse-grown WT plants were wounded by scratching thrice along the leaf blade with a needle. Both, wounded and unwounded control leaves were detached after 24 h and RNA was extracted.

2.9 Putative *NPC* T-DNA insertional mutants of *Arabidopsis thaliana*

Predicted T-DNA insertion mutant lines within *NPC* genes were obtained either from the seed collections of the Salk Institute Genome Analysis Laboratory (SIGnAL) (Alonso et al., 2003) or from Max Planck Institute for Züchtungsforschung Köln (GABI-Kat) (Strizhov et al., 2003).

Table 2.3: T-DNA insertion knockout lines of *NPC* genes

Gene	Knockout	Knockout identification	Binary vector	Selection marker
NPC1 (At1g07230)	*npc1-1*	SALK_027871.43.40.x	pROK2	kanamycin
NPC2 (At2g26870)	*npc2-1*	SALK_115455.17.80.n	pROK2	kanamycin
	npc2-2	SALK_018011.39.70.x	pROK2	kanamycin
NPC3 (At3g03520)	*npc3-1*	SALK_036463.46.20.x	pROK2	kanamycin
	npc3-2	SALK_150666.23.25.n	pROK2	kanamycin
NPC4 (At3g03530)	*npc4-1*	SALK_046713.47.10.x	pROK2	kanamycin
	npc4-2	GK571E10	pAC161	sulfadiazine
	npc4-3	GK539.10.02	pAC161	sulfadiazine
NPC5 (At3g03540)	*npc5-1*	SALK_084476.43.55.x	pROK2	kanamycin
	npc5-2	SALK_045037.53.00.x	pROK2	kanamycin

Seeds were purchased through the Nottingham Arabidopsis Stock Centre (NASC). All insertional mutant lines obtained were from *Arabidopsis thaliana* Columbia 0 background. PCR based strategy was used to identify heterozygous/homozygous mutant lines. Table 2.3 indicates the insertional mutants identified, the binary vector used for the transformation and the selection marker.

2.10 Phenotypic analysis of *npc3* and *npc4* insertion mutants

Homozygous insertion mutants *npc3-1, npc3-2, npc4-1* and *npc4-2* were analysed for their phenotypes in different growth media, nutrient deficient conditions and with exogenously added hormones and signalling components. In all the cases the phenotypes were compared with the *Arabidopsis thaliana* Columbia 0 WT plants given the same conditions of growth.

Sterilized seeds were sown in parallel lines in 20 ml 1% MS agar medium in round petri dishes. The plates were closed with 3M tape (Fa. Micropore) to allow proper air circulation and to reduce ethylene effect. The petri dishes were incubated in a vertical position in growth chamber (18 h light/6 h dark-neon light-TLD 36 W/830, Fa. Phillips, 23°C and 35% RH). After 3-4 days when the seedlings were uniformly grown, they were transferred to 10x10 cm^2 petri dishes containing 60 ml of respective growth media. To each petri dish 4 or 5 uniformly grown seedlings from the knockout line and 4 or 5 seedlings from the WT were transferred. Petri dishes were closed with 3M tape. The plates were again positioned vertically and incubated further at the same growth conditions for 7-14 days depending on the test and the appearance of a phenotype. The documentation was done by scanning at 400 dpi (Fa. Epson, Perception 1650). Root measurements were done by using Carl Zeiss Axioscope 2.1 programme.

2.10.1 Nutrient deficient conditions

T-DNA insertional mutant and WT seedlings were first grown in complete 1% MS agar medium and incubated at above mentioned conditions for 3-4 days until the seedlings were grown uniformly. Then they were transferred to respective nutrient deficient media and to respective nutrient sufficient media as the control growth conditions. Incubation was done as described in 2.10.

2.10.1.1   Phosphate deficient growth condition

Uniformly grown knockout and WT seedlings after 3-4 days of growth in complete 1% MS agar medium were transferred to half strength 1% AM agar with or without 1.25 mM KH_2PO_4 (+Pi or –Pi,) as described in section 2.6.2.2.1. Seedlings were incubated for 7-14 days as described above and documented by scanning. All the glassware and the utensils used were washed with phosphate free detergent (Neodisher LM3, Fa. Roth).

2.10.1.2   Iron deficient growth condition

Uniformly grown knockout and WT seedlings were transferred to each petri dish containing AM medium with or without Fe (+Fe or –Fe) as described in section 2.6.2.2.2. Incubated for 7 days as described in 2.10 and documented by scanning the petri dishes.

2.10.2 Exogenous hormone supplemented growth conditions

2.10.2.1   Auxin (1-naphthaleneacetic acid, 1-NAA) treatment

Seedlings were grown for 3 days initially in complete 1% MS agar medium. Then they were transferred to half strength +Pi or –Pi 1% AM agar both supplemented with 0.03 μM of 1-NAA in 10x10 cm^2 petri dishes and incubated at vertical position as described in 2.10. The seedlings were grown in similar media without 1-NAA as controls but with same end concentration of ethanol that was used as solvent of 1-NAA. Documentation was done by scanning 7 days after transferring the seedlings. In a similar manner phenotypes were tested on 0.03 μM and 0.1 μM 1-NAA supplemented +Pi 1% MS agar media.

2.10.2.2   Cytokinin (zeatin) treatment

Seedlings were grown for 4 days in full strength 1% MS agar medium and then transferred to similar MS medium supplemented with 1 μM zeatin incubated for 7-14 days at the same growth conditions (section 2.10). The control seedlings were grown in MS medium without added zeatin but with same end concentration of solvent. Documentation was done by scanning.

Seedlings were grown for 4 days initially in full strength 1% MS agar medium and then transferred to half strength +Pi 1% AM agar supplemented with 1 μM of zeatin and incubated in vertical position for 7-14 days at the same growth conditions (section 2.10). The control

seedlings were grown in +Pi 1% AM agar without zeatin but with same end concentration of solvent.

The phenotypes of *npc4-1* knockout was tested by growing the seedlings in +Pi 1% AM agar supplemented with different concentration of zeatin (0.5 μM, 1.0 μM and 5 μM) from the beginning of seedling germination. Incubated (section 2.10) and scanned after 14 days of growth.

2.10.2.3   Aminocyclopropane-carboxylic acid (ACC) treatment

Seedlings of *npc3-1*, *npc3-2* knockouts and WT were grown in complete 1% MS agar medium for 4 days and then transferred to half strength +Pi and –Pi 1% AM agar each supplemented with 10 μM ACC. As controls the seedlings were grown in +Pi and –Pi 1% AM agar without ACC and incubated at same growth conditions (section 2.10).   After 7 days of seedling transfer the scanning was done.

2.11 Genomic DNA extraction from *Arabidopsis thaliana*

Phenol-chloroform extraction method was employed to extract genomic DNA from plants. Plant material was flash frozen in liquid nitrogen and ground into a fine powder using sterile mortar and pestle. Ground plant material (50-100 mg) was taken into a 1.5 ml microfuge tube and 700 µl extraction buffer (Appendix VII) and RNAse A (10 mg/ml) were added. The mixture was incubated for 10 min at room temperature (RT) and centrifuged for 5 min at 13,000 rpm. About 500 µl of the cleared supernatant was taken into another sterile microfuge tube and added with 550 µl of phenol solution (phenol / chloroform / isoamyl-mix, 25:24:1). Then mixed for 10 seconds and incubated on ice for 10 min. The mixture was centrifuged at 13,000 rpm for 5 min at RT. To another sterile microfuge tube 475 µl supernatant were taken and 550 µl phenol solution was added, mixed for 10 seconds and centrifuged again at 13,000 rpm for 5 min. The supernatant containing the DNA was taken into another sterile microfuge tube and 500 µl isopropanol was added. DNA was sedimented by centrifuging for 10 min at 13,000 rpm. The sediment was washed with 70% ethanol. After drying, the DNA pellet was dissolved in 40 µl sterile distilled water. The quality of the DNA solution was checked by agarose gel electrophoresis (section 2.20). DNA quantity and quality was also determined photometrically after diluting (section 2.17). DNA samples were stored at -20°C.

2.12 Isolation of total RNA from *Arabidopsis thaliana*

Total RNA was isolated from *Arabidopsis thaliana* plants using Nucleospin RNA II Plant Kit (Fa. Machery-Nagel). Plant material was ground well in liquid nitrogen using a pre-cooled mortar and pestle. Finely powdered samples (85-100 mg) were transferred into pre-cooled 1.5 ml reaction tubes and 350 µl of RA1 buffer, containing 3.5 µl ß-mercaptoethanol, was added to each tube and mixed vigorously. The lysates were filtered through Nucleospin Filter Units by centrifuging 1 min at full speed. To the filtrate 350 µl of ethanol (70%) was added and mixed. The lysates were loaded to Nucleospin RNA column and mixed for 30 seconds at 10,000 rpm. After placing the columns in to fresh collecting tubes 350 µl of membrane desalting buffer was added and the tubes were centrifuged for 1 min at 13,000 rpm to dry the membranes. DNase reaction mixture was prepared adding 10 µl of reconstituted DNase to 90 µl DNase reaction buffer as defined in the kit. DNase reaction mixture (95 µl) was applied directly onto the centre of the silica membrane of the column and incubated for 15 min at RT. Then 200 µl of RA2 was added to each column, incubated at RT for 2 min and centrifuged at 10,000 rpm for 2 min. The columns were placed in fresh collecting tubes and 600 µl of RA3 were added and centrifuged as previously. Again, 250 µl RA3 were added to the column and centrifuged for 2 min at 13,000 rpm to dry the membranes completely. The columns were placed into nuclease-free 1.5 ml reaction tubes and RNA was eluted in 50 µl RNase–free water by centrifugation at full speed for 1 min. Until further use RNA was stored at -70°C. The quality of RNA was checked by running 3 µl of RNA in a 0.8% agarose gel (section 2.20) and the quantity was determined photometrically (section 2.17).

2.13 cDNA synthesis by Reverse Transcription Polymerase Chain Reaction

RT-PCR was performed by using RevertAid^{TM} H minus First Strand cDNA Synthesis Kit (MBI Fermentas). Appropriate volumes of RNA samples were used to get 1-2 µg RNA. For each reaction, the appropriate volume of RNA and 1 µl of Oligo $(dT)_{18}$ primer (0.5 µg/µl) were mixed and deionised nuclease-free water was added to make the final volume 12 µl. The subsequent steps were carried out in a thermocycler (Hybaid PCR express). The reaction mix was incubated initially at 70°C for 5 min and then chilled at 0°C. A second reaction mix consisted of 4 µl of 5x reaction buffer, 1 µl Ribonuclease inhibitor (20 u/µl), 2 µl of 10 mM dNTP mix, and was added to the first reaction mix, and incubated at 37°C for 5 min. RevertAid^{TM} H minus M-MuLV reverse transcriptase (1 µl) was added. The mixture was incubated at 42°C for 60 min and then at 70°C for 10 min. After cooling at 0°C, 1 µl of

RNAse H was added and finally incubated at 37°C for 20 min. The cDNA solution was stored at -20°C.

2.14 Isolation of plasmid DNA from bacteria

DNA was isolated from plasmids either by alkaline lysis mini-preparation (Birnboim and Doly, 1979) or by using a commercial plasmid mini-prep kit (peQLab Biotechnologie GmbH).

2.14.1 Isolation of plasmid DNA by alkaline lysis mini-preparations

E. coli cells containing recombinant plasmids were cultured overnight in 3.0 ml of LB medium supplemented with the appropriate antibiotic at 37°C at 180 rpm (Thermoshaker-Gerhard). From the overnight culture 1.5 ml was transferred into 2.0 ml reaction tubes and centrifuged at 13,000 rpm for 1 min (Sorvall RC 5B plus). The supernatant was removed. The bacteria were resuspended in 0.3 ml of buffer I supplemented with 100 µg/ml RNase A (Appendix IX). Buffer II (0. 3 ml) (Appendix IX) was added to each tube and mixed gently by inverting the tubes 4-6 times and incubated for 5 min at RT. Then 0.3 ml of buffer III (Appendix IX) was added to each tube, mixed by inverting 5-6 times and incubated on ice for 10 min. The mixture was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred into new reaction tubes. Isopropanol (520 µl) was added to each tube, mixed by inverting and centrifuged at 13,000 rpm for 15 min. The supernatant was removed. The DNA sediments were washed with 500 µl of 70% ice cold-ethanol. The tubes were then centrifuged for 10 min at 13,000 rpm. The supernatant was removed and DNA was air dried for 5-10 min. The DNA was re-dissolved in 40 µl of sterilized distilled water and stored at -20°C. The same procedure was used for isolating plasmid DNA from *Agrobacterium tumefaciens* except the incubation of *A. tumefaciens* was at 28°C at 150 rpm.

2.14.2 Isolation and purification of plasmid DNA by Plasmid Miniprep Kit II
(peQLab Biotechnologie GmbH)

Plasmid miniprep kit was used to isolate plasmid DNA for sequencing. *E. coli* culture (1.5 ml) was centrifuged at 6,500 rpm for 10 min (Sorvall RC 5B plus). Bacteria sediment was dissolved in 500 µl of solution I with RNase A (50 µl/ml). Solution II (500 µl) was then added and mixed by inverting 4-6 times. After incubating for 5 min at RT, 700 µl of solution

III was added, mixed by inverting 8-10 times and centrifuged for 5-10 min at 13,000 rpm. Then 800 µl of supernatant was transferred to a column fitted to a collecting tube and centrifuged for 1 min at 13,000 rpm. The flow through was discarded and the remaining supernatant was loaded into the column and centrifuged as before. To the column 500 µl of HB-buffer was added and centrifuged for 1 min at 13,000 rpm. Then 750 µl of DNA-wash buffer was added to the column and centrifuged 1 min at 13,000 rpm. Again, 750 µl of DNA-wash buffer was added to the column and centrifuged 1 min at 13,000 rpm. The accruing solutions in the collecting tube were discarded at each centrifugation step. The column was centrifuged 1 min at 15,000 rpm for drying and afterwards placed in a sterilized microfuge tube and plasmid DNA was eluted in 50 µl of sterilized distilled water by a 1 min centrifugation at 13,000 rpm.

2.15 DNA extraction and purification from the agarose gel

DNA was extracted by Gel Extraction kit (peQLab Biotechnologie GmbH). Digested PCR products and plasmid were separated in a 1% agarose gel (section 2.20) and the specific DNA fragments of vector and insert were excised from the gel with a sharp scalpel. One volume of gel was melted in four volumes of binding buffer at 65°C for 7 min. Then 800 µl of the mixture was loaded to a column fitted to a collecting tube and centrifuged for 1 min at 13,000 rpm (RT). DNA-wash buffer (completed with 1.5 volume of absolute ethanol) 750 µl was added to the column and centrifuged as before. This step was repeated once and the solution collected in the collecting tube was discarded. To dry the membrane, the column was centrifuged for 1 min at 13,000 rpm. DNA was eluted in 35 µl sterile distilled water by centrifugation at 15,000 rpm for 1 min.

2.16 Purification of PCR products

E.Z.N.A. Cycle-Pure Kit (peQLab-Biotechnologie GmbH) was used to purify the PCR products. PCR products were purified to remove any contaminants that may affect cloning. To the total volume of PCR products 4-5 volumes of PC buffer were added. Then 750 µl of the mixture was loaded to the column fitted to a collecting tube and centrifuged for 1 min at 13,000 rpm (RT). DNA-wash buffer (750 µl) was added to the column and was centrifuged for 1 min at 13,000 rpm. This step was repeated. In all the centrifugation steps the solutions in the collecting tubes were discarded. The column was centrifuged again to dry the membranes. The dried column was placed on a sterile reaction tube and 35 µl of sterilized

distilled water was added directly on to the membrane of the column, incubated at RT for 2-3 min and centrifuged at 13,000 rpm to elute the DNA.

2.17 Determination of quantity and purity of DNA, cDNA and RNA

DNA, cDNA and RNA isolations were 1:100 diluted and the absorptions were measured at 230, 260 and 280 nm by photometer (U-3000 Hitachi). DNA, RNA and cDNA concentrations were determined by the following formulas:

$$\frac{E_{260} \times 50 \times \text{dilution factor}}{1000} = \mu g \text{ DNA} / \mu l$$

$$\frac{E_{260} \times 40 \times \text{dilution factor}}{1000} = \mu g \text{ RNA} / \mu l$$

$$\frac{E_{260} \times 33 \times \text{dilution factor}}{1000} = \mu g \text{ cDNA} / \mu l$$

The quotients E_{260}/E_{280} and E_{260}/E_{230} give information about contamination with proteins and polysaccharides respectively. A quotient between 1.8 and 2.0 shows a sufficient purity.

2.18 Polymerase Chain Reaction (PCR)

Polymerase chain reactions (PCRs) were done for the amplification of the promoter regions and for the identification of knockout plants.

2.18.1 Amplification of promoter region

The nucleotide sequence information of *NPC3* (At3g03520) and *NPC4* (At3g03530) was obtained from NCBI. Based on these sequences, oligonucleotide primer pairs were designed to amplify the putative promoters of *NPC3* and *NPC4* having 2025 bp and 1903 bp regions respectively, including transcribed regions but not translated regions. Specificity of the primers was proved by the BLAST programme. Primers were synthesized by Invitrogen life technologies (www.invitrogen.de). Primer sequences are depicted in Table 2.4. The PCR mix consisted of 200 μM of dNTP (TaKaRa), 5 μl of 10x complete buffer (Promega), 1 μl of each primer (5'forward and 3'reverse primer 10 pmol/μl), 0.25 μl (1 unit) pfu polymerase (pfu

Promega) and 1 μl (100 ng/μl) WT DNA template in a final volume of 50 μl. PCR conditions were 94°C 5 min, 94°C 30 sec denaturation, 55°C 30 sec primer annealing and 72°C 2 min extension for 35 cycles (HYBAID PCR express). Promoter regions were subsequently cloned into Gateway pENTRY/TOPO vector (Invitrogen) and sequenced (SeqLab, Göttingen).

Table 2.4: Oligonucleotide primer pairs for amplification of promoter *NPC3* and *NPC4*

Gene	Primer name	Sequence (5'→3')
NPC3 (At3g03520)	5'PNPC3P	5'CACCGAACTTCCACAATTATTTGCACA3'
	3'PNPC3M	5'CAGAAATTTTGACTTGTGTAGTTG3'
NPC4 (At3g03530)	5'PNPC4P	5'CACCGCAACGGTCAAGAAAATTTTCAAAC3'
	3'PNPC4M	5'TCGAATGTTAAACGTTTTGGGTAGAAAC3'

2.18.2 Identification of knockouts

Gene specific primers (5'LP and 3'RP) and T-DNA left border specific primers LBa1 or LBb1 of vector pROK2 for the SALK lines or for the GABI-Kat lines T-DNA left border specific primer 8409 of the pAC161 vector were used in PCR to identify the homozygous or heterozygous mutants. In the first PCR, 5'LP and 3'RP primers were used for the identification of wild type or heterozygous lines. In the second PCR, LBa1 primer in combination with 5'LP or 3'RP primer was used for the identification of heterozygous or homozygous mutant lines. Gene specific primers for each insertion line were selected (Table 2.5), either using SIGnAL tool primer selection protocol (www.signalsalk.com) or by programme Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi) (Rozen and Skaletsky, 1998). After selecting the primers, the specificity was verified by TAIR/BLAST (Altschul et al., 1997).

Table 2.5: Gene specific and T-DNA specific primers used for the identification of knockouts

Gene	Knockout	Primer name	Primer sequence (5'→3')
NPC1 (At1g07230)	*npc1-1*	5'LPnpc1	ACGTTCTTCTTCAAGTCTCTTCGTA
		3'RPnpc1	TATTTGTTGCCTTCACTTACTGTCA
NPC2 (At2g26870)	*npc2-1*	5'LPnpc2-1	TCTACAAGTCTCTTGTCTCCGAGTT
		3'RPnpc2-1	ACGTATTTGAGTTTCCTTAGGCTTT
	npc2-2	5'LPnpc2-2	TTCAACTACTATCCGTCACGATTTT
		3'RPnpc2-1	ACGTATTTGAGTTTCCTTAGGCTTT
NPC3 (At3g03520)	*npc3-1*	5'LPnpc3	GATCCCGGTCACTCTTTTCAG
		3'RPnpc3	CTATGTCTCTTGCCCTAGGAAGTT
	npc3-2	5'LPnpc3	GATCCCGGTCACTCTTTTCAG
		3'RPnpc3	CTATGTCTCTTGCCCTAGGAAGTT
NPC4 (At3g03530)	*npc4-1*	5'LPnpc4-1	AGTCTCTTTCAATCATGGCGA
		3'RPnpc4-1	TGGTGGATTTTACGACCATGT
	npc4-2	5'LPnpc4-1	AGTCTCTTTCAATCATGGCGA
		3'RPnpc4-1	TGGTGGATTTTACGACCATGT
	npc4-3	5'LPnpc4-3	ACCCTAGGCTGGTTTAAAGAACTAAAC
		3'RPnpc4-3	GCTTTCTCAAATTCCTAAAAAGTAAAC
NPC5 (At3g03540)	*npc5-1*	5'LPnpc5-1	TATTGTTGAATCCCGCTAACG
		3'RPnpc5-1	TTTTGTGACGTGCCATACAAG
	npc5-2	3'LPnpc5-2	TAGGGGCCATTTGATAGATCC
		5'RPnpc5-2	ACGTTTCCACGACTACGGAC
		LBa 1	TGGTTCACGTAGTGGGCCATC
		LBb 1	GCGTGGACCGCTTGCTGCAAC
		LB 8409	ATATTGACCATCATACTCATTGC

Genomic DNA was isolated from leaves of potential T-DNA knockout and wild type plants. PCRs were performed with corresponding genomic DNA. PCR mixture (50 µl) was composed of WT or knockout DNA (0.1-100 ng/µl), 10x Taq buffer with MgCl$_2$ (TaKaRa), 40 mM dNTP (TaKaRa), 5'LP and 3'RP gene specific primers (10 pmol/µl) and TaKaRa Ex Taq polymerase 1 unit. PCR conditions were 94°C 5 min, 94°C 30 sec denaturation, 60°C 30 sec annealing and 72°C 2 min extension for 40 cycles (HYBAID PCR express). A second PCR was carried out as above but with LBa1 (10 pmol/µl) and 5'LP or 3'RP primers (10 pmol/µl), depending on the direction of the insertion, to identify the potential homozygous

or heterozygous knockouts. Purified PCR products (section 2.16) of putative homozygous lines were sequenced (SeqLab, Göttingen) with T-DNA specific LBb1 primer in the case of SALK lines and in the case of GABI-Kat with LB 8409 to verify the insertional mutants.

2.19 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.19.1 Identification of transcript zero knockouts

Seeds from each confirmed homozygous knockout line were sown on MS agar selection medium. RNA was extracted from 10 evenly grown seedlings of each knockout line (section 2.12). As control, RNA and DNA were isolated from WT Columbia seedlings grown on MS agar medium. cDNA was synthesized from the total RNA (section 2.13). RT-PCRs were performed for the knockout and WT cDNA and WT DNA with the primer pairs spanning 5' and 3' ends of the corresponding *NPC* gene (Table 2.6).

Table 2.6: Primers used for identification of transcript zero knockouts

Gene	Homozygous knockout	Primer name	Primer sequence (5'→3')
NPC1(At1g07230)	*npc1-1*	5'LPnpc1 3'RPnpc1	ACGTTCTTCTTCAAGTCTCTTCGTA TATTTGTTGCCTTCACTTACTGTCA
NPC2 (At2g26870)	*npc2-1 & npc2-2*	5'LPnpc2-1 3'TZnpc2	TCTACAAGTCTCTTGTCTCCGAGTT TTAAGGTCTTCTTCCGGTGA
NPC3 (At3g03520)	*npc3-1 & npc3-2*	5'LPnpc3 3'RPnpc3	GATCCCGGTCACTCTTTTCAG CTATGTCTCTTGCCCTAGGAAGTT
NPC4 (At3g03530)	*npc4-1 , npc4-2 & npc4-3*	5'LPnpc4-3 3'RPnpc4-1	ACCCTAGGCTGGTTTAAAGAACTAAACC TGGTGGATTTTACGACCATGT
NPC5 (At3g03540)	*npc5-1 & npc5-2*	5'TZnpc5 3'TZnpc5	AGAATGGCCGAGACGAAAA TGACCAAGGAGTAGCATGTG

The PCR mixture (50 μl) was composed of WT or knockout cDNA (100-150 ng/μl), 10x Taq buffer with $MgCl_2$ (TaKaRa), 40 mM dNTP (TaKaRa), 5'LP (forward) and 3'RP (reverse) gene specific primers (10 pmol/μl) and Ex Taq TaKaRa polymerase 1unit per reaction. PCR conditions were 94°C 5 min, 94°C 30 sec denaturation, 60°C 30 sec annealing and 72°C 2 min extension for 40 cycles. Similar PCR were performed with *ACTN2/7* specific primers (McDowell et al., 1996) and cDNA of knockout and WT as control.

2.19.2 *NPC* expression analysis by semi-quantitative RT-PCR

Semi quantitative RT-PCR was performed with *NPC* gene specific primers (Table 2.7) and *ACTN2/7* primers (McDowell et al., 1996) as a standard. Oligonucleotide primer pairs were designed by using primer 3 programe (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi) and consequently subjected to BLAST (www.ncbi.nlm.nih.gov/BLAST) data base to verify the gene specificity. Each of the primer pair was encompassed at least one intron sequence in the corresponding genomic DNA sequence to confirm or exclude any DNA contamination in the cDNA.

First-strand cDNA was synthesised (section 2.13) from each 1-2 μg total RNA (section 2.12) from roots, stems, leaves, flowers and siliques. Semi-quantitative RT-PCR was done. Transcription levels of *NPC* genes were compared by a similar procedure for the chemically treated/untreated (Table 2.2), +Al/-Al (section 2.8.2), +Pi/-Pi (section 2.8.3.1), +Fe/-Fe (section 2.8.3.2) and +S/-S (section 2.8.3.3) seedlings and wounded/unwounded (section 2.8.4) leaves. For the tissue specific expression analysis the PCR mix was consisted of 1 μl (100 ng/μl) cDNA template, 200 μM dNTP (TaKaRa), 5 μl of 10x complete buffer (TaKaRa), 1 μl of each NPC primer (5'forward and 3'reverse 10 pmol/μl), 0.25 μl (1 unit) Ex Taq TaKaRa polymerase in a final volume of 50 μl. PCR conditions were denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 2 min for 35 cycles. With regard to chemical treatments and nutrient deficiency RT-PCR was carried out as described above except that *ACTN2/7* primers were used at a concentration of 0.15 μM as an internal standard together with the gene specific primers at a concentration of 0.3 μM in the same reaction (Montoya, 2002). The number of PCR cycles was 28-40 for amplification of the genes.

Table 2.7: Primers used for *NPC* expression analysis

Gene	Primer name	Sequence (5'→3')	Product size (bp) cDNA	DNA
NPC1 (At1g07230)	5'RTNPC1P	CTTTTTCCGGCTAATGATGA	754	1440
	3'RTNPC1M	ACTTGTTGGTCCCTTCACTG		
NPC2 (At2g26870)	5'RTNPC2P	TCCCTGCCTACACTGTCATT	743	893
	3'RTNPC2M	TTAAGGTCTTCTTCCGGTGA		
NPC3 (At3g03520)	5'RTNPC3P	AGGGTTCCTGCTTTGCTAAT	492	672
	3'RTNPC3M	GATCAACGATCTCATGCTCA		
NPC4 (At3g03530)	5'RTNPC4P	ATCATAACGTACGACGAGCA	736	926
	3'RTNPC4M	ATGGCGAATAAAGCAAGAGA		
NPC5 (At3g03540)	5'RTNPC5P	AAATGGGCCGTACCTAATGT	496	581
	3'RTNPC5M	TGACCAAGGAGTAGCATGTG		
NPC6 (At3g48610)	5'RTNPC6P	TACGAGGCACTTAGGTCGAG	700	1251
	3'RTNPC6M	GTTGTGTGGCCGTGTAGTG		
	5'ACTN	AGGATATTCAGCCACTTGTCTTGT	1120	1430
	3'ACTN	AGAAACATTTCCTGTGAACAATCG		

2.20   Agarose gel electrophoresis

DNA fragments were separated in 0.8-1% agarose gels. An appropriate amount of agarose (Hybaid QualexGold Agarose- AGR500) was melted in 50 ml of 1x TAE (Appendix XII) electrophoresis buffer for 1 min in a microwave (600 W). After cooling down to ~ 50°C, ethidium bromide (0.5 µg/ml) was added. For each 10 µl aliquots of samples, 1/6 volume of 6 x Gel Loading Buffer (Appendix XIII) was added and separated at 100-120 V for 30-35 min in an electrophoresis apparatus (Pharmacia Biotech EPS 3500 XL) using 1 x TAE as buffer system. DNA ladder 1 kb (Gene Craft) was used as size marker. Nucleic acids were visualised in UV light and documented (Fa. Intas gel documentation system).

2.21   Cloning by the Gateway System (Invitrogen)

The technique involves TOPO cloning of the blunt-end PCR product into pENTRY™ TOPO® vector (Appendix Figure 1) to generate an entry clone. Then an expression construct is generated by performing an LR recombination reaction between the entry clone and a Gateway destination vector to introduce the expression construct into the appropriate host (pENTRY Directional TOPO cloning kit instruction manual). The template should contain a protruding CACC at 5' end, and the linearised pENTRY/D entry vector supplied with pENTRYD TOPO cloning kit (Invitrogen) has a protruding GTGG site at 5', allowing the PCR insert to ligate efficiently with the vector.

2.21.1   Cloning into Gateway Entry vector (pENTRY/D-TOPO)

The reaction mixture was composed of 2-4 µl fresh PCR product, 1 µl of salt solution and 0.5 µl pENTRY/D-TOPO vector (Appendix Figure 1). The reaction mix was incubated for 5-30 min at RT and then stored on ice until used for transformation. Cloning reaction (2 µl) was used to transform competent TOP10 *E. coli* cells. The transformation mix was plated on LB medium supplemented with 50 µg/ml kanamycin and incubated overnight at 37°C. Plasmid DNA was isolated by the alkaline mini prep method. PCR was performed with isolated plasmid DNA and sequenced with M13 forward primer (SeqLab, Göttingen) to prove the sequence and the orientation of the insert.

2.21.2   Cloning into the Gateway destination vector pKGWFS7

The destination vector pKGWFS7 (Appendix Figure 2), having T-DNA borders was used to create an expression clone whereby *Arabidopsis* plants can be stably transformed. pKGWFS7 was obtained from VIB Institute, University of Ghent, Belgium (Karimi et al., 2002). This vector contains the GFP-GUS construct for transcriptional promoter analysis. In the LR reaction the entry clone concentration should be 100-300 ng/reaction and destination vector 300 ng/reaction. Entry clone (1 µl) , destination vector pKGWFS7 (3 µl), 5 x LR Clonase™ reaction buffer (1µl) and LR Clonase™ enzyme (0.5 µl) were mixed and incubated overnight at RT. The reaction was stopped by adding 2 µl of the protein kinase K solution and incubation for 10 min at 37°C. From the reaction mix 2 µl were transformed into competent *E. coli* XL1-Blue (section 3.22.1). The plasmids were isolated and presence of the insert was proven by PCR and by sequencing. Then the recombinant destination vector was transformed into *Agrobacteria*.

2.22 Transformation of bacteria

2.22.1 Transformation of *Escherichia coli*

Chemically competent *E. coli* strain XL1-Blue was used for the transformation reaction. The vial containing competent *E. coli* cells was thawed on ice for 3-5 min. The ligation reaction (2 µl) was added to the competent cells and mixed gently. The mix was incubated on ice for 30 min and was heat shocked for exactly 30 seconds at 42°C in a water bath without shaking. The mix was immediately transferred to ice and incubated for 2 min and 500 µl of SOC medium (Appendix X) at RT was added to the mix. The tube was capped tightly and was shaken (WTB binder) at 37°C for 60 min at 180 rpm for antibiotic selection. Then 50 µl volumes of transformation mix were spread on LB agar (Appendix VIII) plates containing ampicillin (50 µg/ml) and incubated overnight at 37 °C. Plasmid DNA was isolated, PCR was done and finally sequencing was done to verify the inserted DNA. Glycerol cultures of the constructs were prepared and stored at -80°C.

2.22.2 Transformation of *Agrobacterium tumefaciens* GV3101

A. tumefaciens was transformed with expression clone following the freeze-thaw method (Weigel and Glazerbrook, 2002). An aliquot of competent *Agrobacterium tumefaciens* (GV3101) was thawed on ice and 10 µl of purified (mini prep) recombinant destination vector was added and the mixture was incubated on ice for 5 min. The mixture was then transferred to liquid nitrogen for 5 min. Afterwards, the mixture was heated to 37°C for 5 min in a water bath. For the expression of antibiotic resistance the mixture was added with 1 ml of LB (Appendix VIII) and incubated overnight at RT at 300 rpm. The cells were collected by spinning briefly in a microcentrifuge, the pellet was dissolved in 100 µl of LB and then spreaded them on LB agar medium supplemented with two antibiotics, one for recombinant plasmid selection (spectinomycin   100 µg/ml) and the other for *Agrobacteria* selection (rifampicin 100 µg/ml). The plates were incubated for 1-3 days at 28°C. Plasmid DNA was isolated and checked by PCR for the presence of the gene of interest and sequenced (SeqLab, Göttingen) for the verification of the inserted DNA. Glycerol cultures were prepared and stored at -80°C.

2.23   Preparation of competent cells

2.23.1 Preparation of competent *Escherichia coli*

Chemically competent *E. coli* strain XL1-Blue were prepared according to the method described by Nishimura et al. (1990). *E. coli* host strain from a glycerol stock was streaked onto LB agar medium supplemented with 12.5 µg/ml tetracycline. A single colony was inoculated into 2.5 ml LB medium and incubated overnight at $37^{o}C$ and 180 rpm. A 100 ml of medium A (Appendix XI) was inoculated with 1 ml of overnight culture and incubated at $37^{o}C$ and 180 rpm until the cell density reaches the mid log phase. Desired cell density of 0.4-0.8 was measured at OD_{600} by photometer (U-3000 Hitachi). The cell suspension was kept on ice for 10 min and then pelleted by centrifugation (Sorval SS34) at $4^{o}C$ for 10 min at 1500 g. The supernatant was removed and the pellet was dissolved in pre-cooled 1.0 ml of medium A. Then 5.0 ml of storage solution B (Appendix XI) was added and mixed well. Bacteria suspension was divided into aliquots of 0.1 ml in pre-cooled microfuge tubes and stored at $-70^{o}C$.

2.23.2 Preparation of *Agrobacterium tumefaciens* GV3101 competent cells for freeze-thaw transformation

Pre-culture of *Agrobacterium tumefaciens* GV3101 was prepared by inoculating 2.5 ml LB medium, supplemented with 100 µg/ml rifampicin with a single colony of the *A. tumefaciens* and was incubated at $28^{o}C$ (180 rpm) overnight. LB medium (200 ml) was inoculated with 1 ml of an overnight culture and incubated at $28^{o}C$ at 200 rpm for 16-20 h until the cells grow to log phase, giving a density of OD_{600} 0.5-0.8. The culture was centrifuged at 5000 rpm for 10 min (Sorval SS34) at RT to pellet the cells. The pellet was washed with sterile TE buffer. The cells were resuspended in 1/10 (2 ml) of the original volume of LB and was aliquoted in 250 µl portions into sterile microfuge tubes. The aliquots were snap-frozen in liquid nitrogen and stored at $-70^{o}C$.

3   RESULTS

3.1   Identification of six putative *Arabidopsis* PC-PLC homologues to bacterial PC-PLC

Sequence comparison data is becoming increasing useful and popular method of investigating the unknown genes. The existence of PC-PLC activity in bacteria species is well known and the amino acid sequences of the *PC-PLC* genes already being identified. The known bacterial PC-PLC sequences were the base of this study to identify the putative *PC-PLC* genes in the *Arabidopsis* genome. Among them three PC-PLC sequences from *Mycobacterium tuberculosis, Burkholderia pseudomallei* and *Pseudomonas aeruginosa* were subjected to search for homologous sequences in the *Arabidopsis* genome using Basic Local Alignment Search Tool (BLAST) provided by National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). Six putative *PC-PLC* genes were identified (Figure 3.1). They were named as non specific phospholipase C1-C6 (*NPC1* to *NPC6*) according to the nomenclature of Nakamura et al. 2005. A putative phosphoesterase family proteins of *Oryza sativa* also showed substantial similarity to the bacterial PC-PLC and high degree of similarity to identified putative *Arabidopsis* PC-PLC sequences.

```
NPC2            ----------------MSIKAFALIQLLSVTILYNHVH----------ATSPIKTIVVV 33
P2 O.sativa     ---------------MAVRRRRPGPVAAAVLLLLAVATQA--------AASPIKTVVVV 36
NPC1            ---------------MAFRRVLTTVILFCYLLISSQSIEFKNSQKPHKIQGPIKTIVVV 44
NPC4            -----------------------------MIETTKGG-------SGSYPIKTIVVL 20
NPC5            ----------------------------MAETKKG-------SESYPIKTIVVL 19
NPC3            ----------------------------MVEETSSGGG-------SSASPIKTIVVL 22
P1 O.sativa     ----------------------------MAAAAAGG-----------KIKTVVVL 16
NPC6            ----------MKPSSASRFSLTFSHFLTLYCLLTQTHVAQGS-----HQWQSPIKTVVVL 45
P3 O.sativa     ----------MG----------RRLLLLFLMLAQAPNSNG---------DSKIKNVVVL 30
B.pseudomallei  ------MTNQNRRDFLRLAAGTAGA-AALQLFPPVIREALAIPANRRTGTIRDVEHIVIL 53
P.aeruginosa    ------MISKSRRSFIRLAAGTVGATVATSMLPSSIQAALAIPAHRRHGNLKDVEHVVIL 54
M.tuberculosis  MGSEHPVDGMTRRQFFAKAAAATTAGAFMSLAGPIIEKAYGAGPCP--GHLTDIEHIVLL 58
                                                                  :: :*::

NPC2            VMENRSFDHMLGWMK-KLNPEINGVDG--SESNPVSVSDP-SSRKIKFGSGSHYVDPD-P 88
P2 O.sativa     VMENRSFDHMLGWMK-RLNPEIDGVTG--GEWNPTNASDP-SSGRVYFGEGAEYVDPD-P 91
NPC1            VMENRSFDHILGWLK-STRPEIDGLTG--KESNPLNVSDP-NSKKIFVSDDAVFVDMD-P 99
NPC4            VQENRSFDHTLGWFK-ELNREIDGVTKSDPKSNTVSSSDT-NSLRVVFGDQSQYVNPD-P 77
NPC5            VQENRSFDHTLGWFK-ELNREIDGVMKSDQKFNPGFSSDL-NSHNVVFGDQSQYVDPN-P 76
NPC3            VQENRSFDHMLGWFK-ELNPEIDGVSESEPRSNPLSTSDP-NSAQIFFGKESQNIDPD-P 79
P1 O.sativa     VMENRSFDHMLGWMK-SLNPEIDGVTG--DEINHLDAADP-TSRAIRFGDGAEYVDPD-P 71
NPC6            VLENRSFDHLLGWMKNSVNPTINGVTG--QECNPVPN----STQTICFTSDAEFVDPD-P 98
P3 O.sativa     ALENRSFDHMLGWMQRLLGLPIDGLTG--AECNPAPGPGPADSLLHCVSPDADLVVPDDP 88
B.pseudomallei  MQENRSFDHYFGKLR--GVRGFGDPRP-LALQNGKSVFHQPVLLGPAELLPFHPDASN-- 108
P.aeruginosa    MQENRSFDHYFGTLK--GVRGFGDRMA-IPLPDGQRVWHQKGSKG--EILPYHFDTST-- 107
M.tuberculosis  MQENRSFDHYFGTLS--DTRGFDDTTPPVVFAQSGWNPMTQAVDPAGVTLPYRFDTTRGP 116
                *******:*:                   :..               :
```

```
NPC2            GHSFQAIREQVFG--SNDTSMD----------PPPMNGFVQQAYSEDPSGNMSA-SVMNG 135
P2 O.sativa     GHSFQEIRQQIFG--SDDASG----------PARMDGFVQQARS--LGDNMTA-AVMRG 135
NPC1            GHSFQAIREQIFG--SNDTSG----------DPKMNGFAQQSES--MEPGMAK-NVMSG 143
NPC4            GHSIQDIYEQVFG--KPWDSGKPD----PNPGHPNMSGFAQNAER--NKKGMSS-AVMNG 128
NPC5            GHSIRDIYEQVFG--KPWDSGHPD----PNPGPATMSGFAQNAER--KMKGMSS-AVMNG 127
NPC3            GHSFQAIYEQVFG--KPFSDESPY----PDP---KMNGFVQNAEA--ITKGMSEKVVMQG 128
P1 O.sativa     GHSMQAIYEQVYG--TPFVDARATPITPPGVPSPPMAGFAQQAEK--EKPGMAD-TVMNG 126
NPC6            GHSFEAVEQQVFG------SGPGQ-------IPS-MMGFVEQALS---MPGNLSETVMKG 141
P3 O.sativa     AHAFEDVLEQLLGFRPNDSTGAAA------SPSDMSGFVRSAVS---VSALLTDAVMRG 138
B.pseudomallei  -LGMQFLQDLPHGWQDMHGAWNKG----------RYDRWIANKG----------TTTMAY 147
P.aeruginosa    -TSAQRVDGTPHTWPDAQQAWNEG----------RMDKWLPAKT----------ERSLGY 146
M.tuberculosis  LVAGECVNDPDHSWIGMHNSWNGG----------ANDNWLPAQVPF-SPLQGNVPVTMGF 165
                  .  .  :                            :                    :


NPC2            FEPDKVPVYKSLVSEFAVFDRWFASVPSSTQPNRMFVHSGTSAGATSNNPISLAKG---- 191
P2 O.sativa     FSPDSVAVYRELVGEFAVFDRWFASVPSSTQPNRLFVHSATSGGATSNNPELLAKG---- 191
NPC1            FKPEVLPVYTELANEFGVFDRWFASVPTSTQPNRFYVHSATSHGCSSNVKKDLVKG---- 199
NPC4            FKPNALPVYKELVQNFAICDRWFASVPASTQPNRLYVHSATSHGATSNDKKLLLEG---- 184
NPC5            FKPDALPVYKELVQNFAICDRWFASVPGATQPNRLFIHSATSHGTTNNERKLLIEG---- 183
NPC3            FPPEKLPVFKELVQEFAVCDRWFSSLPSSTQPNRLYVHAATSNGAFSNDTNTLVRG---- 184
P1 O.sativa     FRPEAVPVYRELVRQFAVCDRWFASNPASTQPNRLFVHSATSHGLVSNDTKLLVAG---- 182
NPC6            FRPEAVPVYAELVKEFAVFDRWFSSIPGPTQPNRLFVYSATSHGSTSHVKKQLAQG---- 197
P3 O.sativa     FTPSRLPAFSALASSFAVFDRWFSSIPGPTQPNRLFLYSATSHGAVAHDKWNLLRG---- 194
B.pseudomallei  LERDDIPFHYQLADAFTICDAYHCSIPSSTDPNRYYMWTGYVGNDGAGGGPVLGNE---- 203
P.aeruginosa    YKEQDIAFQFAMANAFTICDAYHCSFQGGTNPNRLFLWTGTNDPLGQHGGPVTTNDHDSN 206
M.tuberculosis  YTRRDLPIHYLLADTFTVCDGYFCSLLGGTTPNRLYWMSAWIDPDGTDGGPVLIEPN--- 222
                  :.        :.   *  :  *  :..*     *  ***  :   :.


NPC2            ------YPQRTIFDNLDDEEFSFGIYYQNIP--------------------AVLFYQ- 222
P2 O.sativa     ------YPQRTIFDNVHDAGLSFGVYYQDVP--------------------AVLFYR- 222
NPC1            ------FPQKTIFDSLDENGLSFGIYYQNIP--------------------ATFFFK- 230
NPC4            ------FPQKTIFESLDEAGFSFGIYYQFPP--------------------STLFYR- 215
NPC5            ------FPQKTIFESLDEAGFTFGIYYQCFP--------------------TTLFYR- 214
NPC3            ------FPQRTVFESLEESGFTFGIYYQSFP--------------------NCLFYR- 215
P1 O.sativa     ------LPQRTIFDSLHDAGFSFGIYYQYPP--------------------STLFYR- 213
NPC6            ------YPQKTIFDSLHSNDIDFGIYFQNIP--------------------TTLFYR- 228
P3 O.sativa     ------YPQRTIFDSLAADALDYRVYFKTIP--------------------TTLFYR- 225
B.pseudomallei  ---EAGYGWSTYPETLEQAGVSWKIYQDIGTGLDAAGSWGWTQNPYIGNYGDNSLLYFN- 259
P.aeruginosa    GPVEQGYTWTTYPERLQAAGITWRVYQDMAD----------------NFSDNPLIGFR- 248
M.tuberculosis  IQPLQHYSWRIMPENLEDAGVSWKVYQNKLLG----------------ALNNTVVGYNG 265
                      : :      .  : :*  .                            . :.


NPC2            ----SLRKLKYVFK--FHSYGNS--------FKDHAKQGKLPAYTVIEQRYMDTLLEPAS 268
P2 O.sativa     ----NLRKLKYLTK--FHPFHGA--------FRDHAARGSLPNYAVVEQHYMDSKSHPAN 268
NPC1            ----SLRRLKHLVK--FHSYALK--------FKLDAKLGKLPNYSVVEQRYFDIDLFPAN 276
NPC4            ----NLRKLKYLTH--FHQYGIQ--------FKKDCKEGKLPNYVVVEQRWFDLLSTPAN 261
NPC5            ----NLRKLKYLTR--FHDYGLQ--------FKKDCKEGNLPNYVVVEQRWYDLLLNPAN 260
NPC3            ----NMRKLKYVDN--FHQYHLS--------FKRHCKEGKLPNYVVIEPRYFKILSAPAN 261
P1 O.sativa     ----SLRQLKYAGN--FHPFDLA--------FRRHCAEGKLPNYVVVEQRYFDLKMLPGN 259
NPC6            ----NLRQLKYIFN--LHQYDLK--------FKKDAAKGKLPSLTVIEPRYFDLKGLPAN 274
P3 O.sativa     ----RLRTVANAARGTFRRYDAA--------FRDHARRGLLPALSVIEPRYFDLTGTPAD 273
B.pseudomallei  ----QYRNAQPGSP--LYDKARTGTNVSAGGTLFDVLQQDVKNGTLPQVSWICAP--EAY 311
P.aeruginosa    ----QYRAAAPDSP--LIVNGLS-------TWKLDALKRDVLANSLPQVSWIVAP--AKY 293
M.tuberculosis  LVNDFKQAADPRSN--LARFGIS-------PTYPLDFAADVRNNRLPKVSWVLPG--FLL 314
                    :          :              :       :    :: :


NPC2            DDHPS-HDVYQGQKFIKEVYETLRASP-QWNETLLIITYDEHGGYFDHVPTPVRNVPSPD 326
P2 O.sativa     DDHPS-HDVFQGQMLVKEVYETLRASP-QWNQTLMVVTYDEHGGFYDHVPTPVTGVPSPD 326
NPC1            DDHPS-HDVAAGQRFVKEVYETLRSSP-QWKEMALLITYDEHGGFYDHVPTPVKGVPNPD 334
NPC4            DDHPS-HDVSEGQKLVKEVYEALRSSP-QWNEILFIITYDEHGGFYDHVPTPVDGVPNPD 319
NPC5            DDHPS-HDVSEGQKLVKEVYEALRSSP-QWNEILFIITYDEHGGFYDHVPTPLDGVPNPD 318
NPC3            DDHPK-NDVVEGQNLVKEIYEALRASP-QWNEILFVVVYDEHGGYYDHVPTPVIGVPNPD 319
P1 O.sativa     DDHPS-HDVSEGQRFVKEVYEALRGGP-QWEEALLVVTYDEHGGFYDHVPTPVD-VPSPD 316
NPC6            DDHPS-HDVANGQKLVKEVYEALRSSP-QWNETLLVITYDEHGGFYDHVKTPYVGIPNPD 332
P3 O.sativa     DDHPA-HDVANGQRLVKDVYEALRAGP-QWNHTLLIITYDEHGGFYDHVPPPNVGVPSPD 331
B.pseudomallei  SEHPN-WPANYGAWYVEQVLKALTSNPDVWSKTALFITYDENDGFFDHVAPPFAPQSREN 370
P.aeruginosa    SEHPGPSSPIWGAEYTSWVLDALTANPEVWSKTALLVMFDENDGFFDHVAPPAAPSLNKD 353
M.tuberculosis  SEHPA-FPVNVGAVAIVDALRILLSNPAVWEKTALIVNYDENGGFFDHVPPPTPPP-GTP 372
                .:**       *        *  .* *.. :.: :**:.*::*** .*
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NPC2           GIVGPDPFLFQFNR--------------LGIRVPTIAVSPWIEKGTVVHGPNGSPFPSS 371
P2 O.sativa    GIVGPPPYNFAFDR--------------LGVRVPAIVISPWINKGTVVHGPNGSPTATS 371
NPC1           GIIGPDPFYFGFDR--------------LGVRVPTFLISSWIEKGTVIHEPEG-PTPHS 378
NPC4           GILGPPPYNFEFNR--------------LGVRVPTFFISPWIEPGTVIHGPNG-PYPRS 363
NPC5           GILGPPPYNFEFNR--------------LGVRVPTFFISPWIEPGTVLHGSNG-PYLMS 362
NPC3           GLVGPEPYNFKFDR--------------LGVRVPALLISPWIEPGTVLHEPNG-PEPTS 363
P1 O.sativa    GIVSAAPFFFEFNR--------------LGVRVPALFISPWIEPGTVVHRPSG-PYPTS 360
NPC6           GNTGPAPGFFKFDR--------------LGVRVPTIMVSPWIQKGTVVSEAKG-PTESS 376
P3 O.sativa    AIRGPLPFFFRFDR--------------LGVRVPTIMVSPWIRKGTVVGRPPGGPTPTS 376
B.pseudomallei G-LSTVSTAGEIFA----GDATHMAGPYGLGPRVPMLVVSPWTKGGWVCS---------Q 416
P.aeruginosa   GTLRGKTTADATLEWHTKGDIRYRNQPYGLGPRVPMYVISPWSKGGWVNS---------Q 404
M.tuberculosis GEFVTVPDIDSVPG------SGGIRGPIGLGFRVPCLVISPYSRGPLMVH---------D 417
                 .          .                ** ***   :*.:  .    :          .
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```
NPC2           EYEHSSIPATVKKLFN-----LSSPFLTKRDEWAGTFENILQIRKEPRTDCPETLPEP-V 425
P2 O.sativa    EYEHSSIPATVKKLFD-----LPQDFLTKRDAWAGTFESVVQGRTEPRTDCPEQLPMP-M 425
NPC1           QFEHSSIPATVKKLFN-----LKSHFLTKRDAWAGTFEKYFRIRDSPRQDCPEKLPEVKL 433
NPC4           QYEHSSIPATVKTIFK-----LKD-FLSKRDSWAGTFESVIT-RDSPRQDCPETLSTP-I 415
NPC5           QYEHSSIPATVKKIFK-----LKD-FLTKRDSWAGTFESVIT-RNSPRQDCPETLSNP-V 414
NPC3           QFEHSSIPATLKKIFN-----LKS-FLTKRDEWAGTLDAVIN-RTSPRTDCPVTLPEL-P 415
P1 O.sativa    EFEHSSIPATVKKLFN-----LKS-FLTNRDAWAGTFDVVLT-RDAPRTDCPATLPEP-V 412
NPC6           EYEHSSIPATIKKLFN-----LSSNFLTHRDAWAATFEDVVSHLTTPRTDCPMTLPEVAP 431
P3 O.sativa    EYEHSSIPATIKKIFN-----LSSDFLTRRDAWAGTFEHLFTDLDEPRTDCPETLPEIPP 431
B.pseudomallei TFDHTSLLQFIEARFNDRYSVRAPNVTPWRRAVCGDLTSAFN-FSSPDG-SWPQLPDTSG 474
P.aeruginosa   VFDHTSVIRFLEQRFG----VMEPNISPWRRAVCGDLTSAFN-FANPNNEPFPELPDTSQ 459
M.tuberculosis TFDHTSTLKLIRARFG----VPVPNLTAWRDATVGDMTSTFN-FAAPPNPSKPNLDHPR- 471
                ::*:*     :.    *          .   . *       . :   .      *          *
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NPC2           KIRMG---EANEKALLTEFQQELVQLAAVLKGDNMLT--TFPKEISKGMTVIEGKRYMED 480
P2 O.sativa    RIRLT---EANEEAKLSEFQQELVQLASVLNGDHQLS--SLQDTIRDRMNVREGIAYMRG 480
NPC1           SLRPW---GAKEDSKLSEFQVELIQLASQLVGDHLLN--SYP-DIGKNMTVSEGNKYAED 487
NPC4           KLRGT---MAKENAQLSEFQEDLVIMAAGLKGDYKNE--ELIHKLCKETCVADASKYVTN 470
NPC5           KMRGT---VAKENAELSDFQEELVIVAAGLKGDYKNE--ELLYKLCKKTCVSDASKYVTK 469
NPC3           RARDIDIGTQEEDEDLTDFQIELIQAAAVLKGDHIKD--IYPFKLADKMKVLDAARYVEE 473
P1 O.sativa    KMRPAT--EAAEQAALTEFQEELVQLGAVLNGDHADED-VYPRKLVEGMTVAEAASYCNA 469
NPC6           ----MRATEPKEDAALSEFQGEVVQLAAVLNGDHFLSSFPEEIG--KKMTVKQAHEYVKG 485
P3 O.sativa    PSSSSSSTKKEDGGWLSDFQRELVQLAAFLNGDYMLSSFAQEYESRMTMTVKQADAYVRR 491
B.pseudomallei YAPPDRNRHPSYVPVPPAAQSMPKQEAGLRAARALPYELFVLGRIDQSTGKFKLTFANTG 534
P.aeruginosa   ADAIVASQIKLPKPKPPAVAAMPKQEMGIRPARALPYELGVHARYRSGGDALSLTFANTG 519
M.tuberculosis ----------LNALPKLPQCVPNAVLGTVTKTAIPYRVPFPQSMPTQETAPTRGIPSGL 520
                                       .
```

```
NPC2           AMKRFLEAGRMALSMGANKEELVHMKTSLTGR-------------------------- 512
P2 O.sativa    AVKRFFETGMSAKRMGVDDEQIVKMRPSLTTR-------------------------- 512
NPC1           AVQKFLEAGMAALEAGADENTIVTMRPSLTTR-------------------------- 519
NPC4           AFEKFLEESRKARDRGCDENDIVYCVDDDDDH-------------------------- 502
NPC5           AFDKFVEESKKARERGGDENDIVFCVDDDDHN-------------------------- 502
NPC3           AFTRFHGESKKAKEEGRDEHEIVDLSKGSTRH-------------------------- 505
P1 O.sativa    AFKAWMDECDRCRKCGEDGSHIPTVVKPPPPPS------------------------- 502
NPC6           ATSRFIRASKEAMKLGADKSAIVD---------------------------------- 509
P3 O.sativa    AVKSFLEASKRAKRLASSLQGISKPEKKKKMGSGDWGPVLIALVLFVLLTPGLLCQIPGS 551
B.pseudomallei RAGAAFQVTAGNRLDG-PWAYTVEARKRLSDEWSTALTLSIYDLTVYGPNGFLCQFRGST 593
P.aeruginosa   KAGAVFQVFDLLDSENPPKRYTVGARKRLHDSFQGDASG-DYHLEVHGPNGFLRVFRGNL 578
M.tuberculosis C--------------------------------------------------------- 521
```

Figure 3.1: Alignment of predicted amino acid sequences of bacterial PC-PLC from *Mycobacterium tuberculosis* (accession no. P95246), *Burkholderia pseudomallei* (accession no. Q9RGS8) and *Pseudomonas aeruginosa* (accession no. P15713) with putative PC-PLC of *Arabidopsis* NPC1 (accession no. NP_172203), NPC2 (accession no. NP_180255), NPC3 (accession no. NP_187002), NPC4 (accession no. NP_566206), NPC5 (accession no. NP_566207) and NPC6 (accession no. NP_190430) and with putative phosphoesterace family proteins of *Oryza sativa* P1 (accession no. ABF99945), P2 (accession no. XP-463753) and P3 (accession no. BAB92134). Sequences were aligned with ClustalW program (http://www2.ebi.ac.uk/clustalW). Identical, conserved and semiconserved residues are indicated below the alignment by "*****", "**:**" and "**.**" respectively. Small hydrophobic amino acids are indicated in red, acidic in blue, basic in magenta, hydroxyl+amine+basic in green and others in gray.

Conserved domains can be identified in the multiple sequence alignment (Figure 3.1) and shaded in grey colour. The N terminal amino acids "PIKTIVVLVENRSFDH" are highly conserved among all the six *Arabidopsis* sequences and this stretch of amino acids are identical or conserved substitutions among above 3 bacterial species. "ENRSFDH" region was conserved among few other known PC-PLCs of *Burkholderia thailandensis*, *Xanthomonas campestris*, *Burkholderia cepacia* and *Mycobacterium fungorum*. The other conserved regions lie in the middle and C-termini of the deduced sequences. In many of the cases highly conserved regions of amino acids considered to be catalytic sites. But here it is not known that this conserved region is the catalytic site. Comparison of the prospective candidates of *Arabidopsis* PC-PLC domain organization with that of PI-PLC showed no domain similarity to the known X, Y-domains or EF-hand motif in PI-PLC.

M. tuberculosis, B. pseudomallei and *P. aeruginosa* showed low similarity scores in the range of 15% to 18% to that of *Arabidopsis* amino acid sequences. The amino acid sequence similarity scores of the *Arabidopsis* family ranged from 49% (between NPC4 and NPC6, between NPC5 and NPC6) to 83% (between NPC4 and NPC5). Among the other NPCs the identity of the scores varied from 51% to 62%. High sequence similarities in the range of 45% to 64% were observed among the putative phosphoesterase proteins of *O. sativa* and putative PC-PLC of *Arabidopsis* sequences. The highest similarity was observed between NPC2 and *O. sativa* P2 (64%). NPC3 and NPC4 indicated 58% similarity scores with *O. sativa* P1. Among these *O. sativa* proteins similarities were 46% to 57% indicating they are more closely related to the identified putative PC-PLC of *Arabidopsis* than within the *O. sativa* protein group. The sequence similarity scores are presented in Appendix Table 1.

All the bacteria sequences are more or less similar to each other and they fall into one cluster while all the plant sequences fall into another cluster as reflected by unrooted phylogenetic tree (Figure 3.2). NPC3, NPC4 and NPC5 clustered together in which NPC4 and NPC5 again form one sub-group having 83% similarity. This may be due to a recent gene duplication event. Interestingly, four hypothetical proteins of *O. sativa* related to phosphoesterase family showed high homology to the putative PC-PLC of *Arabidopsis*. NPC2, NPC1 and *O. sativa* P2 were clustered together and showed 64% and 57% amino acid similarity score between NPC2 and *O. sativa* P2 and between NPC1 and *O. sativa* P2 respectively. Again NPC6 and *O. sativa* P3 exhibited high similarity making one sub-cluster.
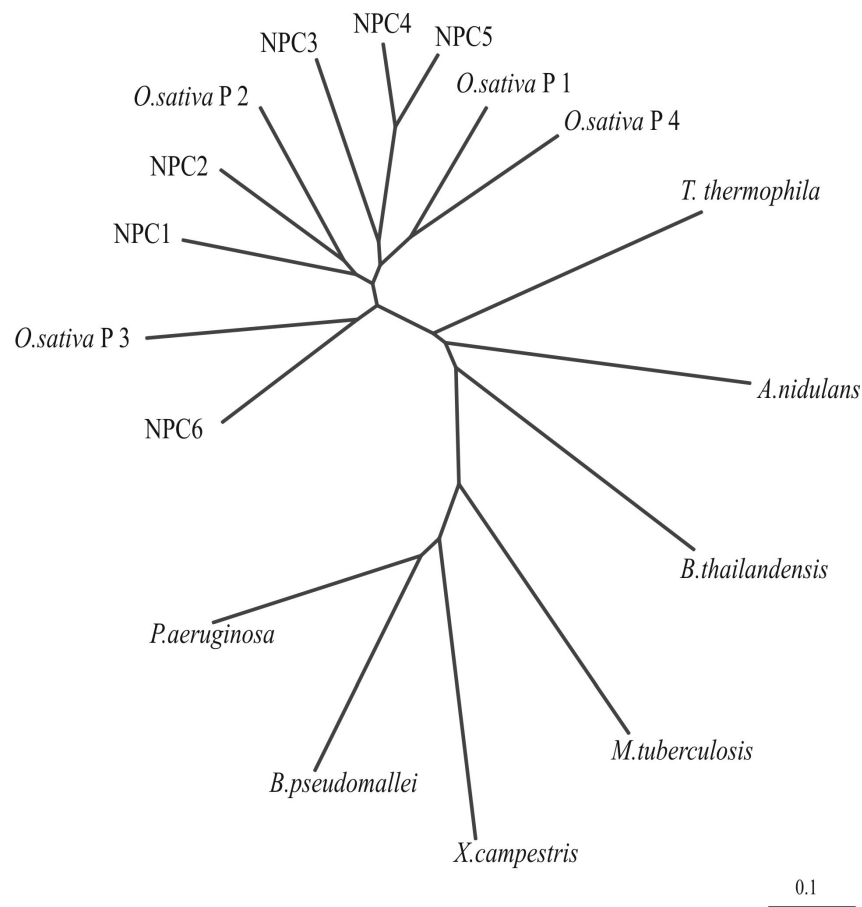
Figure 3.2: Phylogenetic relationship of bacterial PC-PLC [*Mycobacterium tuberculosis* (accession no. P95246), *Burkholderia pseudomallei* (accession no. Q9RGS8), *Burkholderia thailandensis* (accession no. YP_439303), *Pseudomonas aeruginosa* (accession no. P15713) and *Xanthomonas campestris* (accession no.YP_365022)], fungal hypothetical protein related to phosphoesterase family [*Aspergillus nidulans* (accession no. XP_681815)], phosphoesterase family protein of a protozoan [*Tetrahymena thermophilia* (accession no. EAR87012)], phosphoesterase family proteins P1, P2, P3 and P4 of *Oryza sativa* [*O. sativa* P1(accession no. ABF99945), *O. sativa* P2 (accession no. XP_463753), *O. sativa* P3 (accession no. BAB92134) *O. sativa* P 4 (accession no. ABA94565)] and putative PC-PLC of *Arabidopsis* NPC1 (accession no. NP_172203), NPC2 (accession no. NP_180255), NPC3 (accession no. NP_187002), NPC4 (accession no. NP_566206), NPC5 (accession no. NP_566207) and NPC6 (accession no. NP_190430). Phylogenetic tree was produced by the program ClustalW and displayed by the program TREEVIEW.

NPC3, NPC4, NPC5 and *NPC6* are located in chromosome III while *NPC2* and *NPC1* are located in chromosomes II and I respectively. *NPC3, NPC4* and *NPC5* are positioned in tandem in chromosome III. The six putative *PC-PLC* (*NPC)* genes can be classified into two groups depending on amino acid homology and gene structure (Figure 3.3). *NPC1, NPC2* and *NPC6* can be classified into group 1 and *NPC3, NPC4* and *NPC5* into group II.
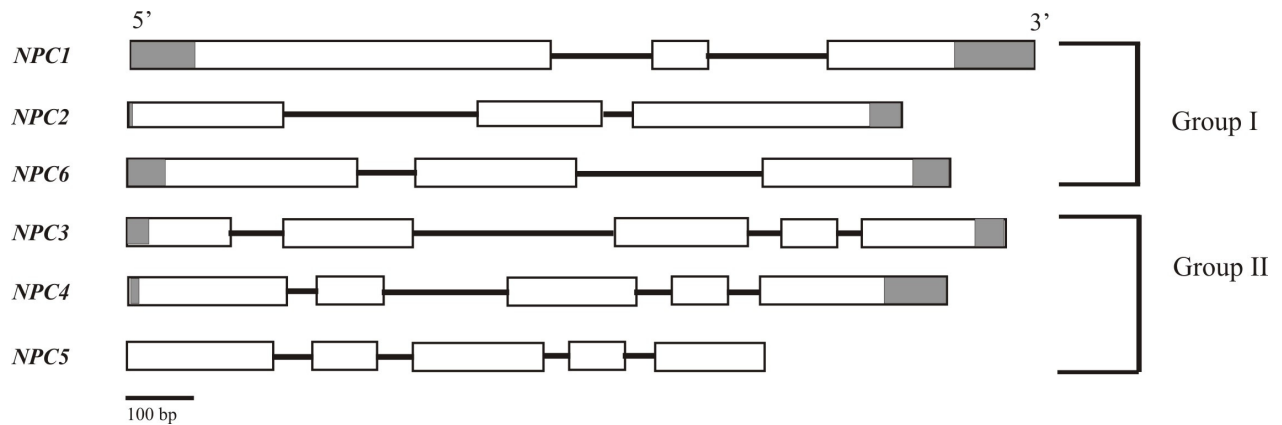
Figure 3.3: Gene structure of the putative *PC-PLC* family (*NPC*) in *Arabidopsis*. Exons are symbolized by white boxes and introns by black lines. Gray boxes indicate the 3' and 5' untranslated regions.

The identified *Arabidopsis* putative PC-PLC consist of 514-538 amino acid residues and molecular weights are approximately 60 KDa (Appendix Table 2). According to the signal P predictions (http://www.cbs.dtu.dk/services/Signal P) NPC6, NPC2 and NPC1 contain signal peptides suggesting to be secreted or membrane-associated while the other members do not contain any signal peptide. According to the PSORT (http://www.expasy.ch/tools) protein localization prediction; NPC4, NPC5 is mainly located in membrane fraction and NPC3 in cytoplasm.

Although extrapolation of sequence homology and background knowledge of bacterial PC-PLC to *Arabidopsis* give a starting point in studying the newly identified putative PC-PLC family, one has to keep in mind that this gene family may be quite a different in terms of substrate specificity, regulation and function. Taken together, it can be concluded that these six genes may be the prospective candidates for *Arabidopsis* PC-PLC. The identification and molecular characterization of these genes will pave the way in determining the functions of PC-PLC in plant kingdom.

3.2 Expression analysis of putative *PC-PLC* (*NPC*) gene family of *Arabidopsis* by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Gene expression is an outcome of a complex chain of reactions which is regulated by development and environment signals. When a plant grows and develops, distinct sets of genes become active depending on the cellular processes. It is well known that transition from juvenile to mature form and from vegetative to reproductive stage distinct sets of genes control the fate of cells. Constitutive enzymes are generally key cellular enzymes required at all growth conditions and are thus synthesized continuously in a growing cell. In other cases, a particular reaction may need only under some conditions and hence the gene products are not synthesized unless needed (Buchanan et al., 2000; Taiz and Zeiger, 2002).

Developmentally and environmentally regulated expression of *PLA, PLD*, *PI-PLC* genes has been reported in *Arabidopsis* and other plant species (Dyer et al., 1994; Hirayama et al., 1995; Fan et al., 1997; Katagiri et al., 2001; Holk et al., 2002; Rietz et al., 2004). Expression pattern of single *NPC* genes which encode putative *PC-PLC* family of *Arabidopsis* was investigated in response to organ specificity, nutrient deficiency and environmental signals such as hormones to get initial insight in elucidating their probable functions.

3.2.1 Organ specific expression of *NPC* genes

To investigate the organ specific expression profile of *NPC* gene members, semi quantitative RT-PCR was done with total RNAs isolated from roots, stems, leaves, flowers and siliques of *in vitro* or greenhouse-grown 30 day old *Arabidopsis thaliana* Columbia 0 wild type (WT) plants.

Multiple *NPC* gene expression was observed in all the plant organs investigated (Figure 3.4). Although *NPC* transcription patterns were broadly overlapping, varied relative expression levels could be identified. *NPC1, NPC2* and *NPC6* that were categorized into group I depending on the gene structure and amino acid sequence similarity, showed relatively higher expression in all the organs observed in comparison to the group II that is consisted of *NPC3*, *NPC4* and *NPC5*. In roots, stems, leaves, flowers and siliques higher expression levels of *NPC1* and *NPC6* were observed. However, expression of *NPC2* was weak in roots and relatively strong expression could be observed in other organs. Transcripts of *NPC3* and *NPC4* were observed in multiple organs while higher level of expression was detected in

roots. The expression of *NPC5* transcript could only be observed in flowers after 35 cycles of PCR but was hardly detectable in roots, stems, leaves and siliques. After 40 PCR cycles, low *NPC5* transcript levels were detected in all the organs.
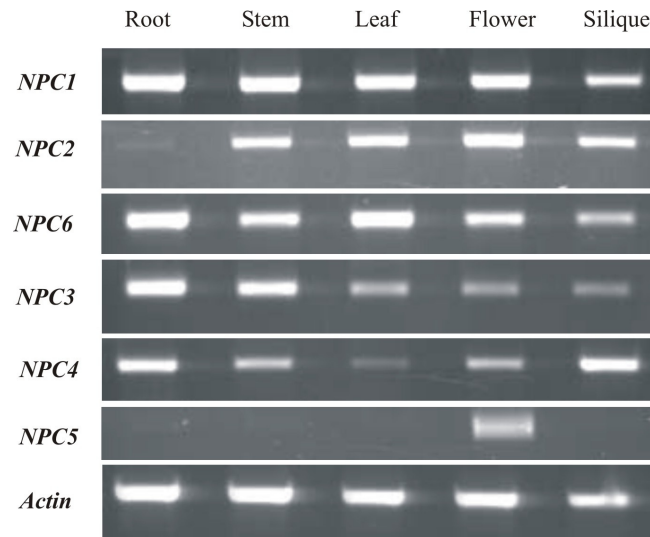

Figure 3.4: Organ specific expression of *NPC* genes. Semi-quantitative RT-PCR analysis was performed with total RNA isolated from root, stem, leaf, flower and silique of 30 d old *in vitro* grown WT plants. *Actin* was used as standard. The gel shows 8 µl of PCR products after 35 cycles.

Taken together, individual members of the *NPC* gene family showed overlapping expression patterns with little variations in relative expression levels. *NPC5* is distinct from others in which expression was very low in all the organs except in flowers.

3.2.2 Transcription of *NPC* genes in response to nutrient deficiency

Differential expression of genes has been reported in response to various nutritional conditions. The expression of *NPC* genes in response to phosphate deficiency (-Pi), sulfur deficiency (-S) and iron deficiency (-Fe) was investigated by semi-quantitative RT-PCR. Expression of each *NPC* gene was compared with the respective control plants grown on nutrient supplemented media (+Pi, +S and +Fe). Significant differences in expression levels between +S and –S were not observed in all the six *NPC* genes. Similarly, significant differences in *NPC* expression levels were not observed between +Fe and –Fe growth conditions. A clear increase in the *NPC4* transcript was observed in –Pi relative to the +Pi (Figure 3.5).

3.2.2.1 Transcription pattern of *NPC* genes in response to phosphate (P$_i$) deficiency

Plants have developed distinct systems to cope with phosphate (P$_i$) deficiency. There are plenty of evidences supporting the coordinated transcriptional regulation of number of genes during phosphate deficiency.

Transcriptional response of *NPC* genes to P$_i$ deficiency was investigated by extracting total RNA from seedlings grown either on 1.25 mM KH$_2$PO$_4$ containing (+Pi) or KH$_2$PO$_4$ lacking (-Pi) media followed by semi-quantitative RT-PCR (section 2.6.2.2.1). The relative transcript abundance of the *NPC1-NPC6* was compared with the level of *actin* (*ACTN6/7*) transcript. Differential expression pattern of *NPC* genes was observed between +Pi and –Pi (Figure 3.5).
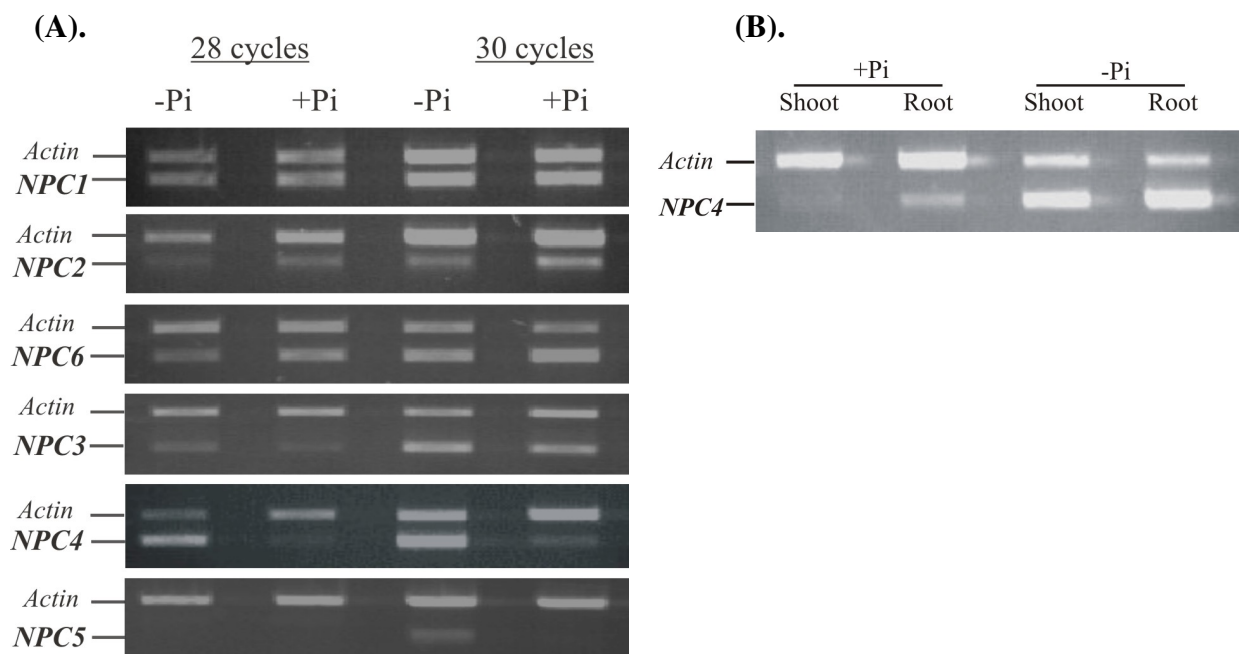

Figure 3.5: RT-PCR analysis of *NPC* expression in response to phosphate deficiency (-Pi).
(A). WT seedlings were grown 10 d on complete MS medium and then transferred to half strength AM either lacking Pi (-Pi) or containing 1.25 mM Pi (+Pi). Plants were harvested after 10 d and RT-PCR was performed with total RNA isolated from whole plants. Primers specific for *NPC* and *actin* were used in the same PCR. The gels show 8 µl of PCR products after 28 and 30 cycles. **(B).** Expression of *NPC4* in shoot and root in response to phosphate deficiency (-Pi). Plants were grown as for (A) and total RNA was isolated from shoot and root separately. Primers specific for *NPC4* and *actin* were used in the same PCR. The gel shows the 8 µl of PCR products after 30 cycles. Similar results were obtained in two independent experiments.

All the *NPC* genes except *NPC5* showed a substantial basal expression under +Pi growth conditions. The level of expression of *NPC1, NPC2* and *NPC6* was higher in comparison to *NPC3* and *NPC4*. A distinct increase in the *NPC4* transcript was observed in –Pi condition relative to the +Pi condition. A weak expression of *NPC5* was observed in response to –Pi

while basal level of *NPC5* expression was barely detected at +Pi status. Differential expression between +Pi and –Pi was not observed in *NPC1*, *NPC2, NPC6* and *NPC3*.

Expression pattern of *NPC4* was investigated in detail by separating shoots and roots in +Pi and –Pi grown plants. As shown in Figure 3.5 (B) both shoots and roots of –Pi grown plants showed significant increase in *NPC4* transcripts in comparison to +Pi grown plants. A recent study reported that NPC4 which hydrolyze PC-PLC was markedly induced both in shoots and roots during phosphate starvation (Nakamura et al., 2005).

3.2.3 Transcription pattern of *NPC* genes in response to phytohormone treatments

In higher plants, regulation of metabolism, growth and morphogenesis often depend on coordinated action of different phytohormones (Taiz and Zeiger, 2002). Transcriptional expression patterns of *NPC* genes were investigated in response to exogenous application of a variety of phytohormones; auxin, brassinolide, cytokinin, salicylic acid (SA), jasmonic acid (JA) and ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC). Differential expression of any of the *NPC* genes could not be observed in SA, JA and ACC treated seedlings compared to the hormone untreated seedlings. Significant increase in *NPC4* transcript level was observed in response to auxin and zeatin treatments (Figures 3.6 and 3.8). In addition, a weak increase in *NPC4* transcript was indicated by brassinolide (Figure 3.7). Differential transcription of other *NPC* genes was not observed in response to any of these hormone treatments.

3.2.3.1 Transcription pattern of *NPC* genes in response to auxin treatment

Auxin plays important roles in plant growth and development at least in part by regulating gene expression and to date several classes of auxin responsive genes have been identified in plants. To see whether *NPC* gene family responds to auxin, RT-PCR analysis was done by treating *Arabidopsis* seedlings with 10 µM of naturally occurring and most abundant auxin indole-3-actic acid (IAA) (section 2.8.1). Further, the induction of *NPC4* was examined by treating the seedlings with 10 µM of other auxins namely 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (1-NAA) for different durations.
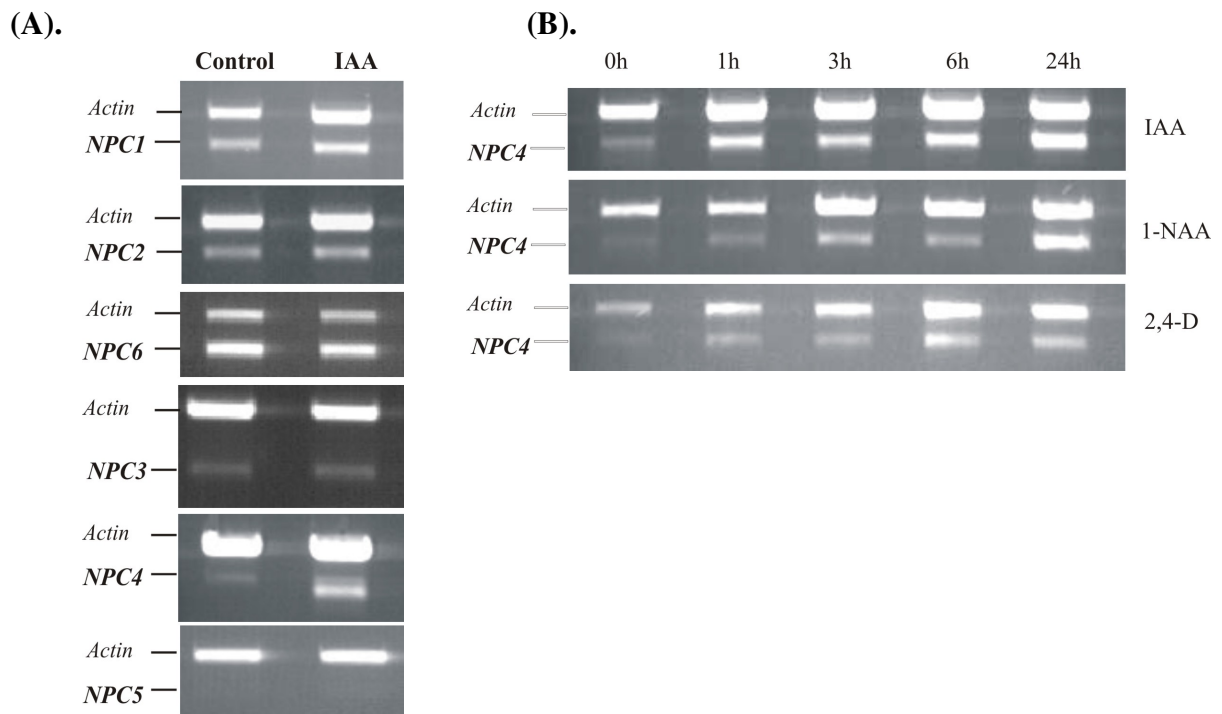
Figure 3.6: RT-PCR analysis of *NPC* expression in response to exogenous auxin application. **(A)** WT seedlings were grown 12 d on AM agar then transferred to liquid AM and treated with 10 µM IAA and total RNA was isolated from whole seedlings after 24 h treatment. Primers specific for *NPC* and *actin* were used in the same PCR. The gels show 8 µl of PCR products after 30 cycles. **(B)** WT seedlings were grown 12 d on AM agar, transferred to liquid AM and treated with 10 µM IAA, 10 µM 1-NAA and 10 µM 2,4-D. Total RNA was isolated from whole seedlings at different time intervals of 0 h, 1 h, 3 h, 6 h and 24 h after the initiation of treatment. Primers for *NPC4* specific and *actin* were used in the same PCR. The gels show 8 µl of PCR products after 30 cycles. Similar results were obtained in two independent experiments.

Increase in *NPC4* transcription (Figure 3.6 A) was observed relative to the auxin untreated control when seedlings were treated with 10 µM IAA for 24 h. Other *NPC* genes were not differentially regulated in response to IAA treatment. Time course study of *NPC4* expression with 10 µM of IAA, 1-NAA and 2,4-D indicated that these three auxin types slightly induced the transcription of *NPC4* after 1 h of treatment in comparison to 0 h control. The transcription levels were increased with increasing duration of the treatment (Figure 3.6 B).

3.2.3.2 Transcription pattern of *NPC* genes in response to brassinolide treatment

Brassinolides are important plant growth regulators in multiple development processes. The modulation of the expression of *NPC* genes in response to brassinolides was studied by RT-PCR by treating *in vitro* grown *Arabidopsis* seedlings with 0.1 µM of epi-brassinolide (BL) for 24 h (section 2.8.1).
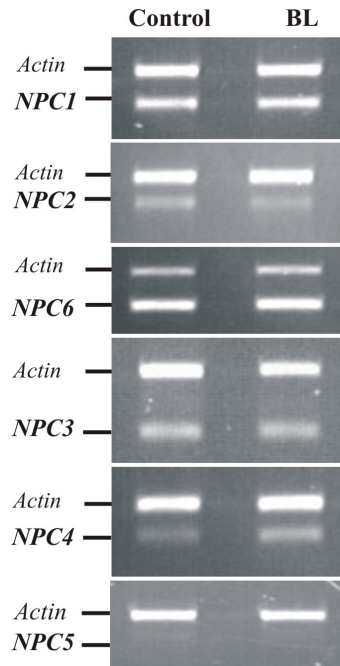
Figure 3.7: RT-PCR analysis of *NPC* expression in response to exogenous epi-brassinolide (BL) application. WT seedlings were grown 12 d on AM agar then transferred to liquid AM and treated with 0.1 µM BL and total RNA was isolated from whole seedlings after 24 h treatment. Primers for *NPC* specific and *actin* were used in the same PCR. The gels show 8 µl of PCR products after 30 cycles.

A striking effect of BL treatment on differential induction of individual *NPC* genes was not observed in comparison to control basal expression levels (Figure 3.7). However, a slight induction of *NPC4* was observed in response to BL treatment indicating probable regulation of this gene by exogenous BL.

3.2.3.3 Transcription pattern of *NPC* genes in response to zeatin treatment

Cytokinins regulate many cellular processes in plant growth and development playing a central role during the cell cycle. It has been shown that exogenous application of cytokinin affect the expression of many different genes in a variety of plant species. To investigate whether *NPC* gene family responds to cytokinin, RT-PCR analysis was done by treating *Arabidopsis* seedlings with 5 µM zeatin, a naturally occurring cytokinin (section 2.8.1).
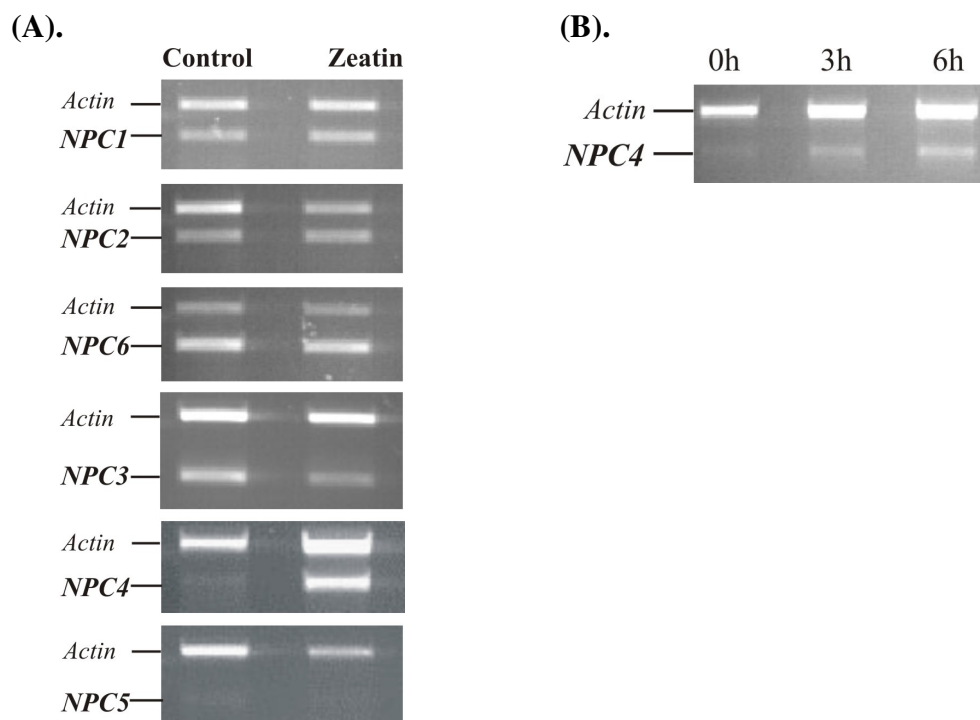
Figure 3.8: RT-PCR analysis of *NPC* expression in response to exogenous zeatin application. **(A)** WT seedlings were grown 14 d on MS agar supplemented with 5 µM zeatin or without zeatin as control. Primers specific for *NPC* and *actin* were used in the same PCR. The gels show 8 µl of PCR products of *NPC1, NPC2* and *NPC6* after 30 cycles and 8 µl of PCR products of *NPC3, NPC4* and *NPC5* after 32 cycles. **(B)** WT seedlings were grown 12 d on AM agar, transferred to liquid AM and treated with 5 µM zeatin for 1 h and 6 h. Primers specific for *NPC4* and *actin* were used in the same PCR. The gel shows 8 µl of PCR products after 32 cycles. Similar results were obtained in two independent experiments.

Compared to the zeatin untreated control seedlings, significant increase in transcription of *NPC4* was observed in zeatin treated seedlings. Differential expression of the other *NPC* genes in response to zeatin was not observed (Figure 3.8 A). The expression of *NPC4* was further analyzed by treating the seedlings with 5 µM of zeatin for 3 h, 6 h and compared with 0 h control transcript level. An increase in *NPC4* transcription could be observed after 3 h of zeatin treatment in comparison to the very low level of basal expression at 0 h. Further increase in the expression level was observed after 6 h of treatment (Figure 3.8 B).

Table 3.1 summarises the transcriptional expression of *NPC* genes in response to various treatments including nutrient deficiencies, exogenous hormone treatments and mechanical damages like wounding.

Table 3.1: Summary of transcriptional expression of *NPC1 to NPC6* by RT-PCR in response to various treatments.   - = no differential expression compared to respective controls, += transcriptional up-regulation compared to respective controls

Treatment	Gene					
	NPC1	*NPC2*	*NPC6*	*NPC3*	*NPC4*	*NPC5*
Nutrient deficiency						
Phosphate deficiency (-Pi)	-	-	-	-	+	+
Sulfur deficiency (-S)	-	-	-	-	-	-
Iron deficiency (-Fe)	-	-	-	-	-	-
Exogenous Aluminium (+Al)	-	-	-	-	-	-
Exogenous phytohormones						
Indole-3-actic acid (IAA)	-	-	-	-	+	-
Epi-brassinolide (BL)	-	-	-	-	+	-
Zeatin	-	-	-	-	+	-
Salicylic acid (SA)	-	-	-	-	-	-
Jasmonic acid (JA)	-	-	-	-	-	-
1-aminocyclopropane-1-carboxylic acid (ACC)	-	-	-	-	-	-
Wounding	-	-	-	-	-	-

Taken together, RT-PCR analysis reveal that expression of group I genes which include *NPC1*, *NPC2* and *NPC6* were dominantly detected in all the organs observed while group II genes which include *NPC3*, *NPC4* and *NPC5* showed relatively low expression. *NPC5* is distinct from all other *NPC*s, that it was expressed only very weak in all the organs except in flowers. In general, these results are in agreement with the microarray data presented by Genevestigator (https://www.genevestigator.ethz.ch) (Zimmerman et al., 2004) and by Arabidopsis massively parallel signature sequence (http://mpss.udel.edu/at/) (Meyers et al., 2004). *NPC4* of the *NPC* gene family is differentially expressed in response to phosphate deficiency, exogenous auxin, zeatin and epi-brassionolide treatments. *NPC5* showed a weak expression in response to phosphate deficient condition. The other members of the *NPC* gene family did not show differential expression in response to nutrient deficiencies, hormone treatments and wounding.

3.3   Histochemical expression analysis of *promoterNPC3::uidA* (*PNPC3*) and *promoterNPC4::uidA* (*PNPC4*)

Genes are expressed in a highly specific and controlled manner during the course of development, differentiation and in response to numerous environmental signals (Galun and Breiman, 1997; Singh, 1998; Buchanan et al., 2000; Taiz and Zeiger, 2002). The process of gene expression is divided crudely into transcription, translation and protein synthesis and therefore can be regulated at either of these levels (Brown, 1998).

Much of the regulatory portion of plant genes is located primarily upstream or 5', from the transcription start site where the RNA polymerase enzymes attach and generally referred to as gene promoters (Buchanan et al., 2000). Promoters play a major role in the regulation of transcription. The structure of eukaryotic promoters are divided generally into two parts, the core or minimal promoter consisting of upstream sequences called *cis*-acting elements required for gene expression and the additional regulatory sequences which control the activity of the core promoter (Buchanan et al., 2000; Taiz and Zeiger, 2002). The minimum promoter typically extends up to 100 bp upstream of the transcription initiation site and includes several sequence elements termed as TATA box, CAAT box and GC box (Stryer, 1995; Taiz and Zeiger, 2002). Many eukaryotic genes contain *cis*-acting DNA elements called enhancers and silencers which are not strictly part of the promoter yet strongly influence transcription (Weaver, 1999; Levin and Tjian, 2003). The promoter sequences vary in length, position, redundancy, orientation in DNA chain and bases showing a vast diversity. Further, a distinct role of the first intron as a potential regulatory element was reported (Jeong et al., 2006). An emerging theme in regulation of gene expression is the identification and analysis of promoters that specify the temporal and spatial expression of all genes in an organism. Genes whose expression has to be monitored can be fused to reporter genes and can be conveniently monitored. Among the many reporter genes, *E. coli uidA* gene encoding β-glucuronidase (GUS) and green fluorescent protein (GFP) from jellyfish *Aequorea victoria* have recently emerged as vital reporter for monitoring gene expression in plants (Jefferson et al., 1987; Mantis and Tague, 2000). To investigate the expression of *NPC3* and *NPC4* genes of the *NPC* gene family in detail, *promoterNPC3::uidA* and *promoterNPC4::uidA* fusions were constructed and introduced into the *Arabidopsis thaliana* Col. 0 WT plants. The transformants were subjected to an array of substances which mimic environmental signals and nutrient deprivations followed by histochemical GUS assay. Further, developmental regulation of these genes was studied at different developmental stages.

3.3.1 Generation of *promoterNPC3::uidA* (*PNPC3*) and *promoterNPC4::uidA* (*PNPC4*) constructs and selection of transgenic plants harbouring *PNPC3* and *PNPC4*

The presumed promoter regions of 2025 bp and 1903 bp upstream of the transcription regions were used to construct ß-glucuronidase (*uidA*) reporter fusions for stable transformants of *NPC3* and *NPC4* respectively (Figure 3.9 A). The promoter region was cloned (section 2.21.2) in front of the GFP-GUS regions of the Gateway-destination vector pKGWFS7 (Figure 3.9 B).
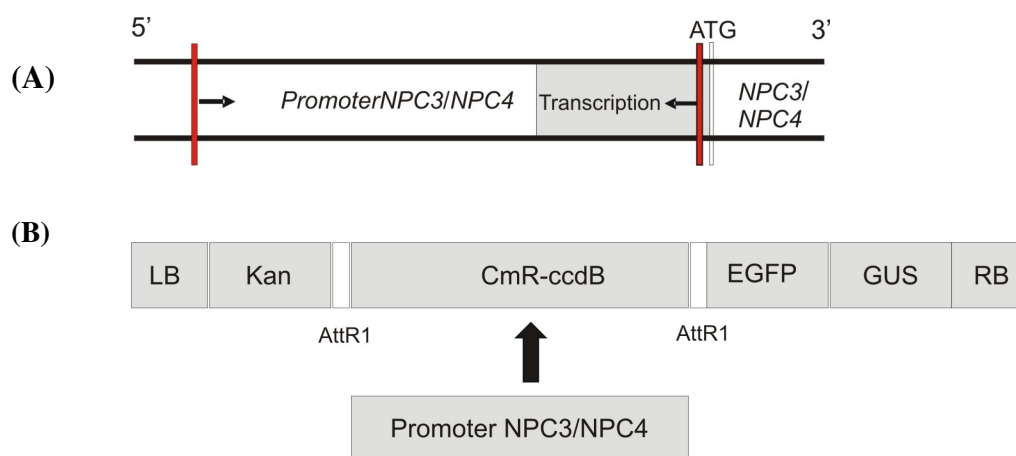

Figure 3.9:   *PromoterNPC3::uidA* (*PNPC3*) and *promoterNPC4::uidA* (*PNPC4*) constructs. **(A)**schematic presentation of deduced putative promoter regions of *NPC3* and *NPC4* in genome of *Arabidopsis thaliana*. Gray box indicates the transcribed region, white vertical bar represent first ATG of the gene and between red vertical bars is the deduced promoter region. **(B)** schematic presentation of cloning site of promoter region in Gateway-destination vector pKGWFS7.

Arabidopsis thaliana Col. 0 WT plants were transformed with the *PNPC3* and *PNPC4* constructs by *A. tumefaciens* mediated floral dip method (section 2.5.3). After harvesting the seeds (T$_1$), they were sown on selective MS-agar medium supplemented with kanamycin (50 µg/ml) and carbenicillin (100 µg/ml) (section 2.5.4). Only those that harbour the *promoter::uidA* construct were able to grow healthy on the selection medium. Non-transgenic seedlings turned into yellow after 7-12 days of sowing while the transgenic seedlings were able to grow in the selection medium with green leaves and developed a root system (Figure 3.10). For each promoter construct several lines of potential transgenic plants were generated. For *PNPC3*, 23 independent transgenic lines were generated while 9 independent lines were generated for *PNPC4*. The number of T-DNA insertions and place of insertion may be

different from transgenic line to line. The T_1 generation of seedlings were grown in the greenhouse. The seeds of self pollinated T_2 lines were used for analysis.
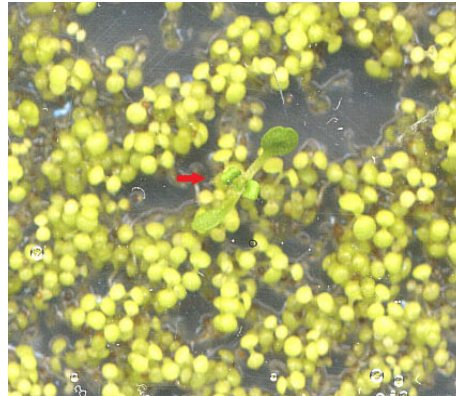

Figure 3.10: T_1 seedlings of *PNPC4* transformed *Arabidopsis thaliana* 12 days after sowing on MS-agar supplemented with 50 μg/ml kanamycin and 100 μg/ml carbenicillin. A potential transgenic seedling having green leaves is shown by an arrow.

3.3.2 Expression analysis of *PNPC3* and *PNPC4* plants

Transcriptional regulation of *PNPC3* and *PNPC4* was investigated in detail during the course of plant growth and in response to various environmental signals followed by histochemical GUS assay.

3.3.2.1 Expression analysis of *PNPC3* and *PNPC4* during plant development

Tissue specific expression of *PNPC3* and *PNPC4* during plant growth was investigated starting from early stages of vegetative growth to reproductive stage. As shown in Figures 3.11 and 3.12 the GUS expression pattern of *PNPC3* and *PNPC4* exhibited high degree of similarity over all the developmental stages. As early as 3 days of seedling growth, GUS staining was observed in the primary root restricted to the tip. Detailed view of the roots revealed that GUS activity was localized around the actively growing meristematic zones of the root tip (Figures 3.11 B and 3.12 D). When plants started to produce lateral roots, GUS activity additionally appeared at the tips of lateral roots. In 14 days old *PNPC3* and *PNPC4* seedlings, GUS staining was always observed at the tips of primary and lateral roots (Figures 3.11 C and 3.12 B). Over the development, GUS activity was observed in the cotyledons and leaves especially around the margins in addition to the tips of primary and lateral roots (Figures 3.11 D and 3.12 C). Again, both promoter constructs showed a similar pattern of GUS expression in 30 day old adult plants.
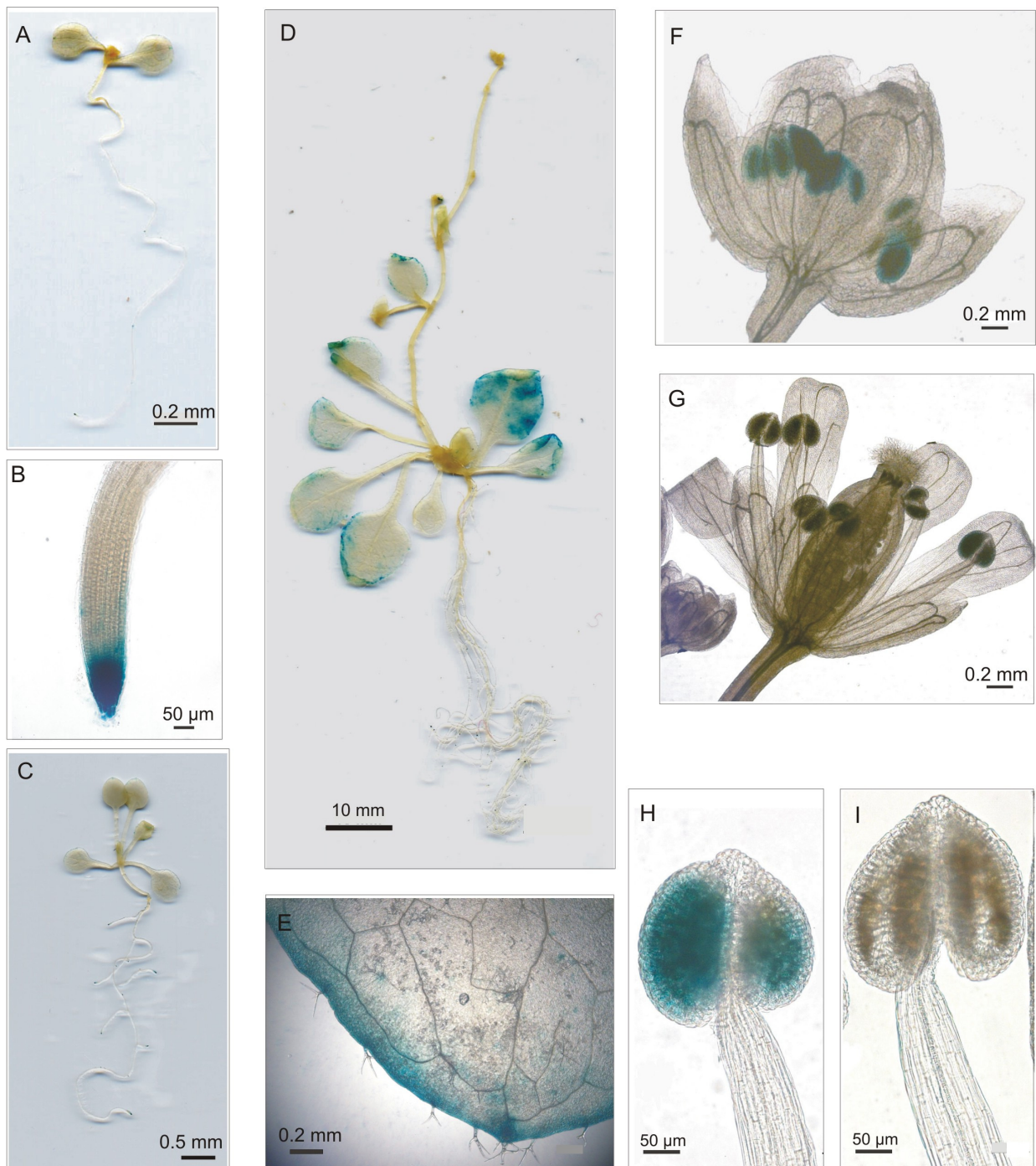
Figure 3.11: Histochemical analysis of *PNPC3* expression at various development stages. **(A)** 6 day old seedling **(B)** close view of tip of the primary root of 14 day old seedling **(C)** 14 day old seedling **(D)** 30 day old plant **(E)** a leaf of 30 day old plant **(F)** young flower **(G)** mature flower **(H)** young anther **(I)** mature anther. The GUS assay was done by incubating the plants in 2 mM X-Gluc at 37°C for 16 h.
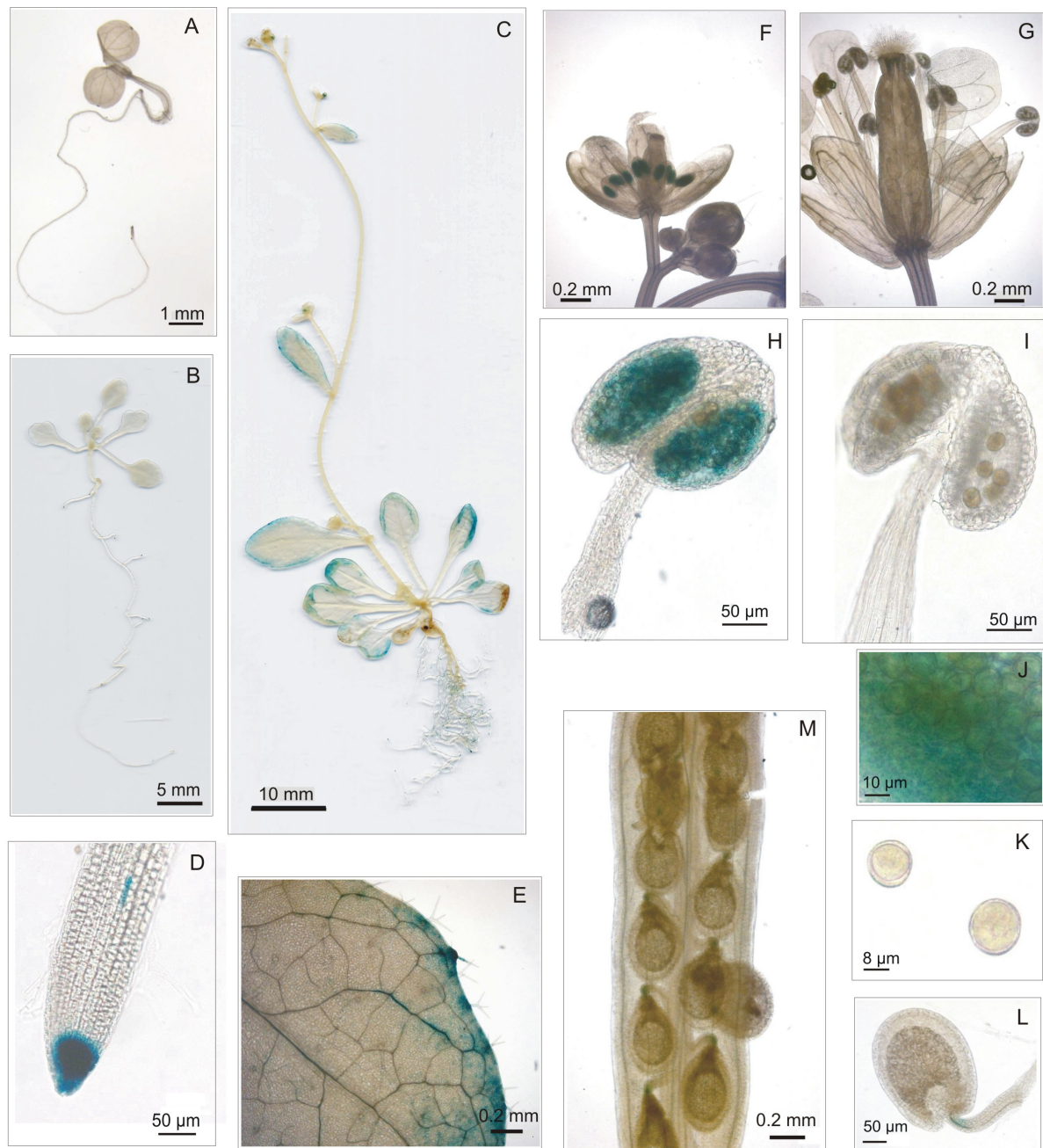
Figure 3.12: Histochemical analysis of *PNPC4* expression at various development stages.
(A) 6 day old seedling (**B**) 14 day old seedling **(C)** 30 day old plant **(D)** close view of tip of the root of 14 day old seedling **(E)** leaf of 30 day old plant **(F)** young flower **(G)** mature flower **(H)** young anther **(I)** mature anther **(J)** young pollen sac tissue of young anther **(K)** pollen grains **(L)** developing seed **(M)** silique. The GUS assay was done by incubating the plants in 2 mM X-Gluc at 37°C for 16 h.

In 30 day old adult plants relatively intense GUS staining was observed both in rosette leaves and in cauline leaves mainly concentrated to the margins. In the root system, as observed in all stages of development, staining was restricted to the root tips indicating a constitutive expression of *PNPC3* and *PNPC4* in the root tips. *PNPC3* and *PNPC4* showed a similar GUS expression pattern in floral organs too. GUS staining was specifically detected in the young anthers in young flowers (Figures 3.11 F, H and 3.12 F, H). In other floral organs namely sepals, petals and pistil, staining was barely detected. Microscopic observation of developing anthers revealed that in *PNPC4* the GUS activity was present in the pollen sac tissues (tapetum) but not in the pollen grains (Figure 3.12 J and K). In mature anthers GUS activity was not observed in any of the tissues (Figures 3.11 I and 3.12 I). In mature siliques *PNPC4* was expressed in the developing seeds and a weak GUS staining was observed in the funiculus (Figure 3.12 L).

Taken together, it can be concluded that the promoters of *NPC3* and *NPC4* are regulated in a similar manner during the course of development given the same conditions of growth. Both the promoters were active in primary and lateral root tips, leaf margins and in young pollen sac tissues. The GUS expression pattern of adult plants is basically consistent with the organ specific RT-PCR analysis of *NPC3* and *NPC4* (Figure 3.4).

3.3.2.2 Induction analysis of *PNPC3*

To get further insight into the transcriptional regulation of *NPC3* in addition to the regulation during development, transgenic plants carrying the *PNPC3* constructs were subjected to a number of signals of plant defence, phytohormones, nutrient deprivation and wounding.

3.3.2.2.1 Induction analysis of *PNPC3* in response to exogenous phytohormone application

As described in section 2.6.2.1, 12-day old *PNPC3* (F_2) seedlings grown *in vitro* were treated with an array of phytohormones and histochemical GUS analysis was done (Figure 3.13).
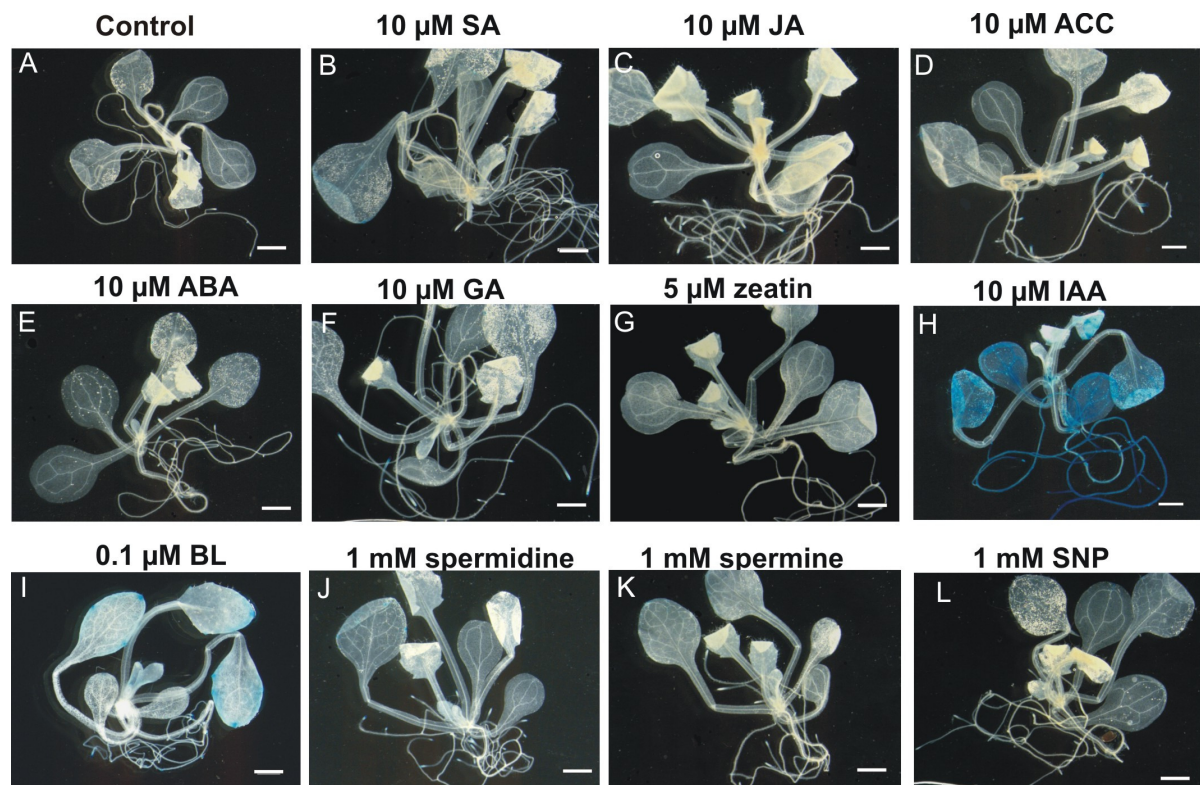
Figure 3.13: Histochemical analysis of *PNPC3* expression after induction with various chemical substances. All the seedlings subjected to treatments were grown initially on AM agar for 12 days. **(A)** mock treated control **(B)** treated with 10 µM SA for 48 h **(C)** treated with 10 µM JA for 48 h **(D)** treated with 10 µM ACC for 48 h **(E)** treated with 10 µM ABA for 48 h **(F)** treated with 10 µM GA for 48 h **(G)** treated with 5 µM zeatin for 48 h **(H)** treated with 10 µM IAA for 24 h **(I)** treated with 0.1 µM BL for 48 h **(J)** treated with 1 mM spermidine for 48 h **(K)** treated with 1 mM spermine for 48 h and **(L)** treated with 1 mM SNP for 48 h. GUS assay was done by incubating the treated seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 1 mm

In 14 day old mock treated control seedlings background GUS activity was very low, showing constitutive GUS expression in the primary and lateral root tips and occasionally scattered weak GUS expression around the margins of some leaves (Figure 3.13A). No or insignificant GUS induction relative to the control was shown by the seedlings treated with phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), gibberellic acid (GA) and zeatin (Figure 3.13 B-G). Further, a relative increase in GUS activity was not observed in polyamines; spermidine and spermine treated seedlings and nitric oxide donor sodium nitropruside (SNP) treated seedlings (Figures 3.13 J-L). In all these cases GUS activity could only be observed in primary and lateral root tips like in the control seedlings. Steroid hormone epi-brassinolide (BL) treated seedlings showed enhanced GUS expression around margins and in the tips of leaves and cotyledons (Figure 3.13 I). However, significantly enhanced GUS expression was

not observed in the root system. A natural auxin, indole-3-acetic acid (IAA) treated seedlings showed a striking GUS activity both in shoots and roots (Figure 3.13 H). Effect of auxin on the expression of *PNPC3* was studied in detail.

3.3.2.2.1.1   Induction analysis of *PNPC3* in response to exogenous auxin

Twelve days old seedlings were treated with 10 µM of different sources of auxins namely naturally occurring indole-3-acetic acid (IAA), synthetic auxin 2,4 dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (1-NAA) for 6 h and 24 h. *PNPC3* induction was determined by histochemical GUS assay.
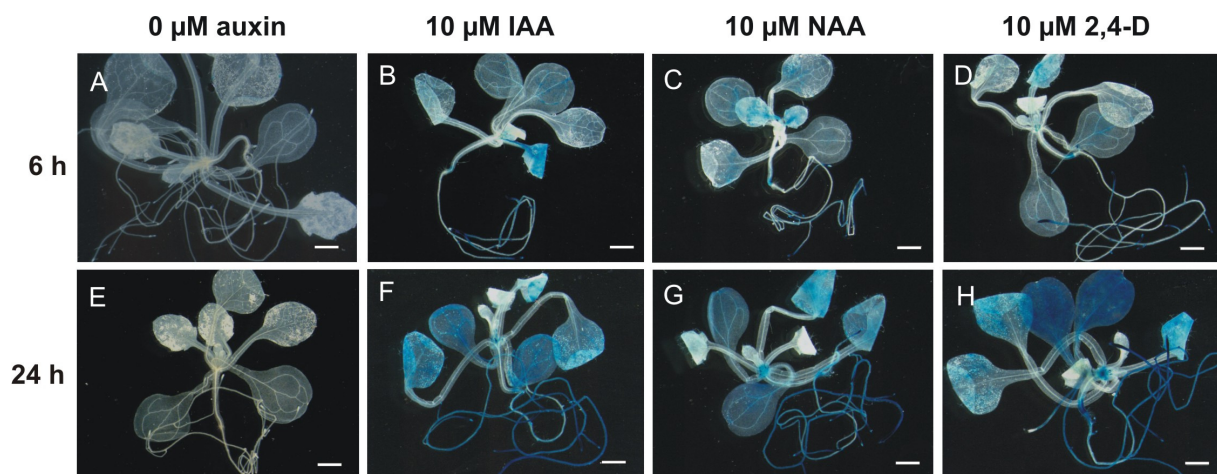
Figure 3.14: Histochemical analysis of *PNPC3* expression after induction with different auxin sources. Seedlings were grown on AM agar for 12 days and then treated with IAA, 1-NAA and 2,4-D for 6 h or 24 h. **(A)** mock treated control for 6 h auxin treatments **(B)** treated with 10 µM IAA for 6 h **(C)** treated with 10 µM 1-NAA for 6 h **(D)** treated with 10 µM 2,4-D for 6 h **(E)** mock treated control for 24 h auxin treatments **(F)** treated with 10 µM IAA for 24 h **(G)** treated with 10 µM 1-NAA for 24 h **(H)** treated with 10 µM 2,4-D for 24 h. GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 1 mm

Dramatic increase in GUS expression was observed in the entire root system and shoots of seedlings treated with natural or synthetic auxin types either for 6 h or 24 h (Figure 3.14). When seedlings were treated with lower concentrations of IAA, 1-NAA and 2,4-D (0.1 µM), roots stained more strongly than shoots indicating *PNPC3* is more sensitive to auxin in roots than in shoots. Detailed microscopic observations of 10 µM 1-NAA induced transgenic *PNPC3* seedlings are depicted in Figure 3.15.
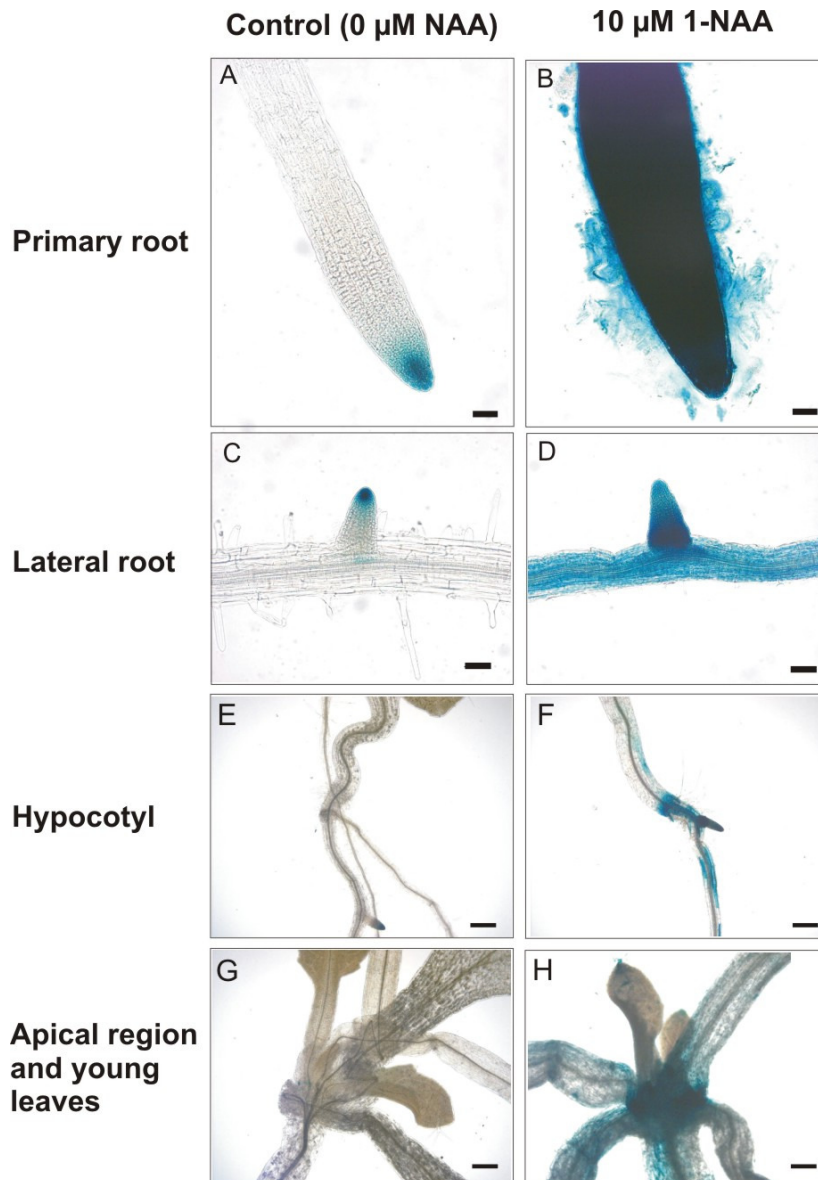
Figure 3.15: Histochemical analysis of *PNPC3* expression in different parts of the seedlings in response to 1-NAA treatment. *PNPC3* seedlings were grown 12 days in AM agar and then treated with 10 µM 1-NAA for 24 h. **(A)** mock treated control primary root tip **(B)** primary root tip of 1-NAA treated **(C)** developing lateral root of control **(D)** developing lateral root of 1-NAA treated **(E)** hypocotyl of control **(F)** hypocotyl of 1-NAA treated **(G)** apical region and young leaves of control **(H)** apical region and young leaves of 1-NAA treated seedlings. GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars A, B, C, D = 50 µm; E, F, G, H = 0.2 mm

Entire primary and developing lateral roots showed strong GUS expression in response to 1-NAA application while in the mock treated control seedlings GUS expression was restricted to primary and lateral root tips. Root shoot junction of the hypocotyls and shoot meristematic region and young leaves showed increased GUS expression indicating strong *NPC3* promoter activity in these sites (Figure 3.15).

3.3.2.2.1.2   Induction analysis of *PNPC3* in response to exogenous brassinolide

The effect of brassinolide on the expression of *PNPC3* was examined by treating 12 d old *PNPC3* seedlings with 0.1 µM epi-brassinolide (BL) for 48 h.
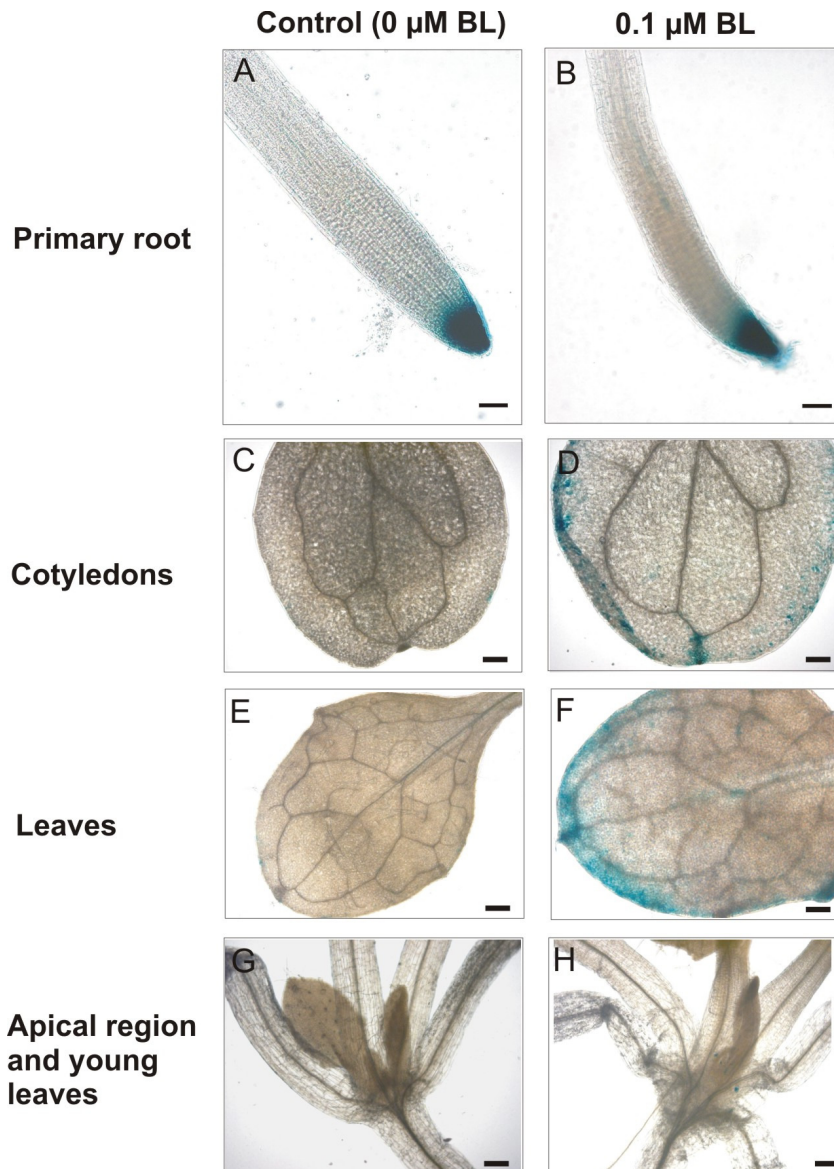
Figure 3.16: Histochemical analysis of *PNPC3* expression in different parts of the seedlings in response to 0.1 µM epi-brassinolide (BL) treatment. *PNPC3* seedlings were grown 12 days in AM agar and then treated with 0.1 µM BL for 48 h. **(A)** primary root tip of mock treated control **(B)** primary root tip of BL treated **(C)** cotyledon of control **(D)** cotyledon of BL treated **(E)** leaf of control **(F)** leaf of BL treated **(G)** apical region and young leaves of control **(H)** apical region and young leaves of BL treated seedlings. GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars A, B = 50 µm; C, D, E, F, G, H = 0.2 mm

In the mock treated control, expression of *PNPC3* was mainly localized to the primary and lateral root tips. A scattered weak GUS expression was sometimes detected in the cotyledons and leaves of the control plants. Treatment with BL significantly induced the GUS expression in the tips and in the margins of cotyledons and leaves (Figure 3.16). The intensity of GUS expression in the leaves was relatively higher than cotyledons. A slight enhancement in the GUS activity in the root tips was observed with BL treatment. In the BL untreated control plants, GUS activity was not observed in the young leaves. In response to BL, apical tips of the developing young leaves exhibited a very weak GUS activity (seen as spots at the tips of leaves). These observations indicate that the *PNPC3* responds to BL mainly in leaves and cotyledons.

3.3.2.2.1.3   Induction analysis of *PNPC3* in response to exogenous ACC

In the primary roots of mock treated control seedlings, GUS activity was restricted to the root apex. In ACC treated seedlings, relatively intense GUS expression was observed in the primary root apex extending to the stele (Figure 3.17). In other parts of the seedling, significant GUS expression was not observed in response to ACC treatment.

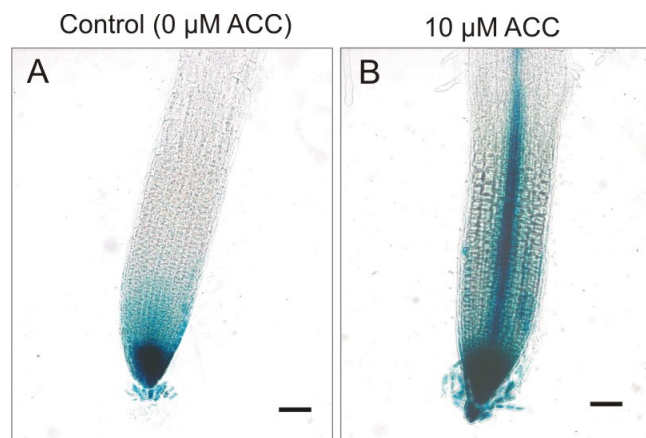
Figure 3.17: Histochemical analysis of *PNPC3* expression in primary root in response to ACC. *PNPC3* seedlings were grown 12 d in AM agar and then treated with 10 µM ACC for 48 h. **(A)** tip of primary root of mock treated **(B)** tip of primary root of ACC treated seedling. GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 50 µm

3.3.2.2.2   Induction analysis of *PNPC3* in response to nutrient deprivation

Transgenic seedlings were subjected to phosphate deficiency (-Pi), iron deficiency (-Fe) and sulfur (-S) deficiency to see whether *PNPC3* respond differentially in comparison to respective nutrient sufficient conditions. Differential induction of GUS activity was not observed in any of the above nutrient deficient conditions (Figure 3.18).
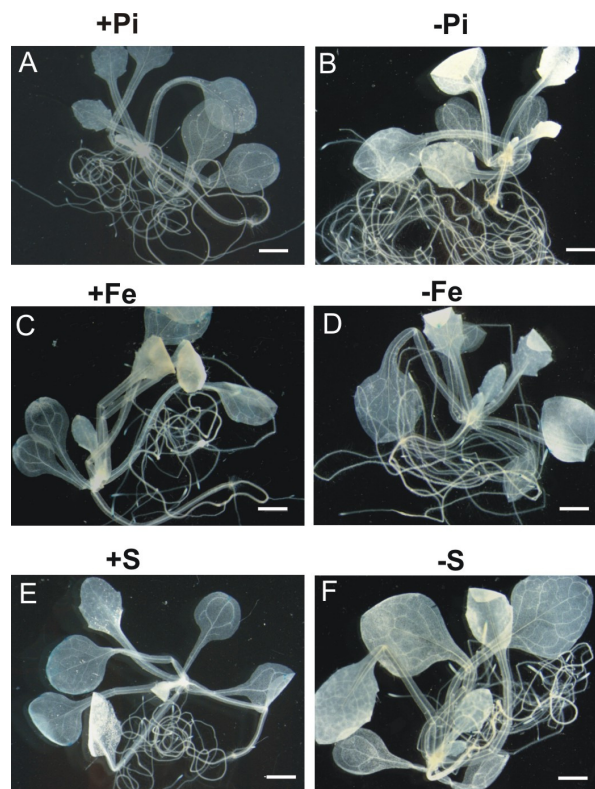
Figure 3.18: Histochemical analysis of *PNPC3* expression in response to nutrient deficient conditions. Seedlings were grown for 10 days in MS agar medium and then transferred and grown in **(A)** Pi containing (+Pi) AM for 10 days **(B)** Pi lacking (-Pi) AM for 10 days **(C)** Fe containing (+Fe) AM for 6 days **(D)** Fe lacking (-Fe) AM for 6 days **(E)** S containing (+S) half strength MS for 6 days **(F)** S lacking (-S) half strength MS for 6 days GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 1 mm

3.3.2.2.3   Induction analysis of *PNPC3* in response to abiotic stresses

The response of *PNPC3* to physical damage was investigated by wounding the leaves of greenhouse-grown 4-5 weeks old plants followed by histochemical GUS assay. In the unwounded control leaf, GUS activity was observed mainly at the margins of the leaf and scattered expression in the leaf blade. Differential GUS activity either local or systemic in response to wounding was not observed in the leaves (Figure 3.19).
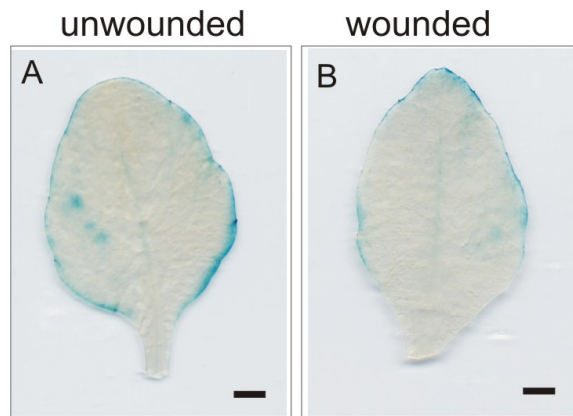
Figure 3.19: Histochemical analysis of *PNPC3* expression in response to wounding. **(A)** unwounded leaf of 4-5 week old plant **(B)** 24 h after wounding a leaf of 4-5 week old plant. GUS assay was done by incubating the leaves in 2 mM X-Gluc buffer at 37^oC for 16 h. Scale bars = 2 mm

Transgenic *PNPC3* did not respond differentially to other abiotic stresses namely cold stress, salt stress and drying in comparison to respective mock treated control seedlings.

Taken together, auxin was the strongest signal for the transcription of promoter of *NPC3* showing intense GUS staining both in roots and shoots. Significant induction of GUS activity was observed in leaves and cotyledons in response to brassinolide. An induction of GUS activity was indicated in root apex when treated with ethylene precursor ACC. All the other hormones including signals of plant defence, nutrient deficiency and abiotic stresses were unable in inducing *PNPC3* driven GUS expression at least in the present experimental conditions. All three independent transgenic lines investigated, showed the same responses towards each different signal tested.

3.3.2.3 Induction analysis of *PNPC4*

All classical hormones as well as Pi, Fe and S nutrient deficient conditions and abiotic factors such as cold stress, salt stress, drying and wounding were tested for their capability to regulate the transcription of the promoter of *NPC4* (*PNPC4*).

3.3.2.3.1 Induction analysis of *PNPC4* in response to exogenous phytohormone application

Transgenic *Arabidopsis* plants carrying *PNPC4* were treated with an array of phytohormones including substances related to pathogen defence to study the transcriptional regulation of *NPC4* gene. As described in section 2.6.2.1, 12-day old seedlings belonging to three independent *PNPC4* transgenic lines were treated with different phytohormones.
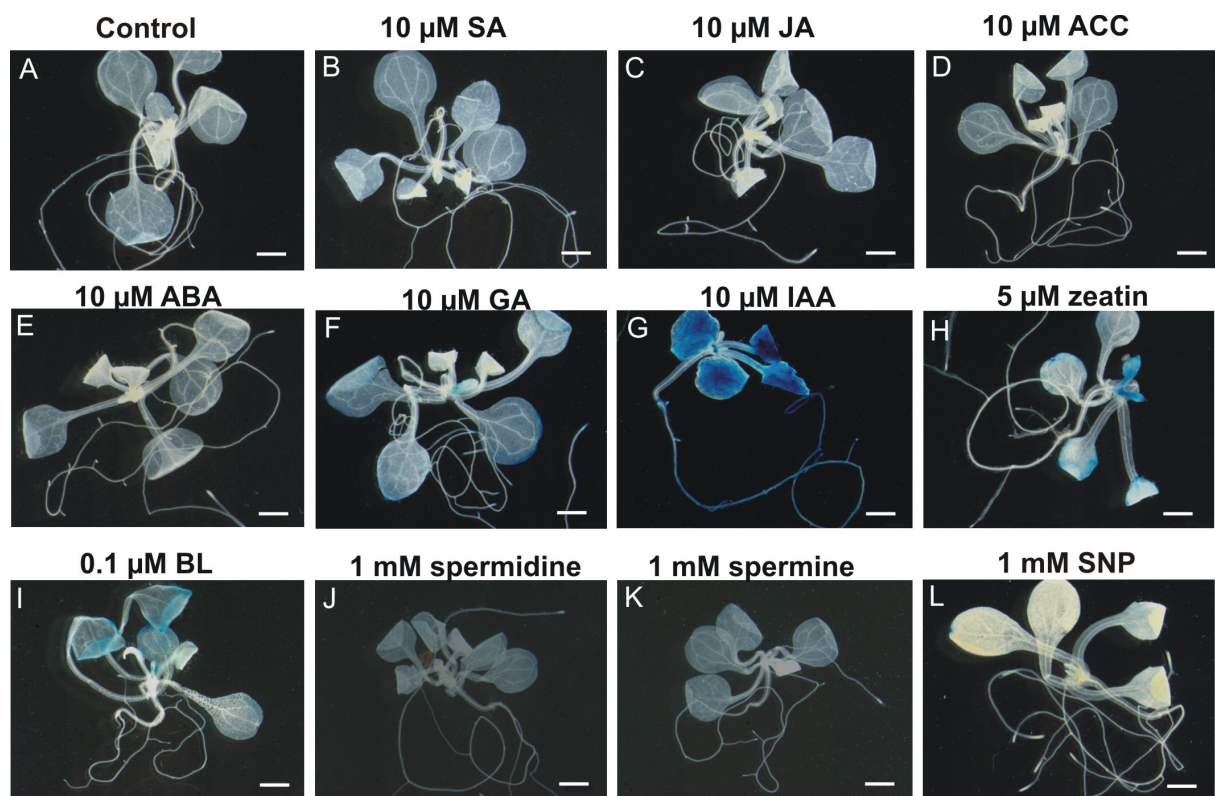
Figure 3.20: Histochemical analysis of *PNPC4* expression after induction with various chemical substances. All the seedlings subjected to treatments were grown initially on AM agar for 12 days. **(A)** mock treated control **(B)** treated with 10 µM SA for 48 h **(C)** treated with 10 µM JA for 48 h **(D)** treated with 10 µM ACC for 48 h **(E)** treated with 10 µM ABA for 48 h **(F)** treated with 10 µM GA for 48 h **(G)** treated with 10 µM IAA for 24 h **(H)** treated with 5 µM zeatin for 48 h **(I)** treated with 0.1 µM BL for 48 h **(J)** treated with 1 mM spermidine for 48 h **(K)** treated with 1 mM spermine for 48 h and **(L)** treated with 1 mM SNP for 48 h. Histochemical GUS assay was done by incubating the treated seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 1 mm

In 14 day old mock treated control *PNPC4* seedlings, the GUS activity was observed consistently in the primary and lateral root tips and occasionally scattered weak activity around the leaf margins (Figure 3.20 A). Similar GUS expression pattern was observed in the *PNPC3* seedlings. A dramatic increase in GUS activity both in roots and shoots was observed in the seedlings treated with 10 µM IAA for 24 h (Figure 3.20 G) as was observed in IAA treated *PNPC3* seedlings. Induction of GUS activity was evident in zeatin treated seedlings. The expression was obvious in the newly emerging leaves (Figure 3.20 H). *PNPC3* did not respond to zeatin treatment. Brassinolide (0.1 µM BL) was found to induce GUS expression in the leaves and cotyledons especially at the tips and margins (Figure 3.20 I) in a similar manner to brassinolide treated *PNPC3* seedlings. Further, a weak GUS activity was observed in the leaves of 10 µM gibberellic acid (GA) treated seedlings (Figure 3.20 F). No or insignificant GUS induction relative to the control was shown by the seedlings treated with other phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC) and abscisic acid (ABA) (Figure 3.20 B-E). Polyamines, spermidine and spermine and nitric oxide donor sodium nitropruside (SNP) were not able to show differential expression of *PNPC4* in comparison to control seedlings (Figure 3.20 J-L).

3.3.2.3.1.1   Induction analysis of *PNPC4* in response to exogenous auxin

Effect of auxin on the expression of *PNPC4* was studied in detail by treating the seedlings with different natural or synthetic auxins namely IAA, 2,4-D and 1-NAA for 6 h and 24 h followed by histochemical GUS assay (Figure 3.21). Strong *NPC4*-driven GUS expression was observed in the entire root system, leaves and apical meristem of the seedling in response to all the three auxin types (Figure 3.21). When seedlings were treated with lower concentrations of IAA, 1-NAA and 2,4-D (0.1 µM), roots stained more strongly than shoots indicating *PNPC4* is more sensitive in roots than in shoots.
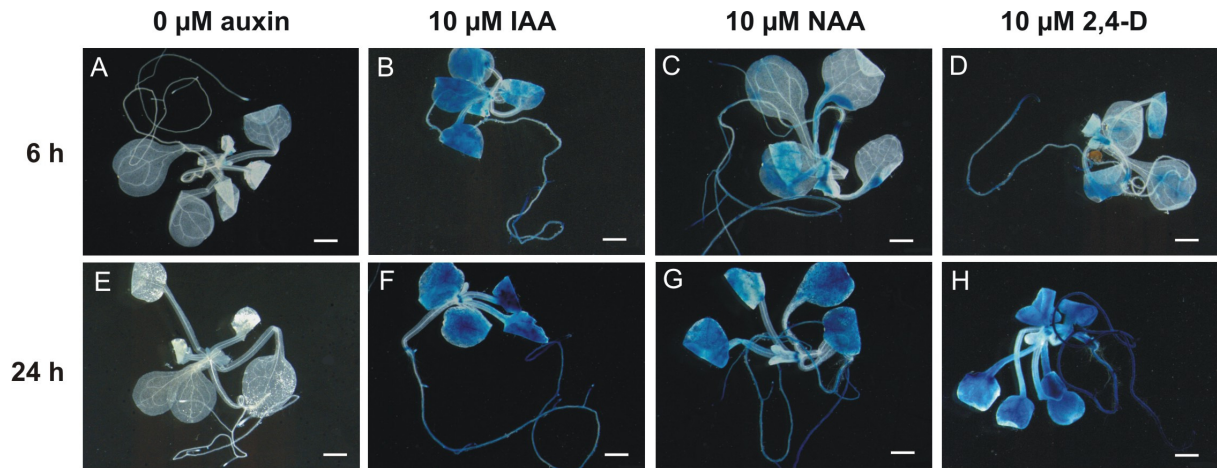
Figure 3.21: Histochemical analysis of *PNPC4* expression after induction with different auxin sources. Seedlings were grown on AM agar for 12 days and treated with IAA, 1-NAA and 2,4-D for 6 h or 24 h. **(A)** mock treated control for 6 h **(B)** treated with 10 µM IAA for 6 h **(C)** treated with 10 µM 1-NAA for 6 h **(D)** treated with 10 µM 2,4-D for 6 h **(E)** mock treated control for 24 h **(F)** treated with 10 µM IAA for 24 h **(G)** treated with 10 µM 1-NAA for 24 h **(H)** treated with 10 µM 2,4-D for 24 h. Histochemical GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 1 mm

Detailed microscopic observation of the GUS expression in different parts of the transgenic *PNPC4* seedlings in response to 10 µM 1-NAA induction for 24 h is depicted in Figure 3.22. In the primary and lateral roots of control seedlings, GUS activity was restricted to the tips. Strong GUS expression was observed throughout the entire primary and lateral roots in response to exogenous 1-NAA (Figures 3.22 B and D). Vascular bundles became strongly stained even after 6 h of auxin treatment. A prominent GUS activity could be observed in root and shoot junction which is absent in the control plants. Even shorter duration of GUS assay (30 min) resulted in *PNPC4* expression in hypocotyl. *PNPC4* expression in the shoot meristem and emerging young leaves was also highly induced by 1-NAA (Figure 3.22 H).
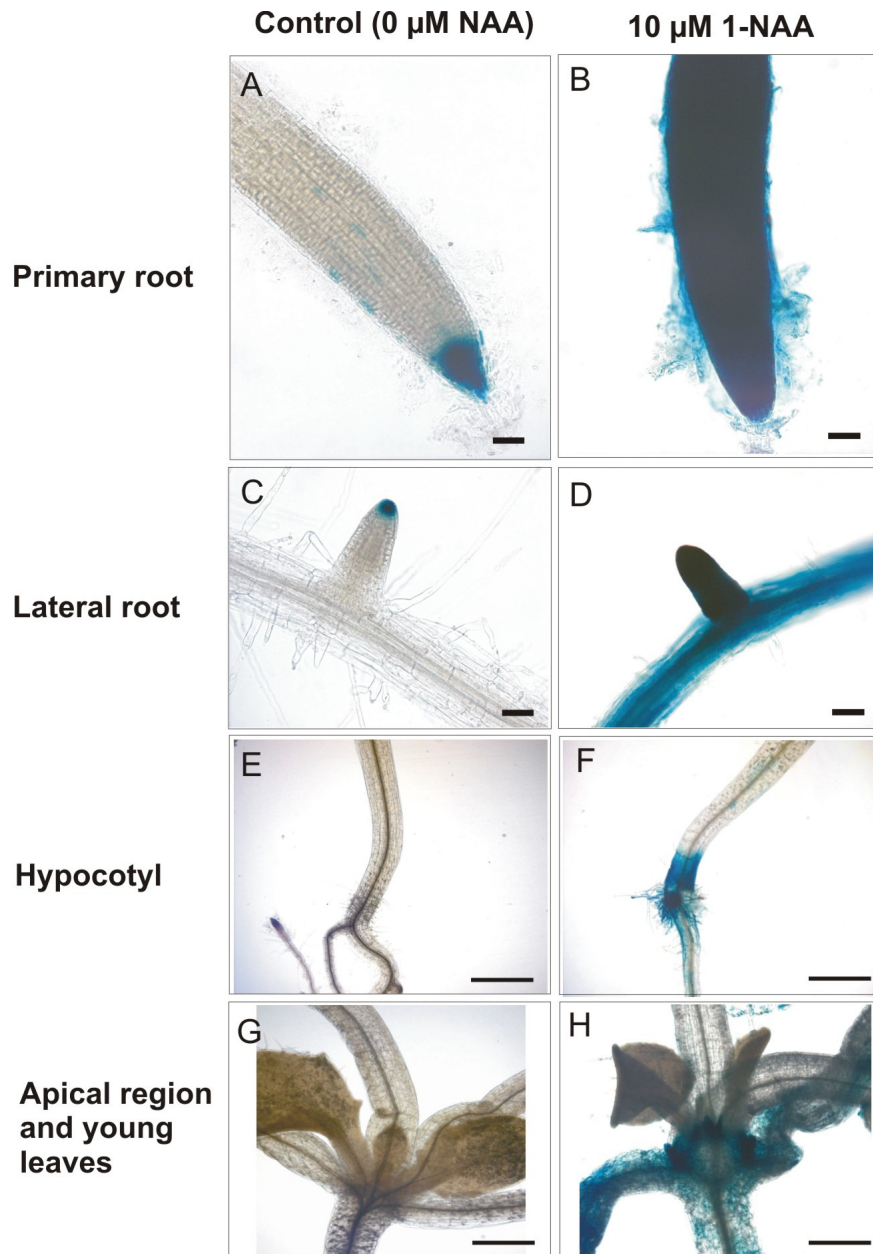
Figure 3.22: Histochemical analysis of *PNPC4* expression in different parts of the seedlings in response to 1-NAA. *PNPC4* seedlings were grown 12 days in AM agar and then treated with 10 µM 1-NAA for 24 h. **(A)** mock treated control primary root tip **(B)** primary root tip of 1-NAA treated **(C)** developing lateral root of control **(D)** developing lateral root of 1-NAA treated **(E)** hypocotyl of control **(F)** hypocotyl of 1-NAA treated **(G)** apical region and young leaves of control seedling **(H)** apical region and young leaves of 1-NAA treated seedlings. GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars A, B, C, D = 50 µm; E, F, G, H = 0.2 mm

3.3.2.3.1.2   Induction analysis of *PNPC4* in response to exogenous zeatin

Expression of the *PNPC4* in response to cytokinine was investigated by treating 12 d old transgenic seedlings with 5 µM of zeatin for 48 h. *NPC4*-driven GUS expression was induced in newly emerging leaves by exogenous zeatin. In the mock treated control seedlings any GUS activity was not observed in the newly emerging leaves. A weak induction of GUS activity was indicated in the developing lateral roots in comparison to the control (Figure 3.23).
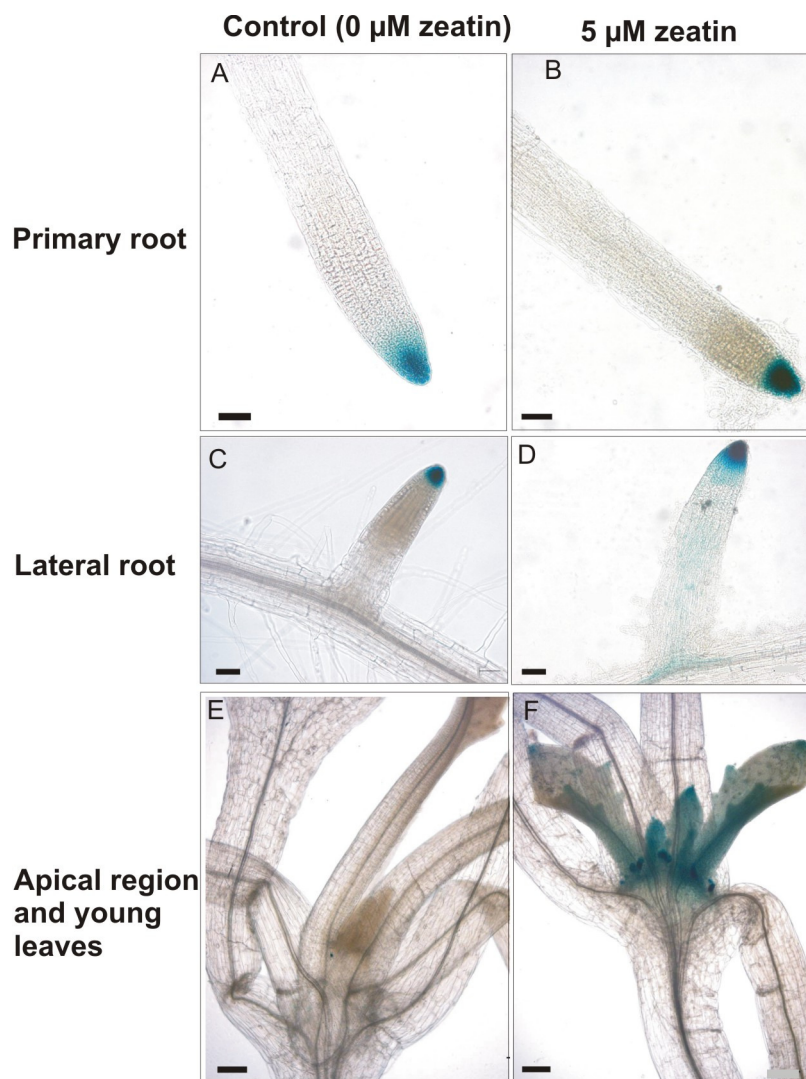
Figure 3.23: Histochemical analysis of *PNPC4* expression in root and shoot in response to exogenous zeatin application. **(A)** primary root tip of mock treated control **(B)** primary root tip treated with 5 µM zeatin for 48 h **(C)** developing lateral root of control **(D)** developing lateral root treated with 5 µM zeatin for 48 h **(E)** apical region and young leaves of control **(F)** apical region and young leaves treated with 5 µM zeatin for 48 h. Histochemical GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars A, B, C, D = 50 µm; E, F = 0.2 mm

3.3.2.3.1.3   Induction analysis of *PNPC4* in response to exogenous brassinolide

The effect of brassinolide on the expression of *PNPC4* was examined by treating 12 d old *PNPC4* seedlings with 0.1 µM epi-brassinolide (BL) for 48 h.
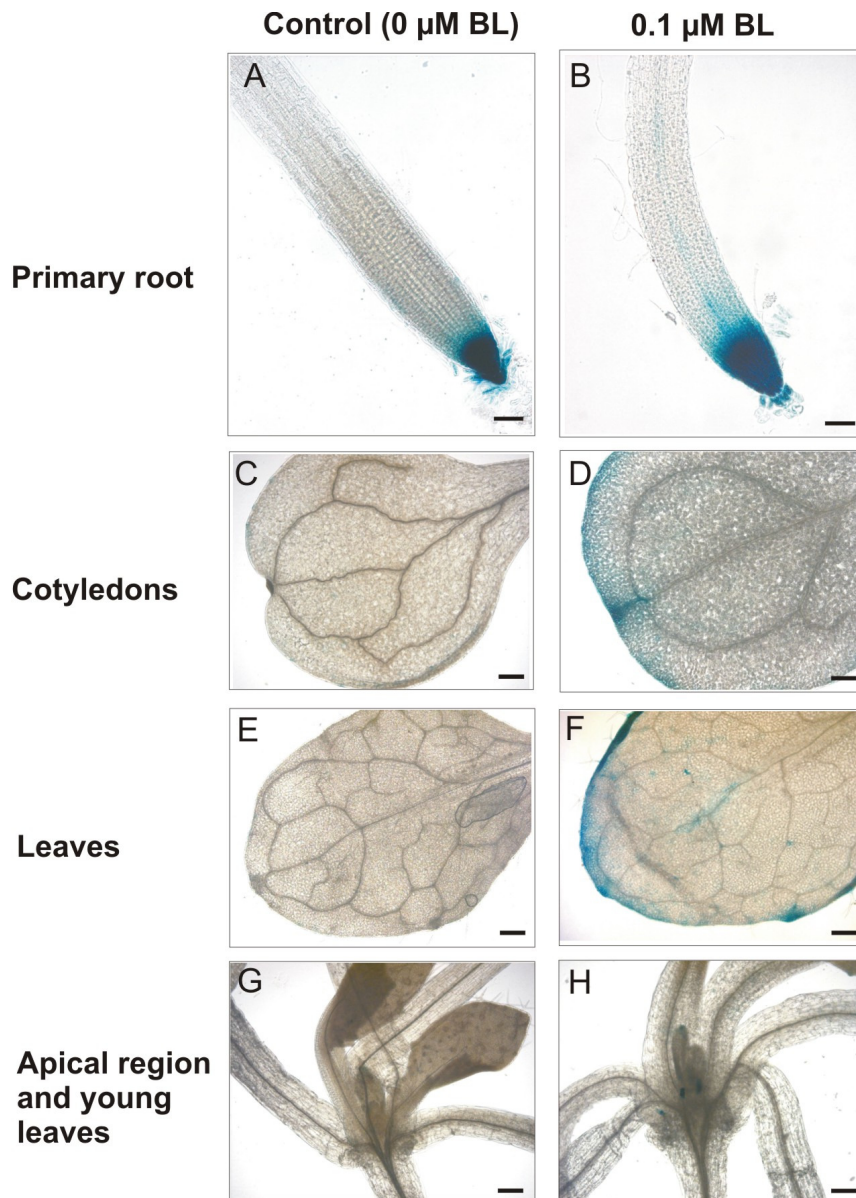
Figure 3.24: Histochemical analysis of *PNPC4* expression in different parts of the seedlings in response to 0.1 µM epi-brassinolide (BL) treatment. *PNPC4* seedlings were grown 12 days in AM agar and then treated with 0.1 µM BL for 48 h. **(A)** primary root tip of mock treated control **(B)** primary root tip of BL treated **(C)** cotyledon of control **(D)** cotyledon of BL treated **(E)** leaf of control **(F)** leaf of BL treated **(G)** apical region and young leaves of control **(H)** apical region and young leaves of BL treated seedlings. GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars A, B = 50 µm; C, D, E, F, G, H = 0.2 mm

In the mock treated control, *PNPC4* was mainly expressed in the primary and lateral root tips. Slightly enhanced GUS activity was observed in the primary root tips in response to BL treatment (Figure 3.24 A and B). A weak and scattered GUS expression was occasionally detected in leaves and cotyledons in control seedlings. A significant GUS activity could be observed in the tips and in the margins of cotyledons and leaves when treated with BL (Figure 3.24 D and F). The intensity of GUS expression in the leaves was relatively higher than cotyledons. In the BL untreated control plants, GUS activity was not observed in the young leaves. In response to BL, apical tips of the developing young leaves exhibited a weak GUS activity (Figure 3.24 H).

3.3.2.3.2   Induction analysis of *PNPC4* in response to nutrient deprivation

PNPC4 seedlings were subjected to phosphate (-Pi), iron (-Fe) and sulfur (-S) deficient conditions. GUS activity was compared with the *PNPC4* seedlings grown in respective nutrient sufficient (+Pi), (+Fe) and (+S) conditions.
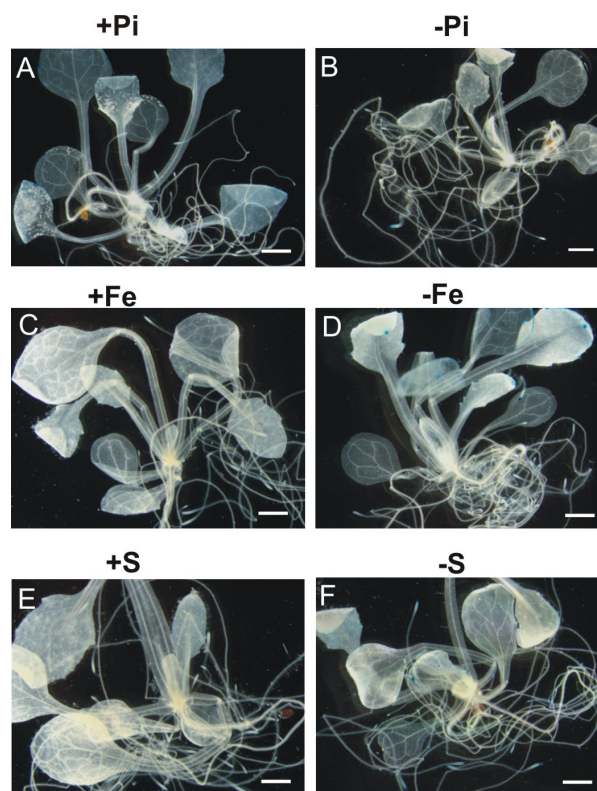
Figure 3.25: Histochemical analysis of *PNPC4* expression in response to nutrient deficient conditions. Seedlings were grown for 10 days in MS agar medium and then transferred and grown in **(A)** Pi containing (+Pi) AM for 10 days **(B)** Pi lacking (-Pi) AM for 10 days **(C)** Fe containing (+Fe) AM for 6 days **(D)** Fe lacking (-Fe) AM for 6 days **(E)** S containing (+S) half strength MS for 6 days **(F)** S lacking (-S) half strength MS for 6 days GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 1 mm

An obvious change in GUS activity was not evident in the whole seedlings grown in Pi, Fe and S deficient growth media in comparison to the seedlings grown in respective nutrient sufficient media (Figure 3.25). However, microscopic observations revealed that phosphate deficiency results in a weak increase in GUS expression in primary root tips, lateral root tips and lateral root primordia (Figure 3.26). Increased GUS expression was not observed in the shoot system even over a long period of growth in phosphate deficient medium.
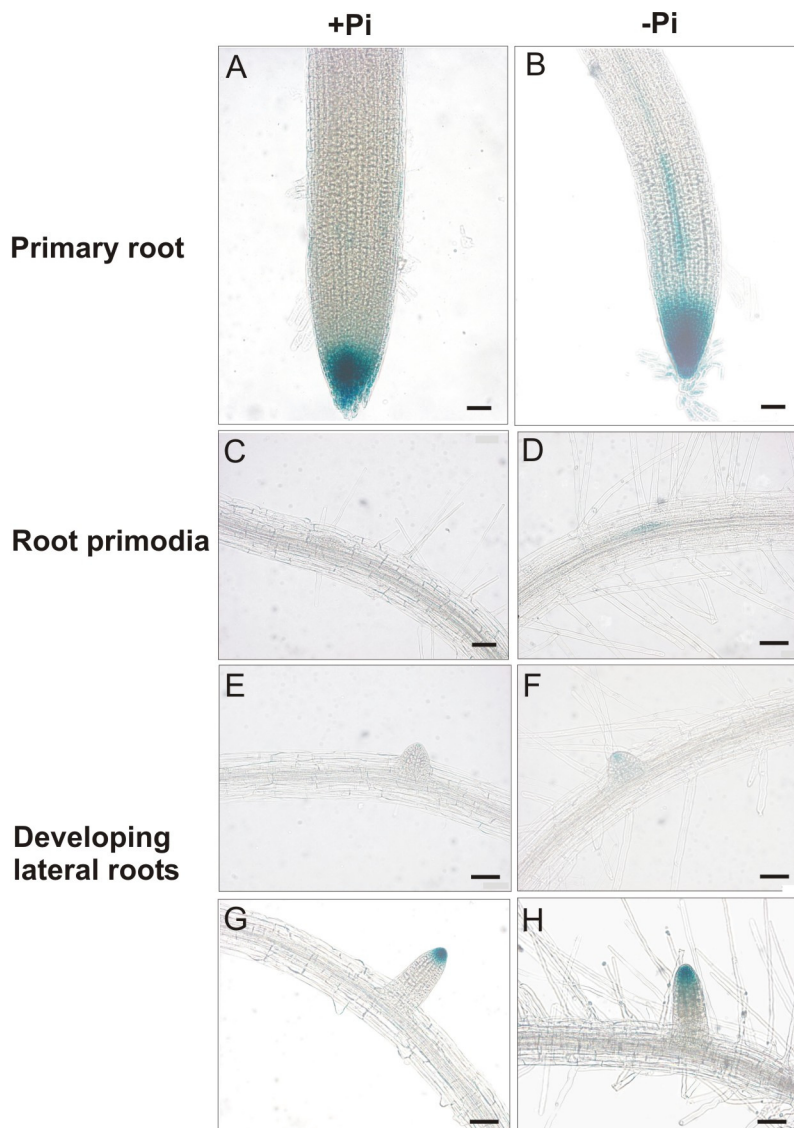
Figure 3.26: Histochemical analysis of *PNPC4* expression in primary and lateral roots at various stages of root development with respect to phosphate deficient growth conditions. Seedlings were grown for 10 days on MS and transferred to Pi containing AM (+Pi) (A,C,E,G) or to Pi lacking AM (-Pi) (B,D,F ,H) and grown for further 10 days. **(A)** primary root apex in +Pi **(B)** primary root apex in –Pi. **(C, E, G)** different stages of developing lateral roots on +Pi **(D,F,H)** different stages of developing lateral roots on –Pi. Histochemical GUS assay was done by incubating the seedlings in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 50 µm

3.3.2.3.3   Induction analysis of *PNPC4* in response to abiotic stresses

Response to a physical damage was investigated by wounding the mature leaves of 4-5 week old greenhouse-grown *PNPC4* plants. Differential expression of GUS activity either local or systemic was not observed in wounded leaves in comparison to unwounded leaves (Figure 3.27).
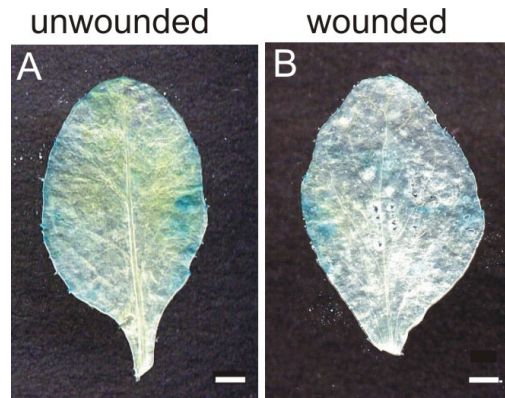
Figure 3.27: Histochemical analysis of *PNPC4* expression in response to wounding. **(A)** unwounded leaf of 4-5 week old plant **(B)** 24 h after wounding a leaf of 4-5 week old plant. GUS assay was done by incubating the leaves in 2 mM X-Gluc buffer at 37°C for 16 h. Scale bars = 2 mm

Transgenic *PNPC4* did not respond to other abiotic stresses namely cold stress, salt stress and drying in comparison to respective mock treated control seedlings.

In summary, promoter of *NPC4* gene responds intensely to exogenous auxin. Strong GUS activity was observed both in roots and shoots. After 6 h of auxin treatment, response of the roots was more intense than the shoots. Like *PNPC3*-driven GUS expression, *PNPC4*-driven GUS expression too indicated a response to brassinolide in the leaves and cotyledons especially throughout the margins. In addition to auxin and brassinolide, *PNPC4* was induced by zeatin specifically in the newly emerging leaves. Further, lack of phosphate in the growth medium weakly induced the promoter of *NPC4* in the primary and lateral roots. A weak enhancement of GUS activity was observed in the primary root apex and developing lateral roots especially in the early lateral root primordia. In phosphate containing growth medium GUS expression was inconspicuous in the lateral root primordia. All the other hormones including signals of plant defence, Fe and S deficiency and abiotic stresses were unable to induce *PNPC4* driven GUS expression. All three independent transgenic lines showed the similar responses towards each different signal tested. Semi-quantitative RT-PCR analysis showed increase in *NPC4* transcripts when treated with exogenous auxin, brassinolide and

zeatin and during phosphate starvation. These results coincide with the GUS expression analysis of *PNPC4*.

3.3.3   Sequence analysis of putative promoter regions of *NPC3* and *NPC4*

Besides qualitative analysis of *PNPC3* and *PNPC4* in response to an array of stimuli, nucleotide sequences of the deduced promoter regions were searched for known regulatory *cis*-elements. The sequence analysis of the deduced promoter regions was done by using the programme PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) (Prestige, 1991; Higo et al., 1999).

Figure 3.28: Schematic presentation of deduced promoter region of *NPC3* gene with possible regulatory elements. The positions of the different elements are shown as coloured vertical bars relative to the translation start site.

Sequence analysis revealed that putative promoter region of *NPC3* contains *cis*-elements with known regulatory functions for different responses (Figure 3.28). Binding sites for W box transcription factors of WRKY family members are present at several different locations in the promoter sequence. W boxes are found as number of different modifications which are responsive to elicitors (Eulgem et al., 2000), salicylic acid (Chen and Chen, 2001; Chen et al., 2002), wounding (Nishiuchi et al., 2004) and gibberellin (Zhang et al., 2004). Putative *cis*-element (ERELEE) that is described in tomato (Montgomery et al., 1993) and carnation (Itzhaki et al., 1994) as responsive to ethylene (ERELEE) and a ACC regulatory element

LECPLEACS2 described in tomato (Matarasso et al., 2005) is found in the *NPC3* promoter sequence. Moreover, sequences similar to auxin regulatory elements found in SAUR 15A-promoter of soybean (Xu et al., 1997), Dof protein NtBBF1 from tobacco (Baumann et al., 1999), SEBF gene from potato (Boyle and Brisson, 2001) are found over the stretch of *NPC3* promoter. The presence of several such auxin inducible *cis*-elements on the promoter region of the gene is in agreement well with the up-regulation of *PNPC3* by auxin.

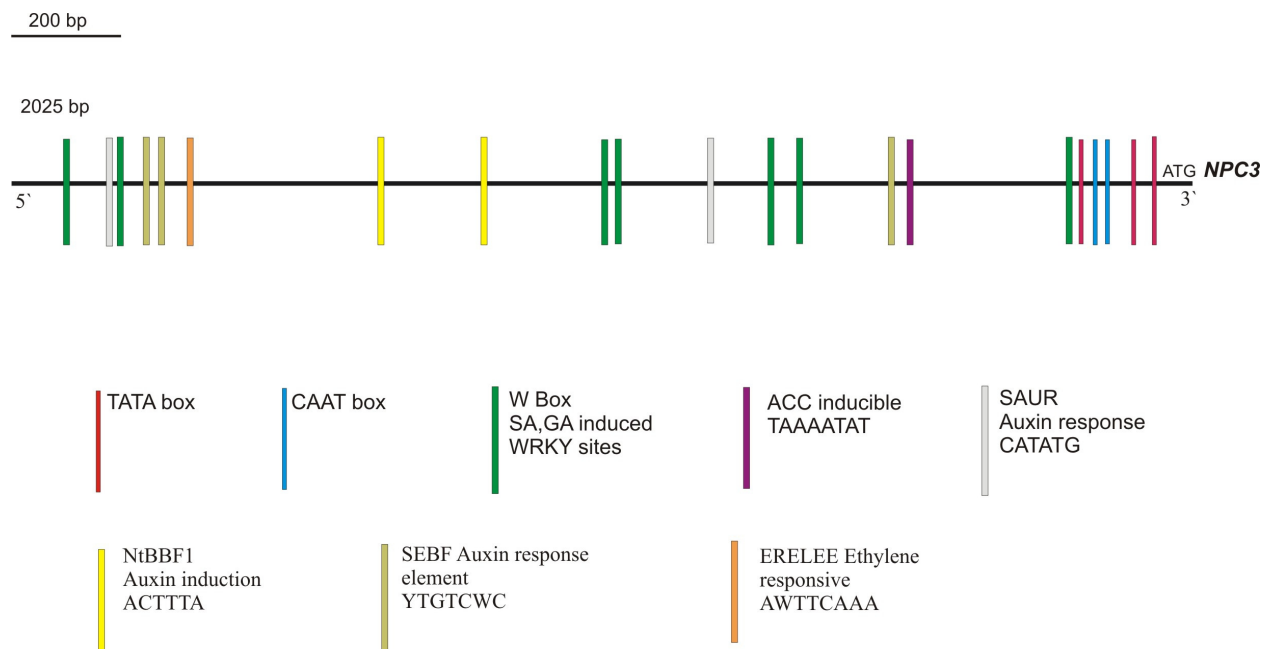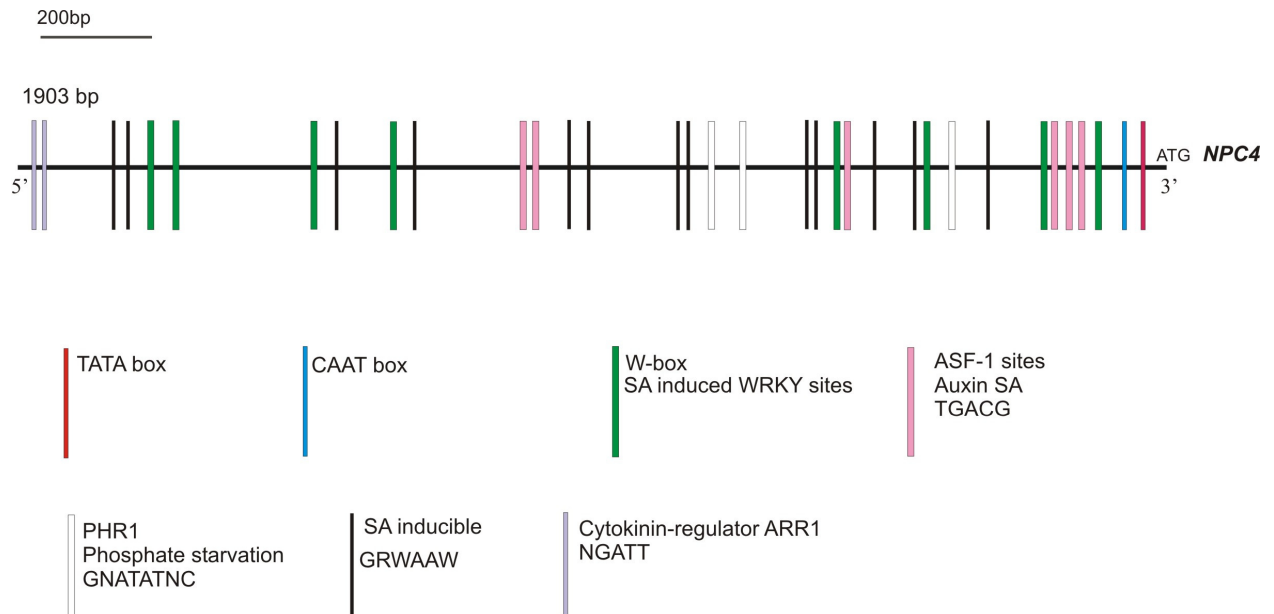**Figure 3.29:** Schematic presentation of deduced promoter region of *NPC4* with possible regulatory elements. The positions of the different elements are shown as coloured vertical bars relative to the translation start site

Several W box transcription factors similar to that of *Arabidopsis* NPR1 gene and recognized specifically by salicylic acid (Chen and Chen, 2001; Chen et al., 2002) are found along the *NPC4* promoter region (Figure 3.29). *cis*-element GTICONSENSUS, that has a role in light and SA regulation in many plant species (Villain et al., 1996) is present in several sites of the *NPC4* promoter region. TGACG motifs found in ASF-1 binding site of CaMV 35S promoter and many other promoters are involved in transcriptional activation of several genes by auxin and /or salicylic acid. This ASF-1 like regulatory element is present at 6 different positions along the *NPC4* region. In addition, conserved sequences of cytokinin regulated transcription factor ARR1 in *Arabidopsis* (Sakai et al., 2000) are found along the sketch of *PNPC4* region. GUS expression of *PNPC4::uidA* in response to auxin and zeatin correlates with the presence of regulatory auxin and zeatin *cis*-elements. Three PHR1 *cis*-elements are found in the *PNPC4*. PHR1 is a binding sequence found in the upstream regions of phosphate starvation

responsive genes from several plant species (Rubio et al., 2001; Shunmann et al., 2004a). The first intron of the *NPC4* was analysed for the presence of any known regulatory *cis*-elements of auxin, cytokinin and phosphate responses. No such predicted regulatory regions were found in the intron region.

3.4 Identification of T-DNA insertional mutants of *NPC* genes

Insertional mutagenesis is one of the methods of disrupting gene function and is based on the insertion of a piece of foreign DNA such as transposable element or T-DNA into the gene of interest (Krysan et al., 1999; Sussman et al., 2000). The insertion of piece of T-DNA on the order of 5 to 25 kb in length generally makes a dramatic disruption of gene function. In the *Arabidopsis* genome a large number of independent T-DNA insertions have been created that represent near saturation of the gene space. Moreover, the precise locations have been determined for many T-DNA insertions allowing one to search for the presence of flanking sequences homologues to any gene of interest (Alonso et al., 2003). Gene knockouts created by insertional mutagenesis are powerful tools in understanding the function of a known specific gene. All the putative knockout lines used in this study were either from the T-DNA collection of SALK institute or from the GABI-Kat of Max Planck Institute for Plant Breeding, Köln. The genetic background of all the lines was *Arabidopsis thaliana* Columbia 0. Polymerase chain reaction (PCR) based screening methods have been developed to identify insertional mutants in a given gene (Krysan et al., 1996). PCR is performed using one gene specific and one T-DNA specific primers, a PCR product is formed only if a T-DNA element has landed either within or very close to the gene of interest.

3.4.1 Identification of homozygous T-DNA insertion knockouts from *NPC1* (At1g07230)

Putative knockout line *npc1-1* (SALK_027871.43.40.x) was from SALK collection and T-DNA is predicted to be inserted in the second intron of *NPC1* (At1g07230) (Figure 3.30).

Figure 3.30: Schematic presentation of *npc1-1* T-DNA insertion site in *NPC 1*. Exons are indicated in white boxes and introns are indicated in black lines. Grey boxes show the 5' and 3' untranslated regions. The triangle indicates the position of the T-DNA insertion. Gene specific primer binding sites are indicated in arrows. T-DNA *npc1-1* is located in the second intron.

PCR strategy was employed to screen all the knockouts. A PCR is done with two gene specific primers spanning over the predicted T-DNA site to prove the uninterruption of the gene of interest. T-DNA insertion in the gene prevents the amplification of the gene. Thus, PCR amplification product is not observed in plants homozygous for the T-DNA insertion. WT as well as plants heterozygous for the T-DNA insertion are able to amplify the corresponding gene. A primer specific for left border of T-DNA (LBa1 or LBb1) in combination with a gene specific primer is used to prove insertion of T-DNA in the gene. The selection of the gene specific primer whether it is 5' (LP) or 3' (RP) orientated depends on the orientation of the T-DNA. Plants homozygous or heterozygous for T-DNA are able to amplify the piece of DNA flanking the two primer binding sites. WT is not able to give amplification product because of absence of T-DNA insertion in the gene of interest.

Genomic DNA was isolated from individual *npc1-1* putative knockout plants and screened by PCR (Figure 3.31). DNA from *Arabidopsis thaliana* Columbia 0 (WT) was used as control.

Figure 3.31: Identification of homozygous knockout *npc1-1* by PCR.
Proof of uninterrupted *NPC1*: PCR with primers 5'LPnpc1 and 3'RPnpc1 amplified 1443 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc1-1*.
Proof of T-DNA insertion in *NPC1*: PCR with primers 5'RPnpc1 and LBa1 amplified 594 bp fragment with genomic DNA of *npc1-1* and no amplification with DNA of WT.
M: 1 kb DNA ladder (Fa. Gene Craft).

WT-DNA produced a calculated 1443 bp amplification product with gene specific primers 5'LPnpc1 and 3'RPnpc1 while no amplification was observed with *npc1-1* DNA (Figure 3.31). Presence of T-DNA insert was tested with T-DNA left border specific primer LBa1 and gene specific 3'RPnpc1. No PCR amplification was observed in WT while calculated 594 bp DNA fragment was observed in *npcI-1* (Figure 3.31) indicating likely a homozygous line. The

PCR product was sequenced with T-DNA left border primer LBb1 to verify the T-DNA insertion in *npcI-1*. T-DNA insertion in the second intron was proved after sequencing. The predicted T-DNA insertion position of *npc1-1* by SIGnAL SALK was same as the position of insertion indicated by sequencing.

3.4.1.1 Analysis of *npc1-1* for transcript zero

Whether the insertion of T-DNA leads to loss of transcription of *NPC1* was examined by RT-PCR. Total RNA was isolated from seedlings of homozygous *npc1-1*. Subsequently, cDNA was synthesized and used as templates in PCR. cDNA synthesised from wild type Columbia 0 (WT) was used as standard template. Primers derived from *Arabidopsis actin* genes, *ACTN 2/7* (McDowell et al., 1996) which were proved to be transcribed in all the plant organs, were used as external standard in PCR.
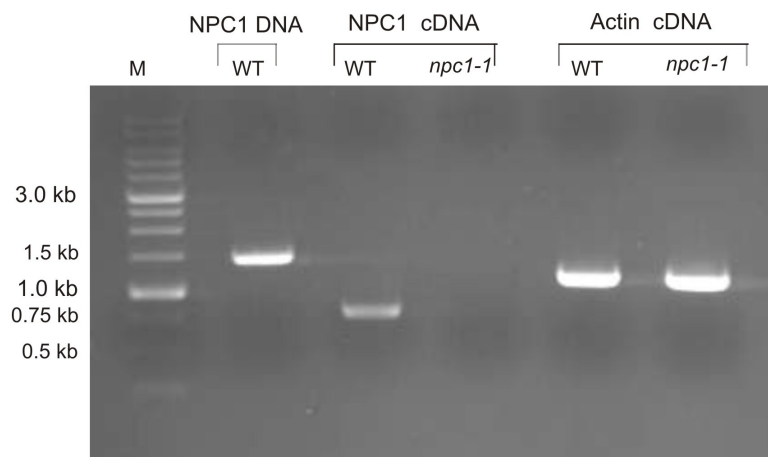
Figure 3.32: Analysis of cDNA of WT and *npc1-1* for *NPC1* transcription.
<u>Transcription of *NPC1*</u>: Primers 5'LPnpc1 and 3'RPnpc1 amplified 1443 bp with genomic DNA of WT, 780 bp with cDNA of WT and no amplification with cDNA of *npc1-1*.
<u>Transcription of *actin*</u>: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT and *npc1-1*.
M: 1 kb DNA ladder (Fa. Gene Craft)

NPC1 specific primers 5'LPnpc1 and 3'RPnpc1 which lie in first and third exons respectively having 2 introns in between (Figure 3.30) were used to test transcription of *NPC1* in *npc1-1*. Genomic DNA of WT showed an amplification product of calculated 1443 bp while cDNA of WT amplified an estimated 780 bp fragment. With cDNA of *npc1-1* no PCR product was observed suggesting *npc1-1* is unable to transcribe *NPC1*. The actin standard showed expected 1120 bp amplification product both in WT and *npc1-1* knockout (Figure 3.32).

3.4.2 Identification of homozygous T-DNA insertion knockouts from *NPC2* (At2g26870)

Two T-DNA insertional knockouts, *npc2-1* (SALK_115455.17.80.n) and *npc2-2* (SALK_018011.39.70.x) predicted to be located in first exon and first intron respectively in *NPC2* was obtained from SALK collection (Figure 3.33).
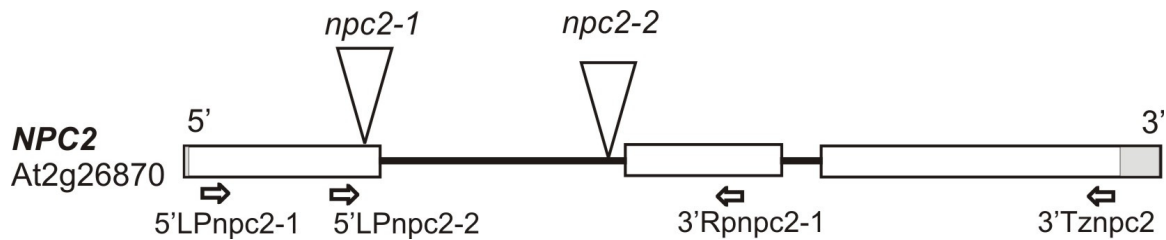
Figure 3.33: Schematic presentation of *npc2-1* and *npc2-2* T-DNA insertion sites in *NPC2*. Exons are indicated in white boxes and introns are indicated in black lines. Grey boxes show the 5' and 3' untranslated regions. The triangles indicate the position of the T-DNA insertion. Gene specific primer binding sites are indicated in arrows. T-DNA *npc2-1* is located in the first exon and *npc2-2* is located in the first intron.

The first PCR was carried out with WT and putative *npc2-1* knockout genomic DNA using 5'LPnpc2-1 and 3'RPnpc2-1 gene specific primers. WT produced a calculated 1264 bp DNA fragment while any amplification was not observed in *npc2-1* (Figure 3.34A). T-DNA specific primer LBa1 and gene specific 3'RPnpc2-1 was used in a second PCR to identify the knockout *npc2-1*. The estimated 1253 bp DNA fragment was produced by *npc2-1* DNA indicating the possible homozygous line (Figure 3.34 A). This DNA amplification was not observed in WT-DNA. Sequencing with T-DNA border primer LBb1 verified the T-DNA insertion and positioned 123 bp away from the predicted site.

Initially, *npc2-2* was identified as heterozygous knockout and homozygous line was identified in the next generation. WT-DNA produced a calculated 861 bp amplification product with gene specific primers 5'LPnpc2-2 and 3'RPnpc2-1 while no amplification was observed with DNA of *npc2-2* DNA (Figure 3.34 B). Gene specific 3'RPnpc2-1 and T-DNA specific LBa1 primers yielded a product of an estimated size of 557 bp in *npc2-2* indicating possible homozygous line (Figure 3.34 B). T-DNA insertion was confirmed by sequencing the PCR product with T-DNA left border specific LBb1 primer. A shift in 4 bp was observed between predicted and investigated position of T-DNA insertion.

(A)   **(B)**

Figure 3.34: Identification of homozygous *npc2-1* and *npc2-2* knockouts by PCR.
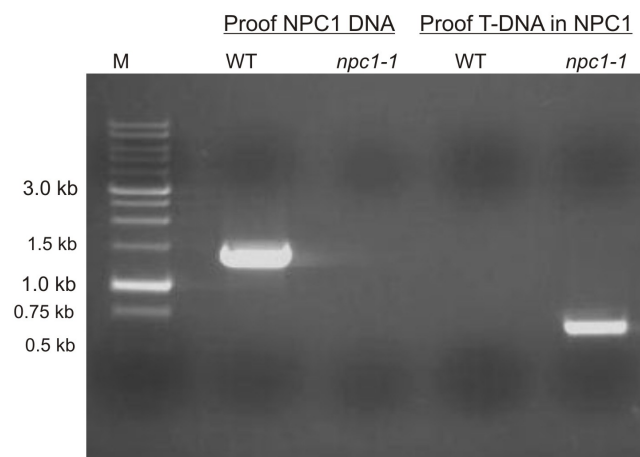**(A)** Identification of homozygous *npc2-1*
Proof of uninterrupted *NPC2*: PCR with primers 5'LPnpc2-1 and 3'RPnpc2-1 amplified 1264 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc2-1*.
Proof of T-DNA insertion in *NPC2*: PCR with primers 3'RPnpc2-1 and LBa1 amplified 1253 bp fragment with genomic DNA of *npc2-1* and no amplification with DNA of WT.
(B) Identification of homozygous *npc2-2*
Proof of uninterrupted *NPC2*: PCR with primers 5'LPnpc2-2 and 3'RPnpc2-1 amplified 861 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc2-2*.
Proof of T-DNA insertion in *NPC2*: PCR with primers 3'RPnpc2-1 and LBa1 amplified 557 bp fragment with genomic DNA of *npc2-2* and no amplification with DNA of WT.
M: 1 kb DNA ladder (Fa. Gene Craft).

3.4.2.1 Analysis of *npc2-1* and *npc2-2* for transcript zero

Homozygous *npc2-1* and *npc2-2* were tested for the transcription of *NPC2* by RT-PCR using primers 5'LPnpc2-1 and 3'TZnpc2 that spans over the first and the third exons of *NPC2* respectively (Figure 3.33). Amplification of 2213 bp and 1522 bp fragments was observed by DNA and cDNA of WT respectively. RT-PCR did not yield any products with cDNA of *npc2-1* and *npc2-2* indicating no transcription of *NPC2*. The actin standards showed expected 1120 bp amplification with cDNA of WT, *npc2-1* and *npc2-2* (Figure 3.35).

(A)

(B)

Figure 3.35: Analysis of cDNA of WT, *npc2-1* and *npc2-2* for *NPC2* transcription.
(A) Analysis of cDNA of *npc2-1*
Transcription of *NPC2*: Primers 5'LPnpc2-1and 3'TZnpc2 amplified 2213 bp with genomic DNA of WT, 1522 bp with cDNA of WT and no amplification with cDNA of *npc2-1*.
Transcription of *actin*: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT and *npc2-1*.
(B) Analysis of cDNA of *npc2-2*
Transcription of *NPC2*: Primers 5'LPnpc2-1and 3'TZnpc2 amplified 2213 bp with genomic DNA of WT, 1522 bp with cDNA of WT and no amplification with cDNA of *npc2-2*.
Transcription of actin: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT and *npc2-2*.
M: 1 kb DNA ladder (Fa. Gene Craft).

3.4.3 Identification of homozygous T-DNA insertion knockouts from *NPC3* (At3g03520)

Putative T-DNA insertional knockouts, *npc3-1* (SALK_036463.46.20.x) and *npc3-2* (SALK_150666.23.25.n) of *NPC3* were obtained from SALK collection. T-DNA insertion locations are indicated in Figure 3.36.
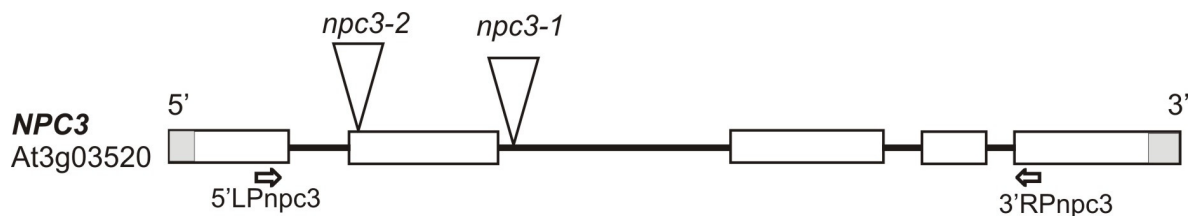
Figure 3.36: Schematic presentation of *npc3-1* and *npc3-2* T-DNA insertion sites in *NPC3*. Exons are indicated in white boxes and introns are indicated in black lines. Grey boxes show the 5' and 3' untranslated regions. The triangles indicate the position of the T-DNA insertion. Gene specific primer binding sites are indicated in arrows. T-DNA *npc3-1* is located the in the second intron and *npc3-2* is located in the second exon.

A DNA fragment of an estimated size of 1997 bp was amplified by WT in a PCR with gene specific primers 5'LPnpc3 and 3'RPnpc3 while no DNA fragment was observed in putative knockout lines *npc3-1* and *npc3-2* (Figure 3.37 A and B). LBa1 and 3'RPnpc3 primers produced an estimated 1639 bp DNA fragment with *npc3-1* (Figure 3.37 A) whereas *npc3-2* produced an estimated 490 bp DNA fragment with LBa1 and 5'LPnpc3 (Figure 3.37 B). The PCR products of insertion mutants were sequenced with T-DNA border primer LBb1 to confirm *npc3-1* and *npc3-2* homozygous knockout lines. There was no difference between the predicted and observed T-DNA insertion position in *npc3-1* while there was a 33 bp difference in observed and predicted insertion sites in *npc3-2*.
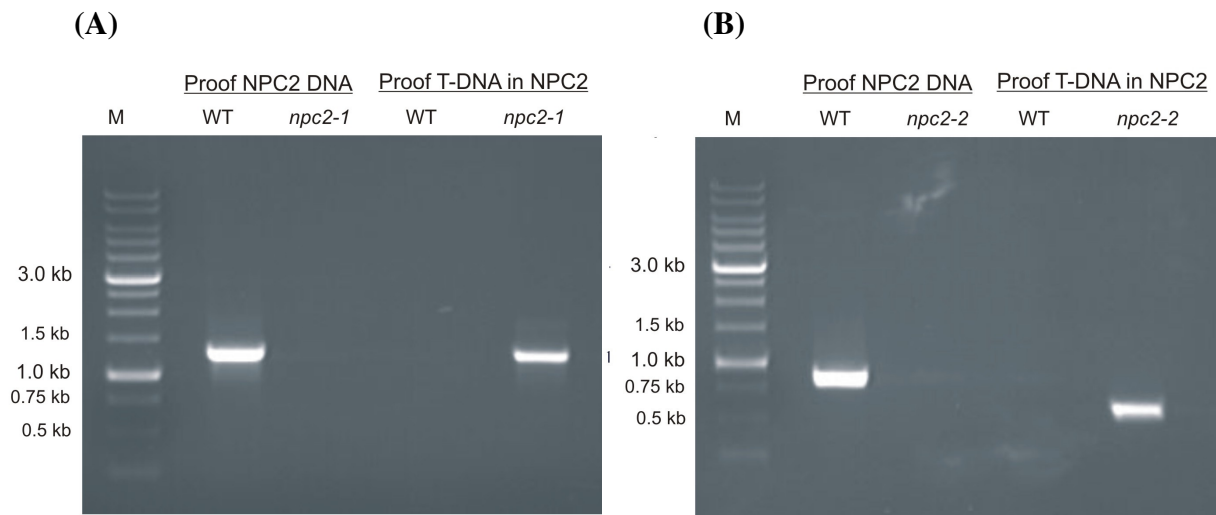

Figure 3.37: Identification of homozygous *npc3-1* and *npc3-2* knockouts by PCR.
(A) Identification of homozygous *npc3-1*
Proof of uninterrupted *NPC3*: PCR with primers 5'LPnpc3 and 3'RPnpc3 amplified 1997 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc3-1*.
Proof of T-DNA insertion in *NPC3*: PCR with oligonucleotides 3'RPnpc3 and LBa1 amplified 1639 bp fragment with genomic DNA of *npc3-1* and no amplification with DNA of WT.
 (B) Identification of homozygous *npc3-2*
Proof of uninterrupted *NPC3*: PCR with primers 5'LPnpc3 and 3'RPnpc3 amplified 1997 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc3-2*.
Proof of T-DNA insertion in *NPC3*: PCR with primers 5'LPnpc3 and LBa1 amplified 490 bp fragment with genomic DNA of *npc3-2* and no amplification with DNA of WT.
M: 1 kb DNA ladder (Fa. Gene Craft).

3.4.3.1 Analysis of *npc3-1* and *npc3-2* for transcript zero

Homozygous *npc3-1* and *npc3-2* were tested for the transcription of *NPC3* by RT-PCR (Figure 3.38).
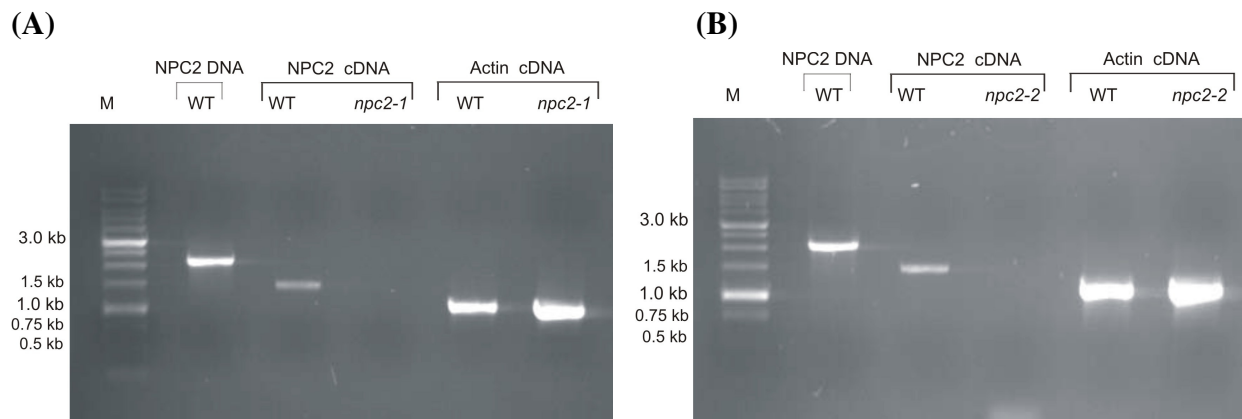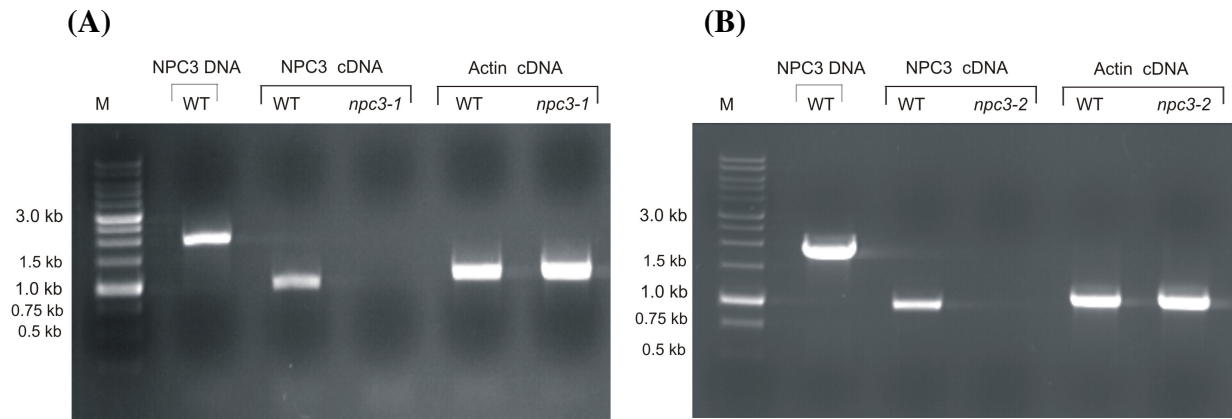

Figure 3.38: Analysis of cDNA of WT, *npc3-1* and *npc3-2* for *NPC3* transcription.
(A) Analysis of cDNA of *npc3-1*
Transcription of *NPC3*: Primers 5'LPnpc3and 3'RPnpc3 amplified 1997 bp with genomic DNA of WT, 1033 bp with cDNA of WT and no amplification with cDNA of *npc3-1*.
Transcription of *actin*: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT and *npc3-1*.
(B) Analysis of cDNA of *npc3-2*
Transcription of *NPC3*: Primers 5'LPnpc3 and 3'RPnpc3 amplified 1997 bp with genomic DNA of WT, 1033 bp with cDNA of WT and no amplification with cDNA of *npc3-2*.
Transcription of *actin*: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT and *npc3-2*.
M: 1 kb DNA ladder (Fa. Gene Craft).

NPC3 specific 5'LPnpc3 and 3'RPnpc3 primers that spans over the first and the fifth exons produced an expected 1997 bp DNA fragment with genomic DNA of WT and the same primers produces expected 1033 bp fragment with cDNA of WT (Figure 3.38 A and B). With the cDNA of homozygous *npc3-1* (Figure 3.38 A) and *npc3-2* (Figure 3.38 B) no PCR amplification was observed indicating no *NPC3* transcription occurred in both the knockouts. The actin standards produced 1120 bp product with cDNA of WT, *npc3-1* and *npc3-2* (Figure 3.38 A and B).

3.4.4 Identification of homozygous T-DNA insertion knockouts from *NPC4* (At3g03530)

T-DNA insertion line *npc4-1* (SALK_046713.47.10.x) was from SALK collection, and T-DNA insertion lines *npc 4-2* (GK571E10) and *npc 4-3* (GK539.10.02) were from GABI-Kat collection. T-DNA insertion positions of each line in *NPC4* are presented in Figure 3.39.

Figure 3.39: Schematic presentation of *npc4-1*, *npc4-2* and *npc4-3* T-DNA insertion sites in *NPC4*. Exons are indicated in white boxes and introns are indicated in black lines. Grey boxes show the 5' and 3' untranslated regions. The triangles indicate the positions of the T-DNA insertions. Gene specific primer binding sites are indicated in arrows. T-DNA *npc4-1* is located in the fourth exon, *npc4-2* in the fifth exon and *npc4-3* in the second intron.

Gene specific 5'LPnpc4-1 and 3'RPnpc4-1 primers were used to screen *npc4-1* and *npc4-2*. Only WT was able to amplify the region flanked by these primers producing calculated 919 bp DNA fragment. DNA of *npc4-1* and *npc4-2* was not able to produce expected 919 bp fragments indicating probable insertion of T-DNA in both alleles (Figure 3.40 A and B). Gene specific 3'RPnpc4-1 and T-DNA left border specific LBa1 amplified an estimated 610 bp fragment with DNA of *npc4-1* (Figure 3.40A). Gene specific 3'RPnpc4-1 and T-DNA left border specific LB8409 amplified an estimated 989 bp fragment with DNA of *npc4-2* (Figure 3.40 B). Similarly, homozygous *npc4-3* was identified. Amplification of *NPC4* flanked by 5'LPnpc4-2 and 3'RPnpc4-2 was observed in WT producing 1042 bp fragment while no amplification was observed in *npc4-3*. Gene specific 5'LPnpc4-2 and T-DNA specific LB8409 primers amplified 868 bp DNA fragment in *npc4-3* indicating *npc4-3* is a probable homozygous line (Figure 3.40 C). To confirm the T-DNA insertions, purified PCR products of *npc4-1*, *npc4-2* and *npc4-3* were sequenced with respective sequencing primers. A shift in 3 bp, 93 bp and 26 bp were identified between predicted and observed in *npc4-1*, *npc4-2* and *npc4-3* respectively.
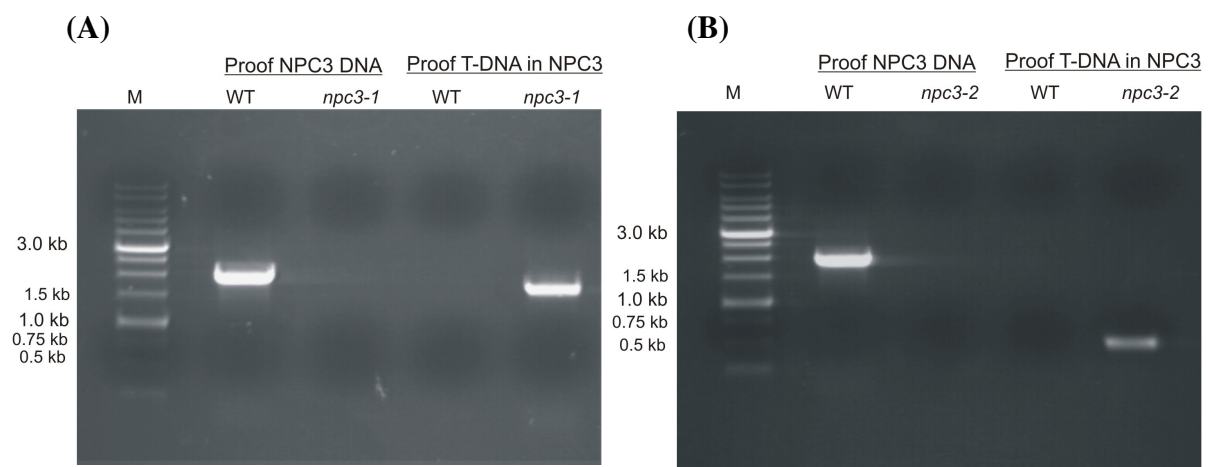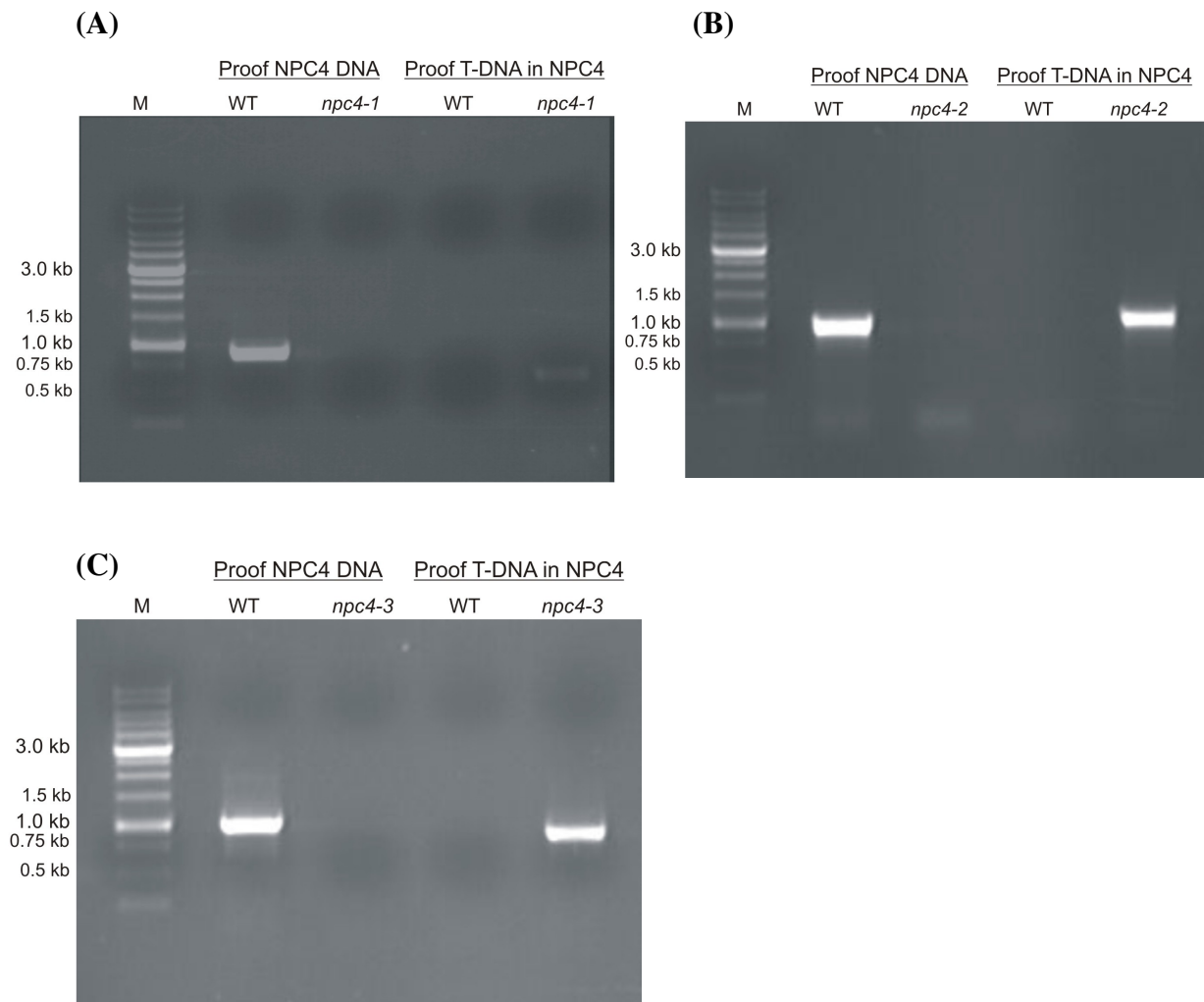
Figure 3.40: Identification of homozygous *npc4-1*, *npc4-2* and *npc4-3* knockouts by PCR.
(A) Identification of homozygous *npc4-1*
Proof of uninterrupted *NPC4*: PCR with primers 5'LPnpc4-1 and 3'RPnpc4-1 amplified 919 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc4-1*.
Proof of T-DNA insertion in *NPC4*: PCR with primers 3'RPnpc4-1 and LBa1 amplified 610 bp fragment with genomic DNA of *npc4-1* and no amplification with DNA of WT.
(B) Identification of homozygous *npc4-2*
Proof of uninterrupted *NPC4*: PCR with primers 5'LPnpc4-1and 3'RPnpc4-1 amplified 919 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc4-2*.
Proof of T-DNA insertion in *NPC4*: PCR with primers 3'RPnpc4-1 and LB8409 amplified 989 bp fragment with genomic DNA of *npc4-2* and no amplification with DNA of WT.
(A) Identification of homozygous *npc4-3*
Proof of uninterrupted *NPC4*: PCR with primers 5'LPnpc4-3 and 3'RPnpc4-3 amplified 1042 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc4-3*.
Proof of T-DNA insertion in *NPC4*: PCR with primers 5'LPnpc4-3 and LB8409 amplified 868 bp fragment with genomic DNA of *npc4-3* and no amplification with DNA of WT.
M: 1 kb DNA ladder (Fa. Gene Craft).

3.4.4.1 Analysis of *npc4-1, npc4-2* and *npc 4-3* for transcript zero

RT-PCR was done with primers 5'LPnpc4-3 which specifically binds to the first exon and 3'RPnpc4-1 which specifically binds to the last exon of *NPC4*. Amplification of *NPC4* was observed with cDNA of WT producing estimated 1539 bp fragment. In contrast, cDNA of *npc4-1*, *npc4-2* and *npc4-3* were not able to amplify the expected 1539 bp region of *NPC4* (Figure 3.41 A and B). The actin standard showed 1120 bp amplification with cDNA of WT and *npc4* lines.
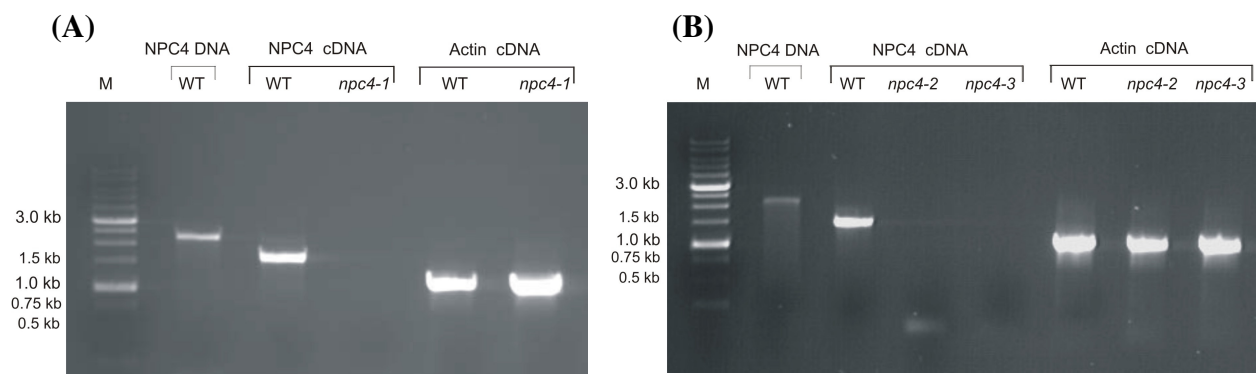
Figure 3.41: Analysis of cDNA of WT, *npc4-1*, *npc4-2* and *npc4-3* for *NPC4* transcription.
(A) Analysis of cDNA of *npc4-1*
Transcription of *NPC4*: Primers 5'LPnpc4-3 and 3'RPnpc4-1 amplified 2205 bp with genomic DNA of WT, 1539 bp with cDNA of WT and no amplification with cDNA of *npc4-1*.
Transcription of *actin*: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT and *npc4-1*.
(B) Analysis of cDNA of *npc4-2* and *npc4-3*
Transcription of *NPC4*: Primers 5LPnpc4-3 and 3'RPnpc4-1 amplified 2205 bp with genomic DNA of WT, 1539 bp with cDNA of WT and no amplification with cDNA of *npc4-2* and *npc4-3*.
Transcription of *actin*: Primers 5'ACTN and 3'ACTN amplified 1120 bp with cDNA of WT, *npc4-2* and *npc4-3*.
M: 1 kb DNA ladder (Fa. Gene Craft)

3.4.5 Identification of homozygous T-DNA insertion knockouts from *NPC5* (At3g03540)

Putative T-DNA insertional knockouts, *npc5-1* (SALK_.084476.43.55) and *npc5-2* (SALK_045037.53.00) were obtained from SALK collection. In *npc5-1* T-DNA is inserted in fifth exon and in *npc5-2* T-DNA is inserted in third exon (Figure 3.42).
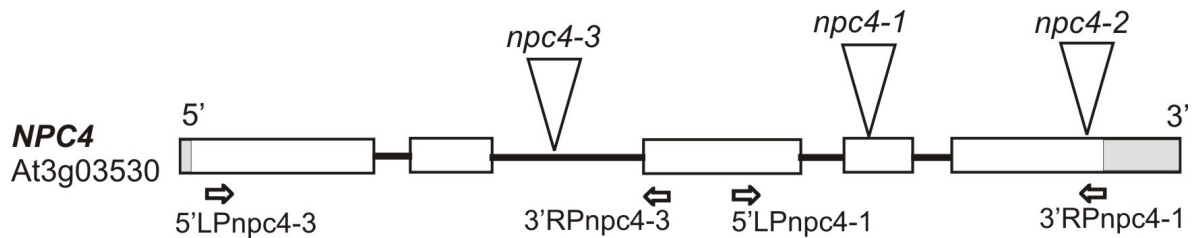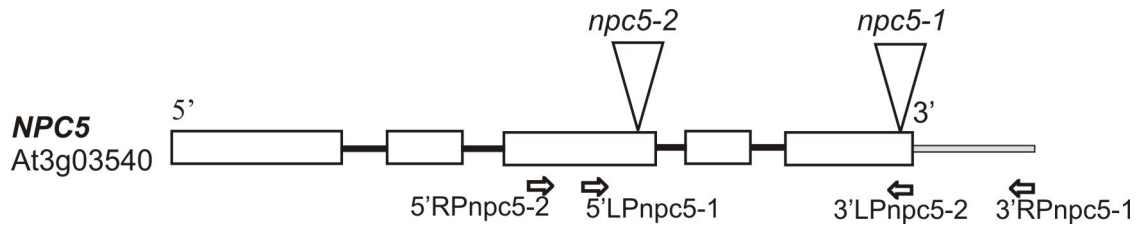
Figure 3.42: Schematic presentation of *npc5-1* and *npc5-2* T-DNA insertion sites in *NPC5*. Exons are indicated in white boxes and introns are indicated in black lines. Grey line shows 3' intergenic region. The triangles indicate the position of the T-DNA insertion. Gene specific oligonucleotide binding sites are indicated in arrows. T-DNA insertion *npc5-1* is located in the fifth exon and *npc5-2* is located in the third exon.

Homozygous *npc5-1* and *npc5-2* were identified by PCR (Figure 3.43).
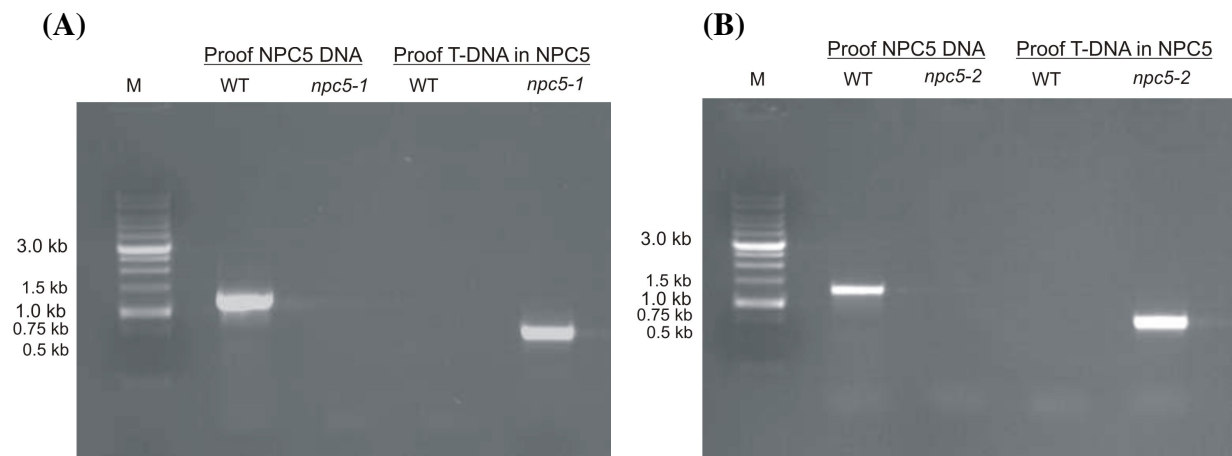
Figure 3.43: Identification of homozygous *npc5-1* and *npc5-2* knockouts by PCR.
(A) Identification of homozygous *npc5-1*
Proof of uninterrupted *NPC5*: PCR with primers 5'LPnpc5-1 and 3'RPnpc5-1 amplified 1303 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc5-1*.
Proof of T-DNA insertion in *NPC5*: PCR with primers 3'RPnpc5-1 and LBa1 amplified 689 bp fragment with genomic DNA of *npc5-1* and no amplification with DNA of WT.
(B) Identification of homozygous *npc5-2*
Proof of uninterrupted *NPC5*: PCR with primers 5'RPnpc5-2 and 3'LPnpc5-2 amplified 1203 bp fragment with genomic DNA of WT and no amplification with genomic DNA of *npc5-2*.
Proof of T-DNA insertion in *NPC5*: PCR with primers 5'RPnpc5-2 and LBa1 amplified 665 bp fragment with genomic DNA of *npc5-2* and no amplification with DNA of WT.
M: 1 kb DNA ladder (Fa. Gene Craft).

Gene specific 5'LPnpc5-1 and 3'RPnpc5-1 primers were used to screen *npc5-1*. Estimated 1303 bp was amplified by DNA of WT and no amplification was observed with DNA of *npc5-1* indicating possible homozygous line. T-DNA insertion in *npc5-1* was observed by amplification of 689 bp fragment with T-DNA specific LBa1 and gene specific 3'RPnpc5-1

(Figure 3.43 A). Sequencing with LBb1 primers confirmed the insertion of T-DNA in the fifth exon of NPC5 as predicted by SIGnAL SALK. The predicted and observed insertion sites were the same. Likewise, gene specific primers 3'LPnpc5-2 and 5'RPnpc5-2 were used in screening *npc5-2* knockout. With the DNA of *npc5-2* any amplification of *NPC5* was not observed while DNA of WT amplified 1203 bp fragment. T-DNA specific LBa1 and 5'RPnpc5-2 amplified an estimated 665 bp fragment indicating T-DNA insertion in third exon as predicted (Figure 3.43 B). Sequencing with LBb1 confirmed that there was no difference in the predicted and observed position of T-DNA insertion.

As with the previous homozygous knockouts, cDNA of homozygous *npc5-1* and *npc5-2* were tested for the transcription of *NPC5*. Transcription of *NPC5* was not observed with cDNA of WT as well as with cDNA of knockouts. Expression of the *NPC5* is generally very low and barely detected in the previous experiments too. Therefore, it is not possible to conclude that *npc5-1* and *npc5-2* homozygous knockouts are transcript zero.

In summary, putative T-DNA insertional knockouts of *NPC* gene family of *Arabidopsis thaliana* obtained either from SALK or GABI-Kat collection were screened by PCR to find homozygous lines. Homozygous knockout lines *npc1-1* of *NPC1*, *npc2-1* and *npc2-2* of *NPC2*, *npc3-1* and *npc3-2* of *NPC3*, *npc4-1*, *npc4-2* and *npc4-3* of *NPC4* and *npc5-1* and *npc5-2* of *NPC5* were identified. *npc4-2* and *npc4-3* knockouts were identified in a later time than the other knockouts because they were available to the public only recently. Proof of T-DNA insertion was done by sequencing with T-DNA specific primers depending on the vector used in transformation. The precise location of T-DNA was determined by sequence comparison. Moreover, homozygous knockouts were further tested for transcript zero by RT-PCR.

3.5 Phenotypic analysis of T-DNA insertional mutants

Gene knockout in which the action of a known gene has been disrupted provides an important tool in determining the function of that particular gene. This approach is commonly known as reverse genetics (Krysan et al., 1996, 1999; Sussman et al., 2000). Generally, the consequences of the mutation on growth and development are determined by analysing phenotypes of confirmed mutants relative to the wild type. However, it has become apparent that many knockout mutants have no readily identifiable phenotype unless grown under specific conditions such as nutrient deficient conditions. Thus, it is often necessary to characterize a variety of phenotypic parameters in a variety of growth conditions which are often unlimited. Functional redundancy among the members of a gene family is a likely reason for the frequently observed lack of identifiable phenotype in a knockout (Hua and Meyerowitz, 1998). Another possible reason for the lack of observable phenotypes is that individual gene family members may have evolved to function only under specific physiological conditions. Once a phenotype has been observed in a knockout it has to be proved that the phenotypic characteristics are indeed controlled by the gene of interest. To prove definitively that the insertional mutation causes the phenotype, additional mutant alleles can be tested for the observed phenotype or complement the mutation by introducing a wild type copy of the gene into mutant plants.

3.5.1 Phenotypic analysis of greenhouse-grown *npc* knockouts

Homozygous knockouts *npc1-1*, *npc2-1*, *npc2-2*, *npc3-1*, *npc3-2*, *npc4-1*, *npc5-1* and *npc5-2* were grown on the soil (Einheitserde) and maintained in greenhouse at 23°C, 65% RH, 9600 lx/m^{2} light intensity and 18 h light/ 6 h dark growth conditions. Phenotypes of the adult knockouts were compared with similarly grown wild type (WT) Columbia 0 adult plants. Neither abnormal growth nor obvious differences in morphology of rosette leaves, upper leaves, stems, flowers and siliques were observed in knockout plants in comparison to WT. Moreover, the flowering time and fertility by producing viable seeds were unaffected in knockouts.
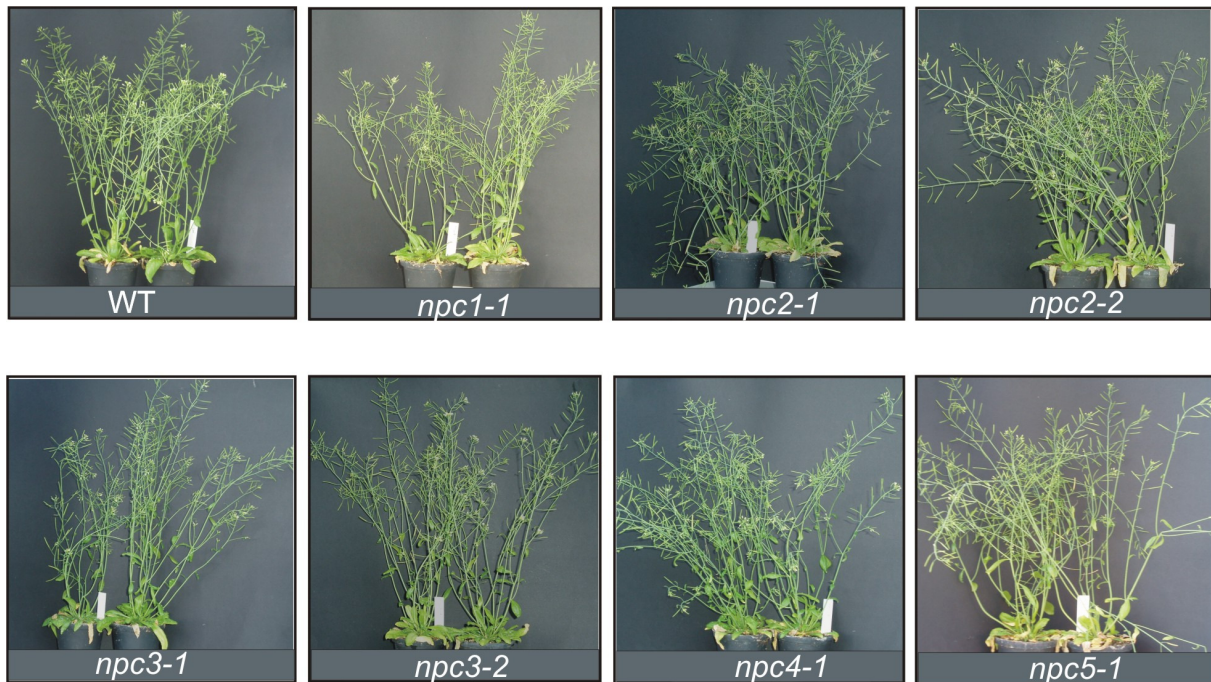
Figure 3.44: Phenotype of 7-8 week old greenhouse-grown knockout and wild type *Arabidopsis thaliana* Columbia 0 plants.

To access the functions of *NPC3* and *NPC4* genes in more detail, already identified T-DNA insertional mutants *npc3-1* and *npc3-2* of *NPC3* and *npc4-1* and *npc4-2* of *NPC4* were analysed for their phenotypes. Plants were subjected to different growth conditions including nutrient deficiency and exogenous hormone supplementation and response phenotype was compared with WT plants that were subjected to similar growth conditions.

3.5.2 Phenotypic analysis of *npc3-1* and *npc3-2*

RT-PCR and *promoterNPC3*::*uidA* (*PNPC3*) analysis had revealed that in the adult *Arabidopsis* plants transcription of *NPC3* occurs in roots, shoots, leaves, flowers and siliques. RT-PCR analysis and genevestigator (http://www.genevestigator.ethz.ch) expression profile data indicated that relative transcription level of *NPC3* was higher in roots. *PNPC3* showed that at the seedling stage *NPC3* is mainly expressed in the roots. Therefore, the potential effect of *NPC3* disruption on growth was investigated *in vitro* paying special emphasis on morphology of root system. *PNPC3* expression pattern indicated that auxin and brassinolides are able to regulate transcription of the *NPC3*. Phenotypes of *npc3-1* and *npc3-2* were studied in detail *in vitro* in response to exogenous application of auxin and brassinolide in phosphate sufficient and deficient growth media. Phenotypes were compared with the WT grown under similar conditions. Other phytohormones namely zeatin, salicylic acid (SA) and ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC) were tested for the phenotypes of

npc3-1 and *npc3-2*. Phenotypic differences were not observed in the knockouts in comparison to WT. Further, phenotypic tests were carried out by growing the knockouts and WT in Fe deficient and S deficient conditions. None of these growth conditions were able to display differential phenotypes in knockouts, compared to similarly grown WT seedlings. These observations indicate either knockouts have no influence on the phenotypes in response to above hormones and nutrient deficient conditions or the test conditions which include age of the plants, hormone concentrations, incubation conditions were not able to show any potential phenotypic differences.

3.5.2.1 Phenotypic analysis of *npc3-1* and *npc3-2* in response to phosphate deficient conditions

Seedlings of *NPC3* knockouts *npc3-1* and *npc3-2* were grown in Arabidopsis agar medium (AM) in vertically oriented petri dishes and compared the morphology of shoot and especially the root system with similarly grown Columbia 0 WT seedlings. Although direct effect of phosphate deprivation on *NPC3* expression was not observed, *npc3-1* and *npc3-2* were investigated for a possible differential responsiveness to phosphate deprivation. Increasing evidence suggests that hormones especially auxin play a key role in mediating the phosphate starvation effects on root system architecture (López-Bucio et al., 2002).

WT, *npc3-1* and *npc3-2* seedlings were grown either on phosphate containing (+Pi) or phosphate lacking (-Pi) AM and after 7 days of growth, root parameters; primary root length, number of lateral roots, lateral root density were scored (section 2.10.1.1). A general retardation of root growth was evident in knockouts both in +Pi and –Pi growth conditions. A reduction in primary root length and lateral root number was obvious in *npc3-1* and *npc3-2* compared to WT in +Pi medium (Figure 3.45 A) as well as in –Pi medium (Figure 3.45 B). In +Pi significant difference in primary root length was observed between WT and *npc3-1* ($P < 0.001$) and between WT and *npc3-2* ($P < 0.001$) while no difference was observed between two knockouts (Figure 3.45 C). Primary roots in *npc3* were on average 11% shorter than WT after 7 days of growth in +Pi. A significant difference in total lateral root numbers over the entire primary roots was displayed by each knockout in comparison to WT ($P < 0.001$) in +Pi (Figure 3.45 D). However, the lateral root densities of *npc3-1* and *npc3-2* were only slightly reduced compared to WT (Figure 3.45 E). Significant differences in hypocotyl lengths were not observed among knockouts and WT (Figure 3.45 F).

Figure 3.45: Effect of phosphate availability on morphology of *npc3-1* and *npc3-2* knockouts.
(**A**) wild type (WT), *npc3-1* and *npc3-2* seedlings were grown for 7 days in Pi containing (+Pi) medium on vertically oriented plates. (**B**) wild type (WT), *npc3-1* and *npc3-2* seedlings were grown for 7 days in Pi lacking (-Pi) medium on vertically oriented plates. (**C**) primary root lengths (**D**) lateral root numbers (**E**) lateral root densities (**F**) hypocotyl lengths of WT, *npc3-1* and *npc3-2* seedlings after 7 days of growth in +Pi and –Pi media. Values shown in C, D, E and F represent mean of 40 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

The aspects of root architecture induced by low phosphorus, such as primary root growth inhibition, increased lateral root lengths and increased lateral root densities were observed both in WT and knockouts (Figure 3.45 B). Primary root lengths of *npc3-1* and *npc3-2* were significantly shorter than WT (P < 0.001) at –Pi conditions (Figure 3.45 C). A 17%-20% reduction in primary root lengths was observed between WT and *npc3* in –Pi after 7 days of growth. In –Pi average 40% reduction in primary root length was indicated by WT when compared with the +Pi grown seedlings. *npc3-1* and *npc3-2* indicated average 44-46% reduction in primary root lengths in –Pi compared to +Pi grown seedlings. In WT seedlings lateral root numbers showed an average of 32% increase when grown –Pi. In contrast, increase in lateral root numbers was not observed in *npc3* seedlings in response to –Pi. Growth in –Pi conditions induced 2.2 fold increase in lateral root density in WT while *npc3-1* and *npc3-2* induced 1.8 to 1.9 fold increase in lateral root density. The increase in lateral root density of the *npc3* knockouts in –Pi was mainly due to the reduced primary root lengths. In –Pi both *npc3* knockouts showed significantly lower lateral root densities than WT (Figure 3.45 E). A general reduction in hypocotyl length was observed at –Pi both in knockouts and WT but differences displayed by knockouts in comparison to WT were not significant (Figure 3.45 F).

These results indicate that disruption of *NPC3* has a effect on primary root elongation and lateral root numbers when grown in normal growth media containing all the nutrients sufficiently including Pi (+Pi). Expression of *PNPC3* in the primary and lateral root tips and root phenotypes of *npc3* suggests potential function/s of *NPC3* in root elongation and development. *npc3* knockouts indicated more or less similar response as WT toward the –Pi mediated inhibition of primary root elongation proposing disruption of *NPC3* has no significant effect on primary root growth in –Pi. The observation that no increase in lateral root numbers in *npc3* in contrast to WT in –Pi, suggest possible involvement of *NPC3* in –Pi triggered lateral root development. Increased lateral root densities of *npc3* propose that *npc3* knockouts respond to -Pi but the response is weaker than WT. Taken together, it can be concluded that *npc3* knockouts have general root growth retardation suggesting potential role of *NPC3* in root development and no significant deficiency in the –Pi response but only a weak –Pi response.

3.5.2.2 Effect of exogenous auxin on phenotype of *npc3-1* and *npc3-2*

Throughout the plant growth, expression of *PNPC3* was obvious in primary and lateral root tips which are important sites of auxin regulation. Dramatic induction of promoter activity was observed with exogenous auxin application further suggesting potential auxin regulatory function/s of *NPC3*. To investigate possible involvement of *NPC3* in auxin response, knockouts of *NPC3* were phenotypically analysed in response to exogenous auxin application. Knockout seedlings were grown in 1-NAA supplemented or non 1-NAA supplemented +Pi and –Pi media and root parameters were compared with similarly grown WT (section 2.10.2.1).

Exogenous auxin has been shown to inhibit the elongation of the primary root and to stimulate lateral root formation. Both, 1-NAA treated WT and *npc3* knockout plants displayed the auxin-mediated root phenotypes (Figure 3.46 A and B). Primary roots of WT and *npc3* grown in +Pi medium supplemented with 0.03 µM of 1-NAA showed reduced lengths compared to control +Pi medium grown seedlings (Figure 3.46 C). The reduction of primary root lengths owing to 1-NAA was 12%- 14% in both WT and knockouts indicating interruption of the *NPC3* gene has no different effect in the response. A significant decrease in primary root length in 0.03 µM of 1-NAA supplemented –Pi growth was not observed in WT and *npc3* knockouts compared to the –Pi grown seedlings (Figure 3.46 C) demonstrating *npc3* has no differential response towards 1-NAA. In all these growth conditions irrespective of Pi availability and 1-NAA supplementation *npc3-1* and *npc3-2* seedlings showed significantly reduced (P < 0.001) primary root lengths compared to WT seedling grown in similar conditions.

Addition of 0.03 µM 1-NAA to either +Pi or –Pi media increased the number of lateral roots and concomitantly lateral root densities both in WT and *npc3* knockouts (Figure 3.46 D and E). 1-NAA supplemented +Pi grown WT and *npc3* knockouts showed about 2.3 fold increase in the lateral root numbers and 2.6 fold increase in lateral root densities compared to respective +Pi grown seedlings. Supplementation of –Pi medium with 0.03 µM of 1-NAA caused about 2 fold increase in the lateral root numbers and about 2.1 fold increase in lateral root density of WT. In a similar manner, about 2.5 fold increase in lateral root numbers and 2.4 fold increase in lateral root density was observed in *npc3* knockouts. These results indicate that even though *npc3* knockouts have similar response as WT to 1-NAA in +Pi
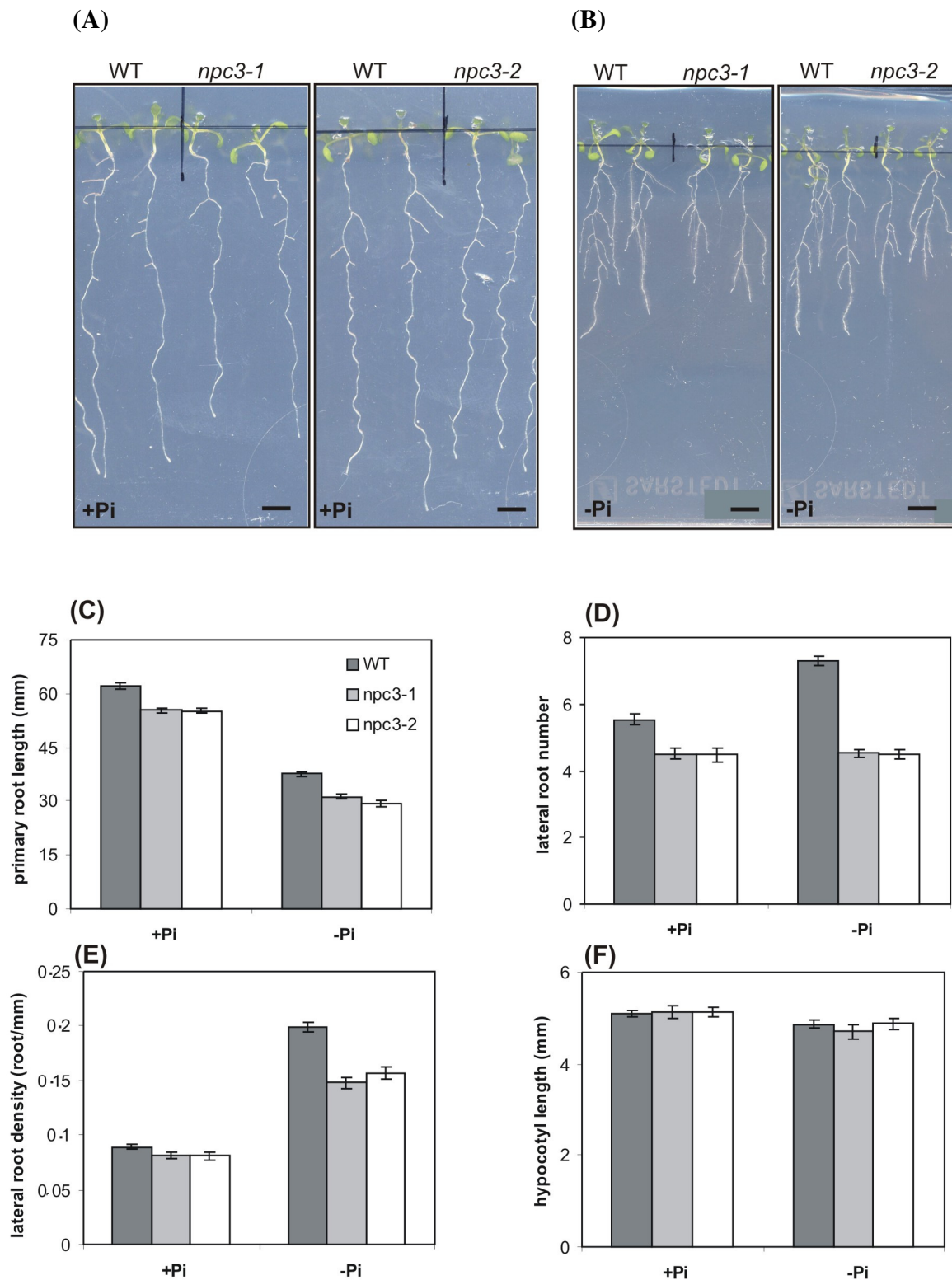
Figure 3.46: Effect of phosphate availability and 1-NAA on morphology of *npc3-1* and *npc3-2* knockouts. **(A)** WT, *npc3-1* and *npc3-2* seedlings were grown for 7 days in Pi containing medium supplemented with 0.03 µM of 1-NAA on vertically oriented plates (+Pi+NAA). **(B)** WT, *npc3-1* and *npc3-2* seedlings were grown for 7 days in Pi lacking medium supplemented with 0.03 µM of 1-NAA on vertically oriented plates (-Pi+NAA). **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities and **(F)** hypocotyl lengths of WT, *npc3-1* and *npc3-2* seedlings after 7 days of growth in +Pi, +Pi+NAA, –Pi and –Pi+NAA media. Values shown in C, D, E and F represent mean of 40 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

growth conditions, *npc3* knockouts have increased sensitivity to 1-NAA in –Pi growth conditions than WT. However, in all the growth conditions lateral root numbers and lateral root densities of both *npc3-1* and *npc3-2* were significantly lower than WT (P < 0.01). A significant difference in hypocotyl lengths were not observed between WT and *npc3* knockouts in any of these growth conditions (Figure 3.46 F).

When WT and *npc3* knockout seedlings were grown in complete Murashige and Skoog agar medium (MS) supplemented with 0.03 µM or 0.1 µM 1-NAA reduction in primary root length, increase in lateral root number and lateral root densities could be observed. WT, *npc3-1* and *npc3-2* showed more or less similar reduction in primary root lengths both in 0.03 µM and 0.1 µM 1-NAA containing MS compared to respective genotypes grown in 1-NAA untreated MS. Significant difference in the lateral root numbers were not observed between these two 1-NAA concentrations both in WT and knockouts. A higher tendency of adventitious root formation could be observed in 0.1 µM 1-NAA. Lateral root densities of WT and *npc3* knockouts were higher in 0.1 µM 1-NAA than in 0.03 µM 1-NAA primarily due to the reduced primary root lengths. Significant difference in the fold increase of lateral root densities were not indicated by either WT or *npc3* knockouts in both the 1-NAA concentrations compared to the control growth conditions without 1-NAA. These results suggest that *npc3* knockouts are not able to differentially respond even to higher 1-NAA concentrations in growth media containing all the nutrients sufficiently including Pi.

Taken together, exogenous 1-NAA application showed typical auxin phenotypes in WT as well as in *npc3* knockouts both in phosphorus sufficient and deficient growth conditions indicating general auxin response was not impaired. Even though *npc3* knockouts have similar response as WT to 0.03 µM 1-NAA in +Pi growth conditions in terms of primary root length, lateral root number and lateral root densities, *npc3* knockouts have slightly increased sensitivity to 1-NAA in –Pi growth conditions than WT.

3.5.2.3 Effect of exogenous brassinolide on phenotype of *npc3-1* and *npc3-2*

Exogenous application of brassinolides at nanomolar to micromolar levels in a number of test systems have shown marked effects on cell elongation and proliferation. Brassinolide was the most effective hormone, besides auxins to stimulate the *NPC3*-promoter driven GUS activity. This finding prompted investigating phenotypes of *NPC3* knockouts *npc3-1* and *npc3-2* in response to exogenous brassinolid application. WT, *npc3-1* and *npc3-2* seedlings were grown for 7 days on 0.05 µM epi-brassinolide (BL) supplemented AM (+Pi) and phenotypes of knockouts were compared with WT.

WT, *npc3-1* and *npc3-2* responded to 0.05 µM of BL by significant reduction in primary root length and wavy root structure compared to respective non-treated genotypes (Figure 3.47 A and B). BL caused on average 48% inhibition of primary root elongation in WT compared to non BL treated controls. In *npc3* knockouts 55%-58% inhibition of root elongation was observed (Figure 3.47 C). A general reduction in lateral root numbers was shown in 0.05 µM BL treated WT and knockouts seedlings (Figure 3.47 D). A clear increase in lateral root densities were observed in BL treated WT and knockouts and were primarily due to the reduction in primary root lengths (Figure 3.47 E). *npc3-1* and *npc3-2* showed significantly lower lateral root densities than WT in the control growth conditions without added BL. In contrast, in 0.05 µM BL supplemented growth conditions lateral root densities of both knockouts were higher than WT. A 1.6 fold increase in the lateral root density in the BL supplemented media compared to the control was shown by WT while the increase was 2-2.2 fold in the knockouts. Older seedlings showed lesser difference between WT and *npc3* knockouts and were more difficult to quantify because of the convoluted roots. A slight reduction in hypocotyl lengths was indicated in all the BL treated seedlings, but significant difference between WT and knockouts was not observed (Figure 3.47 F).

In 0.01 µM BL supplemented growth media 33%, 45% and 50 % inhibition of root elongation was observed in WT, *npc3-1* and *npc3-2* respectively. A significant increase in lateral root densities was shown by all the genotypes in response to 0.01 µM BL. Similar to 0.05 µM BL supplemented growth conditions, increase in the lateral root densities of the knockouts were higher than WT in 0.01 µM BL supplemented growth conditions.

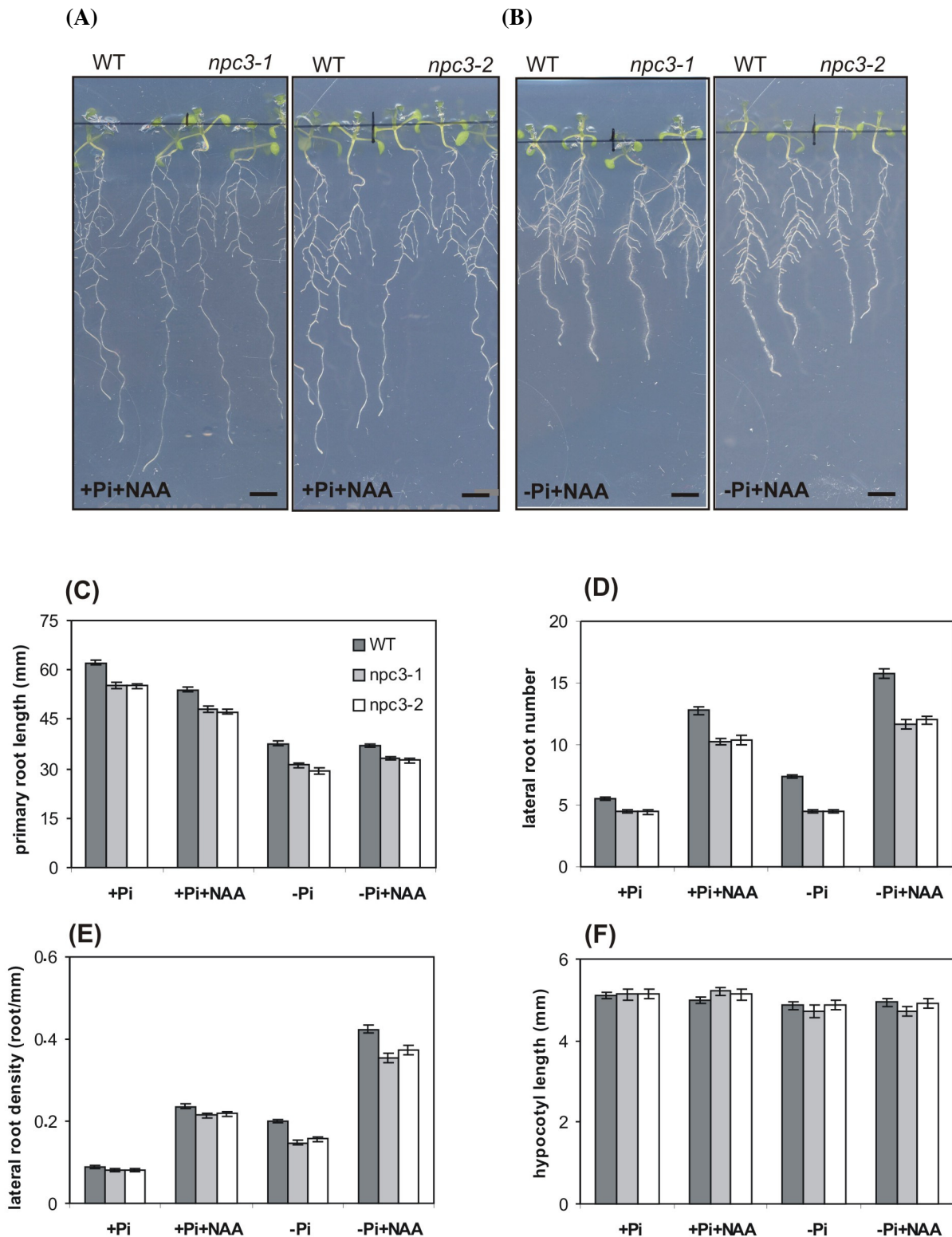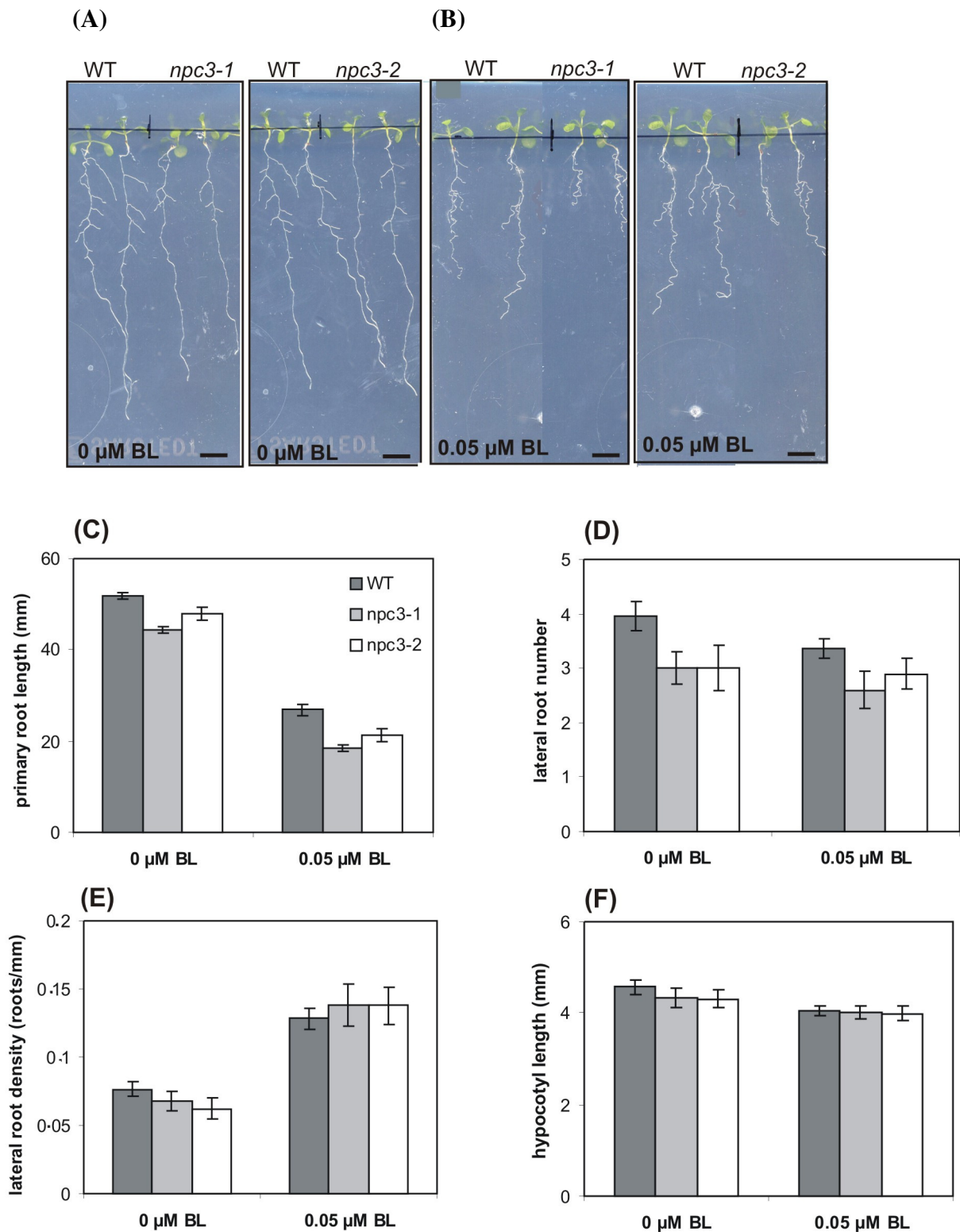**Figure 3.47:** Effect of epi-brassinolide on morphology of *npc3-1* and *npc3-2* knockouts.
(A) WT, *npc3-1* and *npc3-2* seedlings grown for 7 days in Pi containing medium on vertically oriented plates. **(B)** WT, *npc3-1* and *npc3-2* seedlings grown for 7 days in Pi containing medium supplemented with 0.05 µM epi-brassinolide on vertically oriented plates. **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities and **(F)** hypocotyl lengths of WT, *npc3-1* and *npc3-2* seedlings after 7 days of growth in Pi containing Arabidopsis medium with or without 0.05 µM epi-brassinolide. Values shown in C, D, E and F represent mean of 20 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

Taken together, 0.01 and 0.05 µM BL showed relatively higher inhibitory effect on root elongation in knockouts than WT. These results suggest that knockouts are more sensitive to the root phenotypes mediated by BL even though the response is weak.

3.5.3 Phenotypic analysis of *npc4-1* and *npc4-2*

RT-PCR analysis showed *NPC4* is expressed in roots, stems, leaves, flowers and siliques but stronger expression in roots. Moreover, *PNPC4* analysis indicated that GUS activity was observed specifically in primary and lateral root tips at all the stages of development. *PNPC4* expression pattern and RT-PCR analysis showed that phosphate deficient growth conditions, auxin and brassinolides are able to up-regulate transcription of the *NPC4*. Phenotypes of *npc4-1* and *npc4-2* were studied in detail *in vitro* in response to phosphate deficiency, exogenous application of auxin and brassinolide. Results of these analyses are described in sections 3.5.3.1, 3.5.3.2 and 3.5.3.3. *npc4-2* was isolated in a later time than *npc4-1* because of the non availability in the T-DNA knockout collection and hence characterization of *npc4-1* and *npc4-2* was done in separate experiments.

Zeatin and salicylic acid were tested for the phenotypes of *npc4-1* and phenotypic differences were not observed in comparison to WT. Further, phenotypic tests for *npc4-1* were carried out by growing in Fe deficient and S deficient conditions. None of these growth conditions were able to display differential phenotypes compared to similarly grown WT. These observations suggest either disruption of *NPC4* has no influence on the phenotypes in response to above hormones and nutrient deficient conditions or the test conditions employed were not able to show any potential phenotypic differences of the *npc4-1* knockout.

3.5.3.1 Phenotypic analysis of *npc4-1* and *npc4-2* in response to phosphate deficient conditions

Seedlings of *npc4-1* and *npc4-2* were grown in Arabidopsis agar medium containing all the nutrients sufficiently (+Pi) and morphology was compared with similarly grown Columbia 0 wild type in order to see whether disruption of *NPC4* gene causes altered phenotype. Previous RT-PCR experiments revealed enhanced transcriptional induction of *NPC4* during phosphate deficiency. The effect of phosphate starvation on *NPC4* gene was analysed using *npc4* knockouts. As described in section 2.10.1.1 seedlings of *npc4-1* and *npc4-2* were grown in

Arabidopsis medium without phosphorus (-Pi) for 7 days and phenotypes especially root morphology was compared with similarly grown WT seedlings.

Compared to WT seedlings, *npc4-1* seedlings showed a slightly impaired growth in a general growth medium containing all the required nutrients sufficiently (+Pi) (Figure 3.48 A). Primary root length, number of lateral roots and hypocotyl length were affected. In +Pi growth medium significant difference in primary root length was observed between WT and *npc4-1* (P < 0.001) (Figure 3.48 C) and on average knockout roots were 14% shorter than WT after 7 days of growth. A significant difference in total lateral root number (P < 0.001) and lateral root density (P< 0.01) was displayed by *npc4-1* in comparison to WT in +Pi (Figure 3.48 D and E). Similarly, significantly reduced hypocotyl lengths were observed in *npc4-1* (P < 0.001) (Figure 3.48 F).

Phosphate deficiency induced phenotypes such as inhibition of primary root elongation, increased number of lateral roots, increased lengths of lateral roots and high root densities were displayed by WT, *npc4-1* and *npc4-2* when grown in –Pi conditions (Figure 3.48 B). Primary root lengths of *npc4-1* were significantly shorter than WT (P < 0.001) at –Pi conditions (Figure 3.48 C). However, the reduction in primary root lengths owing to phosphate deficiency was approximately 38% both in WT and *npc4-1* indicating *npc4-1* does not differentially respond to phosphate deficiency. Lateral root numbers of WT and *npc4-1* was increased about 1.4 fold in –Pi compared to respective +Pi grown seedlings. Lateral root density was dramatically increased by low phosphate in both WT and *npc4-1* while knockout showing significantly lower lateral root density than WT (P < 0.001) (Figure 3.48 E). However, in –Pi growth conditions the increase in the lateral root densities of WT and *npc4-1* was 2.2 and 2.3 fold respectively. These results indicate that *npc4-1* knockout has no defect in the –Pi response in terms of root phenotypes. A general reduction in hypocotyl lengths was observed in –Pi growth conditions both in WT and *npc4-1* and *npc4-1* showed reduced hypocotyl lengths in comparison to WT (Figure 3.48 F).

npc4-2 seedlings showed similar phenotypes as *npc4-1* in both +Pi and –Pi growth conditions. Compared to WT seedlings, *npc4-2* seedlings too showed a slightly impaired growth in +Pi. Primary root lengths, lateral root numbers, lateral root densities and hypocotyl lengths were significantly lower than WT (Figure 3.49). The percentage inhibition of primary root lengths,

(A)

(B)

(C)

(D)

(E)

(F)
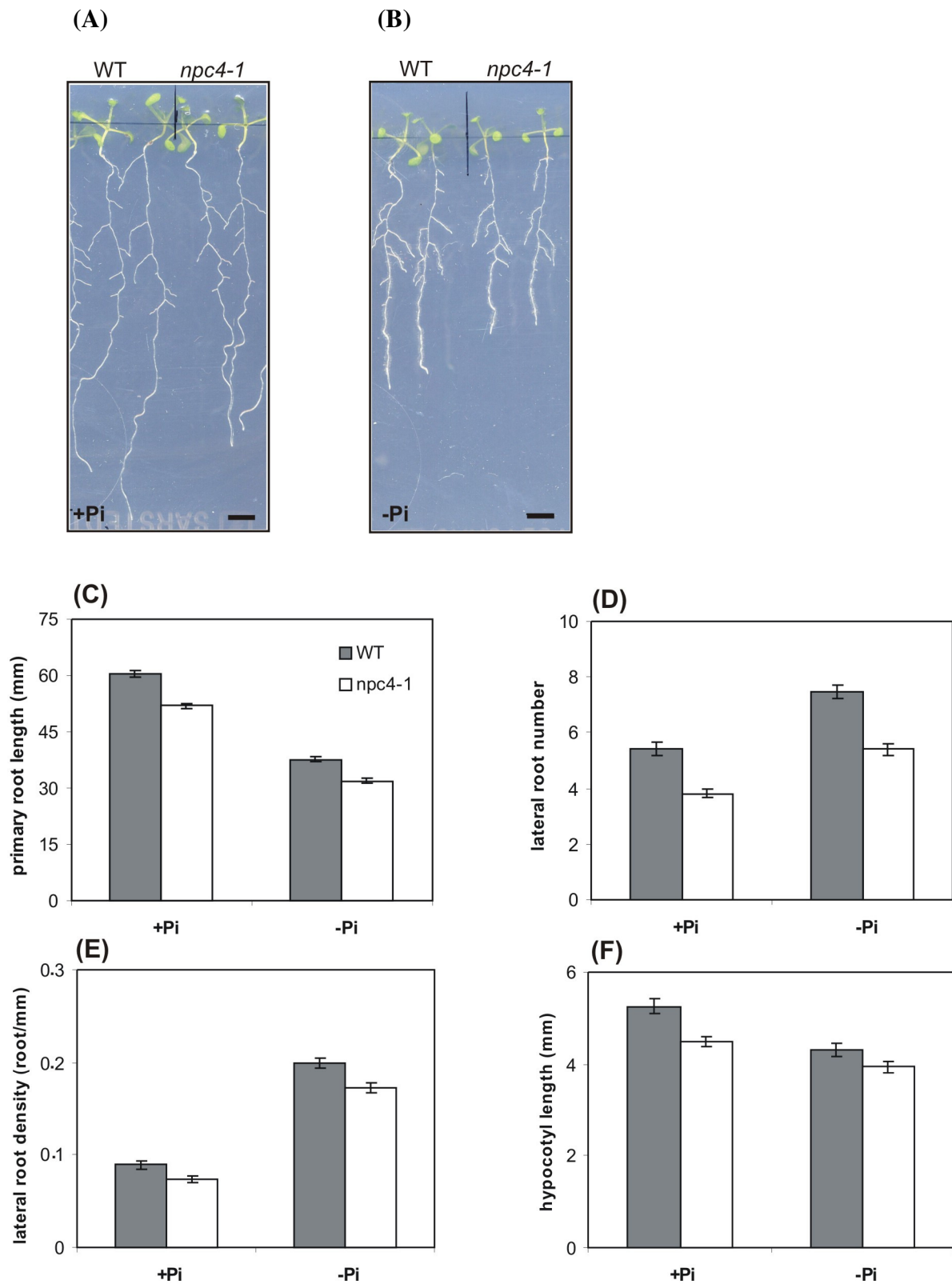
Figure 3.48: Effect of phosphate availability on morphology of *npc4-1* knockout.
(A) Wild type (WT) and *npc4-1* seedlings were grown for 7 days in Pi containing (+Pi) medium on vertically oriented plates. **(B)** Wild type (WT) and *npc4-1* seedlings were grown for 7 days in Pi lacking (-Pi) medium on vertically oriented plates. **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities **(F)** hypocotyl lengths of WT and *npc4-1* seedlings after 7 days of growth in +Pi and –Pi media. Values shown in C, D, E and F represent means of 40 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm
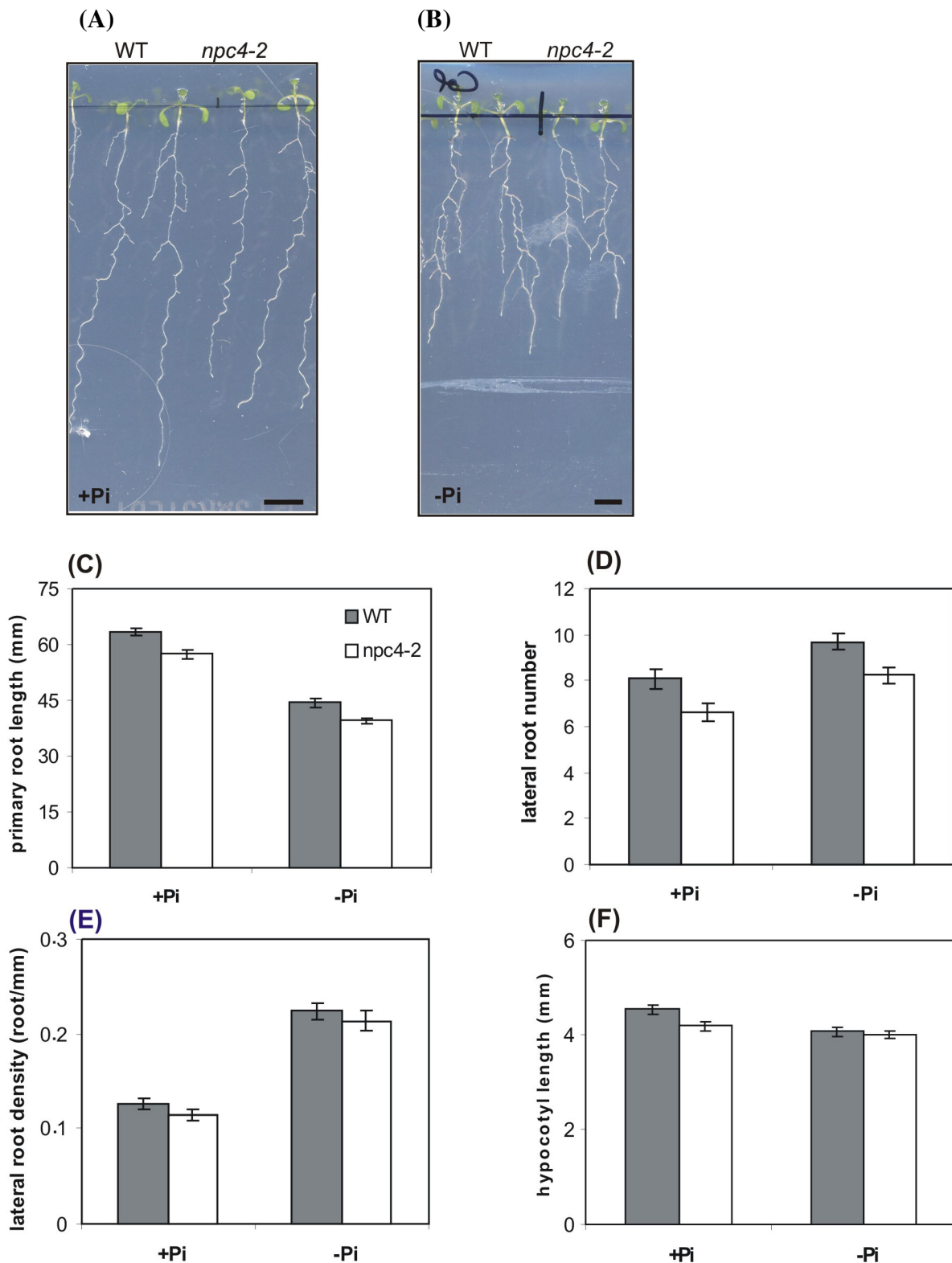
Figure 3.49: Effect of phosphate availability on morphology of *npc4-2* knockout.
(A) Wild type (WT) and *npc4-2* seedlings were grown for 7 days in Pi containing (+Pi) medium on vertically oriented plates. **(B)** Wild type (WT) and *npc4-2* seedlings were grown for 7 days in Pi lacking (-Pi) medium on vertically oriented plates. **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities **(F)** hypocotyl lengths of WT and *npc4-2* seedlings after 7 days of growth in +Pi and –Pi media. Values shown in C, D, E and F represent mean of 40 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

increase in lateral root numbers and lateral root densities owing to phosphate deficiency was almost similar in both WT and *npc4-2* indicating *npc4-2* has no defect in the –Pi response.

It can be seen that *npc4* knockouts do not grow in the same vigour as WT *in vitro* at least in the given conditions and growth stage indicating slightly impaired growth both in the +Pi and –Pi. Although *npc4-1* and *npc4-2* seem to have phenotypic differences in phosphate deficient growth conditions compared to the WT, it can be argued that this effect is due to inherent general growth retardation but not merely due to phosphate deficiency. Taken together, phenotypic observations suggest no significant deficiency of *npc4* in –Pi response and in general, *NPC4* may have potential function/s in root elongation and development.

3.5.3.2 Effect of exogenous auxin on phenotype of *npc4-1* and *npc4-2*

When the transgenic *PNPC4* seedlings were treated with auxin, remarkable increase in GUS activity was observed both in roots and shoots. RT-PCR analysis also revealed that transcription of *NPC4* is induced by auxin. To gain further insight into the effect of auxin in *NPC4* regulation, phenotypes of *npc4-1* and *npc4-2* knockouts were studied in response to exogenous auxin application paying special attention to root system.

Both WT and *npc4-1* knockout plants displayed inhibition of primary root elongation and stimulation of lateral root formation in response to 0.03 µM 1-NAA supplementation to the growth media (Figure 3.50 A and B). The inhibition of primary root length due to 1-NAA in +Pi growth conditions was about 8% both in WT and *npc4-1*. A significant decrease in primary root lengths of WT and *npc4-1* was not observed in –Pi medium supplemented with 0.03 µM of 1-NAA (-Pi+NAA) in comparison to –Pi. These observations demonstrate that *npc4-1* has no differential response towards 1-NAA. In all these growth conditions irrespective of Pi availability and 1-NAA supplementation, *npc4-1* seedlings showed significantly reduced (P < 0.001) primary root lengths compared to WT seedling grown in similar conditions (Figure 3.50 C).

(A)

WT   *npc4-1*

+Pi+NAA

(B)

WT   *npc4-1*

-Pi+NAA

(C)

(D)

(E)

(F)
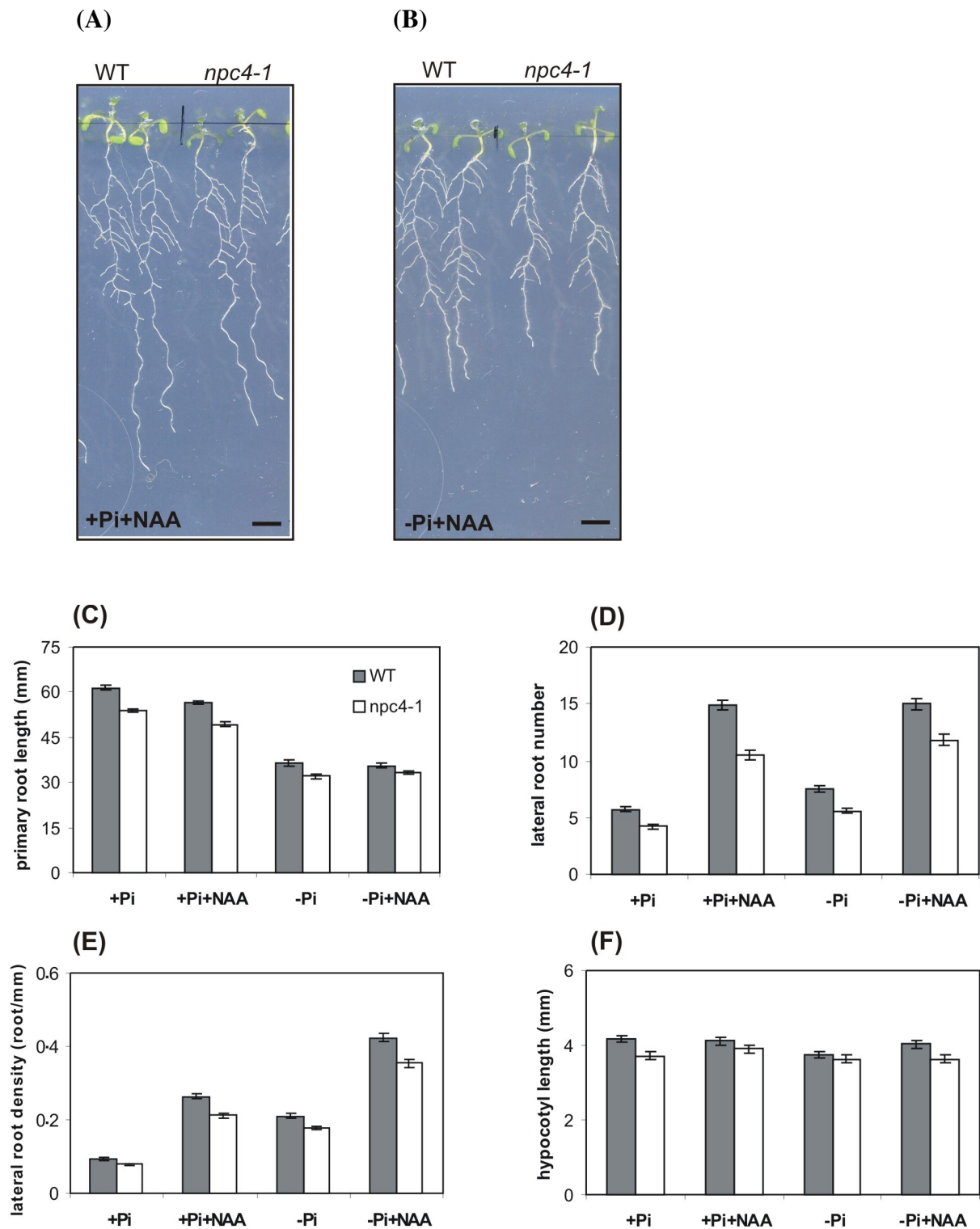
Figure 3.50: Effect of phosphate availability and 1-NAA on morphology of WT and *npc4-1* knockout. **(A)** WT and *npc4-1* seedlings were grown for 7 days in Pi containing medium supplemented with 0.03 µM of 1-NAA on vertically oriented plates (+Pi+NAA). **(B)** WT and *npc4-1* seedlings were grown for 7 days in Pi lacking medium supplemented with 0.03 µM of 1-NAA on vertically oriented plates (-Pi+NAA). **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities and **(F)** hypocotyl lengths of WT and *npc4-1* seedlings after 7 days of growth in +Pi, +Pi+NAA, –Pi and –Pi+NAA media. Values shown in C, D, E and F represent mean of 40 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

(A)

(B)

(C)

(D)

(E)

(F)
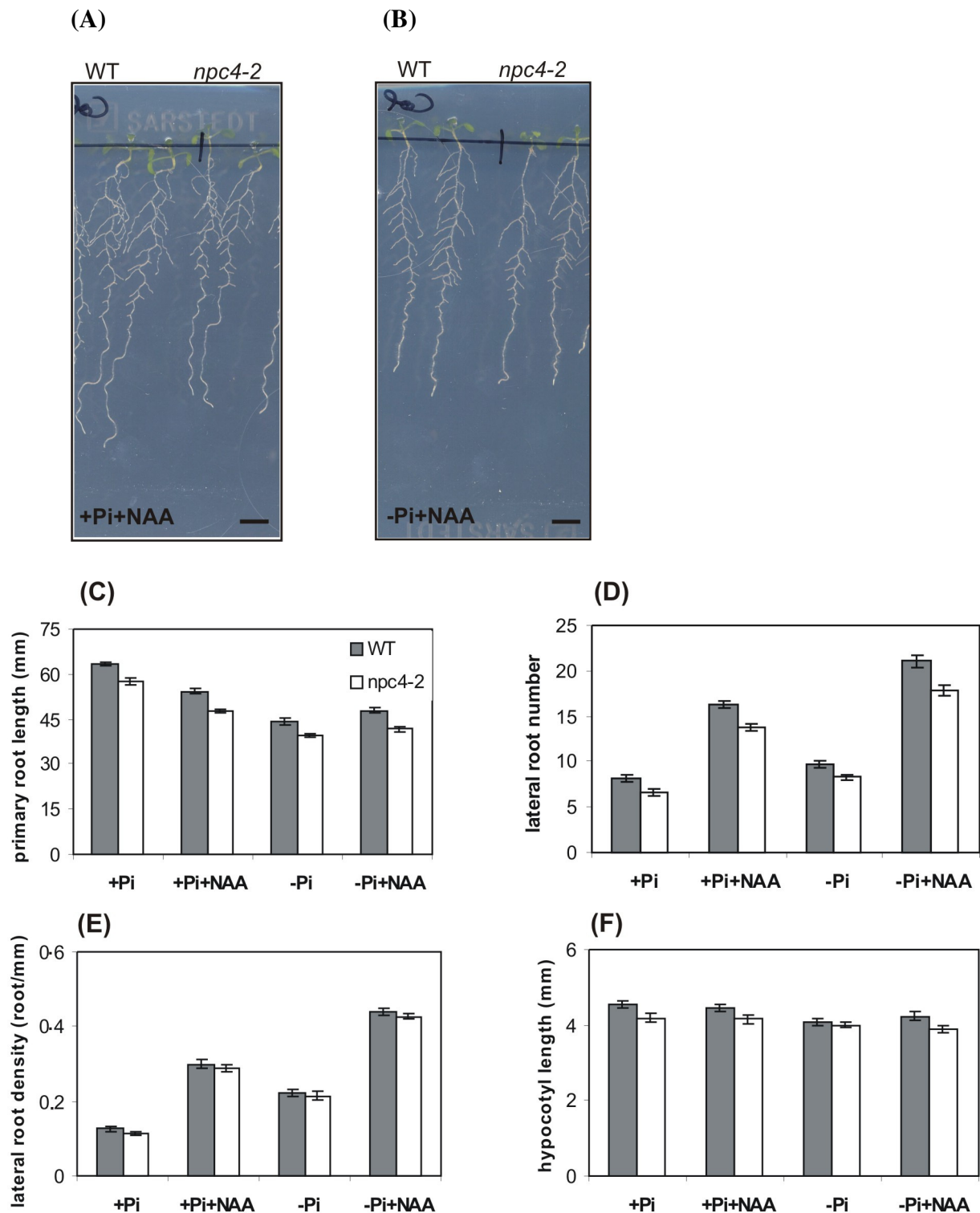
Figure 3.51: Effect of phosphate availability and 1-NAA on morphology of WT and *npc4-2* knockout. **(A)** WT and *npc4-2* seedlings were grown for 7 days in Pi containing medium supplemented with 0.03 µM of 1-NAA on vertically oriented plates (+Pi+NAA). **(B)** WT and *npc4-2* seedlings were grown for 7 days in Pi lacking medium supplemented with 0.03 µM of 1-NAA on vertically oriented plates (-Pi+NAA). **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities and **(F)** hypocotyl lengths of WT and *npc4-2* seedlings after 7 days of growth in +Pi, +Pi+NAA, –Pi and –Pi+NAA media. Values shown in C, D, E and F represent mean of 40 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

Addition of 0.03 µM of 1-NAA to either +Pi or –Pi media increased the number of lateral roots and lateral root densities of WT and *npc4* knockout seedlings compared to the seedlings grown in respective media without added 1-NAA (Figure 3.50 D and E). However, in all the growth conditions lateral root numbers and lateral root densities of *npc4-1* were significantly lower (P < 0.01) than WT (Figure 3.50 D and E).WT and *npc4-1* knockouts showed 2.8 and 2.7 fold increase in the lateral root densities respectively in the 1-NAA supplemented +Pi conditions, compared to +Pi conditions. Supplementation of –Pi medium with 0.03 µM 1-NAA caused about 2 fold increase in the lateral root density of WT and *npc4-1*. Significantly reduced (P< 0.01) hypocotyl lengths in *npc4-1* were observed in +Pi medium but this difference was not very obvious in +Pi+NAA conditions (Figure 3.50 F).

In a similar manner to *npc4-1*, *npc4-2* showed inhibition of primary root elongation and stimulation of lateral root formation in response to 0.03 µM 1-NAA supplementation in +Pi and -Pi growth media (Figure 3.51). The percentage inhibition of primary root length of *npc4-2* and WT due to 1-NAA in +Pi and –Pi, compared to the respective 1-NAA non-supplemented growth conditions was almost similar. Likewise, significantly different increase of lateral root densities in response to 1-NAA was not observed between *npc4-2* and WT in both +Pi and –Pi conditions. In all these growth conditions *npc4-2* showed comparatively reduced primary root lengths, lateral root numbers and densities than WT.

When WT and *npc4-1* knockout seedlings were grown in complete Murashige and Skoog agar medium (MS) supplemented with 0.03 µM or 0.1 µM 1-NAA, differential response of *npc4-1* in terms of primary root inhibition, lateral root number and lateral root density was not observed .

Taken together, when 1-NAA was supplemented to the +Pi or –Pi growth media, the percent increase of lateral root density in WT and *npc4* knockouts was quite similar indicating general auxin response was not impaired. However, in all growth conditions fewer lateral root numbers in knockouts were observed as compared to WT. Therefore, it can be suggested a probable function of *NPC4* in lateral root formation, even though the responsiveness to auxin as such is not impaired.

3.5.3.3 Effect of exogenous brassinolide on phenotype of *npc4-1* and *npc4-2*

Brassinolide (BL) was the most effective hormone, besides auxins to stimulate the *NPC4* promoter driven GUS activity. In addition, transcriptional up-regulation of *NPC4* by BL was observed in RT-PCR analysis. These results prompted investigating phenotypes of *npc4* knockouts in response to BL. Morphology of *npc4*, grown in 0.05 µM BL supplemented Arabidopsis medium was compared with WT grown in similar manner.

Inhibition of primary root elongation, which is a prominent effect of higher concentrations of BL treatment and wavy root structures, could be observed in both WT and *npc4-1* seedlings (Figure 3.52 B). On average a 50% reduction in root length was observed in WT seedlings in BL supplemented growth medium compared to BL lacking control medium after 7 days of growth. Whereas, the reduction of primary root length of *npc4-1* owing to BL treatment was on average 61% (Figure 3.52 C). A reduction in lateral root numbers was observed in both BL treated WT and *npc4-1* (Figure 3.52 D).   A clear increase in lateral root densities were observed in BL treated WT and *npc4-1* (Figure 3.52 E). A 1.4 fold increase in the lateral root density in the BL supplemented media compared to the control was shown by WT, while this increase was 2.1 fold in *npc4-1*. Even though this increase in lateral root density of *npc4-1* was not dramatic, a change from lower lateral root density without BL to a higher lateral root density in the presence of BL in the *npc4-1* relative to the WT was observed. Although weak reduction in hypocotyl lengths was indicated in all the BL treated seedlings, a significant difference between WT and knockouts was not observed (Figure 3.52 F).

Similar to *npc4-1*, a significant inhibition of primary root length was observed in *npc4-2* in response to 0.05 µM BL (Figure 3.53 C). Compared to the WT a clear increase in lateral root density was observed in *npc4-2* in BL treated conditions as opposed to the control growth conditions where the lateral root density of the *npc4-2* was significantly lower than WT (Figure 3.53 E).

The same pattern of change in lateral root densities was indicated by *npc3-1* and *npc3-2* in response to 0.05 µM BL treatment. Prolonged growth in BL supplemented medium showed lesser difference between WT and *npc4* knockouts and were more difficult to quantify because of the convoluted root structures.
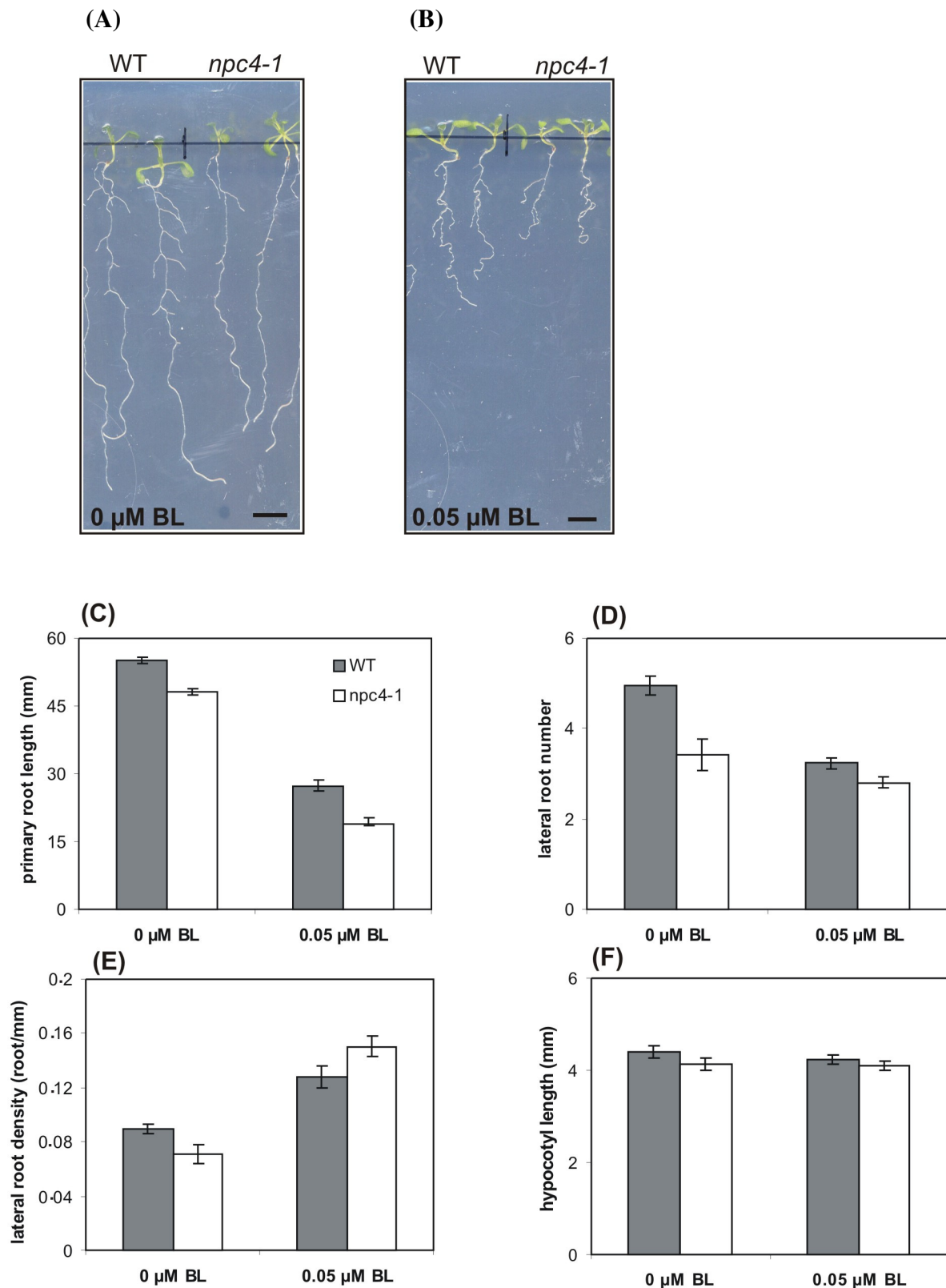
Figure 3.52: Effect of epi-brassinolide on morphology of *npc4-1* knockout.
(A) WT and *npc4-1* seedlings grown for 7 days in Pi containing medium on vertically oriented plates.
(B) WT and *npc4-1* seedlings grown for 7 days in Pi containing medium supplemented with 0.05 µM brassinolide (BL) on vertically oriented plates. **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities and **(F)** hypocotyl lengths of WT and *npc4-1* seedlings after 7 days of growth in Pi containing Arabidopsis medium with or without 0.05 µM BL. Values shown in C, D, E and F represent mean of 30 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm
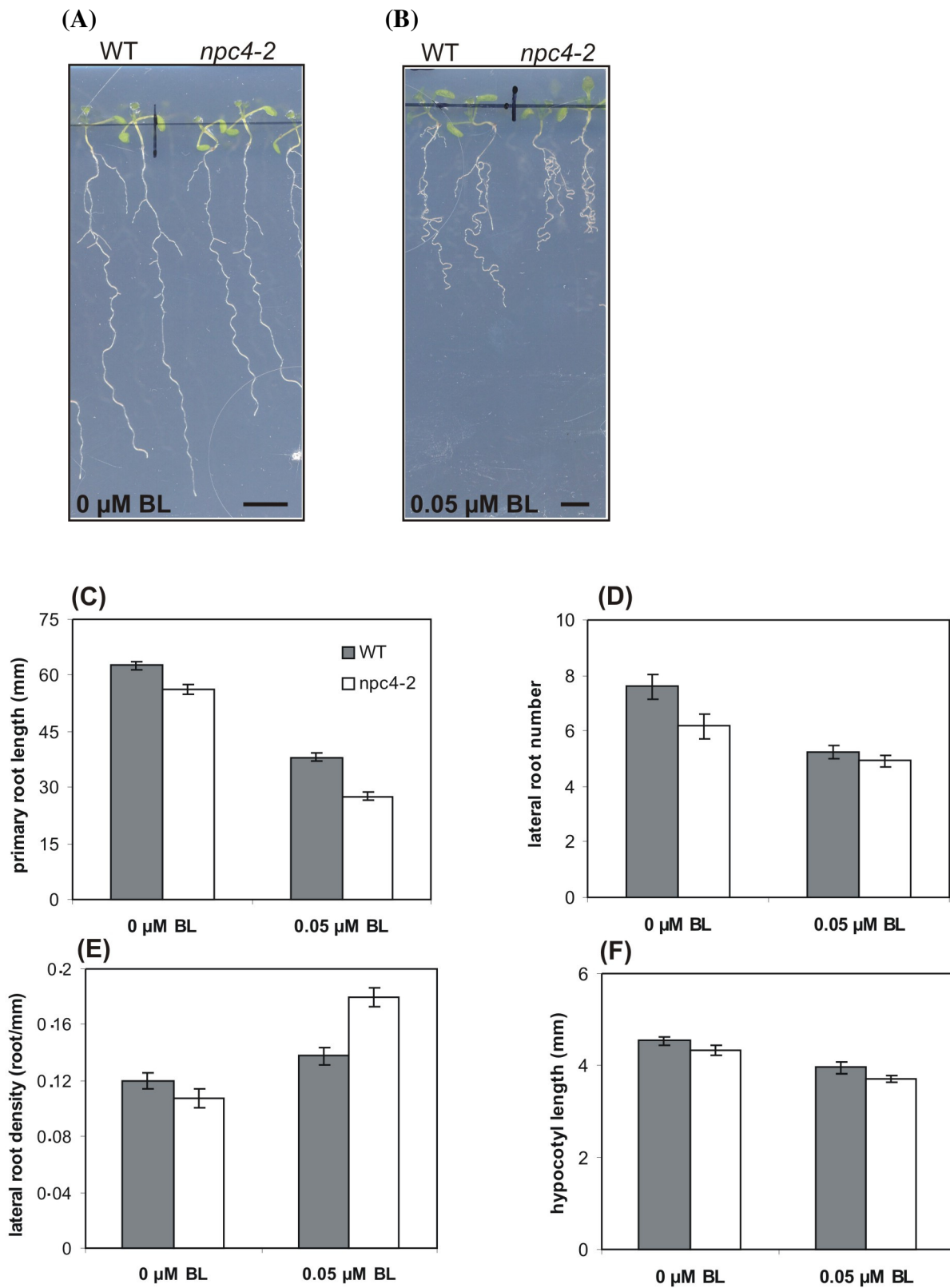
Figure 3.53: Effect of epi-brassinolide on morphology of *npc4-2* knockout.
(A) WT and *npc4-2* seedlings grown for 7 days in Pi containing medium on vertically oriented plates.
(B) WT and *npc4-2* seedlings grown for 7 days in Pi containing medium supplemented with 0.05 µM brassinolide (BL) on vertically oriented plates. **(C)** primary root lengths **(D)** lateral root numbers **(E)** lateral root densities and **(F)** hypocotyl lengths of WT and *npc4-2* seedlings after 7 days of growth in Pi containing Arabidopsis medium with or without 0.05 µM BL. Values shown in C, D, E and F represent mean of 30 seedlings ± SEM. In **A** and **B** scale bar = 5.0 mm

Inhibition of root elongation was also observed in 0.01 µM BL supplemented growth media. In WT and *npc4-1* the reduction of primary root lengths were 35% and 48% respectively. Compared to the BL untreated control growth conditions, 0.01 µM BL caused 1.3 and 1.8 fold increase in lateral root densities in WT and *npc4-1* respectively. The change of the pattern of lateral root density in response to 0.01 µM BL resembles the change observed in 0.05 µM BL.

Taken together, 0.01 and 0.05 µM BL showed relatively higher inhibitory effect on root elongation in *npc4* knockouts than WT. As a result, the lateral root densities of the knockouts were significantly increased above the WT. These results suggest that roots of *npc4* knockouts are more sensitive to BL than the WT. It can be stated that *NPC4* has a BL mediated regulatory role in root elongation.

The phenotypic characteristics of T-DNA insertional mutants of *NPC3* and *NPC4* genes of the *NPC* gene family show considerable similarity in response to a variety of growth conditions. A mild retardation of growth of *npc3* and *npc4* knockouts in comparison to WT was observed when grown *in vitro* providing all the nutrients sufficiently. High degree of similarity in the phenotypes of *npc3* and *npc4* suggest the functional redundancy in the *NPC3* and *NPC4* genes. This similarity in behaviour is reflected in the *PNPC3* and *PNPC4* transcription analysis.

4   DISCUSSION

The existence of phosphatidylcholine hydrolising phospholipases (PC-PLC) in bacteria and animals has long been known and bacterial PC-PLC has been extensively studied in terms of structure, regulation and function. But evidence for the presence of PC-PLC in plant kingdom is lacking except for two recent studies. When glycoprotein and cryptogein elicitors and mastoparan were applied to parsley and tobacco cells, the pool of diacylglycerol (DAG) derived from fluorescent labelled phosphatidylcholine (PC) was strongly and rapidly decreased indicating down regulation of PC-PLC and its involvement in signal transduction (Scherer et al., 2002). Moreover, based on amino acid sequence similarity to known bacterial PC-PLC, six putative PC-PLCs were identified in the *Arabidopsis* genome. A recent study by Nakamura et al. (2005) showed that *NPC4* codes for functional PC-PLC and it is transcriptionally activated in response to phosphate deprivation in *Arabidopsis*. Despite these informations, no other experimental evidence regarding gene regulation and biological functions of plant PC-PLCs are available to date. However, sequence comparison and expression patterns of the putative *PC-PLC* gene family of *Arabidopsis* provide some functional clues. The present study was initiated to investigate the temporal and spatial transcription pattern and functional characterization of this putative *PC-PLC* (*NPC*) gene family.

4.1   Putative *PC-PLC* gene family (*NPC*) members show differential transcription regulation during development

By definition, a gene family is a set of genes coding for diverse proteins which, by virtue of their high degree of sequence similarity, are believed to have evolved from a single ancestral gene (Hurles, 2004). Six genes coding for proteins showing amino acid sequence similarity to some known PC-PLC of bacteria were identified from the *Arabidopsis* genome and are referred to here as *NPC* gene family. Depending on the amino acid sequence and the gene structure the gene family members were subdivided into two groups; *NPC1*, *NPC2* and *NPC6* classified into group I and *NPC3*, *NPC4* and *NPC5* classified into group II the latter of which are arranged in tandem in chromosome III (Figure 3.3). RT-PCR analysis revealed that all the *NPC* gene members except *NPC5* showed overlapping expression in roots, stems, leaves, flowers and siliques but with varied intensities of expression (Figure 3.4). Expression of *NPC5* was very low in all the organs and transcript was observed only in flowers. As

indicated in Table 4.1 this pattern of observation is in general agreement with microarray analysis data provided by Genevestigator and MPSS.

Table 4.1: *NPC* **gene family, predicted localization, organ specific and growth stage specific microarray expression profiles predicted by Genevestigator and MPSS**

Gene name	Localization[a]	Transcription intensity in organs Genevestigator[b] MPSS[c] ( )					Transcription intensity during growth (days)[d]			
		Root	Stem	Leaf	Flower	Silique	1-14	14-21	21-28	28-45
NPC1	Secretory	1097 (32)	1853	1381 (15)	1312 (71)	1683 (65)	2143	2271	2449	2974
NPC2	Secretory	318 (0)	499	483 (9)	550 (5)	814 (27)	1370	828	924	951
NPC6	Secretory	740 (23)	1396	2056 (32)	1425 (55)	1489 (35)	3726	3451	3039	3359
NPC3	Cytosolic	2367 (138)	681	348 (11)	331 (17)	265 (1)	1466	1884	459	718
NPC4	Cytosolic	na (67)	na	na (5)	na (2)	na (0)	na	na	na	na
NPC5	Cytosolic	437 (0)	156	79 (0)	282 (11)	199 (0)	221	430	165	449

[a] Predicted by the Signal P program (available at www.cbs.dtu.dk/services/SignalP) and PSORT (available at http://www.expasy.ch/tools)

[b] Organ-specific expression profile (Gene Atlas, 22k full genome Arabidopsis affymetrix gene chip) provided by Genevestigator (available at https://www.genevestigator.ethz.ch). Signal intensity values are arbitrary units (Zimmerman et al., 2004). na – data not available

[c] Normalized abundance of transcription in different organs of adult Arabidopsis provided by Arabidopsis massively parallel signature sequences (MPSS) (available at http://mpss.udel.edu). Expression values are in parts per million (Meyers et al., 2004).

[d] Growth stage specific expression profile of Arabidopsis provided by Genevestigator (available at https://www.genevestigator.ethz.ch). na – data not available

Over the growth cycle, *NPC1* and *NPC6* showed relatively high expression with little variation in expression intensities at all the growth stages. *NPC2* also showed a quite uniform expression levels during all the development stages suggesting potential function/s of these genes throughout the plant development.

NPC3 and *NPC4* having high sequence resemblance show similar organ specific expression patterns and highest expression was observed in roots. In addition to RT-PCR, transcriptional fusion of promoter of *NPC3* (*PNPC3*) and promoter of *NPC4* (*PNPC4*) to GUS reporter gene showed high degree of similarity in tissue specific transcription profiles (Figures 3.11 A-D and 3.12 A-D). *PNPC3* and *PNPC4* showed expression in roots is restricted to the tips of primary and lateral roots at every stage of development. The root tip contains actively growing and rapidly dividing cells and it is one of the major sites of high auxin content. Transgenic *Arabidopsis* with GUS fused to the highly active synthetic auxin-response element referred to as *DR5* shows a similar pattern of expression in roots indicating a high free auxin concentration (Aloni et al., 2003, 2006). It can be assumed that *NPC3* and *NPC4* may be associated with auxin-related root meristematic activity. In addition, expression of *PNPC3* and *PNPC4* in both rosette and cauline leaves prominently around the leaf margins including hydathodes and in lamina, in parenchyma cells in the anthers and walls of the pollen sac (Figures 3.11 C-F and 3.12 C-F) resembles the *DR5::GUS* expression pattern (Aloni et al., 2003,2006) suggesting potential auxin-related function of *NPC3* and *NPC4*.

Taken together, overlapping transcription profiles observed in the *NPC* gene family members suggest functional redundancy among the *NPC* family gene members. However, differential expression pattern observed in group I and II members suggest each group may be involved in some specific functions in addition to common functions which cannot be further specified on the data gathered here. Relatively low expression levels and predicted cytoplasmic/membrane association suggest group II members may have signal transduction functions but not ruling out any metabolic functions. High transcription levels observed throughout the life cycle and predicted coding for secreted proteins suggest that group I members may be involved in general metabolic activities.

4.2   Transcriptional regulation of *NPC3* and *NPC4* genes by auxin and potential auxin related function

Auxin is involved in virtually every aspect of plant growth and development from embryogenesis to senescence. Some of the key functions associated with auxin are stimulation of cell elongation, cell division, vascular tissue differentiation, mediating gravitropism and phototropism, shoot and root formation and branching, floral bud development and fruit development (Timpte et al., 1995; Taiz and Zeiger, 2002; Friml, 2003). Genome-wide profiling experiments have revealed a wealth of auxin-induced genes and they

share common sequences referred to as Auxin-Responsive Element (AuxRE) in putative regulatory regions (Ulmasov et al., 1997).

Histochemical GUS analysis showed that, when *PNPC3* and *PNPC4* transgenic seedlings were treated with exogenous auxin, either natural or synthetic, a dramatic increase in GUS activity throughout shoot and root occurred (Figures 3.14 and 3.21). However, shorter incubation or staining periods and lower auxin concentrations displayed relatively more intense GUS activity in roots as compared to shoots, indicating a more sensitive auxin response in roots than in shoots (Figures 3.14 and 3.21). In the mock-treated control seedlings transcription was observed in the primary and lateral root tips only. This staining pattern was similar to that observed in *DR5::GUS* seedlings (Ulmasov et al., 1997; Nakamura et al., 2003). Other genes, related to auxin biosynthesis, *CYP79B2* and *CYP79B2* (Ljung et al., 2005) showed similar patterns implying that *PNPC3* and *PNPC4* share some common regulatory features with genes of auxin biosynthesis. The shoot apex which consists of the apical meristem and young leaf primordia is an active site of cell division and a primary site of auxin synthesis (Taiz and Zeiger, 2002). *PNPC3* and *PNPC4* exhibited strong GUS expression in the shoot apex in response to auxin treatment in a similar manner to *DR5::GUS*. This observation again hints a possible participation of the *NPC3* and *NPC4* in auxin funtions.

Promoter sequence analysis revealed that putative promoter regions of *NPC3* and *NPC4* contain known auxin responsive sequence elements like *SAUR, SEBF* and *ASF*, further strengthening argument for auxin related functions of these genes. Moreover, RT-PCR analysis revealed transcriptional induction of *NPC4* in response to exogenous auxin even after 1 h of treatment (Figure 3.6). These results together with promoter studies indicate a likely role of *NPC4* in auxin related functions. Although auxin effects on *NPC3* transcription are not supported by RT-PCR, the promoter-GUS studies strongly suggest its probable association with auxin related functions. However, these observations are not sufficient enough in assigning exact function/s of the genes and understanding the mechanism of action because of functional redundancy of the genes and complex nature of functional interaction of biological processes.

4.3   Do *NPC3* and *NPC4* have an auxin related function in lateral root development?

The importance of auxin in many aspects of root development including initiation and emergence of lateral roots, patterning of the root apical meristem, root elongation and gravitropism has been described extensively (Malamy and Benfey, 1997; Berleth and Sachs, 2001). Dramatic influence of external cues such as nutritional status and light in lateral root development indicate the complicated interaction of extrinsic and intrinsic factors in this process (Zhang and Forde, 2000; Bhalerao et al., 2002).

Lateral root formation involves two major steps, namely stimulation of cell cycle reactivation in the xylem pericycle and establishment of a new meristem (Calenza et al., 1995; Laskowski et al., 1995; Malamy and Benfey, 1977). Activation and progression through the major phases of the cell cycle are governed by the control of cyclin-dependent kinases and auxin also plays a central role in reactivation of cell cycle (Beeckman et al., 2001). A GUS reporter construct for the G2 to M transition associated gene *CYCB1;1* is strongly expressed in pericycle cells undergoing the first divisions of lateral root initiation (Beeckman et al., 2001; Himanen et al., 2002). Expression of *CDKB1;1* starts from the S phase into the G2 to M transition (Menges and Murray, 2002). With auxin treatment, pericycle cells showed very strong GUS activity for both markers, hinting that auxin increases cell cycle activities (Vanneste et al., 2005). These results indicate that lateral root formation is regulated by auxin mediated cell cycle activities.

With regard to auxin dependency, lateral root formation in young roots can be basically divided into two phases: an initiation phase that is dependent on a root tip-localized source of auxin, and an emergence phase that is dependent on a leaf derived source (Bhalerao et al., 2002). Hydrolysis of auxin conjugates in the germinating *Arabidopsis* seed leads to an accumulation of IAA at the root tip between 1-3 days after germination. The emergence of lateral root primordia (LRP) in early *Arabidopsis* seedling development of 4-7 days after germination is highly dependent on auxin derived from aerial plant organs but in older seedlings of 7-10 days after germination LRP emergence was less dependent on shoot-derived auxin as roots gain competence to synthesize IAA and supply the primary root apex with IAA (Bhalerao et al., 2002; Ljung et al., 2001, 2005). The prominent role of auxin in mediating lateral root development has been successfully exploited by screening several mutants in auxin signalling, transport and homeostasis (De Smet et al., 2006). Mutations that disrupt the distribution of IAA between its aerial source and root sink such as *tir3* (Ruegger et al., 1997),

stm1 (Casimiro et al., 2001) and *aux1* (Hobbie and Estelle, 1995; Marchant et al., 2002) lead to reduced lateral root numbers. Mutants of auxin responses like *shy2* (Tian and Reed, 1999) and mutants of reduced auxin biosynthesis such as the *cyp79B2 cyp79B3* double mutant (Ljung et al., 2005) showed reduced lateral root formation. Auxin overproducing mutant lines *sur1-3* and *sur2-1,* defective in IAA biosynthesis, showed auxin-accumulating phenotypes with exorbitant lateral root meristem activity (Barlier et al., 2000). This evidence indicates that mutants impaired in any of the processes in auxin regulation show altered lateral root phenotypes signifying an important correlation between auxin and lateral root forming activities.

Both *npc3* and *npc4* knockout seedlings showed significantly reduced numbers of lateral roots and lateral root densities than WT (Figures 3.45 and 3.48). The altered lateral root phenotype observed in these knockouts may be due to impairment in any of the processes of the cell cycle leading to lateral root initiation, emergence and development functions that depends on root or shoot-derived auxin. Inasmuch as auxin is the most important factor in lateral root formation, *NPC3* and *NPC4* are likely involved in this auxin function. Since auxin responsiveness is not impaired in these mutants (see below) this function is downstream of auxin signal transduction.

Application of exogenous auxin induces lateral formation in *Arabidopsis* (Laskowski et al., 1995). The phenotypes of many of the lateral root mutants affected in a specific part of the auxin pathway have been rescued or mimicked through auxin application (Tian and Reed, 1999; Marchant et al., 2002). *npc3* and *npc4* knockouts were tested for any altered root response to auxin by growing them in media supplemented with 0.03 µM of 1-NAA. Both knockouts showed relatively less numbers of lateral roots and lateral root densities compared to WT in response to 1-NAA but the percentage of increase of lateral root numbers and densities in knockouts and WT were almost similar relative to the growth without 1-NAA (Figures 3.46 A, 3.50 A and 3.51 A). Similarly, the percentage inhibition of primary root elongation in response to 1-NAA was not significantly different between knockouts and WT. These observations indicate that the main responses to the auxin signal transduction in roots, primary root growth inhibition and increase in lateral root density, are not impaired in *npc3* and *npc4* knockouts. Since cell cycle activation and root length inhibition by auxin are responses downstream of auxin signal transduction, these may be impaired by the loss of functional *NPC3* and *NPC4*. Alternatively, it can be argued external auxin supplementation may lead to altered signalling to rescue any defects in auxin mediated lateral root

development in knockouts. However, to determine the exact auxin-related function/s of *NPC3* and *NPC4* genes in root development further investigations are necessary.

4.4   Transcriptional regulation of *NPC4* gene by cytokinin and potential cytokinin related function

Cytokinins have been implicated in many aspects of plant growth and development including shoot organogenesis, leaf senescence, vascular development and photomorphogenic development (Mok and Mok, 1994; Taiz and Zeiger, 2002). Some cytokinin functions are executed primarily through the control of cell cycle activity. Cytokinins affect the expression of many different genes in a variety of plant species (D́ Agostino et al., 2000).

RT-PCR analysis revealed enhanced expression of *NPC4* in the seedlings grown on zeatin supplemented growth medium while other members of the *NPC* gene family did not show differential expression (Figure 3.8).   Transcriptional up-regulation of *NPC4* was observed after 3 and 6 hours of zeatin treatment. Moreover, enhanced GUS activity in the emerging young leaflets and weak increase in GUS activity in lateral root tips were observed when *PNPC4* seedlings were treated with zeatin (Figure 3.23). The expression profiles especially enhanced induction in mitotically active sites suggest potential function of *NPC4* in the cytokinin functions perhaps in cell cycle regulation. Phenotypic differences either in shoots or roots was not observed in *npc4* in response to exogenous zeatin application. This suggests that *npc4* knockouts are not impaired in their zeatin response. However, it should not be excluded that the mode of tests, including growth stage, growth media and incubation conditions employed in assessing the phenotypes may have hindered observing any morphological response differences between knockout and WT.

4.5   Transcriptional regulation of *NPC3* and *NPC4* genes by brassinolides and their implication in brassinolide-related functions

Brassinolides (BL), plant steroid hormones, regulate many of the same growth and developmental processes as auxin, including cell division and expansion, vascular differentiation, root growth, senescence and photomorphogenesis (Clouse et al., 1996; Clouse and Sasse, 1998; Bishop and Koncz, 2002). A number of physiological studies demonstrated the interaction between BLs and auxin in many cellular processes but little is known about underlying molecular mechanisms (Clouse and Sasse, 1998; Nakamura et al., 2003; Halliday, 2004). Recently, a comprehensive comparison of BL and auxin regulated genes have been done in *Arabidopsis* as well as in soybean and tomato (Clouse et al., 1996; Goda et al., 2004). Investigations have shown many genes are only BL-or auxin-stimulated, indicating distinct regulation, while some are stimulated by both BL and auxin, indicating overlap of auxin and BL regulated genes. Several early auxin-inducible genes, belonging to *SAUR*, *GH3* and *IAA* gene families are induced by auxin and BL treatment in *Arabidopsis* but induction kinetics are often slower for BL (Goda et al., 2002).

RT-PCR analysis showed a weak transcription induction of *NPC4* in the 0.1 µM of brassinolide (BL) treated whole seedlings. Moreover, the promoter analysis of *NPC3* and *NPC4* demonstrated enhanced GUS activity in response to exogenous application of 0.1 µM BL although the response is comparatively much lower than after application of auxin (Figures 3.13 and 3.20). In the cotyledons and leaves, intense GUS expression was observed throughout the margins, in tips, hydathode regions and faint expression towards lamina and veins. When *DR5::GUS* reporter fusion *Arabidopsis* plants were treated with 0.01 µM and 0.05 µM of BL, enhanced GUS activity was mainly exhibited in the tips and along the margins of cotyledons and leaves (Nakamura et al., 2003; Bao et al., 2004). The GUS expression pattern of *PNPC3* and *PNPC4* is more or less similar to the *DR5::GUS* expression pattern in response to exogenous BL, possibly suggesting some common features in gene activation. Moreover, Nakamura et al. (2003) reported that early auxin genes *IAA5* and *IAA19* are induced by exogenous BL in a similar way to *DR5* and, hence, BL mediated activation of these *IAA* genes at least in part through the AuxRE *cis* elements.

Transcriptional induction of *NPC3* and *NPC4* in response to BL might be related to a growth function like cell division and perhaps cell expansion similar to auxin function. However, this

postulate needs further evidence. Studying *npc3* and *npc4* insertional mutants may provide insights into probable function of these genes in relation to BL.

4.6   *NPC3* and *NPC4* may have a brassinolide related function in root development

Several studies have shown that brassinolides (BL) are important regulatory substances in roots in addition to their known regulatory functions in shoot growth. A number of BL-deficient and BL-insensitive mutants have been identified in *Arabidopsis*, pea, tomato and rice (Clouse et al., 1996; Bishop and Koncz, 2002; Fujioka and Yokota, 2003) and a prominent feature of the phenotype of these mutants is dwarfism. The other obvious characteristics of these mutants are dark green leaves, reduced fertility, a prolonged life span and abnormal skotomorphogenesis. Based on the morphological characteristics, BL mutants can be subdivided into severe, intermediate and weak alleles (Noguchi et al., 1999). Studies have indicated that dwarf mutants of *Arabidopsis* defective in BL biosynthesis such as *dwf1-6*, *det2*, *cpd* and *cbb3* can be rescued by exogenous application of BLs (Li et al., 1996; Choe et al., 2002; Müssig et al., 2003) whereas mutants such as *bri1*, *dwf12* and *bin2* that are defective in perception of BL or in signalling components and they cannot be rescued by exogenous BLs (Clouse et al., 1996; Li et al., 2001; Choe et al., 2002).

Low concentrations ranging from 0.05 to 0.1 nM of exogenous BLs have been shown to promote root elongation in *Arabidopsis* seedlings (Clouse et al., 1996; Li et al., 2001; Müssig et al., 2003; Bao et al., 2004; Li et al., 2005), dark grown cress (Yopp et al., 1981) and maize (Kim et al., 2000). In contrast, 1.0 nM or higher concentrations of exogenous BLs have been shown to inhibit root elongation in *Arabidopsis* (Clouse et al., 1996; Müssig et al., 2003; Bao et al., 2004; Kim et al., 2006; Villar, 2006), mung bean, wheat and maize (Roddick and Ikekawa, 1992). However, 1 nM of BL clearly stimulated the root growth of *dwf1-6* and *cbb3* mutants of *Arabidopsis* and higher concentrations such as 10 nM of BL was necessary to inhibit root growth (Müssig et al., 2003). Bao et al. (2004) reported that in *Arabidopsis*, lateral root density is increased in response to 1-100 nM BL with 10 nM being optimal. A significant reduction in lateral root numbers was observed in 10 day old *Arabidopsis* seedlings when grown in 1 µM of BL (Kim et al., 2006). The quantification of lateral roots is very difficult at high BL concentrations because of the highly convoluted growth and shape of the roots. It has been shown that at low levels of auxin, BLs and auxin have synergistic effects on the promotion of lateral root formation (Bao et al., 2004).

In the present study, *npc3* and *npc4* knockouts, as well as WT seedlings responded to exogenously added 0.05 μM brassinolide (BL) by significant reduction in primary root lengths compared to respective mock-treated control genotypes (Figures 3.47 and 3.52). These results are consistent with previous reports (Müssig et al., 2003; Bao et al., 2004) that showed inhibition of primary root elongation at 0.01-0.05 μM of BL. Further, with BL treatment a significantly higher inhibition of primary root elongation was observed in knockouts compared to WT. This observation indicates that both *npc3* and *npc4* knockouts are more sensitive to 0.05 μM of BL than WT in root elongation response. Total lateral root number was slightly reduced in WT, *npc3* and *npc4* knockouts in 0.05 μM BL, compared to the control growth conditions. However, a considerable increase of the lateral root densities was observed in 0.05 μM BL treated WT and knockouts, and the lateral root densities of BL treated *npc3* and *npc4* seedlings were higher than in WT seedlings while in the BL untreated *npc3* and *npc4*, the lateral root densities were lower than WT. The observations indicate that these knockouts have altered response to exogenous BL suggesting potential role of *NPC3* and *NPC4* in regulating root growth triggered by BL.

BL is one of the many factors that regulate root growth. The dwarf phenotype of BL-deficient and BL-insensitive mutants clearly indicate that BL being a positive regulator of root elongation. Slightly impaired root growth observed in *npc3* and *npc4* knockouts may be due to either low endogenous levels of BL or decreased sensitivity to the endogenous BL and, thus, suggest positive regulatory function of *NPC3* and *NPC4* in root development.

The biphasic effect of BL, either promoting root elongation or inhibiting root elongation depends on the concentrations of the BL. *npc3* and *npc4* showed significantly stronger reduction in primary root elongation when grown in exogenous 0.05 μM BL as compared to growth reduction with endogenous BL only, which is a high concentration to cause inhibition of root growth. However, significant inhibition of root elongation observed in both *npc3* and *npc4* compared to WT indicate that interruption of *NPC3* and *NPC4* makes the seedlings become more sensitive to 0.05 μM BL. Thus, *NPC3* and *NPC4* may have negative regulatory effect on BL sensitivity.

Taken together, it can be hypothesised that *NPC3* and *NPC4* have both positive and negative regulatory roles in BL response. These two genes may act differently depending on the intensity of exogenous BL signal. Low exogenous BL concentrations may lead to low sensitivity in *npc3* and *npc4* thereby shifting the influence positively that finally leads to less

root inhibition, but high exogenous BL concentrations may lead to high sensitivity thereby shifting the influence negatively that finally leads to more root inhibition. This interpretation can be verified by testing the root response in a wide range of exogenous BL concentrations. Another postulate for the observed phenotype may be by a secondary effect of shift in auxin sensitivity induced by BL altering the levels of endogenous auxin or by enhancing the sensitivity to auxin.

4.7   Transcriptional induction of *NPC4* in response to phosphate limitation and its implications in phosphate associated functions

Phosphorous is an essential nutrient for plant growth, development and reproduction. To cope with low availability of soluble inorganic phosphate (P_i), the assimilated form of phosphorous, plants have evolved elaborate developmental and metabolic adaptations to enhance P_i acquisition (Raghothama, 1999, 2000; Abel et al., 2000; López-Bucio et al., 2002). In *Arabidopsis* a large number of P_i starvation-responsive genes have been identified and functional classification revealed that these genes include those involved in cell biogenesis, cellular organization, cellular transport, cell division, signal transduction and response to stress to name a few of the wide range of functions (Hammond et al., 2003; Wu et al., 2003). However, the *NPC* genes were not included in these data bases. Since a large number of genes with diverse functions are regulated upon P_i starvation it is difficult to assign precise functions of each gene in phosphate regulation.

RT-PCR analysis showed transcriptional up-regulation of *NPC4* in both roots and shoots after 10 days of P_i starvation (Figure 3.5). A significant differential expression pattern between P_i sufficient and deficient conditions was not observed in other members of this gene family except a very weak induction of *NPC5* in P_i deficient conditions (Figure 3.5). Nakamura et al. (2005) reported that transcriptional induction of *NPC4* upon 6 days of P_i starvation. An increased DAG production was observed when crude protein extracts of P_i starved *Arabidopsis* roots were incubated with [^{14}C] PC. Moreover, investigations using anti-NPC4 antibody revealed that NPC4 protein was enriched in the plasma membrane fraction (Nakamura et al., 2005). Based on these findings and P_i starvation- mediated transcriptional expression of monogalactosyldiacylglycerol (MGDG) synthase genes, it was proposed that NPC4 plays an important role in the supply of P_i and DAG in digalactosyldiacylglycerol (DGDG) synthesis during P_i starvation (Nakamura et al., 2005). Because *NPC4* is not significantly present in photosynthetic tissues, NPC4 protein is doubtful to be involved

largely in DGDG synthesis. The antibody raised against the NPC4 was shown also to react against the putative phosphoesterase, polypeptide 2, of oat (Andersson et al., 2005). When the lipase activities of isolated oat plasma membranes were assayed against exogenous substrates, the activities more closely resembled those of PLD in conjunction with PA phosphatase, rather than PLC (Andersson et al., 2005). Therefore, the non specificity of this antibody makes the identification and characterization of NPC4 protein questionable. It can be rather suggested that *NPC4* may be involved in signalling a low P_i status. If PC-PLC is involved in the decrease of PC and increase of DGDG, the group I *NPC* genes (*NPC1*, *NPC2* and *NPC6*) would be the prime candidates for this function because of their much higher expression than group II *NPC* genes. Even without regulation of their activity the lack of P_i could decrease re-synthesis of PC and thus shift the percentage of PC in membranes. This would not explain the increase in DGDG.

When *PNPC4* seedlings were subjected P_i deprivation, a weak enhancement of GUS activity was observed in the root system restricted to primary and lateral root tips and lateral root primordia at various developmental stages (Figure 3.26). A similar GUS expression pattern was observed in the root system of *DR5::GUS* in response to P_i starvation (Lopez-Bucio et al., 2005; Nacry et al., 2005). It can be hypothesised that *NPC4* responds to P_i deficiency in an auxin-regulated manner, as auxin regulation of root responses to P_i starvation was suggested by the analysis of Nacry et al. (2005).

In the putative promoter region of the *NPC4*, four copies of P-responsive MYB transcription factor PHR1 sites could be found. Unlike the promoter region of phosphate transporter *Pht* of barley, that contains PHR1 motifs (Rubio et al., 2001; Schünmann et al., 2004a, 2004b), *PNPC4* expression in response to P_i deficiency was only slightly induced in roots and no induction in shoots. A requirement of additional promoter elements could serve as an explanation for the discrepancy of RT-PCR data and *PNPC4* data in response to P_i starvation.

4.8 *NPC3* and *NPC4* may not have specific functions in phosphate deficiency triggered root system architecture

In *Arabidopsis*, pronounced changes of the root system in response to low phosphorous availability include reduced primary root growth, increased lateral root density and length and increased root hair density (Williamson et al., 2001; López-Bucio et al., 2002, 2005; Linkohr et al.,2002; Al-Ghazi et al., 2003; Müller and Schmidt, 2004; Nacry et al., 2005). All these alterations in the root system are adaptive responses for efficient P_i forage. Studies with *pho2*, an *Arabidopsis* mutant affected in phosphate allocation, have shown shoot phosphate status influences the response of the root system architecture to phosphate starvation (Williamson et al., 2001). Phytohormones and their interactions are known to play key roles in mediating the P_i starvation effects on root system architecture (Martin et al., 2000; López-Bucio et al., 2002; Nacry et al., 2005). Auxin plays an important role in P_i starvation triggered inhibition of primary root growth and promotion of lateral root development (López-Bucio et al., 2002; Nacry et al., 2005).

Expression studies with cell cycle marker *CycB1;1::GUS* and the quiescent centre (QC) identity marker *QC46::GUS* have shown that in low P_i conditions, cell proliferation in the primary root is affected and induces an irreversible shift from an indeterminate to determinate root growth (Sánchez-Caldeón et al., 2005). Role of auxin in triggering the inhibition of elongation of primary root and modification in lateral root initiation and development during P_i starvation is been demonstrated by studies using auxin resistant mutants and *DR5::GUS* (Al-Ghazi et al., 2003; López-Bucio et al., 2005; Nacry et al., 2005).

WT as well as *npc3* and *npc4* knockout seedlings showed typical P_i starvation triggered root architectural changes such as reduced primary root elongation, increased lateral root density, lateral root length and increased root hair density when grown in P_i depleted media (Figures 3.45 and 3.48). In general, these observations indicate that disruption of *NPC3* and *NPC4* genes do not have major effects in the P_i deficiency mediated root adaptive responses. Both WT and *npc3* knockouts showed more or less similar inhibition of primary root elongation in –Pi relative to the +Pi growth conditions although primary root lengths of knockouts were significantly shorter than WT in both +Pi and –Pi (Figure 3.45 C). However, when taken into consideration the percentage increase of lateral root densities of *npc3* knockouts (80-94%) and WT (124%) due to –Pi, it can be assumed that *npc3* has a weak impairment in lateral root development. In phosphate deficient growth conditions *NPC3* may have a minor contribution

towards the modified lateral root development probably by altering auxin sensitivity or auxin transport (Nacry et al., 2005).

The reduction of primary root lengths, increase in lateral root numbers and lateral root densities owing to –Pi were indistinguishable in *npc4* knockouts and WT proposing *NPC4* is not a potential candidate involved in Pi deficiency mediated altered root responses at least in the present experimental conditions. On the contrary, enhanced GUS activity apparent in lateral root primordia, lateral root tips and primary roots of P_i starved *PNPC4* resembling the expression pattern to P_i starved *DR5::GUS* and transcriptional up-regulation of *NPC4* in P_i starved roots may suggest a regulatory function of *NPC4* in roots, perhaps mediated by auxin.

4.9   Do the *NPC3* and *NPC4* have functions of signal transduction or metabolism?

NPC4 showed significant up-regulation both in roots and shoots in response to P_i starvation. Nakamura et al. (2005) showed that the PC hydrolysing activity to produce DAG was highly induced specifically in the roots during P_i starvation. It was proposed that *NPC4* is involved in supplementation of both P_i and DAG from membrane localized phospholipids for DGDG synthesis. These authors suggested that *NPC4* plays an important role in phosphate metabolism. However, relatively low transcription levels of *NPC4* in photosynthetic organs and high expression levels in the root system specific localized around the actively growing meristematic zones of root tips cast doubts on *NPC4* as a potential candidate for phospholipid metabolism. Especially, the activity increase in P_i starvation could have been due to other genes of the gene family.

In contrast, comparatively low transcription of *NPC3* and *NPC4* over the plant growth cycle than the other members of the *NPC* gene family, the predicted cytosolic localization and potential Ca^{2+} binding sites at C terminal suggest that they have signal transduction functions rather than metabolic functions. Rapid down regulation of DAG observed in elicitor treated plant cell cultures with only a minor amount of PA as a product indicated presence of putative PC-PLC in plants. The rapidity of the time course indicates the association of PC-PLC in signal transduction (Scherer et al., 2002). Although this study does not give direct evidence for *NPC* genes having signal transduction functions it can be stated that one or more plant PC-PLCs have signalling functions like animal PC-PLCs. Gene structure and localization and transcription profiles suggest that *NPC1*, *NPC2* and *NPC6* may have metabolic functions.

However, based on the existing evidences it is not yet possible to define the exact function of each gene of the *NPC* family.

DAG, generated as a direct hydrolytic product of PLC or as an indirect product of PA dephosphorylation is an important second messenger in animals that activates protein kinase C. Protein kinase C (PKC) is an integral part in the cell signalling machinery in animal systems. PKC comprises of a large family with multiple isoforms and each of which is said to be regulated in a different manner to play distinct roles for the control of cellular functions (Nishizuka, 1995). Conversely, in plants, existence of PKC has not been verified to date. However, some scattered evidences are reported for the presence of PKC in plants. An isoform of mammalian PKC has been identified in potato tubers and was shown to be important in elicitor-mediated defence response (Subramaniam et al., 1997). A protein having PKC activity has been purified from maize and shown to be related to conventional serine/threonine PKC of animals (Chandok and Sopory, 1998). How DAG function as a signalling component in the absence of PKC in plants is an open question. Plants may use its phosphorylated derivative PA as a second messenger in mediating cellular functions (Munnik, 2001). Increasing evidences are present for the involvement of PA in signalling cascades of plants. A second messenger role of phosphocholine for the mitogenicity of growth factors has been proposed in animal systems (Nishizuka, 1995). But any role of phosphocholine in plants is not known.

4.10 Conclusions and future perspectives

The more or less similar transcription profiles observed for *NPC3* and *NPC4* during the growth cycle and in response to various environmental signals suggest functional redundancy of these two genes. Transcriptional up-regulation by auxin, brassinolides and zeatin localized to meristematic tissues imply potential functions of *NPC3* and *NPC4* in cell cycle regulation. Slight growth retardation observed in seedlings of T-DNA insertional knockouts *npc3* and *npc4* may be due to impairment in regulation of auxin and/or brassinolide or their interaction.

Investigations on root development in response to exogenous auxin and phosphate deficiency did not show any significantly altered phenotypes in *npc3* and *npc4* as compared to WT response suggesting *NPC3* and *NPC4* do not play key roles in auxin and phosphate deficiency mediated root development. However, significant inhibition of root elongation and increased lateral root densities shown by these knockouts in response to exogenous brassinolide suggest

a potential role of *NPC3* and *NPC4* genes in brassinolide triggered root development. Further studies are needed to answer the question how these gene products regulate brassinolide responses in roots.

The rapidity of production of DAG when treated with brassinolide can be investigated with purified *NPC3* and *NPC4* enzymes and it would give a clue for potential brassinolide mediated signal transduction functions of these genes. *NPC3* and *NPC4* overexpressing and RNAi-mediated silencing transgenic plants will be important tools in understanding the roles of these genes in plant growth and development. Recombinant expression of each of the NPC proteins and *in vitro* tests with purified enzymes can provide valuable information about the properties of these enzymes.

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6   APPENDIX

Appendix I: Composition of Murashige and Skoog medium (MS)
(Murashige and Skoog, 1962)

Micro elements

$MnSO_4. 1H_2O$	0.10	mM
H_3BO_3	0.10	mM
$ZnSO_4.7 H_2O$	29.91	µM
$Na_2MoO_4.2H_2O$	1.03	µM
$CuSO_4. 5H_2O$	0.10	µM
$CoCl_2.5H_2O$	0.11	µM
KI	5.00	µM

Macro elements

KH_2PO_4	1.25	mM
$CaCl_2.2 H_2O$	2.99	mM
NH_4NO_3	20.61	mM
$MgSO_4.7 H_2O$	1.50	mM
KNO_3	18.79	mM

Fe Solution

$FeSo_4. 7 H_2O$	0.1	mM
Na_2EDTA	0.1	mM

Vitamin solution

Nicotinic acid	4.06	µM
Thiamine HCl	0.30	µM
Pyridoxine HCl	2.43	µM
Myo-Inositol	0.56	mM

Saccharose 20g/l ,   Bacto Agar (BD France) 10g/l
pH 5.8 adjusted with KOH

Appendix II-A: Composition of Arabidopsis medium (AM)

KNO_3	5 mM
KH_2PO_4	2.5 mM
$MgSO_4$	2.0 mM
$Ca(NO_3)_2$	2.0 mM
Fe-NaEDTA	50 µM
H_3BO_3	70 µM
$MnCl_2$	14 µM
$CuSo_4$	0.5 µM
$ZnSo_4$	1 µM

NaMoO$_4$ 0.2 µM
NaCl   10 µM
CoCl$_2$ 0.01 µM

Saccharose 20g/l ,   Bacto Agar (BD France) 10g/l
pH 5.8 adjusted with KOH

Appendix II-B: Composition of ½ concentrated Arabidopsis medium (AM)

Composition is at half concentration as Appendix IV-A and with 1% Saccharose, 1% Bacto Agar (BD , France), 20 mM Mes pH 5.8

Appendix III: Composition of seed sterilization solution

Sodiumhypochlorite   4.0%
Triton X-100   0.2%

Appendix IV: Composition of infiltration medium

MS salt   ½ x concentrated
B5 vitamin   1 x concentrated
Saccharose   5%
Benzylaminopurin (BAP)   0.044 µM
Tween 80   0.15% (v/V)
pH 5.7 adjusted with KOH
BAP and Tween 80 added after autoclaving

Appendix V: Composition of selection medium

1x concentrated MS salt and vitamin
Agar   0.8% (v/V)
Saccharose   2%   (v/V)
pH 5.7 adjusted with KOH
After autoclaving 100 µg/ml carbenecillin and 50 µg/ml kanamycin were added

Appendix VI: Composition of X-Gluc buffer

5-Bromo-4-Chloro-3-Indoyl-ß-D-Glucoronic acid (X-Gluc)   1 mM
(dissolvedin 250 µl of DMSO)
NaPO$_4$ buffer pH 7.0   50 mM
(1 M Na$_2$HPO$_4$ and 1 M NaH$_2$PO$_4$)
Triton-X   0.5%
K-Ferrycyanide   0.5 mM
K-Ferrocyanine   0.5 mM

Appendix VII: Composition of DNA extraction buffer from plants

Tris/HCl (pH 9.0)	0.2 M
LiCl	0.4 M
EDTA	0.25 M
SDS	10%

Appendix VIII: Composition of LB medium (1L)

NaCl	10.0 g
Yeast extract	5.0 g
Tryptone	10.0 g

Add distilled H_2O_2 to 1 L
pH 7.0 adjusted with 5 N NaOH

LB-Agar: LB-medium + 2% Bactoagar

Appendix IX: Composition of buffers used for isolation of plasmid DNA from alkaline miniprep

Buffer 1:

Tris/HCl (pH 8.0)	50 mM
EDTA	10 mM
RNAse A (4^0C)	100 µg/µl

Buffer II:

NaOH	200 mM
SDS	1.0% (v/V)

Buffer III:

K-Acetate (pH 5.5)	3.0 M

Appendix X: Composition of SOC medium

Bactotrypton	2.0%
Yeast extract	0.55%
NaCl	10.0 mM
$MgCl_2$	10.0 mM
$MgSO_4$	10.0 mM
Glucose	20.0 mM

pH 7.0 adjusted with 5 N NaOH

Appendix XI: Composition of media used for preparing competent cells

Medium A

LB medium
$MgSO_4.7H_2O$ 10 mM
Glucose   0.2%
pH 7.0 adjusted with 5 M NaOH

Medium B

LB medium
Glycerol   36%
PEG (MW 7500)   12%
$MgSO_4.7H_2O$ 12 mM
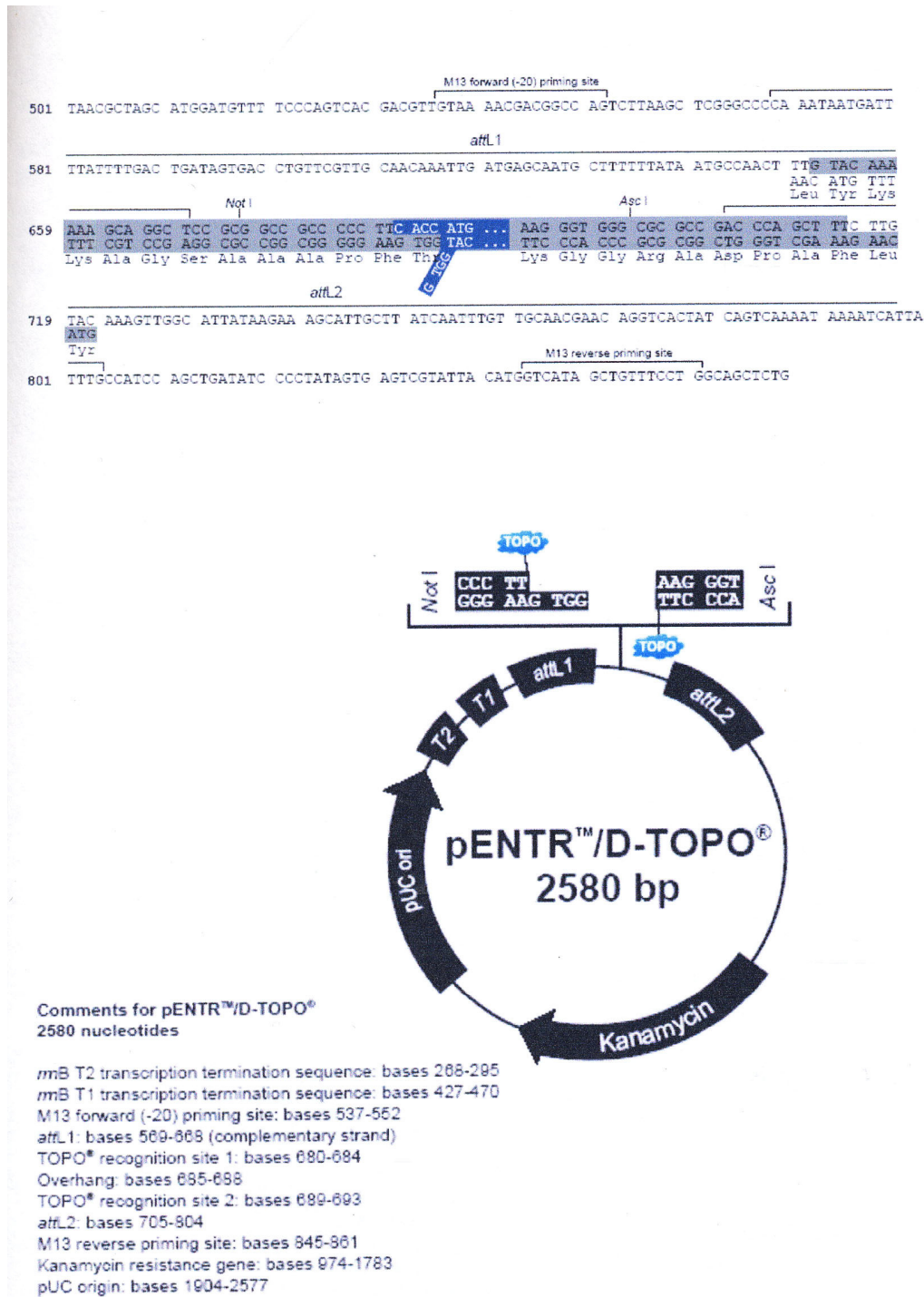pH 7.0 adjusted with 5 M NaOH

Appendix XII: Composition of 50 x TAE buffer

Tris/Acetat (pH 8.0)   40.0   mM
EDTA   1.0   mM
pH 8.0 adjusted with 5 N NaOH
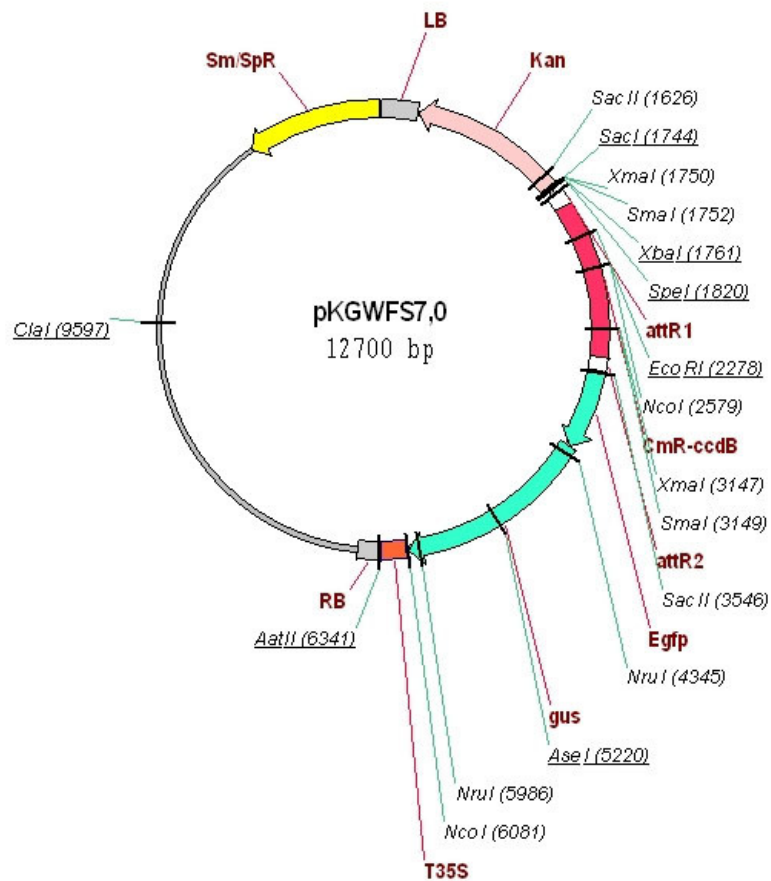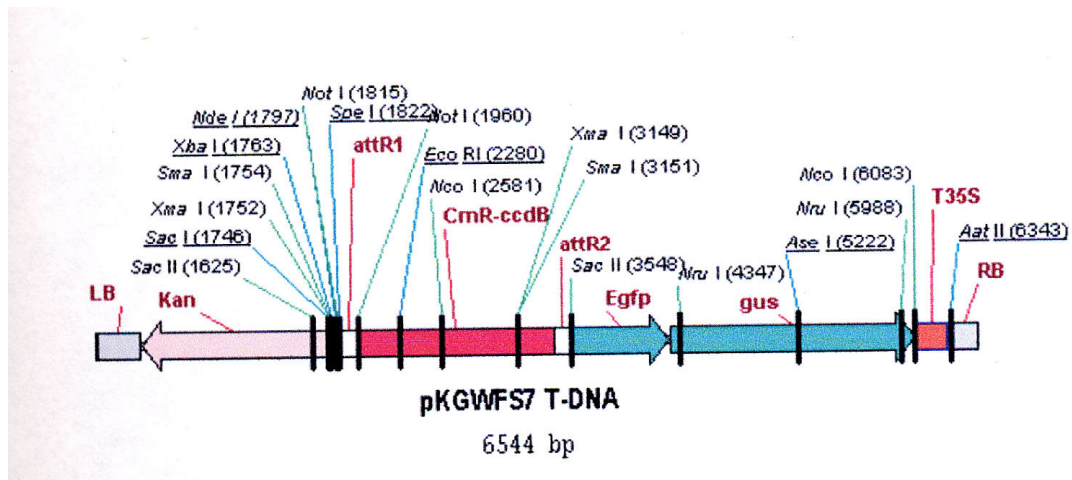

Appendix XIII: Composition of 6 x Gel loading buffer

Bromphenol blue   0.25% (v/V)
Xylencyanol   0.25% (v/V)
EDTA (pH 8.0)   7.5 mM
Glycerin v/V   50%   (v/V)

Appendix Figure 1: Map of Gateway entry vector pENTRY^{TM}/D-TOPO

Appendix Figure 2: Map of Gateway expression vector pKGWFS7

Appendix Table 1: Similarity score of putative PC-PLC of bacteria, *Arabidopsis* and putative phosphoesterase proteins of *O.sativa*

```
SeqA Name          Len(aa) SeqB Name        Len(aa)  Score
========================================================================
1    B.pseudomallei  700     2    M.tuberculosis   521      35
1    B.pseudomallei  700     3    P.aeruginosa     692      49
1    B.pseudomallei  700     4    NPC3             523      18
1    B.pseudomallei  700     5    NPC4             538      18
1    B.pseudomallei  700     6    NPC5             521      18
1    B.pseudomallei  700     7    NPC6             520      18
1    B.pseudomallei  700     8    NPC2             514      16
1    B.pseudomallei  700     9    NPC1             533      15
1    B.pseudomallei  700     10   P1 O.sativa      527      16
1    B.pseudomallei  700     11   P2 O.sativa      520      18
1    B.pseudomallei  700     12   P3 O.sativa      593      15
2    M.tuberculosis  521     3    P.aeruginosa     692      33
2    M.tuberculosis  521     4    NPC3             523      17
2    M.tuberculosis  521     5    NPC4             538      20
2    M.tuberculosis  521     6    NPC5             521      19
2    M.tuberculosis  521     7    NPC6             520      19
2    M.tuberculosis  521     8    NPC2             514      18
2    M.tuberculosis  521     9    NPC1             533      17
2    M.tuberculosis  521     10   P1 O.sativa      527      17
2    M.tuberculosis  521     11   P2 O.sativa      520      18
2    M.tuberculosis  521     12   P3 O.sativa      593      21
3    P.aeruginosa    692     4    NPC3             523      16
3    P.aeruginosa    692     5    NPC4             538      15
3    P.aeruginosa    692     6    NPC5             521      14
3    P.aeruginosa    692     7    NPC6             520      16
3    P.aeruginosa    692     8    NPC2             514      13
3    P.aeruginosa    692     9    NPC1             533      17
3    P.aeruginosa    692     10   P1 O.sativa      527      15
3    P.aeruginosa    692     11   P2 O.sativa      520      16
3    P.aeruginosa    692     12   P3 O.sativa      593      14
4    NPC3            523     5    NPC4             538      62
4    NPC3            523     6    NPC5             521      59
4    NPC3            523     7    NPC6             520      51
4    NPC3            523     8    NPC2             514      55
4    NPC3            523     9    NPC1             533      53
4    NPC3            523     10   P1 O.sativa      527      58
4    NPC3            523     11   P2 O.sativa      520      52
4    NPC3            523     12   P3 O.sativa      593      44
5    NPC4            538     6    NPC5             521      83
5    NPC4            538     7    NPC6             520      49
5    NPC4            538     8    NPC2             514      54
5    NPC4            538     9    NPC1             533      54
5    NPC4            538     10   P1 O.sativa      527      58
5    NPC4            538     11   P2 O.sativa      520      54
5    NPC4            538     12   P3 O.sativa      593      43
6    NPC5            521     7    NPC6             520      49
6    NPC5            521     8    NPC2             514      53
6    NPC5            521     9    NPC1             533      53
6    NPC5            521     10   P1 O.sativa      527      57
6    NPC5            521     11   P2 O.sativa      520      53
6    NPC5            521     12   P3 O.sativa      593      44
7    NPC6            520     8    NPC2             514      57
7    NPC6            520     9    NPC1             533      55
7    NPC6            520     10   P1 O.sativa      527      54
7    NPC6            520     11   P2 O.sativa      520      55
7    NPC6            520     12   P3 O.sativa      593      55
8    NPC2            514     9    NPC1             533      61
8    NPC2            514     10   P1 O.sativa      527      56
8    NPC2            514     11   P2 O.sativa      520      64
8    NPC2            514     12   P3 O.sativa      593      48
9    NPC1            533     10   P1 O.sativa      527      53
9    NPC1            533     11   P2 O.sativa      520      57
9    NPC1            533     12   P3 O.sativa      593      43
10   P1 O.sativa     527     11   P2 O.sativa      520      57
10   P1 O.sativa     527     12   P3 O.sativa      593      46
11   P2 O.sativa     520     12   P3 O.sativa      593      49
```

Appendix Table 2: Summarizing table of the six members of the putative PC-PLC of Arabidopsis as revealed by NCBI *Arabidopsis* protein data base.

Gene name	Gene identification	Protein accession no.	Description	Amino acid no.	Mol. weight
NPC1	At1g07230	NP_172203	Low similarity to phospholipase C2 precursor of Mycobacterium	533	60.03
NPC2	At2g26870	NP_180255	Low similarity to non – hemolytic phospholipase C precursor Burkholderia pseudomallei	514	57.68
NPC3	At3g03520	NP_187002	Low similarity to phospholipase C2 precursor of Mycobacterium	523	59.09
NPC4	At3g03530	NP_566206	Low similarity to phospholipase C2 precursor of Mycobacterium	538	60.72
NPC5	At3g03540	NP_566207	Similar to phospholipase C2 precursor of Mycobacterium	521	59.07
NPC6	At3g48610	NP_190430	Low similarity to phospholipase C2 precursor of Mycobacterium	520	57.91

Curriculum Vitae

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Publications and Conference presentations

Publications

Zahn, M., **Wimalasekera, R**., Göbel, C., Feussner, I., Holk, A. and Scherer, G. F. E. (2005). Expression of Arabidopsis phospholipase A genes in Petunia x hybrida. Increased hypersensitive-like response after infection with Botrytis cinerea and Pseudomonas syringae pv. Tomato DC3000 demonstrates a function for phospholipase A in pathogen defence. *Physiol. Mol. Plant Pathol.* 67: 2-14

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