Molecular mechanisms of root hair growth induced by Pi deficiency in *Brassica carinata*

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Zusammenfassung

Unterschiedliche Phosphateffizienz bei *Brassica carinata* konnte auf längere Wurzelhaare in dem Pi effizienten Genotypen Bale im Vergleich zu dem Pi ineffizienten Genotypen Bacho zurückgeführt werden. Ziel dieser Arbeit war die Identifizierung und die Charakterisierung von Genen, die das Wurzelhaarwachstum unter Pi Mangel regulieren. Zwei unterschiedliche Ansätze wurden hierbei verfolgt: eine Subtraktionshybridisierung reicherte die bei Pi Mangel differentiell exprimierten Sequenzen beider Genotypen bzw. die durch Pi Mangel induzierten Gene in dem Pi effizienten Genotypen an. Zudem wurde mit Hilfe eines whole genome microarrays von *Arabidopsis thaliana* eine Transkriptomanalyse durchgeführt. Die Selektion der Kandidatengene erfolgte aufgrund ihrer möglichen Funktion im Wurzelhaarwachstum oder im Pi Signalweg. Im Folgenden wurden Wurzelhaarphänotyp und Genexpressionsmuster unter Pi, N und K Mangel und nach Änderungen im Pi and N Angebot untersucht. Ebenso wurde die Transkription in verschiedenen Pflanzenorganen und entlang der Wurzel analysiert, um eine Relation der Kandidatengene mit dem durch Pi Mangel induzierten Wurzelhaarwachstum herzustellen.

Pi und N Mangel führten zu längeren Wurzelhaaren in Bale, während K Mangel keinen Einfluss hatte. In Bacho konnte kein Effekt von Pi, N und K Mangel festgestellt werden. Transfer in Pi und N Mangel führte bei Bale zu einem verlängerten Wurzelhaarwachstum nach 4h während vice versa die Zugabe von Pi und N die Wurzelhaarbildung nach 2h bzw. 6.5h reduzierte. Die Expressionsmuster von HRGP (hydroxyproline rich protein), LRR (leucine rich repeat receptor like protein kinase), XTH (xyloglucan endotransglucosylase) und IPS (Induced by Pi starvation) reagierten übereinstimmend mit Änderungen in der Wurzelhaarentwicklung und könnten damit eine Funktion in der Regulation des Wurzelhaarwachstums bei Pi und N Mangel haben. Die Expression des vermutlichen Zellwandproteins HRGP wurde durch Pi und N Mangel herunterreguliert und die erhöhte Transkription in dem Teil der Wurzel, wo keine weitere Streckung der Wurzelhaare erfolgt, lässt auf eine negative Regulation durch Pi und N Mangel und eine Funktion in der Festigung der Wurzelhaarzellwand schließen. In Übereinstimmung wurde HRGP im Genotypen Bacho nicht reguliert. LRR weist Ähnlichkeiten zu einer Rezeptorkinase auf und war positiv durch Pi und N Mangel reguliert. Die verstärkte Transkription in den Wurzelspitzen, wo die Wurzelhaarinitiierung und -elongation stattfinden, könnte auf eine Funktion in der frühen Wurzelhaarentwicklung deuten, die durch Pi und N Mangel induziert wird. Die Expression von XTH wurde rasch nach Pi Zugabe herunterreguliert und die Induktion durch -Pi in beiden Genotypen lässt eine Funktion in der Wurzelhaarentwicklung schließen, die in beiden Genotypen vorkommt. IPS wurde früh nach Zugabe von Pi herunterreguliert. Eine direkte Funktion im Wurzelhaarwachstum kann indes aufgrund einer fehlenden Regulation bei N Stress und entlang der Wurzelzonen ausgeschlossen werden. Die entgegen gesetzte Regulation von LRX1 (leucine rich/ extensin protein) und F-box in Bale und Bacho unter Pi Mangel lässt eine Bedeutung bei der Wurzelhaarentwicklung vermuten. Beide waren verzögert nach Pi Zugabe im Vergleich zur Wurzelhaarentwicklung herunterreguliert. Während für LRX1 eine Funktion in der Endphase der Wurzelhaarelongation aufgrund einer hohen differentiellen Regulation in den entsprechenden Wurzelzonen bei -Pi und +Pi vermutet wird, könnte F-box aufgrund seiner Funktion bei der Degradation von Zielproteinen zu einem späteren Zeitpunkt der Wurzelhaarentwicklung beteiligt sein.

Schlüsselwörter: Wurzelhaare, Pi Mangel, Pi Effizienz

Abstract

Different phosphorus efficiency in Ethiopian mustard (*Brassica carinata)* genotypes was attributed to longer root hairs in cv. Bale (Pi efficient) compared to cv. Bacho (Pi inefficient). The present study aims at the identification and characterization of genes regulating root hair growth during Pi starvation. Two different approaches identified the candidate genes: first, Suppression Subtractive Hybridization (SSH) enriched differentially expressed sequences in both cultivars during Pi deprivation as well as Pi starvation responsive genes in cultivar Bale. Secondly, transcriptional profiling was performed by using *Arabidopsis thaliana* whole genome microarrays. Candidate genes were selected according to a putative function in root hair development or Pi signaling. Root hair phenotype as well as gene expression pattern were evaluated in response to Pi, N and K starvation and after changing Pi and N supply. Moreover, transcription was determined in different organs and along the root intending to clarify the Pi starvation induced mechanisms in enhancing root hair growth.

Root hair length was enhanced during Pi and N starvation with no effect of K deprivation in cv. Bale while cv. Bacho responded to none of the examined nutrient stresses. Transferring seedlings of Bale to Pi and N deprived nutrient solution resulted in enhanced root hair length after 4h and vice versa a reduction in root hair length was observed 2h and 6h after resupplying Pi and N. The expression patterns of HRGP (hydroxyproline rich glycoprotein), LRR (leucine rich repeat receptor like protein kinase), XTH (xyloglucan xyloglycosyltransferase) and IPS (induced by Pi starvation) corresponded in temporal agreement to changes in root hair length and might have a function in regulating root hair growth. The putative cell wall protein HRGP was downregulated during Pi and N stress and higher expressed in non elongating parts of the root suggesting a negative regulation in root hair growth probably by strengthening the cell wall. Furthermore, HRGP was not regulated in Bacho. The root-specific LRR with similarities to a receptor-like protein kinase and was upregulated in response to Pi and N deprivation and most pronounced expressed in root tips where root hair initiation and elongation occur. This could relate LRR to a function in early stages of root hair development induced by Pi and N starvation. XTH was rapidly downregulated after resupply of Pi and an enhanced expression in both cultivars during -Pi suggested a function for XTH in root hair development by remodelling cell walls during growth processes in both cultivars. The non coding molecule IPS responded rapidly to changes in Pi nutrition but a relation to root hair growth can be excluded since there was no differential expression along the root tip and no response to N stress. The function of IPS is not clear. The converse regulation of LRX1 (leucine rich/ extensin protein) and F-box in both cultivars during Pi depletion suggests a function in root hair growth. However, both genes were downregulated with delay to Pi resupply compared to the reduction in root hair length assuming a function in later stages of root hair growth. F-box proteins are obviously involved in regulated degradation of target proteins and may control root hair length at a later stage.

Keywords: root hair, Pi deficiency, Pi efficiency

Contents

Abbreviations

A	adenine
ACC	aminocyclopropane carboxylic acid
At	Arabidopsis thaliana
ATP/ ADP	adenosintriphosphate/ adenosindiphosphate
B.	Brassica
BLAST	Basic Local Alignment Search Tool
bp	base pair
C	cytosin
cDNA	copyDNA (Desoxyribonucleic acid)
C(t)	cycle treshhold
C-terminus	carboxy-terminus
cv.	cultivar
Cy	cyanine
dCTP	desoxycytidine triphosphate
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleosidtriphosphate
E.	Escherichia
e.g.	for example
EST	Expressed Sequence Tag
et al.	et alia
Fig.	Figure
G	guanine
GEO	gene expression omnibus
GUS	β-glucuronidase
H position /H cell	hair cell
i.e.	id est
Km	Michaelis constant
LB	lysogeny broth medium
miRNA	microRNA
N position/ N cell	non hair cell
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
nt	nucleotide
N-terminus	amino-terminus
P1BS	PHR1 binding site
PCR	polymerase chain reaction

PEP	Phosphoenolpyruvat
Pi	inorganic phosphate
PMT	Photomultipliertube
PSI	phosphate starvation induced
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RhoGTPase	Rho guanosine triphosphatase
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
ROS	reactive oxygen species
Rpm	rounds per minute
SE	standard deviation
SSH	Suppression Subtractive Hybridization
SUMO	small ubiquitin-like modifier
T	thymin
Tm	melting temperature
TF	transcription factor
U	units
UV (light)	ultraviolet
X-Gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

Gene Abbreviations

AGP	Arabinoglactan protein
AP	APETALA
bHLH	basic Helix Loop Helix
CHI	chitinase
CESA	Cellulose Synthase
CPC	CAPRICE
EGL	ENHANCER OF GLABRA
EXO	Exocyst subunit
EXP	EXPANSIN
FER	FERONIA
GL	GLABRA
HRGP	Hydroxyproline Rich Glycoprotein
IPK	inositol polyphosphate kinase
IPS	INDUCED BY PHOSPHATE STARVATION
KUP	Potassium Uptake Permease
LPI	LOW PHOSPHORUS INSENSITIVE
LRR	Leucine-Rich Repeat
LRX	LRR-Extensin
LTP	Lipid Transfer Protein
MAPK	Mitogen-Activated Protein Kinase
MIZ	msx2-Interacting Zinc finger
MRH	MORPHOGENESIS OF ROOT HAIR
PHO	Phosphorus-deficient mutant
PHR	PHOSPHATE STARVATION RESPONSE
PLD	Phospholipase D
PME	Pectinmethylesterase
PRP	proline rich protein
RHD	ROOT HAIR DEFECTIVE
RHL	ROOT HAIRLESS
RLK	receptor-like protein kinase
ROP	Rho GTPase
RSL	ROOT HAIR DEFECTIVE 6-LIKE
SAP	Scaffold Attachment Factor
SCM	SCRAMBLED
SIZ	SAP/MIZ
THE	THESEUS

TOE	TARGET OF EARLY ACTIVATION TAGGED
TRY	TRIPTYCHON
TTG	TRANSPARENT TESTA GLABRA
UBQ	Ubiquitin
WER	WEREWOLF
XTH	Xyloglucan endotransglucosylase/hydrolase
XTR	Xyloglucan endotransglucosylase related

General Introduction

1. Phosphate in the soil

Plants acquire phosphate as inorganic phosphate (Pi) from the soil solution. The formation of insoluble complexes with cations, particularly iron and aluminium in acidic soils and calcium in calcareous soils, contributes to only a small fraction of free total Pi in soil solution (Gerke, 1992; Vance et al. 2003). Consequently, even in fertile soils the availability of Pi for the plants is low (2-10 µM) thereby limiting crop yield (Barber et al. 1962; Reisenauer, 1966; Bieleski, 1973). Furthermore, a significant portion (20-80%) of Pi in the soil is present in organic form and thus not readily available for plants (Jungk et al. 1993; Richardson, 1994). Fertilizers were applied to overcome Pi deficiency and to increase crop yield but rock phosphate as the common non-renewable resource is expected to deplete within the next 50-80 years (Isherwood, 2000; Vance, 2001). Increasing world population and extensive agriculture even in low-fertility soils will further increase the demand on Pi fertilizers.

2. Phosphate in plants

Phosphate is an essential macronutrient for plant growth and development (Bieleski, 1973). It is a structural component of nucleic acids, phospholipids and ATP (Vance et al. 2003; Raghothama and Karthikeyan, 2005; Jain et al. 2007; Schachtman and Shin, 2007). Due to phosphorylation and dephosphorylation of proteins it participates in signal transduction and is a regulatory factor in photosynthesis and oxidative metabolism (Raghothama, 1999).

The Pi ions move to the uptake sites of the roots by diffusion rather than mass flow because of strong reactions with soil components (Fitter and Hay, 2002). They have to be taken up along a steep concentration gradient across the plasma membrane as cytosolic Pi concentrations are about 1000-fold higher (5-17 mM) than available Pi in the soil solution (Mimura et al. 1996). For this reason, the acquisition of Pi occurs through an energy-mediated co-transport driven by protons (Sakano et al. 1992). In the root, both high- and low-affinity uptake mechanisms exist depending on Pi concentration in the soil solution with an increased expression of high affinity Pi transporters (with low Km) during Pi deficiency

(Bieleski, 1973; Furihata et al. 1992; Muchhal et al. 1996). In the plant, Pi enters metabolism via non-dedicated pathways. The most important route is via synthesis of ATP from ADP and Pi. Excess of Pi is stored in the vacuole where it helps to maintain Pi homeostasis (Mimura et al. 1990).

3. Responses to Pi deficiency

Plants have evolved physiological and morphological adaptations to enhance Pi efficiency (Raghothama 1999; Vance et al. 2003; Richardson et al. 2009). These strategies aim at conserving and mobilizing internal Pi (Pi utilization efficiency) or increase the availability and uptake of Pi from the soil (Pi uptake efficiency; Smith, 2001).

Pi utilization efficiency is enhanced by mobilization and translocation of Pi in the plant (Duff et al. 1991; Bariola et al. 1994; del Pozo et al. 1999; Raghothama, 1999; Haran et al. 2000; Baldwin et al. 2001; Miller et al. 2001; Li et al. 2002). An improved uptake of Pi is realized by the expression of high affinity Pi transporters in the root (Duff et al. 1994; Gilroy and Jones, 2000; Lynch and Brown, 2001). Protons and organic acids like malate and citrate are secreted under low Pi to break down complexed Pi (Neumann et al. 1999; Vance et al. 2003; Shen et al. 2005). Furthermore, starvation of Pi induces changes in the root system architecture including a reduced growth of the primary root and an increased number and length of root hairs and lateral roots resulting in an increased root-shoot ratio (Foehse et al. 1991; Bates and Lynch, 1996; Gahoonia and Nielsen, 1998; Ma et al. 2001; Williamson et al. 2001; Lopez-Bucio et al. 2002). Root hair growth was reported to respond to local Pi availability in the root (Bates and Lynch, 1996; Martin et al. 2000) while lateral and primary root growth is obviously dependent on whole Pi status (Williamson et al. 2001; Ticconi et al. 2004). Changes in root architecture contribute to explore a greater soil volume and increase the effective root absorptive area for Pi uptake.

3.1. Molecular mechanisms of root hair formation

Root hairs are tubular-shaped tip-growing protrusions of specialised epidermal cells functioning in nutrient and water uptake (Peterson and Farquhar, 1996; Gilroy and Jones, 2000; Jungk, 2001). They are responsible for up to 90% of Pi uptake under low Pi (Foehse et al. 1991; Gahoonia and Nielsen, 1998) and can contribute to the uptake surface by 77% (Parker et al. 2000; Jungk, 2001). The formation of root hairs starts in the differentiation zone after elongation has ceased and can be subdivided into three parts: cell fate determination, swelling formation/ initiation and tip growth (Dolan et al. 1994; Bibikova and Gilroy, 2003).

3.1.1. Cell fate determination

Root epidermal cells in the family *Brassicaceae* develop into hair-bearing cells (trichoblasts) when located over the junction of two underlying cortical cells (H position) whereas cells lying over a single cortical cell normally adopt the non-hair cell fate (N position; atrichoblasts; Dolan et al. 1994; Galway et al. 1994). This position-dependent determination is already present during embryogenesis and implies a signal mechanism arising from the underlying cells that directs the epidermal cell fate (Schiefelbein, 2000). Recently, SCRAMBLED (SCM) encoding for a LRR receptor-like protein kinase was identified as a receptor of positional signals from underlying root cortical cells and thereby regulating the abundance of transcription factors that determines the epidermal cell fate (Kwak et al. 2005). Within the meristematic zone, SCM itself is present in all cell files and the distribution of the ligand could contribute to the positional determination (Kwak et al. 2005).

For the specification of non-hair cells in the meristematic region some transcription factors were identified: GLABRA2 (GL2) encodes a homeodomain-Zip-related transcription factor (Rerie et al. 1994; Di Cristina et al. 1996), TRANSPARENT TESTA GLABRA1 (TTG1) encoding for a WD40 protein (Galway et al. 1994; Walker et al. 1999), WEREWOLF (WER), a Myb transcription factor (Lee and Schiefelbein, 1999) and two bHLH transcription factors, GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3; Bernhardt et al. 2003). Mutations in these genes resulted in more hair bearing cells in ectopic position (Galway et al. 1994;

Masucci et al. 1996; Lee and Schiefelbein, 1999). Recently, another Myb transcription factor closely related to WER, MYB23, was identified in non-hair cells. It is positively regulated by WER and can also substitute WER function during specification of the epidermal cells (Kang et al. 2009).

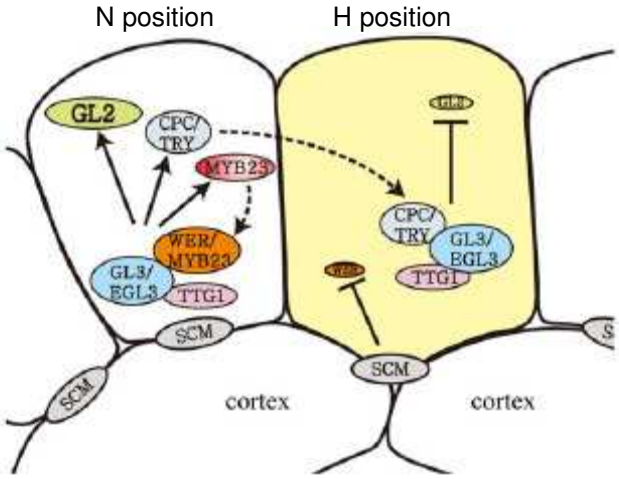


Fig. 1 Scheme of the interactions of transcription factors in determining epidermal cell fate. Solid lines with arrowheads indicate transcriptional regulation with positive effect while blunt-ended lines indicate a negative regulation. The dashed lines indicate movements. Kang et al. 2009

The Myb proteins CAPRICE (CPC) and TRIPTYCHON (TRY) were shown to be positive regulators of hair cell fate since mutations produced a “bald” root phenotype (Wada et al. 1997; Kirik et al. 2004; Simon et al. 2007). Probably, SCM inhibits WER transcription in the H cells (Fig. 1; Kwak and Schiefelbein, 2007; 2008). In the consequence, high WER levels result in the non-hair cell fate (Lee and Schiefelbein, 2002; Kwak and Schiefelbein, 2007). It has been discussed that WER, GL3, EGL3, and TTG1 act as a complex together to induce GL2 as well as CPC and TRY expression in the N cells (Fig. 1; Lee and Schiefelbein, 2002; Wada et al. 2002; Schiefelbein, 2003; Kirik et al. 2004; Ryu et al. 2005). The GL2 protein promotes then the non-hair cell fate by negatively regulating root-hair specific genes and is obviously the farthest downstream gene to regulate cell fate (Masucci et al. 1996; Lee and Schiefelbein, 1999; 2002). Targets of GL2 are the genes ROOT HAIR DEFECTIVE (RHD6) required for root hair initiation (Masucci and Schiefelbein, 1994; Masucci et al. 1996),

phospholipase D1 (PLD1) involved in root hair outgrowth and elongation (Ohashi et al. 2003), a cellulose synthase (CESA5) and a xyloglucan endotransglucosylase (XTH) both involved in cell wall synthesis during root hair development (Tominaga-Wada et al. 2009). These genes are either positively or negatively regulated by GL2 containing all a conserved L1-box region in their promoter region (Ohashi et al. 2003; Tominaga-Wada et al. 2009). Transcripts of CPC and TRY are preferentially transcribed and translated in N cells and then the proteins move to the H cells inducing hair cell fate (Fig. 1). The interaction of CPC with EGL3 and GL3 could compete with WER for binding to these two bHLH proteins (Lee and Schiefelbein, 1999; Kirik et al. 2004; Ryu et al. 2005; Tominaga et al. 2007; Ishida et al. 2008). Probably an inhibiting complex of CPC, GL3 and TTG1 acts in H position to repress GL2 transcription and root hair formation is not longer suppressed (Fig. 1; Schiefelbein and Lee, 2006).

3.1.2. Root hair initiation

Root hair initiation is characterized by actin reorganization in the cytoplasma and changes in cell wall pH at the site of root hair emergence, the apical end of the trichoblasts (Emons and Derksen, 1986; Masucci and Schiefelbein, 1994; Bibikova et al. 1998). Local acidification throughout the initiation process facilitates cell wall loosening through the activity of expansins thereby inducing swelling formation by a turgor-driven mechanism (Cosgrove, 1997; Bibikova et al. 1998; Baluska et al. 2000). Expansins are small secreted proteins binding to cellulose and disrupting the noncovalent bonds between cellulose and polysaccharides (McQueen-Mason and Cosgrove, 1995). The gene ROOT HAIR DEFECTIVE1 (RHD1) restricts swelling size (Schiefelbein and Somerville, 1990; Parker et al. 2000). A localized cell wall loosening and bulge formation might additionally be influenced by the activity of xyloglucan endotransglycosylases (XTH) at the site of root hair initiation (Fry et al. 1992; Vissenberg et al. 2001). Xyloglucan as a structural polysaccharide crosslinked to cellulose microfibrils is cleaved and rejoined by XTHs thereby enabling both cell wall loosening and rearrangement (Fry et al. 1992; Nishitani and Tominaga, 1992; Vissenberg et

al. 2005). Other genes involved in root hair initiation are ROOT HAIRLESS (RHL1, RHL2, RHL3). Fewer root hairs were formed by mutations in these genes but these hairs elongate normally suggesting a function of these genes in root hair initiation (Schneider et al. 1997; Grierson et al. 2001).

3.1.3. Root hair tip growth

Root hair tip growth requires a directed delivery of cell wall and cell membrane material to the growing tip, followed by wall assembly and crosslinking (Carol and Dolan, 2001). Tip growth is characterized by influx of Ca^{2+} at the growing tip and a high cytosolic Ca^{2+} gradient controls direction of growth (Bibikova et al. 1997; Wymer et al. 1997). The involvement of Ca^{2+} channels is suggested and activation requires tip-localized reactive oxygen species (ROS) produced by an NADPH oxidase (RHD2; Foreman et al. 2003). ROS are concentrated at the elongating tip and are absent when growth ceases (Carol and Dolan, 2006). Furthermore, a small Rho guanosine triphosphatase (Rho GTPase; ROP2) was reported to be involved in tip growth due to activation of RHD2 (Jones et al. 2002). Moreover, ROP2 was detected at the future root hair initiation site proposing also a function in this process (Jones et al. 2002). Additionally, two other ROOT HAIR DEFECTIVE (RHD3, RHD4) genes are involved in correct elongation of root hairs (Schiefelbein and Somerville, 1990; Galway et al. 1997).

After root hair tip growth the cell wall expansion ceased and rigidification occurs by forming crosslinks between polysaccharides and protein components (Cosgrove, 1997). Peroxidases in the cell wall can oxidize various substrates in the cell wall thereby forming crosslinks (reviewed in Passardi et al. 2004). One key protein responsible for strengthening the cell wall is the family of extensins or hydroxyproline rich glycoprotein (HRGP). Crosslinks with themselves or other cell wall components leads to insolubilization and to an increase in tensile strength (Knox, 1995; Brady et al. 1998; Held et al. 2004).

3.1.4. Molecular mechanisms of root hair formation induced by Pi deficiency

The deficiency of Pi can result in longer and more root hairs even in atrichoblast position (Bates and Lynch, 1996; Lopez-Bucio et al. 2003; Müller and Schmidt, 2004). Starvation of Pi obviously affects root hair development after the epidermal pattern mechanism since meristematic characteristics of hair and non-hair cells were unaltered (Müller and Schmidt, 2004). Very recently, the bHLH transcription factor ROOT HAIR DEFECTIVE 6-LIKE4 (RSL4) has been identified controlling cell growth and size in root hairs. Furthermore, this transcription factor was regulated in response to low Pi availability and induces hair cell elongation (Yi et al. 2010).

Moreover, two other transcription factors triggering alterations in root hair growth during Pi deficiency (WRKY75, BHLH32) were identified (for review see Valdés-López and Hernández, 2008; Lin et al. 2009). The expression of WRKY75 is induced during Pi deficiency and acts as a positive regulator of many Pi starvation induced (PSI) genes. However, under sufficient Pi supply it is a negative regulator of lateral root development and root hair number (Devaiah et al. 2007). Likely, bHLH32 acts as a negative regulator of root hair formation, anthocyanin synthesis and PSI gene expression (Chen et al. 2007). Furthermore, bHLH32 can interact with transcription factors involved in root epidermal patterning resulting in the formation of tricho- and atrichoblasts (Chen et al. 2007; Schiefelbein et al. 2009).

However, a potential link of a Pi signal and the increased number and length of root hairs even in developmentally non-hair position is less understood.

3.2. Molecular mechanisms of Pi response

Many adaptive responses to Pi starvation are regulated at the transcriptional level (Raghothama, 1999; Raghothama and Karthikeyan, 2005). Transcriptional profiling in several plants has revealed genes with altered expression during Pi starvation (Hammond et al. 2003; Wasaki et al. 2003a; 2006; Wu et al. 2003; Misson et al. 2005; Jain et al. 2007; Morcuende et al. 2007; Müller et al. 2007; Tesfaye et al. 2007). Hammond et al. (2003; 2004) determined two transcriptional programs: they clustered early genes (induced within hours)

considered as an unspecific general stress response in comparison to late genes (after several days or show a gradual induction during Pi depletion) that determined a Pi specific response and affect morphological and physiological changes of the plant. However, the time interval characterizing early and late responses is not consistently used among different authors (Hammond et al. 2003; Wu et al. 2003; Misson et al. 2005). Early genes may be activated when external concentration of Pi is decreasing and later gene expression may be initiated by a reduced level of internal signal (Schachtman and Shin, 2007).

Several studies described *cis*-regulatory elements in the promoter of genes regulated after different time intervals. A *cis*-element is the binding site for transcription factors and conserved *cis*-elements give a hint for a common regulation (Lachtman, 1997). In *Arabidopsis* a *cis*-regulatory element was observed that is conserved in genes upregulated 4h after removal of Pi in shoots where no binding partner is up to now identified (Hammond et al. 2003). Moreover, Wu et al. (2003) found a *cis*-regulatory sequence in the promoter of genes upregulated after 48h and another element in genes downregulated 24h after starting Pi depletion in leaves. The best studied *cis*-regulatory element in the promoter region in a number of Pi inducible genes is P1BS (PHR1 binding site: GNATATNC; Rubio et al. 2001). The binding partner for P1BS is PHOSPHATE STARVATION RESPONSE1 (PHR1) encoding for a Myb-type transcription factor that seems to play a role in a global regulation of Pi responsive genes. However, although PHR1 is a positive regulator of Pi starvation responses, the formation of root hairs seems to be independent of PHR1 in *Arabidopsis*, suggesting several Pi starvation induced pathways (Martin et al. 2000; Rubio et al. 2001). The PHR1 transcript itself is present in the nucleus irrespective of the Pi status of the plant suggesting a posttranslational regulation or the requirement of another Pi responsive regulatory factor for proper Pi starvation response (Franco-Zorilla et al. 2004; Nilsson et al. 2007). A candidate for posttranslational modifications of PHR1 is AtSIZ1, a small ubiquitin-like modifier (SUMO) E3 ligase upregulated by Pi depletion (Miura et al. 2005). Sumoylation regulates protein-protein or protein-DNA interactions and protects proteins against ubiquitin-mediated degradation (Yuan and Liu, 2008). Since PHR1 has a sumoylation site it could

therefore be a target for the regulation by AtSIZ1 (Miura et al. 2005). However, a dual function was suggested since AtSIZ1 may act as a positive regulator of Pi starvation responses by sumoylating PHR1 or as a negative regulator in other aspects of Pi response including lateral root and root hair formation or anthocyanin accumulation independent of PHR1 (Miura et al. 2005). Genes induced by PHR1 are phosphatases, RNAses, genes involved in anthocyanin and organic acid synthesis, Pi transport as well as genes involved in Pi homeostasis (Rubio et al. 2001).

Genes with function in Pi homeostasis include non-protein encoding RNAs, microRNAs (miRNA) and members of the IPS1 (INDUCED BY PHOSPHATE STARVATION1) family with homologues in different plant families (Burleigh and Harrison, 1997; 1998; 1999; Liu et al. 1997; Martin et al. 2000; Wasaki et al 2003b). A target for the Pi deficiency induced microRNA miR399 is PHO2, an ubiquitin conjugating E2 enzyme negatively regulating Pi transporters (Fuji et al. 2005; Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Doerner, 2008). Members of the IPS1 family have one sharing 22-24 nt-long-motif with striking sequence complementary to miR399 (Burleigh and Harrison, 1999; Martin et al. 2000; Jones-Rhoades et al. 2006; Mallory and Vaucheret, 2006; Shin et al. 2006a). The function of IPS1 members is not fully known, but the partial complementary allows an inhibition of the effect of miR399 on cleaving PHO2 mRNA by binding of IPS1 through a mechanism called target mimicry (Franco-Zorilla et al. 2007). The fine tuning mechanism between miR399 and IPS1 offers a possibility to regulate Pi starvation response and homeostasis.

Taken together, Pi signal transduction could be regulated at different levels; the level of gene expression, posttranscriptional modifications, the level of proteins or even posttranslational adjustments by several regulatory molecules.

4. Hypotheses

The efficiency of Pi was attributed in several plants to the formation of longer root hairs in more Pi efficient genotypes. Little is known about the molecular processes and signals underlying the formation of root hairs in response to Pi deficiency. The present study aimed

at the identification and characterization of genes regulating root hair growth induced by Pi starvation. *Brassica carinata* cultivars Bale and Bacho differing in root hair length during Pi deficiency served for the genetic background. Two different approaches were used for the identification of candidate genes: first, Suppression Subtractive Hybridization (SSH) enriched differentially expressed sequences in both cultivars during Pi deprivation as well as in the Pi efficient cultivar Bale comparing sufficient and deficient Pi supply. Second, a whole genome microarray analysis was reasonable since *B. carinata* belongs to the same family as the fully sequenced *Arabidopsis thaliana*. The candidate genes were further characterized for a potential function in root hair development by evaluating expression patterns in response to changes in nutrient supply, in different organs and along the root axis.

The hypotheses developed as a consequence were

- Suppression Subtractive Hybridization (SSH) reveals differentially expressed genes in cultivars Bale and Bacho during Pi starvation contributing to the formation of longer root hairs
- Identification of differentially expressed genes in the Pi efficient cultivar Bale comparing Pi deficient and sufficient conditions by SSH presents genes with a function in root hair development and/ or Pi signaling
- Microarray analysis confirms candidate genes identified by SSH and determines other genes eventually missed by SSH
- Both Pi and N deficiency enhance root hair growth and a common regulation of candidate genes hints at a function in root hair formation
- Changes in gene expression correspond temporally to changes in root hair growth
- Gene expression in different sections along the root predicts a function of candidate genes in the particular stage of root hair development

CHAPTER I

Identification and characterization of genes involved in Pi starvation induced root hair growth in *Brassica carinata* cultivars

Abstract

Different phosphorus efficiency in Ethiopian mustard (*Brassica carinata)* cultivars was attributed to longer root hairs in the Pi efficient cultivar. Transcriptomic analysis using an SSH approach was performed to reveal differentially expressed genes in the Pi efficient cv. Bale and the Pi inefficient cv. Bacho during Pi deficiency. Genes validated as upregulated in cv. Bale were LTP1 (lipid transfer protein), XTR (xyloglucan endotransglucosylase related), KUP (potassium uptake permease) and 14-3-3 while CHI (chitinase), HRGP (hydroxyproline rich glycoprotein) and LTP2 (lipid transfer protein) could be confirmed as upregulated in cv. Bacho by semi-quantitative RT-PCR. Root hair growth and gene expression were investigated in both cultivars in response to Pi, N and K starvation, by changing Pi and N supply. Furthermore, transcript level was examined along the root.

In Bale root hair length was enhanced besides Pi during N starvation and this phenotype was observed 4h after removing Pi and N while readdition of Pi and N resulted in reduced root hair length after 2h and 6.5h, respectively. In Bale gene activity of the putative cell wall protein HRGP was downregulated by Pi and N depletion and upregulated by Pi and N resupply corresponding to an enhanced and reduced root hair length. Further, no change in transcription level was observed in the Pi inefficient cv. Bacho with shorter root hairs and transcription was higher in mature root parts in cv. Bale. Therefore, HRGP could contribute to rigidification of the cell wall negatively regulating root hair growth. In Bale, an enhanced expression in response to Pi depletion was found for XTR, 14-3-3 and LTP2 but no effect of N deprivation. Furthermore, the temporal expression was delayed in response to Pi depletion compared to the formation of longer root hairs indicating no function in root hair growth. However, a role in general root growth was suggested since expression was highest in the root tips and declined to older parts. The activity of CHI was downregulated under Pi and N starvation and was more pronounced in mature root parts proposing a negative role in Pi and N signaling. However, a physiological role in root hair formation remains elusive. For LTP1 and KUP a function in -Pi induced root hair growth could be ruled out since no impact on altered Pi and N supply was obvious in the Pi efficient Bale.

Keywords: root hair, Pi starvation, Pi efficiency, hydroxyproline rich glycoprotein, SSH

1. Introduction

Phosphorus (Pi) is one of the essential macronutrients required for plant growth and development. Although Pi is relatively abundant in many soils, low Pi availability is one of the major factors limiting plant productivity (Wissuwa and Ae, 1999; Vance et al. 2003). Consequently, plants cope with Pi limitation with morphological and physiological adaptations to enhance Pi availability in the soil and to increase its uptake by roots (Raghothama, 1999; Vance et al. 2003). One morphological response is the formation of longer root hairs to explore a greater soil volume (Bates and Lynch, 1996; Williamson et al. 2001; López-Bucio et al. 2002). Changes in root hair length were associated with Pi uptake efficiency in Ethiopian mustard (Eticha and Schenk, 2001), barley (Wissuwa and Ae, 2001) and peanut (Gahoonia and Nielsen, 2004). Little is known about the processes and signals regulating the architecture of roots during Pi deficiency. In contrast, regulation of physiological adaptation mechanisms to low Pi environment could be related to differential gene expression (Hammond et al. 2003; Wasaki et al. 2003a; 2006; Wu et al. 2003; Misson et al. 2005; Morcuende et al. 2007). Some transcription factors were identified as positive or negative regulators of the Pi starvation response in higher plants (reviewed by Lin et al. 2009). The *Arabidopsis* gene PHR1 (PHOSPHATE STARVATION RESPONSE1) encoding for a Myb transcription factor was first identified as a positive mediator of Pi starvation responses and is required for the induction of a number of Pi responsive genes (Rubio et al. 2001). PHR1 acts through binding to the P1BS (PHR1 specific binding sequence) element (GNATATNC) existing in the promoter of many Pi starvation induced (PSI) genes (Rubio et al. 2001; Franco-Zorrilla et al. 2004). However, PHR1 did not affect the number and length of root hairs under Pi depletion (Rubio et al. 2001; Nilsson et al. 2007) whereas overexpression of PHR1 homologues in rice resulted in enhanced root elongation and enhanced root hair growth independent of the Pi status (Zhou et al. 2008). PHR1 could be post-transcriptionally modified by a small ubiquitin-like modifier (SUMO) E3 ligase, AtSIZ1, thereby affecting positively some Pi dependent responses together with PHR1. In contrast, it was suggested

that AtSIZ1 negatively regulates root hair number and length as well as anthocyanin accumulation independent from PHR1 (Miura et al. 2005). Furthermore, a microRNA (miR399) was specifically induced by Pi deprivation and was shown to affect the transport of Pi within the plant but root architecture seems not affected (Aung et al. 2006; Bari et al. 2006). Instead, some transcription factors were identified as new components of the Pi signaling pathway affecting negatively root hair formation. BHLH32 repressed a range of Pi starvation induced processes including anthocyanin synthesis, total Pi content and root hair length and was able to interact with transcription factors involved in determining the pattern of trichoblasts and atrichoblasts in roots of *Arabidopsis* (Chen et al. 2007; Schiefelbein et al. 2009). Furthermore, the WRKY75 transcription factor was induced during Pi deprivation but was shown to regulate negatively anthocyanin synthesis, lateral root formation and root hair number (Devaiah et al. 2007). Moreover, the inositol polyphosphate kinase AtIPK1 functioned in regulating root hair development irrespective of Pi nutrition (Stevenson-Paulik et al. 2005). The molecular function of these genes is still unknown but the observations suggest that many independent signaling pathways exist in regulating Pi response.

In this study, two Ethiopian mustard (*Brassica carinata*) genotypes differently responding in root hair length under Pi deficiency were used for a SSH approach to identify genes involved in the Pi signal transduction pathway. In addition, SSH derived genes with a putative function in -Pi induced root hair development were further characterized by their nutrient specific and spatio-temporal expression profile along the root.

2. Materials and Methods

2.1. Plant material and cultivation

Seeds of *Brassica carinata* were vernalized in the dark for three days at 4°C and then germinated for 5 days in the growth chamber with 16h light (20°C)/8h dark (15°C) photoperiod, a photosynthetically active radiation of 200 µmol photons $m^{-2}s^{-1}$ and 70% relative humidity. Uniformly germinated seedlings were transferred to a continuously aerated nutrient solution containing 1 mM Pi as KH_2PO_4 or free of Pi. The other nutrient composition in the solution was (in mM): 2.25 $Ca(NO_3)_2$; 2.5 K_2SO_4; 1 $MgSO_4$; 0.25 KCl and (in µM) 25 H_3BO_3; 1.5 $MnSO_4$; 1.5 $ZnSO_4$; 0.5 $CuSO_4$; 0.025 $(NH_4)_6Mo_7O_{24}$ and 35.8 Fe (Fe^{III}- EDTA) and pH 5.5. After five days in nutrient solution lateral root tips of 2 cm length, stems and leaves of six plants were harvested 2h after the light period started (for reduction of possibly occurrence of diurnal rhythmic), frozen immediately in liquid nitrogen and stored at -80°C before use. Seedlings were also transferred to nutrient solution lacking nitrogen by substituting $Ca(NO_3)_2$ by $CaCl_2$ or potassium by omitting K_2SO_4 and KCl and substituting KH_2PO_4 by NaH_2PO_4. The transfer experiment was initiated by transferring 10-days-old seedlings grown 5 days in complete nutrient solution to Pi or N free solution and vice versa. Some plants remained in the original solution as control (0, 24h). Root tips of 1.5 cm length were harvested after 0.5, 1, 2, 4, 8, 24, 48, 72, 96h for RNA isolation. Additionally, 1 cm root sections (0-1 cm, 1-2 cm, 2-3 cm, Fig. 1) beginning from the root tip were harvested from plants grown in +Pi and -Pi after five days in nutrient solution. Pictures of root phenotype were taken with the AxioCam MRc (Zeiss) under the Axioskop (Zeiss) after staining with 1% acid fuchsin in A. dest.

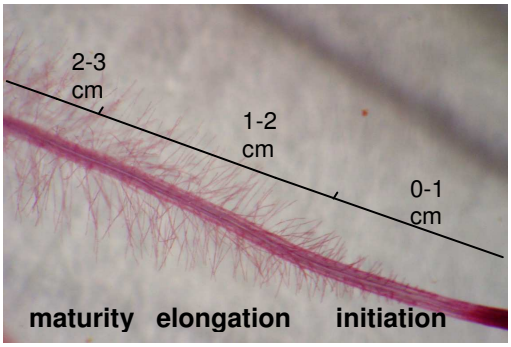


Fig. 1 Sections of 1 cm length were harvested to characterize the gene expression pattern along the root hair differentiation stages

2.2. Suppression Subtractive Hybridization (SSH)

Total RNA was isolated from frozen root tips of *B. carinata* plants cv. Bale and Bacho subjected 5 days to Pi deficient (-Pi) nutrient solution using the NucleoSpin RNA Plant kit (Macherey & Nagel, Düren, Germany) following the manufacturer's instructions. The Super SMART PCR cDNA synthesis kit (BD Biosciences Clontech Palo Alto, CA) was then used to generate tester and driver double-stranded cDNA from 1 µg total RNA according to the manufacturer's protocol. Two subtractive cDNA libraries were generated: in the forward run of SSH cDNA of *Brassica carinata* cv. Bale grown under -Pi served as tester and cDNA of cultivar Bacho grown under -Pi as driver, and in a second reverse run *B. carinata* cv. Bacho -Pi was the tester and Bale -Pi the driver. SSH was performed using the Clontech PCR-select cDNA subtraction kit (BD Clontech) following the manufacturer's instructions. After reverse transcription of tester and driver, the cDNA populations were digested with RsaI (10 U/µl) and then the tester cDNAs were divided into two portions, each ligated with a different adaptor. Two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed sequences. The subtracted PCR amplified cDNAs were cloned directly into the T/A cloning vector pCR2.1 TOPO using TOPO TA cloning kit (Invitrogen, USA) and then transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, USA), producing the resultant subtractive cDNA library. Transformed cells were plated onto LB agar medium containing 50 µg/ml kanamycin and 40 µg/ml X-Gal, and incubated at 37℃

overnight. White colonies (putative positive clones) were obtained the next day, and each colony was picked individually and checked for presence and size of individual inserts by colony PCR using flanking M13 forward and reverse primers (forward 5´-GTAAAACGACGGCCAG-3´ reverse 5´-CAGGAAACAGCTATGAC-3´). The final reaction volume of 25 µl contained 20.54 µl distilled water, 2.5 µl 10x buffer, 0.5 µl dNTP mix (10 mM each, Roth), 0.63 µl each of M13 primers (10 pm/µl), 0.2 µl SupraTherm™ Taq DNA polymerase (5 U/µl, GeneCraft, Köln, Germany) and a tip bacterial culture. The PCR was carried out using the following cycling conditions: 3 min at 94°C, and then 33 cylces of 15 sec at 94°C, 30 sec at 56°C and 1 min at 70°C. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized by UV fluorescence (312 nm; INTAS Gel iX Imager, Göttingen). Positive clones were grown overnight in 3 ml liquid LB medium with 50 µg/ml kanamycin. The clones were stored as glycerol stocks at -80°C.

2.3. Sequence analysis

Clones were sent for sequencing in 0.8% LB agar medium supplemented with 50 µg/ml kanamycin (IIT Biotech, Bielefeld, Germany). The obtained expressed sequence tag (EST) sequences were compared by sequence similarity searches to the GenBank database using BLASTX and BLASTN of the *Arabidopsis* information resource TAIR (www.arabidopsis.org/wuBLAST/index2.jsp) to identify their putative functions. The cDNAs were named according to homologous sequences in the database. Functional classification of ESTs was performed according to the annotations in the databases. The sequences of ESTs identified in this work were deposited in supplementary material.

2.4. Expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from stems, leaves or lateral root tips using NucleoSpin RNA Plant kit (Macherey & Nagel). RNA quality was checked by gel electrophoresis and then quantified with the Nanophotometer (IMPLEN, München, Germany). For expression analysis by semi-quantitative RT-PCR, 1 µg total RNA of each sample was reverse transcribed with oligo (dT)-primers using the RevertAid [TM] H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and was performed at 42°C for 60 min with a final denaturation at 70°C for 10 min. Afterwards RNA was removed by RNAse H (10 U/µl, Epicentre, Madison, WI, USA) treatment for 20 min at 37°C. Concentration and purity of cDNA was determined with the Nanophotometer (IMPLEN). Aliquots containing 800 ng of first strand cDNA were used as a template in a 50 µl PCR reaction containing 40.7 µl distilled water, 5 µl 10x buffer, 1 µl dNTP mix (10 mM each), 1 µl each of primer (reverse and forward) and 0.3 µl SupraTherm™ Taq DNA Polymerase (Genecraft). PCR was carried out in the presence of gene-specific primers designed by Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) based on the sequenced ESTs of *B. carinata* identified in the SSH library. After initial denaturation at 94°C for 3 min, PCR was carried out for different number of cycles of 94°C for 15 sec, 52°C-56°C for 30 sec depending on Tm of primers and 70°C for 1 min. To monitor equal amounts of cDNA, the constitutively expressed actin gene was amplified simultaneously (forward 5´-AGGATATTCAGCCACTTGTCTGTG-3´ reverse 5´-AGAAACATTTCCTGTGAACAATCG-3´). Samples were removed after a diverse number of cycles and amplification state was monitored on a 1.2% agarose gel stained with ethidium bromide. The primer sequences and predicted amplicon sizes are given in table 1:

Table 1: used RT-PCR primer sequences derived from sequenced SSH clones

EST clone	Sequence (5´- 3´) forward reverse	Tm	Product size (bp)
BcBal-1	GCCGTATAAATCCGGCATAC TGCCGATACACACACAAACC	68 68	238
BcBal-2	TCACGCAAACACGGAGAATA ACCCAGAAGATGACCCCTTT	66 68	116
BcBal-3	TGCGTGTGTTATCGCATCTC GAGGTTCCCTCAGGGTTTTG	68 70	196
BcBal-4	GCAGAAAACAACCTGGAAGC TCACAAAGGGAAGCAAGGTC	68 68	192
BcBal-6	GTCGCGGCTGTAGAATTGAT GCGGGGATACAAGAAACATC	68 68	122
BcBal-8	GTGGTGCCAAGAAGAGGAAG ACCAATATTCCGATGCCAAT	60 54	321
BcBal-17	TTCATGATGTCGTCGAAGGA CTCTGCCAAAACAAGCCTTT	56 56	241
BcBal-19	TTTGGCTGAAGAGACCTGTG TCCACACGAATGACCAGGTA	68 68	182
BcBal-31	CACTCTCTGGACATCCGACAT CTTCAACATGGAAAGAAACAAAGA	71 67	153
BcBal-32	GGAAGTTCACCTCGATCCAA CGACAGAGCTCGGAGGTAAG	68 72	196
BcBal-35	GAGAAAACAGCAACAAGAGAATCA GGGCTTGTGAAGAAGGACAA	69 68	168
BcBal-40	GCTGGAATCGCCCTTAGC TATTCAACTTAGGCGCATGG	69 66	154
BcBac-2	TCTCCACCTCCTCCATACCA CATGCATGAAGGGACAACTC	70 68	244
BcBac-4	ACATCGCACCATCTTTTGGT TCAGATGGCTTCGAGAGCTT	66 68	188
BcBac-10	GGACAAAGCAAACTTGCACA GATGGAAACCGTTATTGGTGA	66 67	246
BcBac-14	ATTTGGGGCCACCATTAGAG AGAGACGACCGTCCAACAAT	68 68	179
BcBac-24	AACACGGGCATCAACAATCT GAACGATTCCTTGGACCTGA	66 68	222
BcBac-28	AGGAAGATCCCTCGTGGATT TTATGCATGGGCACACTTTT	68 64	195
BcBac-32	TGACATTGACGGATGGATGT CGGAATCACCATTCTCCATT	66 66	201

3. Results

3.1. Phenotypic characterization of B. carinata cultivars under Pi deficiency

Previous work revealed a Pi efficient cultivar Bale and an inefficient cultivar Bacho. Under Pi starvation the Pi efficient cultivar Bale had longer root hairs in both soil and nutrient solution (Eticha and Schenk, 2001). In this work, *Brassica carinata* cv. Bale and cv. Bacho seedlings were grown in complete nutrient solution and under -Pi conditions. As previously observed under Pi starvation root hairs of *B. carinata* cv. Bale were significantly longer and denser compared to cv. Bacho after 5 days (Fig. 2). At this time differential gene expression analysis by constructing an SSH library was performed.

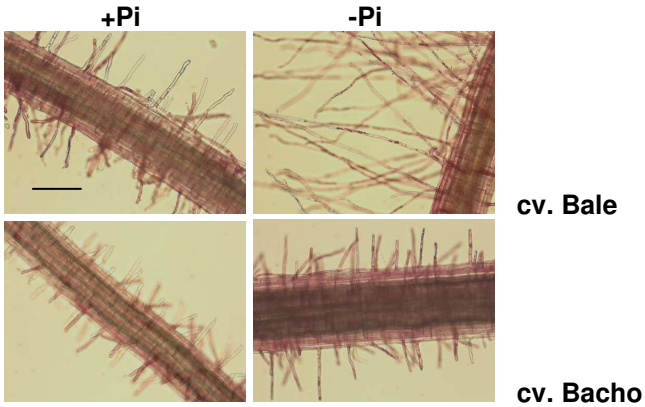


Fig. 2 Root hair phenotype under Pi deficiency of *Brassica carinata* cv. Bale (top panel) and Bacho (bottom panel). Bar = 300 µm

3.2. Identification of differential expression of Pi responsive genes by SSH

In order to understand the molecular mechanisms leading to increased root hair length under low Pi availability in Ethiopian mustard, an SSH library was constructed to identify Pi starvation responsive genes. Each pool of reverse transcribed cDNA was used as tester and driver in two different runs of SSH for the identification of up and downregulated genes. A total of 168 (upregulated in Bale) and 218 (upregulated in Bacho) bacterial colonies were picked up from the SSH library and analyzed for presence and size of the insert by colony PCR. The cDNA inserts varied in size from 250 bp to 750 bp with a clear cumulation around

500 bp. The gene inserts were sequenced and the obtained expressed sequence tags (ESTs) were classified according to their annotated function indicated by database analysis into 5 functional categories: metabolism, signal transduction, stress or defence response and cell wall (Table 2, 3). Some clones were similar to proteins with unknown function or without significant homology to known proteins and were grouped into the category miscellaneous. The largest group of genes with known function upregulated in Bale contained homologies to genes in *Arabidopsis* involved in the general metabolism (19), followed by the group of stress and defence responsive genes (11). We also identified 5 clones involved in signal transduction and 4 genes that have a function in cell wall assembly. Only one clone could not be assigned to a known function. On the other hand, the identified ESTs upregulated in the Pi inefficient cultivar Bacho were classified in the general metabolism (17) followed by stress and defence response (12). The group of ESTs involved in cell wall assembly contained 5 genes and 4 clones were categorized in signal transduction. However, in this reverse SSH run the class of unknown proteins (11) was larger compared to the result of the forward run identifying upregulated genes with a putative function to increase Pi efficiency.

Table 2 Identification of upregulated cDNA sequences during Pi starvation in the Pi efficient *B. carinata* cv. Bale compared to cv. Bacho

Clone	putative gene identity	Homologues of *Brassica* ESTs in *Arabidopsis*	E-value	length (bp)
Metabolism				
BcBal-2	Methylentetrahydrofolate-hydrolase (MTHFR1)	At3g59970	2.1e-19	198
BcBal-4	inorganic Diphosphatase	At1g01050	1.4e-103	587
BcBal-6	vacuolar ATP synthase subunit B	At1g20260	7.6e-54	425
BcBal-7	Nudix hydrolase homolog 16	At3g12600	9.5e-56	469
BcBal-9	caffeoyl-CoA 3-O-methyltransferase	At4g34050	7.7e-23	205
BcBal-12	SEC14 cytosolic factor family protein	At1g14820	3.5e-34	274
BcBal-13	aminotransferase class I and II family protein	At2g22250	2.7e-09	169
BcBal-17	K+ transporter/ KUP	At4g33530	1.9e-19	239
BcBal-18	xylem cystein peptidase	At4g35350	3.0e-45	379
BcBal-21	tonoplast intrinsic protein	At5g47450	1.2e-08	101
BcBal-22	Embryo defective protein	At2g03150	8.0e-52	413
BcBal-24	Ubiquinol-Cytochrome C Reductase	At3g52730	1.4e-19	237
BcBal-25	ABC transporter family protein	At5g06530	7.4e-02	131
BcBal-26	Calcineurin B-like protein (AtCBL)	At4g26570	3.1e-18	168
BcBal-27	cytochrome c oxidase-related	At4g37830	1.8e-16	189

BcBal-28	Alpha-tubulin	At4g14960	2.4e-10	204
BcBal-29	phospholipase A2-alpha	At2g06925	6.4e-03	141
BcBal-30	Histone H3.2 (HTR8)	At4g40040	3.3e-09	340
BcBal-37	ATPase III subunit	AtCg00140	8.5e-13	80
Signal transduction				
BcBal-8	ubiquitin extension protein	At1g23410	3.0e-52	436
BcBal-15	zinc finger protein (C3HC4-type RING finger)	At1g63840	4.3e-12	257
BcBal-19	myb transcription factor	At1g74840	1.4e-14	214
BcBal-31	14-3-3 protein (GRF2)	At1g78300	2.8e-12	210
BcBal-35	eukaryotic translation initiation factor SUI1	At4g27130	2.1e-09	217
Stress or defence response				
BcBal-1	non specific lipid transfer protein (nsLTP) (AIR1)	At4g12550	4.3e-16	422
BcBal-5	heat stable protein 1 (HS1)	At3g17210	4.3e-13	247
BcBal-11	hydrophobic protein (RCI2B)	At3g05890	9.8e-29	229
BcBal-14	cytosolic thioredoxin H-type 3	At5g42980	2.1e-12	207
BcBal-20	Bet v I allergen family protein	At1g70880	4.0e-24	398
BcBal-23	phosphatase	At1g73010	8.9e-15	247
BcBal-32	Exordium-like protein 2 (EXL4)	At5g64260	7.6e-46	433
BcBal-34	CBL-interacting protein kinase 3	At2g26980	1.7e-10	160
BcBal-36	DNA damage repair protein (DRT100)	At3g12610	1.3e-05	80
BcBal-38	stress responsive protein	At4g37220	3.5e-42	247
BcBal-40	non specific lipid transfer protein (nsLTP)	At4g12520	1.6e-19	284
Cell wall				
BcBal-3	xyloglucan endotransglycosylase (XTR9)	At4g25820	8.9e-12	243
BcBal-10	proline rich extensin protein	At1g23720	4.0e-19	237
BcBal-16	arabinogalactan-protein (AGP3)	At4g40090	1.4e-14	213
BcBal-39	arabinogalactan-protein (AGP9)	At2g14890	1.7e-31	417
Miscellaneous				
BcBal-33	integral membrane yip1 family protein	At4g30260	2.9e-10	127

Table 3 Identification of upregulated cDNA sequences during Pi starvation in the Pi inefficient *B. carinata* cv. Bacho compared to cv. Bale

Clone	putative gene identity	Homologues of *Brassica* ESTs in *Arabidopsis*	E-value	length (bp)
Metabolism				
BcBac-4	cystathionine gamma-synthase	At3g01120	1.0e-40	294
BcBac-7	ADP, ATP carrier protein (ANT1)	At5g13490	6.0e-04	101
BcBac-8	atpF ATPase I	AtCg00130	5.0e-26	158
BcBac-9	tetratricopeptide repeat (TPR)-containing protein	At3g16760	9.3e-21	136
BcBac-16	aspartate-aminotransferase isoenzyme (Asp3)	At5g11520	7.9e-20	218
BcBac-17	Enolase	At2g36530	2.0e-15	267
BcBac-22	vesicle-associated membrane protein (VAMP)	At3g60600	1.2e-10	209
BcBac-23	caseinolytic protease	AtCg00670	1.1e-07	478
BcBac-25	DERLIN-2.2	At4g04860	2.0e-17	110
BcBac-30	nicotianamine synthase	At5g04950	8.0e-19	262
BcBac-38	calcium-binding EF hand family protein	At5g28900	7.8e-08	225
BcBac-39	ATPase III subunit	AtCg00140	2.8e-16	99

BcBac-42	protein phosphatase 2C family protein	At4g38520	4.6e-02	311
BcBac-45	NAD(P)H dehydrogenase subunit H protein	AtCg01110	1.4e-47	285
BcBac-47	mitochondrial ATP Synthase D Chain	At3g52300	1.9e-22	215
BcBac-48	AtNADP-ME4 malic enzyme	At1g79750	3.5e-09	258
BcBac-49	AtRBL2, rhomboid protein	At1g63120	4.7e-24	242
Signal transduction				
BcBac-13	ubiquitin-specific protease (UBP14)	At3g20630	1.2e-21	165
BcBac-21	Leucine rich repeat protein	At1g25570	2.1e-19	315
BcBac-31	fibrillarin 2(FIB2)	At4g25630	2.1e-05	167
BcBac-46	ATHB30	At5g15210	2.3e-01	129
Stress or defence response				
BcBac-11	gluthathione S-transferase	At1g78370	2.1e-10	176
BcBac-14	class IV chitinase (CHI)	At3g47540	7.0e-20	224
BcBac-15	Beta glucosidase BGLU22	At1g66280	8.7e-54	395
BcBac-18	putative thioredoxin (ATH8)	At1g69880	6.1e-10	156
BcBac-26	ACCELERATED CELL DEATH 11	At2g34690	6.3e-11	250
BcBac-28	non specific lipid transfer protein (nsLTP)	At4g12520	1.9e-22	285
BcBac-32	1-aminocyclopropane-1-carboxylate oxidase (ACCoxi)	At2g19590	1.3e-37	291
BcBac-34	glyceraldehyde-3-phosphate dehydrogenase (GAPC-2)	At3g04120	5.6e-07	184
BcBac-37	peroxidase 50	At4g37520	6.5e-10	188
BcBac-40	flavin mononucleotide-binding flavodoxin-like quinone reductase	At5g54500	1.9e-19	234
BcBac-43	lipid transfer protein (LTP)	At4g12545	3.3e-16	303
BcBac-44	transaldolase-like protein	At5g13420	2.5e-41	247
Cell wall				
BcBac-2	hydroxyproline-rich glycoprotein (HRGP)	At1g76930	1.6e-46	530
BcBac-10	hydroxyproline-rich glycoprotein (HRGP)	At1g21310	1.6e-50	367
BcBac-24	actin-related protein 3 (ARP3)	At1g13180	1.7e-58	318
BcBac-33	proline-rich extensin protein	At1g26250	8.3e-16	224
BcBac-35	microtubule motor, kinesin motor protein-related	At5g23910	7.0e-15	220
Miscellaneous				
BcBac-1	unknown protein	At1g65845	1.1e-05	489
BcBac-3	expressed protein NuLL	At4g32020	7.0e-19	392
BcBac-5	senescence-associated protein	At2g44670	4.7e-07	196
BcBac-6	expressed protein	At5g40690	1.8e-01	134
BcBac-12	ycf1 hypothetical protein	AtCg01130	8.9e-84	636
BcBac-19	unknown protein	At5g61340	6.5e-27	184
BcBac-20	MD-2-related lipid recognition protein	At3g11780	5.4e-17	273
BcBac-27	jacalin lectin family protein	At2g39330	3.5e-05	269
BcBac-29	dentin sialophosphoprotein-related	At5g52530	1.4e-01	143
BcBac-36	unknown protein	At5g43830	1.7e-01	103
BcBac-41	unknown protein	At3g19200	2.0e-26	299

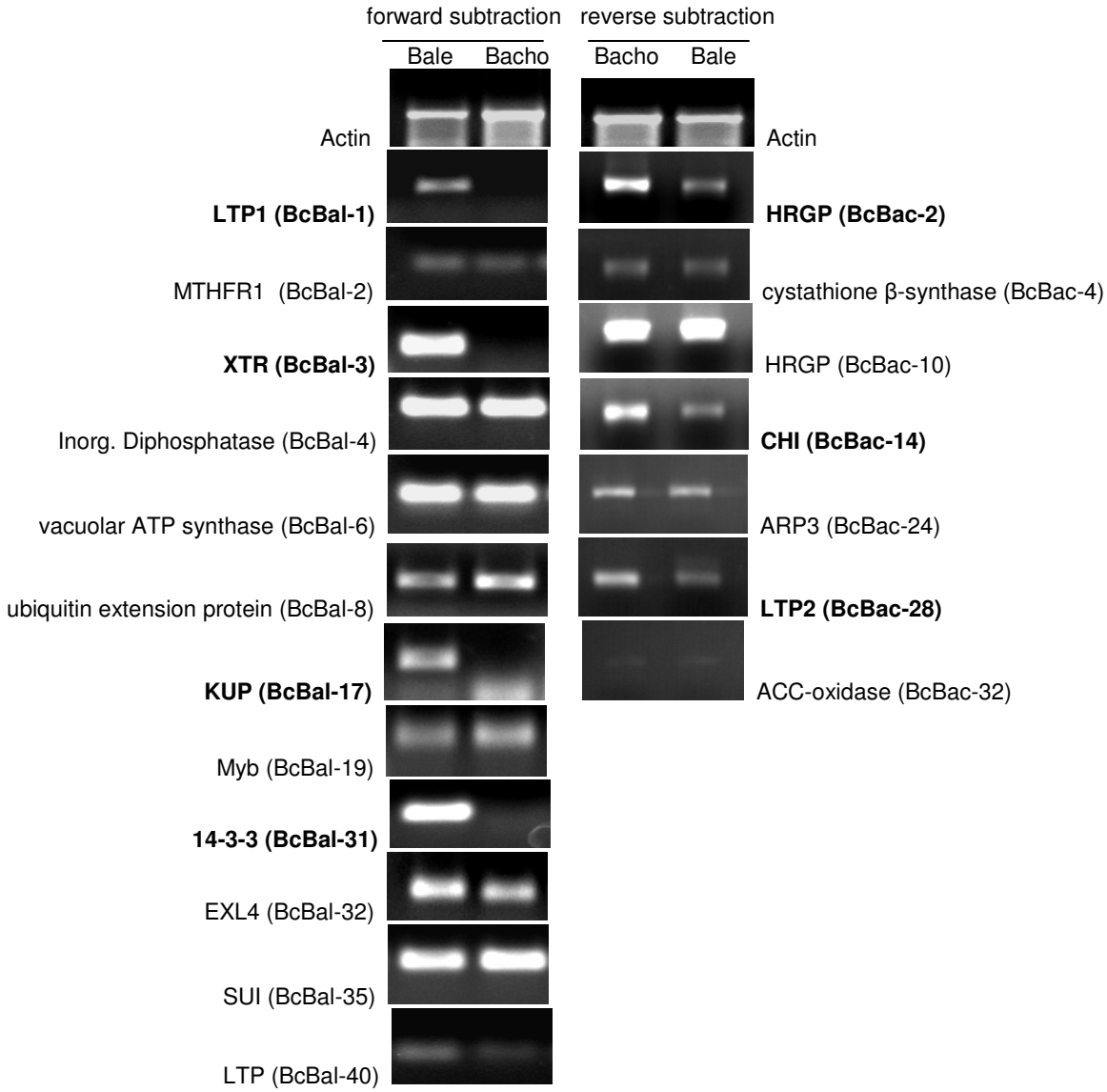


Fig. 3 Differential expression of genes in roots of *B. carinata* cv. Bale and cv. Bacho after 5 days Pi starvation resulting from SSH library as verified by semi-quantitative RT-PCR. Genes used for further analysis were indicated in bold letters. For abbreviations see Table 2 and 3

3.3. Validation of putative Pi responsive genes by semi-quantitative RT-PCR

Differential regulation of gene transcripts in roots of *Brassica carinata* varieties under low Pi supply obtained by SSH was further investigated by semi-quantitative RT-PCR. The candidate genes were chosen based on their putative function in root hair development or for reported response to Pi starvation. Of the genes identified in the forward subtraction, only four genes belonging to different functional groups were confirmed upregulated in cv. Bale (Fig. 3): LTP1 (lipid transfer protein), XTR (xyloglucan endotransglucosylase related), KUP

(K^+-uptake permease) and 14-3-3. In the SSH library originating from the reverse subtraction upregulation for three genes designated as HRGP (hydroxyproline rich glycoprotein), CHI (chitinase) and LTP2 (lipid transfer protein in the reverse run) in Bacho was confirmed. These genes were selected for further characterization. The semi-quantitative PCR resulted in no product for LTP1, XTR, KUP and 14-3-3 with Bacho cDNA as template. However, a PCR with genomic DNA of Bale and Bacho confirmed the annealing of primers designed from the sequenced ESTs from Bale and the existence of the corresponding genes in Bacho (Fig. 4). For all other genes semi-quantitative PCR showed the presence of a PCR product by using the designed primers in both cultivars.

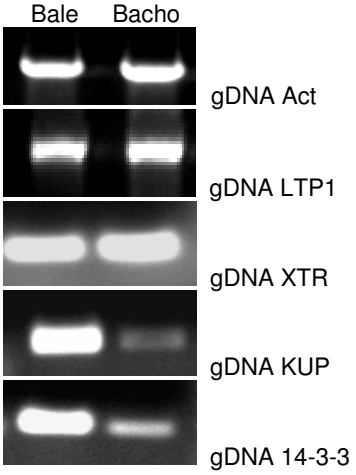


Fig. 4 Presence of LTP1, XTR, KUP and 14-3-3 in the genomic DNA of two *B. carinata* cultivars.

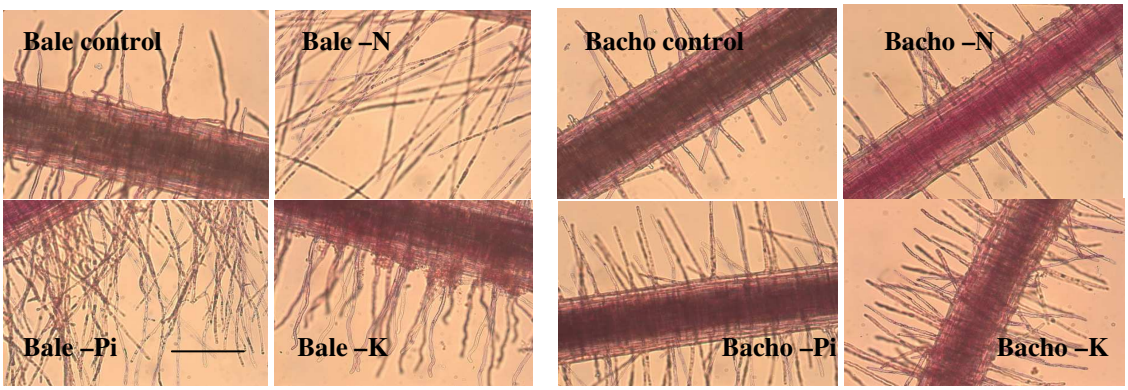
3.4. Transcriptional regulation of putative Pi responsive genes under nutritional stress

To study the effect of other nutritional stress factors on root hair phenotype and the transcription of candidate genes, cultivars Bale (Pi efficient) and Bacho (Pi inefficient) were subjected to phosphate (Pi), nitrogen (N) and potassium (K) starvation. The phenotypic analysis exhibited in Bale an increased root hair length under Pi and N deprivation whereas during K starvation a similar root hair phenotype as in control conditions was observed. The Pi inefficient cv. Bacho differed not in root hair length in all deficiency treatments (Fig. 5A).

Transcript level in the root was monitored for differentially expressed genes by semi-quantitative RT-PCR. Under Pi deficiency in Bale a slight increase of the transcript abundance was visible for LTP1, XTR, 14-3-3, and LTP2 while low Pi supply resulted in a downregulation of HRGP. The expression level of KUP and CHI was not affected. Upregulation in Pi starved Bacho plants was also observed for 14-3-3, KUP, CHI and LTP2 whereas Pi depletion was not effective for HRGP. LTP1 and XTR transcripts were not detected in all treatments (Fig. 5B). In Bale N starvation declined gene expression of LTP1, 14-3-3, CHI, LTP2 and HRGP compared to control conditions, weakly increased XTR transcript level or had no impact as for KUP. In Bacho N starvation decreased the transcript level of 14-3-3, CHI and LTP2 or was not effective in changing expression level of HRGP.

A slight induction during N limitation was visible for KUP. In Bale K starvation resulted in a downregulated expression of 14-3-3, KUP and LTP2 or had no effect on LTP1, XTR, CHI and HRGP expression. In Bacho K starvation led to a weak upregulation of KUP and a decline in expression level for CHI. All other genes were not affected. Pi and N starvation both induced the formation of longer root hairs in Bale, and XTR was upregulated whereas HRGP was downregulated in response to both nutrient stresses. In cv. Bacho where Pi and N depletion did not enhance root hair growth, expression level of XTR and HRGP were not affected by nutrient supply. All other candidate genes were regulated differentially by Pi and N starvation suggesting no link to root hair growth a common respond to Pi and N starvation. Therefore, XTR and HRGP could be involved in regulation of root hair growth.

A



B

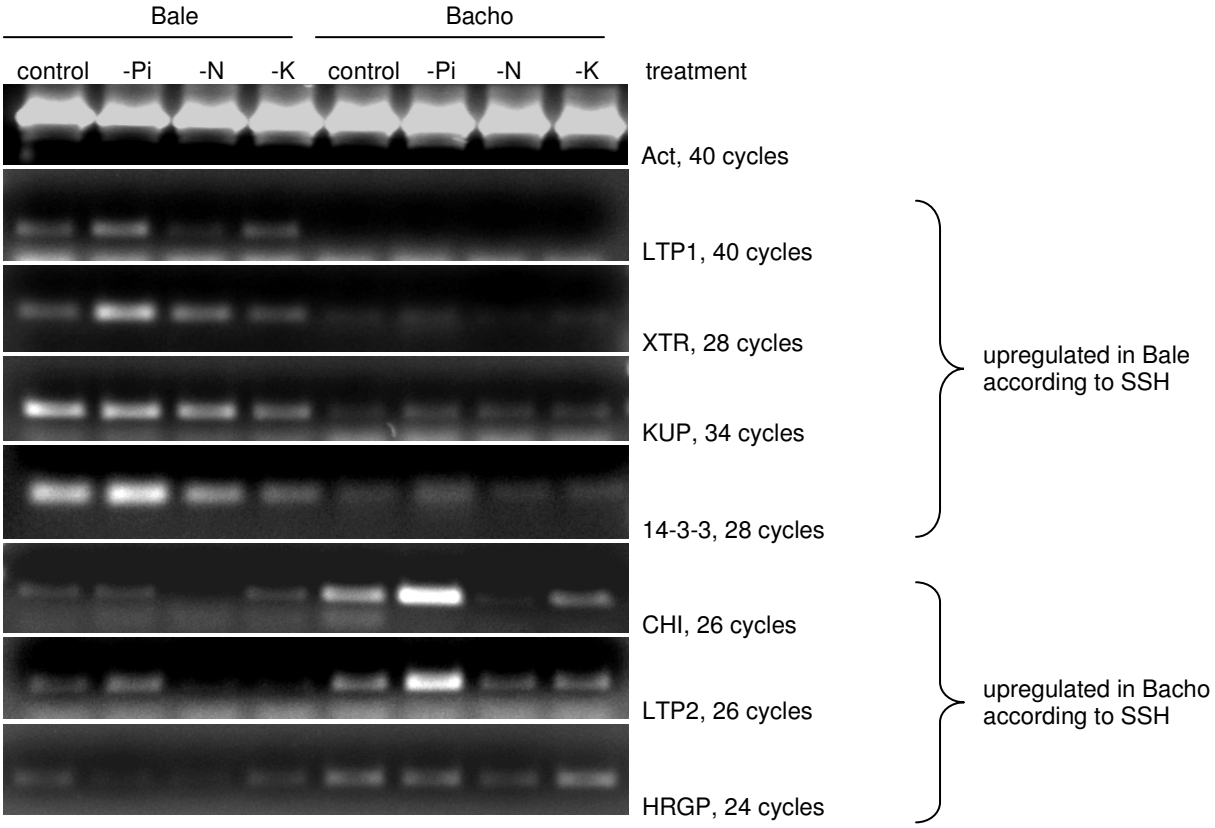


Fig. 5 Effect of five days starvation of Pi, N and K on (A) root hair phenotype and (B) differential gene expression in roots of *Brassica carinata*. Bar = 400 µm

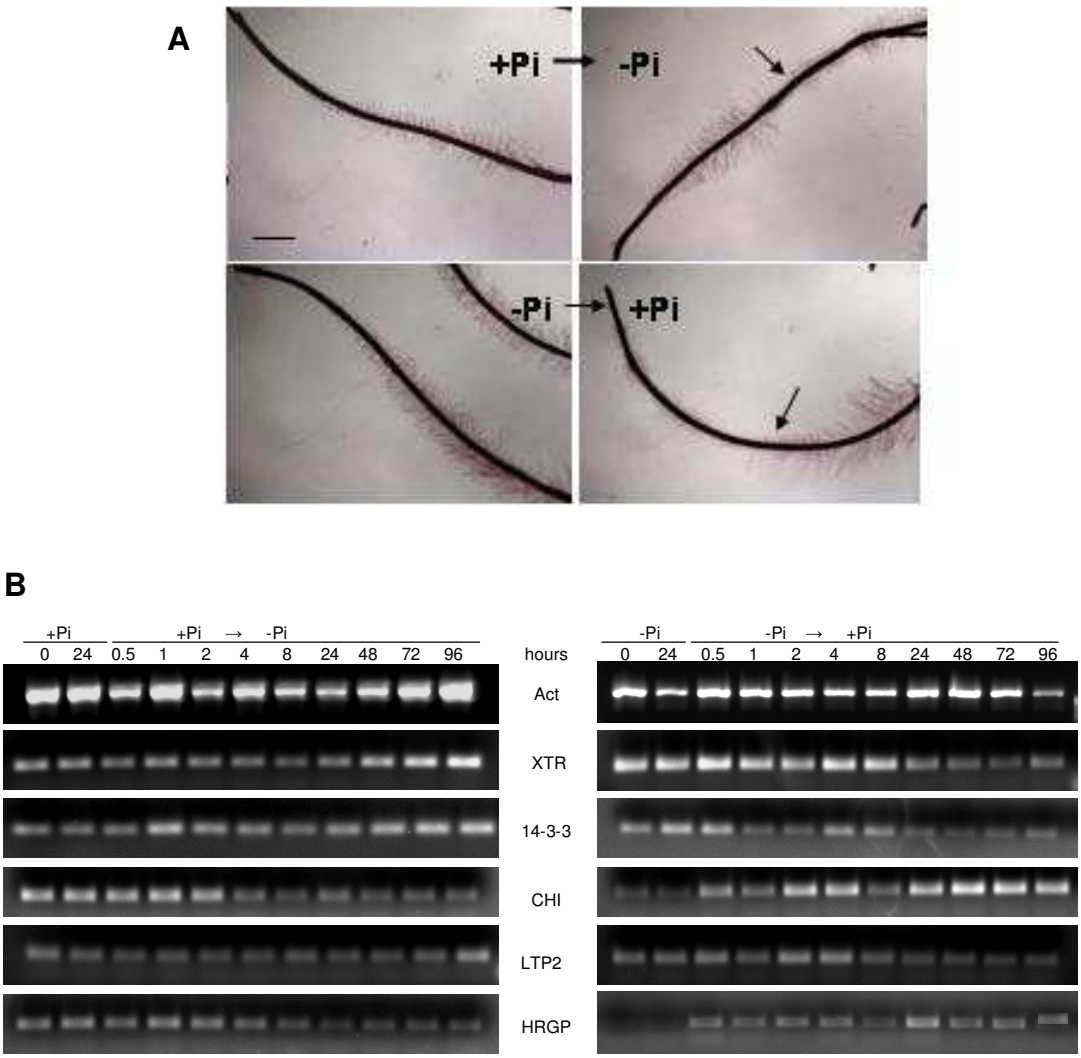
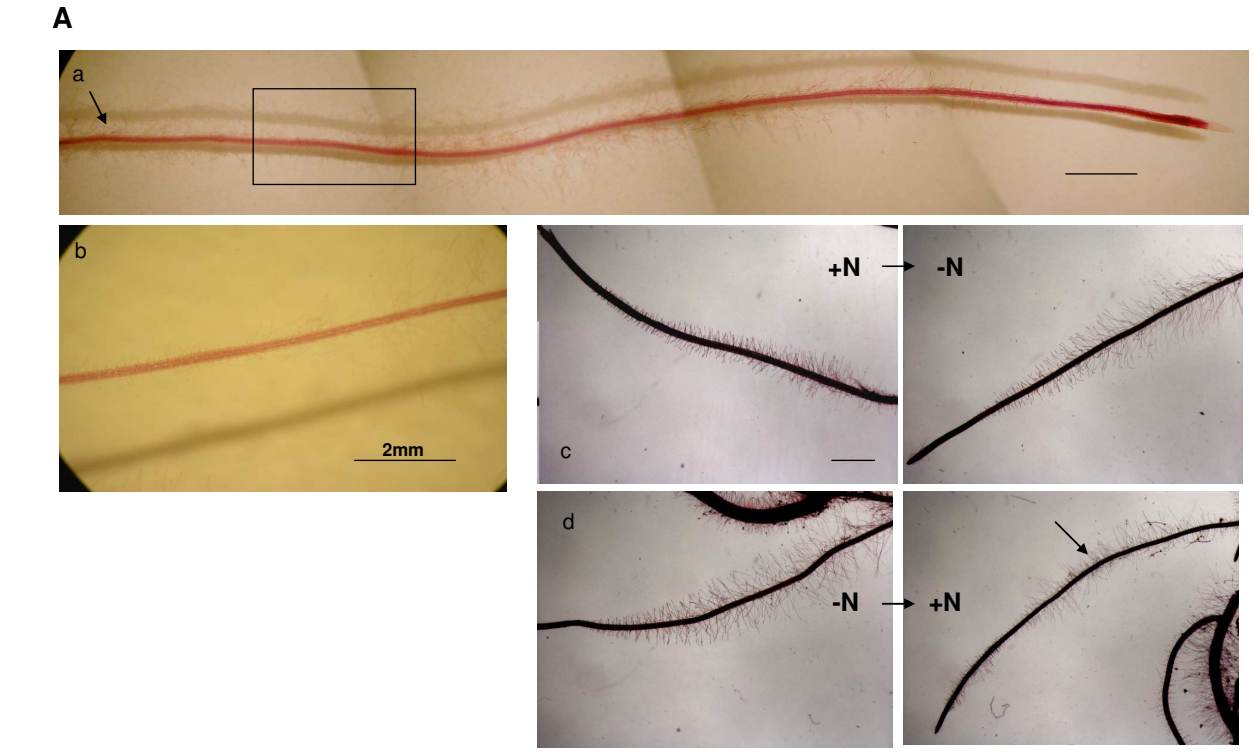


Fig. 6 Influence of varied Pi supply on (A) root hair phenotype of *Brassica carinata* cv. Bale 24h after changing Pi supply and (B) transcript level of XTR, 14-3-3, CHI, LTP2, and HRGP in roots as affected by time after change of Pi supply. Arrows indicate time point of transfer with growth rates of 1 cm/day. Bars = 1.6 mm

To determine the velocity of the response to Pi deprivation, plants of Bale (Pi efficient) were transferred to -Pi after 5 days of growth in +Pi nutrient solution and vice versa. Bacho was not included in this experiment since no further information was expected regarding a molecular link between root hair formation and gene expression. From root growth rates (1 cm day $^{-}$1) root hair length was calculated to be induced or repressed 4h and 2h, respectively (Fig. 6A). Changes in root hair development starting from time point of transfer were observed more rapid by resupply of Pi (2h) than by withdrawal (4h). With depletion of Pi the

transcript level of XTR was enhanced after 48h followed by a continuous increase up to 96h and decreased with resupply of Pi within 24h. The expression profile of 14-3-3 was fluctuating due to changes in Pi supply starting after 1h with a final increase with Pi starvation and a decrease after Pi supply. Conversely, the gene transcription of CHI and HRGP responded to withholding Pi with a downregulation after 4h and 8h, respectively, and stayed then on a constant level. Resupply of Pi resulted in an increased expression after 0.5h followed by some intermediate fluctuations. The transcription of LTP2 displayed a slow and weak reaction to Pi removal with a first decrease in expression level after 0.5h and an increase after 96h. Pi resupply resulted in a short term (0.5h) increase and a following decline in gene expression after 8h (Fig. 6B). It was obvious that changes in expression level were more rapid by resupply of Pi compared to inducing Pi starvation similar to the root hair response. There was no regulation of KUP and LTP1 regarding Pi resupply or removal and therefore no impact on root hair development could be attributed to these genes (data not shown).

N starvation induced a similar root hair phenotype and therefore it was supposed that it could follow the same signaling cascades. For this reason, *B. carinata* cv. Bale seedlings were exposed to the same experimental design as with Pi and the gene expression profile was examined in response to changing N supply. After 4h of changed N supply, root hair length was increased due to depletion of N and a reduction of root hair length was observed 6.5h after addition of N (Fig. 7A). The growth rate of roots was highest in roots transferred to N depleted nutrient solution (2.6 cm/24h) compared to all other transfer experiments (1 cm/24h).



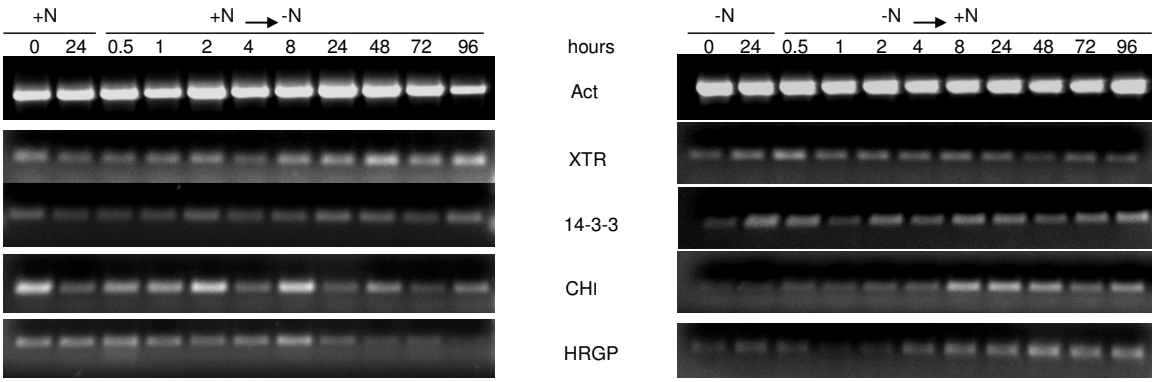


Fig. 7 Influence of changing N supply on (A) root hair phenotype of *B. carinata* 24h after changing N nutrition and (B) differential transcript level of candidate genes in roots as affected by time. (a) Merge of +N → -N transfer and box with higher magnification (b) and at root tip (c). Phenotype of root tip after transfer –N → +N (d). Arrow indicates root tip at time point of transfer with growth rates of 1 cm/day for –N → +N. Growth rate of +N → -N was 2.6 cm/day. Bar = 1.7 mm if not otherwise declared

XTR was upregulated after 48h withholding N but remained constant after N resupply. However, the change in expression was not that strong as with Pi supply. The expression of 14-3-3 was fluctuating and followed no clear trend. The abundance of CHI and HRGP mRNA

was reduced after removal of N and enhanced after N resupply within 0.5h and 4h, respectively (Fig. 7B). However, no differential expression regarding changing N nutrition could be detected for LTP2 (data not shown). In general, the expression patterns resembled those after changing Pi supply.

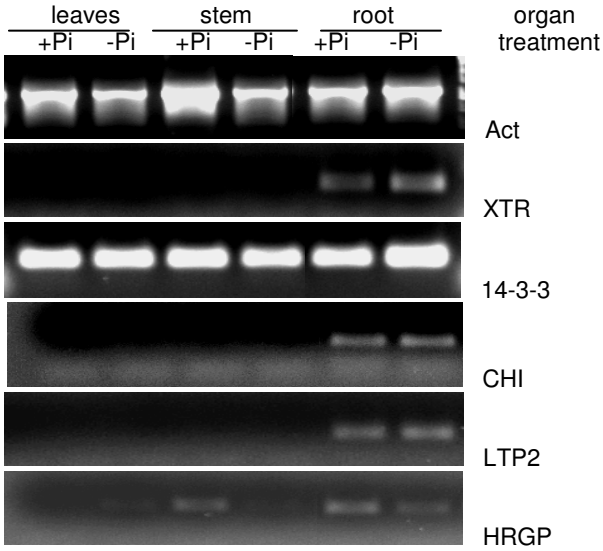


Fig. 8 Spatial expression of differentially expressed genes in *B. carinata* cv. Bale as affected by Pi supply

3.5. Spatial expression of Pi responsive genes

To evaluate the spatial expression of the candidate genes leaf, stem and root samples were harvested from plants grown 5 days with complete nutrition and under Pi deficiency, and semi-quantitative PCR was carried out. This revealed a root-specific expression for XTR, CHI and LTP2 (Fig. 8) with an enhanced expression for XTR under -Pi conditions compared to control conditions. 14-3-3 occurred in all organs and was not affected by Pi supply. The mRNA of HRGP was observed in all organs but in leaves Pi depletion resulted in a weak upregulation, in stem and root in a downregulation of the transcript level. Additionally, the expression pattern was examined along the root of Bale in one cm sections representing the initiation (0-1 cm), elongation (1-2 cm) and maturity (2-3 cm) stage of root hair development

(Fig. 9). Pi deprivation enhanced the transcript level of XTR in section 1-2 cm compared to Pi sufficient nutrition while it was unaffected in other sections. The transcript level of 14-3-3 was enhanced under -Pi in section 0-1 cm and decreased in section 2-3 cm. For both Pi levels, expression of XTR and 14-3-3 was more distinct in the section 0-1 cm compared to the mature parts of the root (Fig. 9). In contrast, limited Pi supply decreased the expression of CHI, LTP2, and HRGP with the most prominent difference in section 0-1 cm for CHI and HRGP. Regardless of Pi supply transcript abundance of CHI and HRGP was higher in older root sections while the opposite was observed for the expression of LTP2 (Fig. 9).

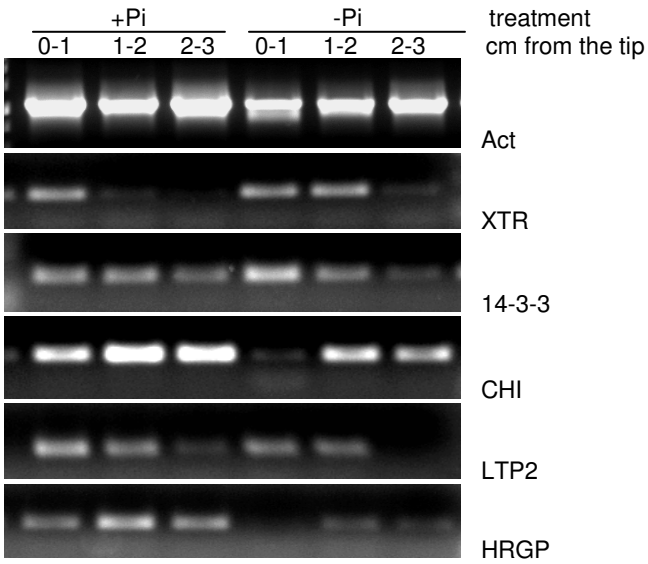


Fig. 9 Expression profile along the root of *Brassica carinata* cv. Bale seedlings as affected by Pi supply

4. Discussion

4.1. Differential gene expression in Brassica carinata cultivars under Pi deficiency

Suppression subtractive hybridization (SSH) method was carried out to identify differentially expressed genes in the Pi efficient cv. Bale and the Pi inefficient cv. Bacho under low Pi. The ESTs of the resulting cDNA libraries were classified according to their annotated function in databases into five groups: metabolism, signal transduction, stress or defence response, cell wall and miscellaneous. In the following those genes are discussed where a differential expression during Pi starvation was previously reported or a relationship with root hair growth could be assumed.

The group **metabolism** displayed genes upregulated in Bale with homologies to an inorganic Diphosphatase (BcBal-4), calcineurin B-like protein (BcBal-26), cytochrome c oxidase related (BcBal-27) and phospholipase A2 alpha (BcBal-29). Pi starvation induced expression for these genes was also reported in rice roots (Wasaki et al. 2003a). These enzymes participate in the degradation of proteins and internal nucleic acid substrates for reutilisation of Pi (Abel et al. 2002). Furthermore, a member of the ABC transporter family (BcBal-25) was induced in Bale in response to Pi limitation. Similar results were observed for ABC transporter in rice and common bean (Wasaki et al. 2003a; Tian et al. 2007). Another ABC transporter was regulated during iron deficiency in tomato suggesting a molecular cross talk in nutrient signaling (Wang et al. 2002b). Additionally, a vacuolar ATP synthase subunit B (BcBal-6) involved in hydrolase activity was induced under low Pi as also observed in *Arabidopsis* (Wu et al. 2003). Instead, a gene encoding for an enolase (BcBac-17) was upregulated in Bacho that catalyzes the interconversion of 2-phosphoglycerate to PEP and therefore plays an important role in glycolysis (Lal et al. 1998; Wang et al. 2002b). Further, in Bacho a gene with similarity to a protein phosphatase (BcBac-42) was upregulated compared to cv. Bale contributing to recycle Pi from internal pools (Duff et al. 1994; del Pozo et al. 1999; Abel et al. 2002). The Pi starvation induced expression of phosphatases was also reported in rice, *Arabidopsis* and bean (Wasaki et al. 2003a; Wu et al. 2003; Misson et al. 2005, Hernandez et al. 2007; Morcuende et al. 2007; Tian et al. 2007). In Bacho, the

expression of a nicotianamine synthase (BcBac-30) was enhanced as it was also reported for Pi starved roots in tomato (Wang et al. 2002b). This enzyme is involved in biosynthesis of phytosiderophores (Herbik et al. 1999; Higuchi et al. 1999). In contrast to own observations, no regulation during Pi starvation was reported for an ubiquitin extension protein (BcBal-8) functioning in protein modification (Callis et al. 1990) and a K^+- transporter (BcBal-17) required for root hair elongation (Rigas et al. 2001; Desbrosses et al. 2003). Both genes were upregulated in Bale and a function in root hair growth was assumed.

In the second group **signal transduction** a number of transcription factors were found to be upregulated in Bale and Bacho that could modify the regulation of genes positively or negatively in Pi starvation induced root hair formation. A Myb transcription factor (BcBal-19) was induced in response to Pi starvation suggesting a positive regulation in Pi signaling. Similar results with other Myb transcription factors were obtained in other studies (Wu et al. 2003; Hammond et al. 2003; Misson et al. 2005; Hernandez et al. 2007; Morcuende et al. 2007; Müller et al. 2007). Within the family of 14-3-3 proteins an induction in response to Pi depletion was found for Bale (BcBal-31). An upregulation of 14-3-3 proteins during Pi, K and Fe starvation was also reported in tomato roots (Wang et al. 2002b). As well as for zinc finger proteins a -Pi induced expression in Bale (BcBal-15) was observed and could contribute to a regulation of transcription during Pi starved conditions. Zinc finger proteins were reported to be both up and downregulated triggered by Pi deprivation in roots of common bean (Hernandez et al. 2007). A higher expression in response to Pi limitation was observed in Bale for a translation initiation factor (BcBal-35). Similarly, Venkatachalam et al. (2009) observed a translation initiation factor induced during Pi depletion in *Lolium multiflorum*.

In the present work, genes of the category **stress and defence** upregulated in Bale are two lipid transfer proteins (BcBal-1; BcBal-40) and an exordium like protein (BcBal-32) functioning in cell expansion processes (Schroeder et al. 2009) and in Bacho, a glutathione-S-transferase (BcBac-11), a chitinase (BcBac-14), other lipid transfer proteins (BcBac-28; BcBac-43), a peroxidase (BcBac-37) and a putative thioredoxin protein (BcBac-18). For the

upregulated genes in Bacho similar results during Pi stress were reported for rice, *Arabidopsis* and ryegrass (Wasaki et al. 2003a; Misson et al. 2005; Morcuende et al. 2007; Venkatachalam et al. 2009). These genes were induced by stress and are assumed to have a function in unspecific defence mechanism (Misson et al. 2005). The induction of peroxidases was also attributed to the production of reactive oxygen species (ROS) during Pi starvation (Juszczuk et al. 2001). The upregulation of a glyceraldehyde-3-phosphate dehydrogenase (BcBac-34) suggest an involvement of pathways that could result in the secretion of more organic acids during Pi starvation as it was previously reported (Shen et al. 2002; Uhde-Stone et al. 2003; Wasaki et al. 2003a). However, in previous experiments no differences in organic acid exudation in Bale and Bacho were detected (Eticha and Schenk, 2001). Moreover, an ACC oxidase (BcBac-32) was identified as low Pi inducible in Bacho and similarly Hernandez et al. (2007) found an upregulation of ACC in roots of common bean. ACC oxidases are known as key enzymes in ethylene biosynthesis (Yang and Hoffman, 1984). An induction of non-specific lipid transfer proteins (nsLTPs) in response to Pi starvation was previously reported in rice (Wasaki et al. 2003a). The family of nsLTP is highly diverse and is originally defined by their capacity to transfer lipids between membranes *in vitro* (Kader 1996; 1997). Their precise physiological functions are mainly unknown.

Upregulation of a number of cell wall proteins involved in **cell wall** modifications was observed in Bale and Bacho. Cell wall proteins contribute to cell wall loosening and enable cell wall elongation while others function in strengthening cell wall structure, both processes potentially affecting root hair growth. A member of the family of xyloglucan endotransglucosylases/ hydrolases (XTH, BcBal-3) related genes (XTR) was induced during Pi depletion and similar results were obtained in roots of *Arabidopsis* and rice during Pi stress (Wu et al. 2003; Wasaki et al. 2003a). XTHs catalyze the cleavage and re-joining of xyloglucan molecules in the primary cell wall among cellulose microfibrills thus helping to maintain plasticity for cell growth and expansion (Fry et al. 1992) as well as strengthening the cell wall (Vissenberg et al. 2001). Furthermore, a -Pi induced upregulation of hydroxyproline

rich glycoproteins (HRGPs) in Bacho (BcBac-2, BcBac-10) was observed. This result corresponded to an upregulation in roots of common bean during Pi deprivation (Hernandez et al. 2007). Proline rich proteins (PRPs) were also upregulated in Bale (BcBal-10) and in Bacho (BcBac-33) in agreement with results obtained by Hernandez et al. (2007) in response to Pi stress. However, Hernandez et al. (2007) reported as well about a downregulation of other PRPs by Pi removal. HRGPs and PRPs are structural components of cell walls with enhanced expression due to abiotic stress (Showalter, 1993; Cassab, 1998). Likewise, Jones et al. (2006) indicated a function and high abundance of HRGPs related to root hair elongation.

The results obtained from the subtractive cDNA library were validated by RT-PCR for genes putatively related to Pi starvation induced root hair growth or having most abundant ESTs in the cDNA library. In Bale an enhanced expression of LTP1 (BcBal-1), XTR (BcBal-3), KUP (BcBal-17) and 14-3-3 (BcBal-31) was affirmed compared to Bacho under Pi deficient conditions (Fig. 2). In Bacho an upregulation of a HRGP (BcBac-2) a chitinase (BcBac-14), and LTP2 (BcBac-28) was observed compared to Bale under Pi deficiency (Fig. 2). LTP1, XTR, 14-3-3 and KUP were not expressed in Bacho since primers annealed to genomic DNA of Bacho (Fig. 3).

4.2. Nutrient specific expression profile

Root hair phenotype and expression of candidate genes were investigated during Pi, N and K starvation (Fig. 5). Pi and N deprivation but not K starvation induced the formation of longer root hairs in the Pi efficient cultivar Bale while the root hair length was not altered in Bacho in response to all nutrient stresses examined (Fig. 5A). Similar results regarding Pi and N induced root hair elongation were already reported (Foehse and Jungk, 1983; Bates and Lynch, 1996). In roots of Bale during Pi starvation an upregulation of LTP1, XTR, 14-3-3 and LTP2 was observed whereas these genes were not altered or even suppressed under N starvation (Fig. 5B). This suggests that response of genes is specific for Pi starvation. However, for 14-3-3 and LTP2 a relationship with root hair growth is questionable since both

Pi and N depletion enhanced root hair length. This conclusion is strengthened by the observation that 14-3-3 and LTP2 were also upregulated during Pi deficiency in Bacho although this variety produced no elongated root hairs. Expression of LTP1 and XTR was only detected in cultivar Bale indicating a genotypic regulation. The expression profiles of KUP and CHI indicated no function in root hair development (Fig. 5B). In contrast, the expression profile of HRGP declined in Bale during both Pi and N starvation indicating a common negative role in Pi and N signal transduction and a direct control of root hair elongation. This was strengthened by the fact that in Bacho no differential expression of HRGP was observed in all treatments. Thus, for HRGP a negative function in root hair growth seems a reasonable assumption.

Differences in root hair length were visible 2h and 4h after resupply and removal of Pi and 6.5h and 4h, respectively after changing N supply in cv. Bale (Fig. 6A, 7A). Similarly, Bates and Lynch (1996) showed an increased and reduced root hair elongation in *Arabidopsis* 10h after removal and resupply of Pi, respectively. Therefore, genes involved in Pi and N induced root hair extension were expected to respond before visible changes occurred. The expression of XTR and LTP2 by removing Pi supply was upregulated after 48h and 96h, respectively, while in low N supply only a weak (after 48h, XTR) or no (LTP2) upregulation occurred (Fig. 6B, 7B). This expression pattern suggested no function in root hair growth since the temporal concurrence of gene activity and altered root hair length was not observed. In contrast, a XTH, which has similarities to XTR, altered the expression within 4h after withdrawal of Pi in shoots of *Arabidopsis thaliana* (Hammond et al. 2003). 14-3-3 was rapidly up (1h) and downregulated (1h) with subsequent fluctuations in response to withdrawal and resupply of Pi indicating a complex regulation during Pi starvation but relation to root hair growth was already disputed (Fig. 5). In *Arabidopsis*, Cao et al. (2007) observed a diverse regulation of different members of the 14-3-3 family due to Pi deficiency. CHI was down (4h) and upregulated (0.5h) by Pi depletion and Pi supply, respectively as well as regulated by N supply (Fig. 7B). However, a relation to root hair elongation was already ruled out. The function of CHI is still unclear since chitinases were induced as a general stress

response that is in disagreement with the upregulation by resupplying Pi and N. Instead, the downregulation of HRGP (8h, 24h) by both Pi and N starvation corresponded to the time frame where a regulation of genes for root hair growth can be supposed. Furthermore, this expression pattern suggested a negative regulation in a common pathway for Pi and N in root hair elongation (Fig. 6B, 7B). HRGP is potentially involved in strengthening the cell wall but the rapid response (0.5h) after Pi resupply may suggest an up to now unknown function in early Pi signaling.

Removal of Pi supply delayed the response of root hair elongation and gene expression compared to the more rapid alterations by Pi resupply. This could be explained by a lagged sensing of Pi starvation due to dilution progress in the root tissue and was also expected for N. However, N depletion resulted in a faster adaptation in root hair length than N resupply. Determination of growth rates within 24h revealed a 2.6 times higher rate compared to the resupply of N making a more rapid response possible.

4.3. Organ specific expression profile

The root-specific expression of XTR, CHI and LTP2 could indicate an organ and developmental specific profile where isoforms of a gene family accomplish different functional roles in different tissues (Fig. 8; Thoma et al. 1994; Soufleri et al. 1996; Vignols et al. 1997; Clark and Bohnert, 1999; Arondel et al. 2000; Imoto et al. 2005; Osato et al. 2006). 14-3-3 and HRGP were expressed throughout the plant suggesting a function not restricted to the root. While 14-3-3 showed no differential expression in response to low Pi, HRGP was higher expressed in root and stem in Pi sufficient conditions and weakly upregulated in leaves under Pi depletion (Fig. 8). This indicated an opposite gene regulation in different organs as already known for genes involved in epidermal cell pattern either in leaves resulting in trichomes or the converse regulation in roots producing root hairs (Schellmann et al. 2002). In contrast to the present study, the transcripts of many HRGP genes have been detected exclusively in the root (Bucher et al. 1997; Merkouropoulos et al. 1999).

The expression profiles of XTR, 14-3-3 and LTP2 suggested a function in early stages of root development since the expression was highest in the sections 0-1 cm and 1-2 cm and declined to mature root parts (Fig. 9). However, the delayed expression of XTR and LTP2 in response to changes in Pi supply was contradictory to a function in early Pi stress induced root hair growth (Fig. 6B). However, in *Arabidopsis* initiation of root hair growth was coupled with an activity increase in XTH which has similarities with XTR (Vissenberg et al. 2001). Similarly, Jones et al. (2006) demonstrated a very high expression of XTH activity in roots of *Arabidopsis* wild type plants compared to rhd2 mutant plants were root hair elongation was suppressed and overexpression of XTH1 in *Brassica campestris* led to enhanced stem elongation (Shin et al. 2006b). Also for 14-3-3 isoforms and nsLTPs the expression was detected in young leaves, stems and elongating cotton fibers, the latter having growth pattern like root hairs (Thoma et al. 1994; Szopa et al. 2003; Shi et al. 2007; Boutrot et al. 2007). In contrast, HRGP and CHI had an enhanced transcription in mature root sections with a higher expression in Pi sufficient conditions compared to low Pi conditions. These observations suggested a function in later stages of root hair development presumably to strengthen the cell wall (Fig. 9). Previously, crosslinking of HRGPs in the cell wall was reported to restrict cell elongation and to increase the mechanical strength in roots of *Arabidopsis* (Bradley et al. 1992; De Cnodder et al. 2005). Correspondingly, overexpression of a HRGP in transgenic *Arabidopsis* plants resulted in an enhanced stem thickness (Roberts and Shirsat, 2006).

4.4. Conclusion

The expression of HRGP was negatively related to root hair elongation. The transcript was found in leaves, stem and root but regulation was converse during Pi deficiency with a downregulation in root and stem and an upregulation in leaves. Furthermore, expression was enhanced in mature root parts and the temporal response to changes in Pi and N supply corresponded to variation of root hair length. It was reported that HRGPs function in strengthening cell walls by crosslinking (Showalter, 1993).

XTR, LTP2 and 14-3-3 could not be related to root hair development although the expression was highest in the root tip. It is conceivable that these genes have a function in root growth but a relation to root hair elongation was ruled out since the response to changing Pi supply was delayed. For CHI no physiological function for root hair growth was evident. The root specific and most pronounced expression in older root parts together with the rapid response to changing Pi and N supply suggested a negative role in signaling of nutrient starvation. However, chitinases were reported to be a general stress response and therefore positively regulated by stress (Wasaki et al. 2003a; Misson et al. 2005; Morcuende et al. 2007). For LTP1 and KUP no effect on root hair development was discovered since no regulation during change in Pi and N supply was observed. For further characterization of the involved genes additional research has to be done to clarify the role in Pi signaling.

Chapter II

Identification and characterization of differentially expressed genes involved in root hair growth during Pi deficiency in *Brassica carinata*

Abstract

Phosphate (Pi) starvation induced the formation of longer root hairs in *Brassica carinata*. Suppression subtractive hybridization (SSH) was carried out to identify differentially expressed genes in roots with a putative function in root hair growth during Pi starvation. The resulting subtractive cDNA library revealed genes with similarities to a leucine rich receptor-like kinase (LRR), a MADS box transcription factor (MADS), a proline rich protein (PRP) and a Pi starvation inducible molecule with unknown function (IPS) in *Arabidopsis thaliana* that were confirmed by semi-quantitative RT-PCR. The expression patterns were examined in response to Pi, N and K starvation, by changing Pi and N supply and along the root to reveal a function in root hair growth.

Besides Pi, root hair length was increased during N depletion while K starvation had no effect on root hair length. Additionally, a reduction in root hair length was observed 4h after removal of Pi and N as well as an enhanced root hair length after 2h and 6.5h, respectively, after resupply of Pi and N. The expression of LRR was enhanced after 24h and 2h in response to Pi and N starvation and the temporal concurrence suggests a function in root hair development considering a dilution effect by sensing Pi depletion in the root tissue. This was strengthened by the fact that LRR expression was most pronounced in the root sections where root hairs start to develop. As LRR putatively encodes for a receptor protein kinase a function in perceiving a common signal in Pi and N signaling to induce root hair growth is conceivable. For IPS a highly Pi specific expression throughout the plant was shown. The early response after removal (24) and resupply (8h) of Pi may contribute to an up to now unknown mechanism with no direct relation to root hair growth. The expression of PRP was weakly regulated by Pi supply and the delayed expression after transfer excludes a function in early root hair development. Though the transcription was highest in root tips a role in root growth is suspected since PRPs contribute to cell wall assembly. The transcript level of MADS was not controlled by Pi or N starvation and implies no function in root hair development.

Keywords: root hair, Pi starvation, SSH, LRR, IPS, Pi efficiency

1. Introduction

Phosphorus is an essential macronutrient for plant growth and development. It is involved in a wide range of physiological and biochemical processes including energy conservation, signal transduction, photosynthesis, and respiration as well as phosphorus is a component of nucleic acids and biomembranes. Despite the fact that phosphorus is abundant in most soils, the availability of inorganic phosphate (Pi) for plant uptake is often low because of strong interactions with soil components. Consequently, plants have developed morphological and physiological adaptations in response to Pi deficiency (Vance et al. 2003; Richardson et al. 2009). These strategies aim at increasing uptake of Pi from the soil or at maintaining Pi homeostasis. Morphological adaptations imply changes in root architecture and formation of root hairs to explore a greater soil volume (Bates and Lynch, 1996; Ma et al. 2001; López-Bucio et al. 2002). Furthermore, Pi starvation induces in roots expression of Pi transporters, RNases and acid phosphatases as well as release of organic acids and protons to increase the availability of Pi in the soil (Raghothama, 1999; Baldwin et al. 2001; Bozzo et al. 2002; Vance et al. 2003; Rausch and Bucher, 2002). Transcriptional profiling in several plants has revealed genes with altered expression during Pi starvation (Hammond et al. 2003; Wasaki et al. 2003a; Ticconi and Abel, 2004; Morcuende et al. 2007; Müller et al. 2007). The promoter region in a number of -Pi inducible genes revealed a conserved domain that seems to play a role in a global regulation of Pi responsive genes. This motif (GNATATNC) is the binding site of PHR1 (PHOSPHATE STARVATION RESPONSE1) encoding for a Myb-type transcription factor (Rubio et al. 2001). PHR1 is a positive regulator of Pi starvation responses but the formation of root hairs seems to be independent of PHR1 in *Arabidopsis*, suggesting a different Pi starvation induced pathway (Martin et al. 2000; Rubio et al. 2001). PHR1 responsive genes contain members of the IPS1 (INDUCED BY PHOSPHATE STARVATION1) family (Rubio et al. 2001). These non-protein encoding RNAs are highly induced during Pi stress. The function of these genes is largely unknown but a role in Pi allocation between root and shoot was suggested. Due to a sensible target mimicry mechanism IPS1 is supposed to regulate the effect of Pi depletion inducible microRNA

because both contain a motif of high complementary (Burleigh and Harrison et al. 1999; Shin et al. 2006a; Franco-Zorilla et al. 2007). These microRNAs (miRNA) are important for the transcriptional or translational regulation for genes with function in Pi homeostasis (Chiou et al. 2006; Doerner, 2008).

Root epidermal cells in the family *Brassicaceae* develop into hair-bearing cells when located over the junction between two underlying cortical cells (the H position) whereas cells lying over a single cortical cell normally adopt the non-hair cell fate (N position). This position-dependent determination requires a signal mechanism arising from underlying cells that direct the epidermal cell fate (Schiefelbein, 2000). Several genes involved in a regulatory network to produce hair and non-hair cell fate in the *Arabidopsis* root epidermis have been identified. TRANSPARENT TESTA GLABRA1 (TTG1), WEREWOLF (WER), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3), and GLABRA2 (GL2) are necessary to specify the non-hair cell fate (Galway et al. 1994; Masucci et al. 1996; Lee and Schiefelbein, 1999; 2002; Bernhardt et al. 2003) while determination of hair-bearing root cells occurs through the genes CAPRICE (CPC) and TRIPTYCHON (TRY)(Wada et al. 1997; Schellmann et al. 2002; Kirik et al. 2004). It seems that in N cells TTG1, WER, GL3 and EGL3 act in a complex regulating positively GL2 expression and also CPC and TRY expression. The products of the latter genes move then to the H position repressing the activity of GL2 in a complex with GL3 and TTG1 (reviewed in Schiefelbein et al. 2009). Pi starvation leads to the development of hairs in the non-hair position and therefore has an impact on epidermal gene expression (Müller and Schmidt, 2004).

In this study, an Ethiopian mustard (*Brassica carinata*) genotype responded to Pi deficiency with longer root hairs. An SSH approach was performed to identify differentially expressed genes in the Pi starvation response that were putatively involved in the formation of root hairs. Expression of candidate genes was investigated regarding temporal nutrient stress and in a spatial pattern along the root axis.

2. Materials and Methods

2.1. Plant material and cultivation

Seeds of *Brassica carinata* cv. Bale were vernalized in the dark for three days at 4°C and then germinated for 5 days in the growth chamber with a 16h (20°C) day/ 8h (15°C) night cycle. The photosynthetically active radiation was 200 µmol photons $m^{-2}s^{-1}$ and relative humidity was adjusted to 70%. Uniform seedlings were transferred to a continuously aerated nutrient solution containing 1 mM KH_2PO_4 or free of Pi. The composition of other nutrients was in mM: 2.25 $Ca(NO_3)_2$; 2.5 K_2SO_4; 1 $MgSO_4$; 0.25 KCl; and in µM: 25 H_3BO_3; 1.5 $MnSO_4$; 1.5 $ZnSO_4$; 0.5 $CuSO_4$; 0.025 $(NH_4)_6Mo_7O_{24}$ and 35.8 Fe (Fe^{III}- EDTA) at pH 5.5. After five days of cultivation lateral root tips of 2 cm length were harvested 2h after begin of light period, frozen in liquid nitrogen and stored at -80°C before use. Seedlings were also starved for N by substituting $Ca(NO_3)_2$ by $CaCl_2$ and K by omitting K_2SO_4 and KCl and substituting KH_2PO_4 by NaH_2PO_4 and lateral roots were harvested after five days. For transfer experiments seedlings were grown five days in complete nutrient solution before transfer to a nutrient solution lacking Pi or N, respectively, and vice versa. Some plants remained in the original solution as control. Root tips of 1.5 cm length were harvested at 0.5, 1, 2, 4, 8, 24, 48, 72, and 96h after transfer or 0 and 24h for the control plants. Additionally, stem and leaf samples and 1 cm root sections (0-1; 1-2; 2-3 cm) beginning from the tip of plants grown at +Pi and -Pi were harvested after five days of cultivation for RNA isolation. Pictures of root phenotype were taken with the AxioCam MRc (Zeiss) under the Axioskop (Zeiss) after staining with 1% acid fuchsin (in A. dest.).

2.2. Suppression subtractive hybridization (SSH)

Total RNA was isolated from root tips subjected five days to Pi sufficient and Pi depleted conditions using the NucleoSpin RNA Plant kit (Macherey & Nagel, Düren, Germany) following the manufacturer's instructions. The Super SMART PCR cDNA synthesis kit (BD Biosciences Clontech Palo Alto, CA) was used to generate tester and driver double-stranded cDNA from 1 µg total RNA according to the manufacturer's protocol. For identification of Pi

responsive genes cDNA from roots grown in 1 mM Pi served as driver and cDNA from plants grown without Pi as tester. SSH was performed using the Clontech PCR-select cDNA subtraction kit (BD Clontech) following the manufacturer's instructions. After reverse transcription of tester and driver, the cDNA populations were digested with RsaI (10 U/µl) and tester cDNA ligated to two different adaptors separately. Two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed sequences. The subtracted PCR amplified cDNAs were cloned directly into the T/A cloning vector pCR2.1 TOPO using TOPO TA cloning kit (Invitrogen, USA) and then transformed into chemically competent *E. coli* TOP10 cells (Invitrogen) producing the resultant subtractive cDNA library. Transformed cells were plated onto LB agar medium containing 50 µg/ml kanamycin and 40 µg/ml X-Gal, and incubated at 37°C overnight. White colonies (putative positive clones) were obtained the next day, and each colony was picked individually and checked for presence and size of individual inserts by colony PCR using flanking M13 forward and reverse primers (forward 5´-GTAAAACGACGGCCAG-3´ reverse 5´-CAGGAAACAGCTATGAC-3´). The final reaction volume of 25 µl contained 20.54 µl distilled water, 2.5 µl 10x buffer, 0.5 µl dNTP mix (10 mM each, Roth), 0.63 µl each of M13 primers (10 pm/µl), 0.2 µl SupraTherm^{TM} Taq DNA polymerase (5 U/µl, GeneCraft, Köln, Germany) and a tip bacterial culture. The PCR was carried out using the following cycling conditions: 3 min at 94°C, and then 33 cycles of 15 sec at 94°C, 30 sec at 56°C and 1 min at 70°C. The PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide, and visualized under UV light (312 nm; INTAS Gel iX Imager, Göttingen). Positive clones were grown overnight in 3 ml liquid LB medium with 50 µg/ml kanamycin. The clones were stored as glycerol stocks at -80°C.

2.3. Sequence analysis

Clones were sent for sequencing in 0.8% LB agar medium supplemented with 50 µg/ml kanamycin (IIT Biotech, Bielefeld, Germany). The obtained expressed sequence tags (ESTs) were compared by sequence similarity searches to the GenBank database using BLASTN and BLASTX of the *Arabidopsis* information resource (TAIR;

(www.arabidopsis.org/wuBLAST/index2.jsp) to identify their putative functions. The ESTs were named according to most homologous sequences in the databases. Functional classification was arranged appropriately to annotations in the databases. The sequences of ESTs identified in this work were deposited in supplementary material.

2.4. Expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from stem, leaves, or lateral roots using NucleoSpin RNA Plant kit (Macherey & Nagel). RNA quality was checked by gel electrophoresis and quantified by the Nanophotometer (IMPLEN, München, Germany). For expression analysis by semi-quantitative RT-PCR, 1 µg total RNA of each sample was reverse transcribed with oligo (dT)-primers using the RevertAid [TM] H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and was performed at 42°C for 60 min with a final denaturation at 70°C for 10 min. Afterwards RNA was removed by RNAse H (10 U/µl, Epicentre, Madison, WI, USA) treatment for 20 min at 37°C. Concentration and purity of cDNA was determined with the Nanophotometer (IMPLEN). Aliquots containing 800 ng of first strand cDNA were used as template in a 50 µl PCR reaction containing 40.7 µl distilled water, 5 µl 10x buffer, 1 µl dNTP mix (10 mM each, Roth), 1 µl each of primer (10 pm/µl, reverse and forward) and 0.3 µl SupraTherm^{TM} Taq DNA Polymerase (5 U/µl, Genecraft). Gene specific primers were designed by Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) based on resulting sequences from cDNA library and new cDNA served as template. After initial denaturation at 94°C for 3 min, PCR was carried out for different number of cycles of 94°C for 15 sec, 52°C-56°C for 30 sec depending on Tm of primers and 70°C for 1 min. To monitor equal amounts of cDNA, the constitutively expressed actin gene was amplified simultaneously (forward 5´-AGGATATTCAGCCACTTGTCTGTG-3´ reverse 5´-AGAAACATTTCCTGTGAACAATCG-3´). Samples were removed after a diverse number of cycles and amplification state monitored on a 1.2% agarose gel stained with ethidium bromide. The primer sequences and predicted amplicon sizes were as follows (Table 1):

Table 1: used RT-PCR primer sequences derived from sequenced SSH clones

EST clone	Sequence (5´- 3´) forward reverse	Tm	Product size (bp)
BcBal-2	TTGACTACAAATCGCCACCA AGCCAAAGAATCCATTCCAA	66 64	218
BcBal-4	AAATCTCCACCACCAGCTTC GCCATGTTATTAGTCCACACTCTC	60 70	151
BcBal-7	TGAACTAGTTGTCGCGGATG GATGAGAAGGAACATGCTTGG	68 69	223
BcBal-8	GGCCGAATAACTCCGACATA CCCATAATCCCTTGTTGCAT	60 58	222
BcBal-17	CACAAACCAGAGATTCAGAACG TCGAGGAGTGGAGGACAGAT	69 70	210
BcBal-19	CACCCACGTGTATGTGTATGC ATTCAACCTTAGGAGATGGT	64 56	217
BcBal-20	GCCGTATAAATCCGGCATAC GGGAATGAACTTGCCGATAC	60 60	249
BcBal-21	GGCGCAATGATGTAACAAGA CAGGTACTCCGTCGTTTTGG	66 70	241
BcBal-30	GCAAGCCCTATGTGCAGAGT CACGTTGCACCAGATAATCC	62 60	196
BcBal-32	TCAAAGGGAGAAAGCATGAAA AAGGCTGACGATGAAGAGGA	58 60	169
BcBal-36	AGAGGCAGTGTCTATGTCAAAGC TGCTTTGCTTTGCTTTTGTG	68 56	202

3. Results

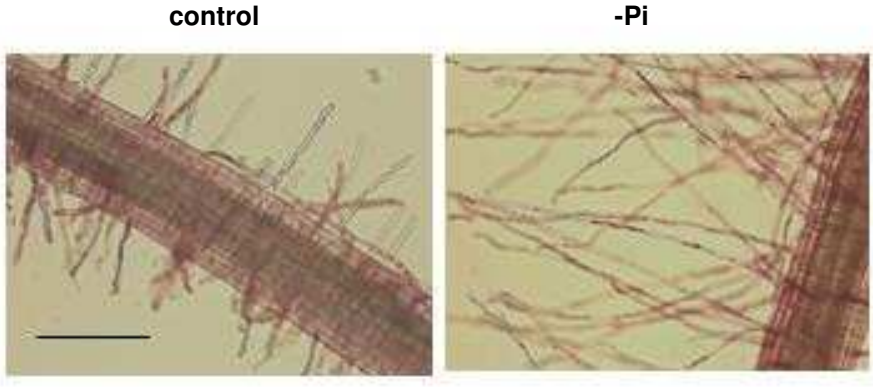


Fig. 1 Root hair phenotype of *B. carinata* cv. Bale during control (1 mM) and Pi deficient (0 mM) conditions. Bar = 400 µm

3.1. Identification of Pi regulated cDNA sequences by Suppression Subtractive Hybridization (SSH)

Brassica carinata cv. Bale plants responded to Pi depletion with longer and more root hairs (Fig. 1). Using cDNA from roots grown five days under Pi deprived conditions as tester and cDNA from roots of plants grown in complete nutrient solution as driver, a SSH was performed to obtain differentially expressed genes with a putative function in root hair elongation. The resulting cDNA library of expressed sequence tags (ESTs) revealed a range in size of about 200 bp to 1000 bp with a cumulation around 400 bp. A total of 125 individual clones were picked from the cDNA library and checked for existence of an insert. After sequencing 36 differentially expressed clones were identified by comparing to homologous sequences using BLAST programs. The resulting ESTs were classified according to their function in five categories: metabolism, cell wall, stress/ defence response and signal transduction. Clones without attributed function or homology to known proteins were classified in the category miscellaneous (Table 2). The largest group of putatively regulated genes during Pi deficiency belonged to the group metabolism (14). The other classes contained 4-7 genes. Particular attention was given to genes which are potentially involved in root hair growth and differential expression of selected ESTs was validated by semi-quantitative RT-PCR (Fig. 2). The following cDNA fragments displayed a regulated

expression regarding Pi supply and were chosen for further analysis: a gene encoding for a proline rich protein (PRP), a non-specific lipid transfer protein (LTP), a gene reported to be

Table 2 Identification of Pi starved upregulated cDNA sequences in *B. carinata* cv. Bale by SSH

Clone	putative gene identity	Homologues of *Brassica* ESTs in *Arabidopsis*	E-value	length (bp)
Metabolism				
BcBal-5	mitochondrial ATP synthase	At4g29480	5.4e-18	210
BcBal-9	plasma membrane intrinsic protein 1B (PIP1;2)	At2g45960	2.0e-69	423
BcBal-10	nucleosome assembly protein (NAP) family	At3g13782	2.7e-59	417
BcBal-11	ACT domain-containing protein	At5g04740	2.1e-119	642
BcBal-13	adenosylhomocysteinase	At4g13940	9.2e-19	179
BcBal-15	methyl-CpG-binding domain containing protein	At3g46580	6.2e-04	65
BcBal-18	cytosolic glyceraldehyde-3-phosphate dehydrogenase	At3g04120	4.4e-13	211
BcBal-22	hydrogen-exporting ATPase (AHA7)	At3g60330	3.1e-51	394
BcBal-23	lactoylgluthathione lyase	At1g11840	5.2e-09	139
BcBal-25	carbohydrate transmembrane transporter/ sugar:hydrogen symporter	At5g16150	8.5e-50	372
BcBal-27	phosphatase	At1g17710	9.8e-54	509
BcBal-28	putative type 1 membrane protein (PMP)	At3g24160	4.4e-23	279
BcBal-34	DNA-dependent ATPase	At3g06400	1.4e-48	268
BcBal-35	vacuolar ATPase subunit F family protein	At4g02620	2.5e-15	212
cell wall				
BcBal-2	proline rich extensin like family protein (PRP)	At1g23720	6.3e-55	357
BcBal-4	proline rich extensin like family protein (PRP)	At3g28550	9.3e-28	326
BcBal-19	hydroxyproline rich glycoprotein (HRGP1)	At3g54590	2.8e-58	390
BcBal-21	arabinogalactan-protein (AGP3)	At4g40090	4.2e-09	198
Stress and defence response				
BcBal-8	non specific LTP (nsLTP)	At4g12545	4.1e-16	352
BcBal-14	pollen Ole e 1 allergen and extensin family protein	At4g02270	3.5e-22	282
BcBal-16	acid phosphatase class B family protein	At5g44020	2.1e-28	196
BcBal-20	non specific LTP (nsLTP, AIR1)	At4g12550	6.1e-18	444
BcBal-24	thioredoxin 3	At5g42980	4.7e-11	224
BcBal-30	non specific LTP (nsLTP)	At3g18845	3.8e-23	207
BcBal-32	AtIPS (induced by Pi starvation)	At3g09922	7.5e-30	373
Signal transduction				
BcBal-1	F-box family protein	At3g19560	2.5e-01	208
BcBal-6	DNA-binding family protein	At1g29850	1.5e-10	121
BcBal-7	MADS box family protein	At4g37940	2.2e-26	185
BcBal-12	26S proteasome non-ATPase	At5g05780	2.0e-45	298
BcBal-17	leucine rich repeat receptor-like protein kinase (LRR)	At2g28970	7.6e-53	545
BcBal-33	20S proteasome alpha subunit B	At1g79210	3.4e-18	224
BcBal-36	auxin binding / ubiquitin-protein ligase	At3g26810	0.19	359
Miscellaneous				
BcBal-3	unknown protein	At1g55340	2.7e-26	252
BcBal-26	unknown protein	At2g31480	1.3e-36	254
BcBal-29	unknown protein	At3g14280	7.2e.-72	535
BcBal-31	unknown protein	At3g01130	2.3e-40	370

specifically induced by Pi deprivation (IPS), a putative MADS box transcription factor (MADS) and a leucine rich repeat receptor-like protein (LRR). However, MADS was downregulated in response to Pi starvation thereby conversely regulated as expected from SSH result (Fig. 2). Additionally, a reverse run with inverted tester and driver cDNA was performed resulting in downregulated genes during Pi deficiency (data not shown). Not only that no MADS EST was detected in the resulting library but also no relevant genes with relation to root hair growth were sequenced.

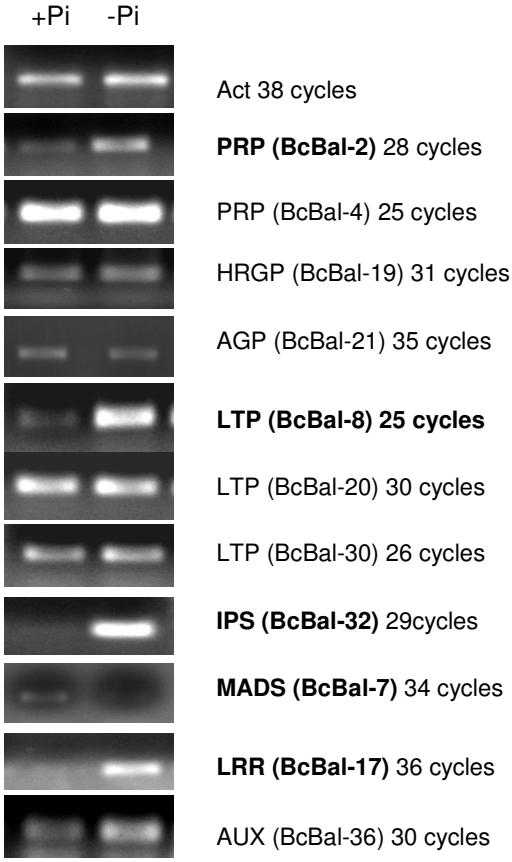


Fig. 2 Differential expression of genes resulting from SSH library as validated by semi-quantitative RT-PCR. Genes used for further analysis were indicated in bold letters. For abbreviations see Table 2

3.2. Nutrient specific and temporal expression pattern of Pi responsive genes after changing Pi and N supply

To examine the influence of other nutrients on root hair length and expression level of the candidate genes, plants were subjected to Pi, N and K depletion for 5 days. Root hair length was increased during Pi and N deficiency but not during K starvation (Fig. 3A). The expression of LRR was upregulated during Pi and N deficiency compared to control and K starvation. Gene activity of IPS was strongly induced by Pi starvation while no mRNA accumulation was detected in all other treatments. The deficiency of Pi resulted in a slight increase of PRP and LTP transcript level while N and K starvation were not effective. Instead, in Pi, N and K starved roots the expression of MADS was declined weakly to the level in control conditions (Fig. 3B).

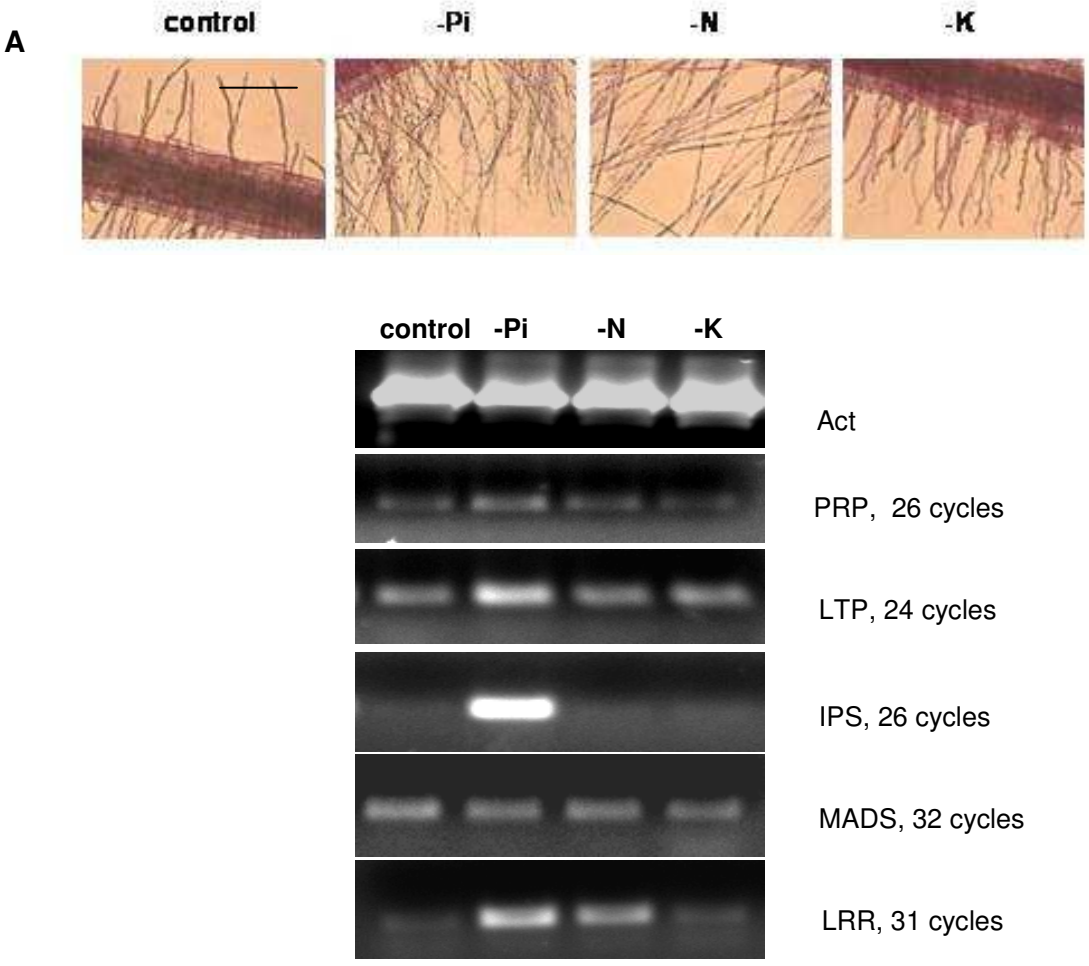


Fig. 3 Effect of five days starvation of Pi, N and K on (A) root hair phenotype and (B) differential gene expression in roots of *Brassica carinata*. Bar = 300 µm

To evaluate the temporal response of root hair growth and gene expression during Pi deficiency, plants were transferred after 5 days of growth in complete nutrient solution to Pi depleted nutrient solution and vice versa. Root hair length was increased or reduced within 4h or 2h, respectively by transferring the plants to Pi depleted or Pi repleted media, respectively (Fig. 4A). However, change in root hair phenotype was delayed (4h) after removal of Pi but occurred immediately 2h after Pi resupply. Correspondingly, expression of IPS and LRR was induced 24h after removal of Pi but decreased already 8h and 4h, respectively, after adding Pi (Fig. 4B). Meanwhile, the transcript abundance of PRP and LTP was slightly upregulated 48h after starting Pi starvation and raised continuously up to 96h while Pi readdition reduced transcript level after 24h. Expression level of MADS was not significantly affected by changing Pi supply.

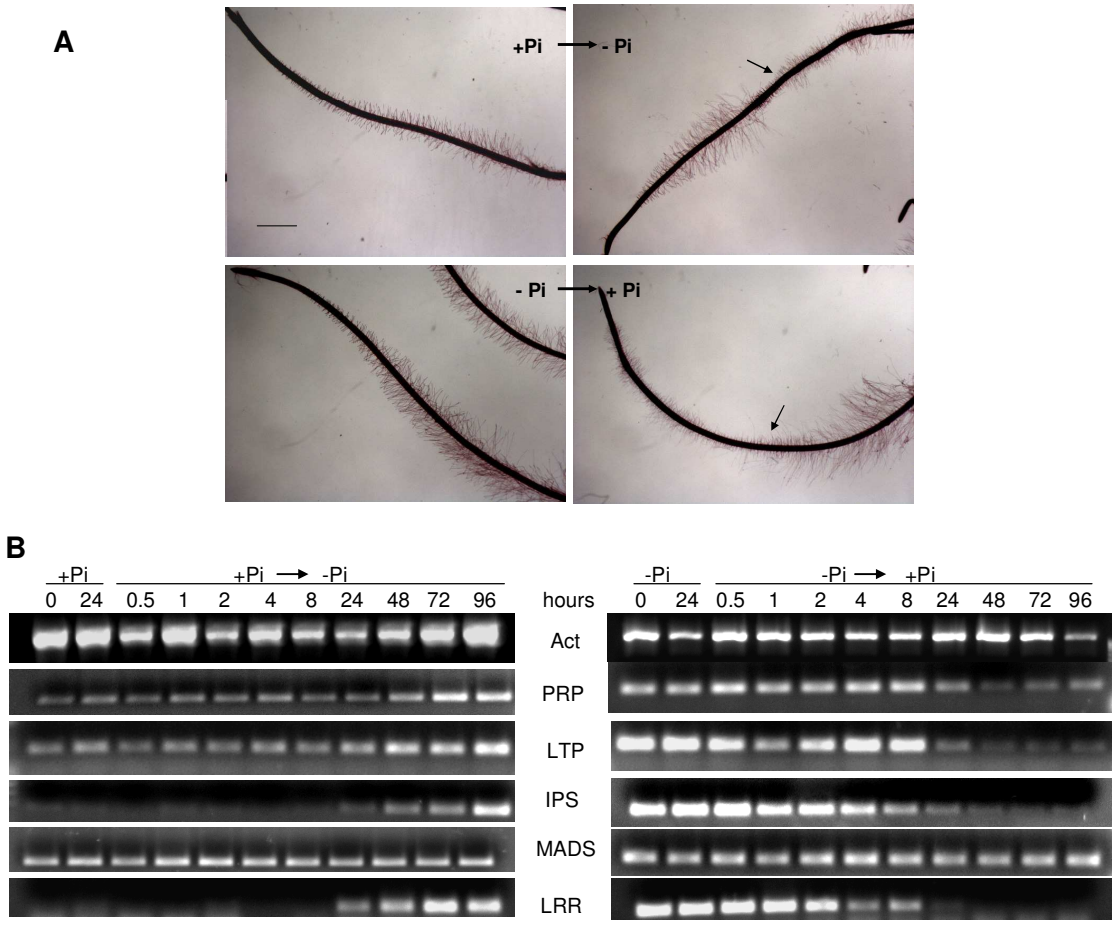


Fig. 4 Influence of changing Pi supply on (A) root hair phenotype of *B. carinata* 24h after changing Pi nutrition and (B) differential gene expression of candidate genes in roots as affected by time. Arrows indicates root tip at time point of transfer with growth rates of 1 cm/day. Bar = 1.6 mm

Since the root hair phenotype of *B. carinata* during Pi and N stress was quite similar the effect of changing N supply on root hair length and transcriptional regulation was evaluated in the same experimental design as with Pi. Enhanced (4h) or reduced (6.5h) root hair length was visible by removing or adding N, respectively, comparable to the phenotypic observations during Pi starvation (Fig. 5A). It is noteworthy that the growth rate after transfer to low N supply was 2.6 times higher compared to both Pi transfers and N resupply.

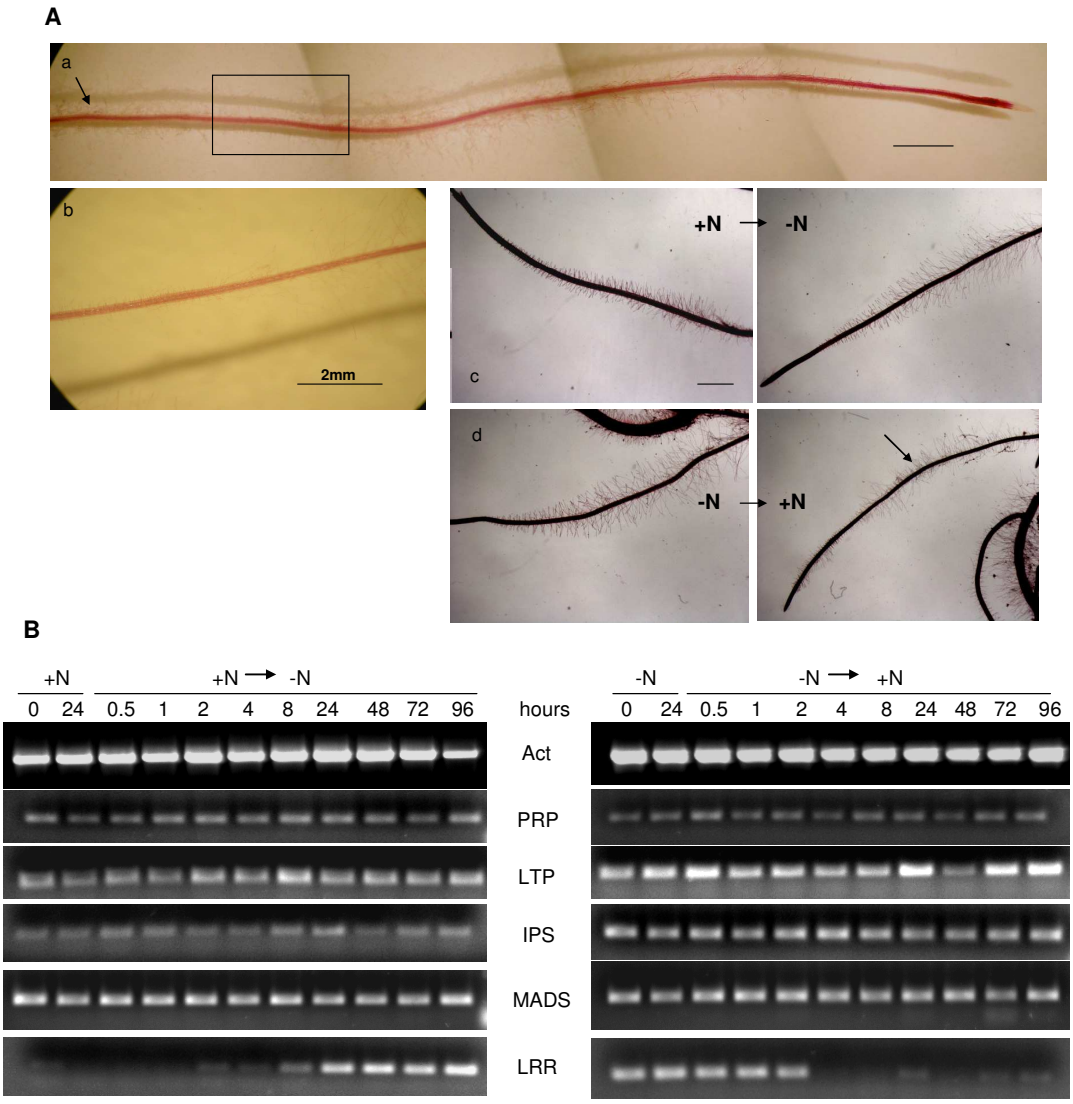


Fig. 5 Influence of changing N supply on (A) root hair phenotype of *B. carinata* 24h after changing N nutrition and (B) differential transcript level of candidate genes in roots as affected by time. (a) Merge of +N → -N transfer and box with higher magnification (b) and at the root tip (c). Root hair phenotype after transfer -N → +N (d). Arrow indicates root tip at time point of transfer with growth rates of 1 cm/day for -N → +N. Growth rate of +N → -N was 2.6 cm/day. Bar = 1.7 mm if not otherwise declared

There was a clear upregulation of LRR starting 2h after initiating N deprivation and increasing up to 24h whereas supply of N to N starved roots suppressed the transcript level of LRR after 4h. For LTP a weakly increased expression was observed 2h after removal of N and a fluctuating increase after N resupply (Fig. 5B). These changes in gene expression occurred faster compared to Pi transfer. However, no change in expression level was observed for PRP, IPS and MADS with varying N supply (Fig. 5B).

3.3. Spatial expression of candidate genes

For PRP, LTP, MADS and LRR genes a root-specific expression was observed with an upregulation of PRP, LTP and LRR and a weak downregulation of MADS during low Pi supply. However, for IPS in all organs an upregulation in response to Pi deficiency was observed (Fig. 6). Additionally, the expression pattern was examined along the root in sections representing initiation (0-1 cm), elongation (1-2 cm) and maturity (2-3 cm) stage of root hair development (Fig. 7). Transcription level of PRP, LTP, MADS and LRR was highest in sections 0-1 cm and 1-2 cm. Pi deficiency enhanced expression of PRP, LTP and LRR but was not effective for MADS. In contrast, IPS gene activity responded similarly to Pi deficiency in all root zones (Fig. 7).

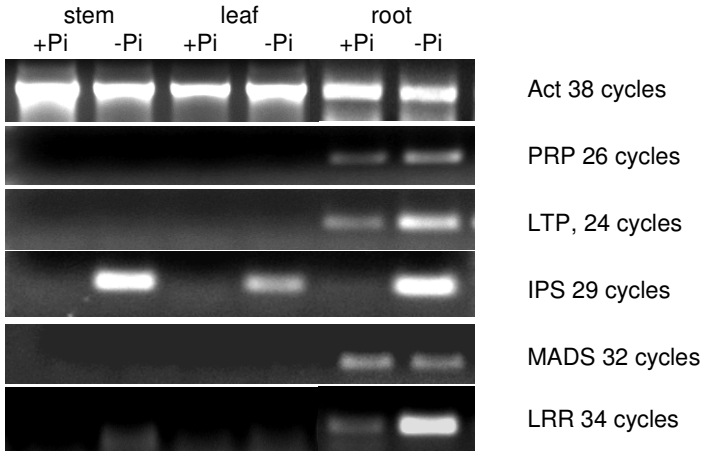


Fig. 6 Tissue-specific expression of differentially expressed genes in *B. carinata* as affected by Pi supply.

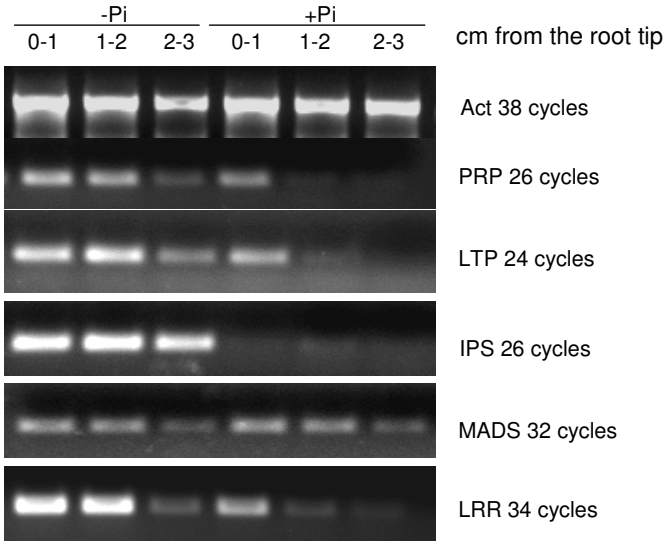


Fig. 7 Gene expression level along the root of *B. carinata* seedlings as affected by Pi supply.

4. Discussion

Pi deficiency in *Brassica carinata* cv. Bale resulted in more and longer root hairs (Fig. 1) which required a complex regulation of numerous genes. In this study, differentially expressed genes in response to Pi starvation in roots of *Brassica carinata* cv. Bale were identified by SSH analysis. The resulting ESTs were classified according to their annotated function in databases into five groups: metabolism, cell wall, stress and defence response, signal transduction and miscellaneous (Table 2). Those genes will be discussed which putatively have a function in Pi signaling or root hair development. In the group **metabolism** no ESTs were found to have an obvious function in root hair growth. In the group **cell wall** upregulation of two proline rich proteins (PRP), a hydroxyproline rich glycoprotein (HRGP) and an arabinogalactan protein (AGP) was found. Previously, PRPs and HRGPs were reported to be upregulated by Pi deficiency in common bean (Hernandez et al. 2007) and Hammond et al. (2003) detected an AGP in shoots of *Arabidopsis* after 4h of Pi depletion. Cell wall proteins are involved in composition and restructuring of the cell wall during growth processes (Showalter, 1993; Cassab, 1998). In the category **stress and defence response**, several non specific lipid transfer proteins (nsLTP) were upregulated in low Pi supply and this was also shown in rice (Wasaki et al. 2003a). The family of nsLTP is highly diverse and was originally defined by their capacity to transfer lipids between membranes *in vitro* and is regulated by diverse stress factors (Kader, 1996; 1997). Furthermore, a member of the IPS gene family (IPS) was induced by low Pi supply. IPSs are non coding molecules putatively involved in Pi homeostasis (Burleigh and Harrison, 1999; Martin et al. 2000; Shin et al. 2006a; Franco-Zorilla et al. 2007). The group **signal transduction** revealed a regulation of a MADS box transcription factor (MADS) and a member of this family was associated with N signaling before (Zhang and Forde, 1998) suggesting a function in Pi signaling as well. Additionally, Pi starvation enhanced expression of a member of the leucine rich repeat receptor-like protein kinases (LRR-RLK) which might play a role in signal perception and protein-protein interactions (Kobe and Deisenhofer, 1994; Torii, 2004). However, in rice

leaves a LRR receptor-like kinase was found to be downregulated in long-term Pi starvation (Wasaki et al. 2003a).

The previously discussed genes of the subtractive cDNA library were validated by semi-quantitative RT-PCR for differential expression. This confirmed an upregulation of PRP (BcBal-2), LTP (BcBal-8), IPS (BcBal-33) and LRR (BcBal-18) during Pi starvation. However, MADS (BcBal-7) was downregulated in response to Pi deprivation opposite as expected (Fig. 2).

4.1. Root hair phenotype in response to nutrient deficiencies

Pi and N depletion but not K starvation enhanced root hair length (Fig. 3A) which was in agreement with observations in *Arabidopsis* by Bates and Lynch (1996). Additionally, root hair length was calculated to be increased and reduced 4h and 2h after removal and resupply of Pi, respectively assuming constant growth rates (Fig. 4A). The later response of root hair length after transfer from +Pi to -Pi supply might be caused by a dilution effect in the root tissue leading to a delayed sensing of Pi starvation. Furthermore, the rapid response (within hours) suggested a local sensing of Pi availability. Likely, alterations in root hair growth were observed 10h after changing Pi supply in *Arabidopsis* and were attributed to a regulation at the root level (Bates and Lynch, 1996). The resupply of N to N starved roots reduced root hair length after 6.5h while removal of N resulted in longer root hairs after 4h (Fig. 5A). However, this latter transfer resulted in 2.6 times greater growth rates compared to the other transfer experiments with a constant growth rate of 1 cm/ day. This might explain the more rapid morphological response by withdrawal compared to resupply of N since the dilution effect of N in root tissue should be the same as with Pi.

Genes with a putative function in root hair growth induced by changes in Pi and/or N supply could respond in a temporal concurrence to alterations in the phenotype. Therefore, dynamics of gene expression were investigated after changing Pi and N supply (Fig. 4B, 5B) and relationship with root hair development will be evaluated.

4.2. The expression of LRR is correlated to root hair growth

The expression of the putative receptor molecule LRR was highly upregulated in roots in response to Pi and N depletion (Fig. 3B). Transfer of well-fed plants to both Pi and N deficiency induced upregulation of LRR within 24h and 2h, respectively and was rapidly repressed (4h) after resupply of Pi and N (Fig. 4B, 5B). The expression of LRR was restricted to the root (Fig. 6) and most pronounced in Pi starved root tip where root hairs start to differentiate and elongate (Fig. 7). The temporal concurrence of the expression pattern with the development of root hairs (Fig. 4A, 5A) suggested a common signaling pathway involving LRR in regulating root hair growth during Pi and N depletion. Furthermore, the faster response to changes in N supply may refer to a direct signal transduction with nitrate as signal molecule (Fig. 5B; Zhang et al. 2007) whereas Pi itself was not suggested to be the initial signaling molecule in Pi starvation processes (Hoffland et al. 1992; Jeschke et al. 1997; Burleigh and Harrison, 1999). However, changes in root hair length were observed more rapidly after changing Pi supply than N supply questioning the velocity of LRR expression pattern.

The high expression in the root tips during Pi stress strengthened a role for LRR in early root hair development. Jones et al. (2006) described a LRR-RLK (MRH1) that is required for root hair elongation but not for initiation. Additionally, a positive influence on root length was reported for SUNN (Schnabel et al. 2005). However, two examples of LRR receptor kinases were given for a role in inhibiting cell growth and therefore have a contrasting effect as here hypothesized: THESEUS1 (THE1) inhibited cell elongation if cellulose synthesis is perturbed (Hématy et al. 2007). Another membrane receptor (FERONIA) arrested the pollen tube growth upon arrival on female gametophyte (Escobar-Restrepo et al. 2007). In summary, LRRs can have positive or negative function in growth processes.

According to the expression pattern by changing Pi and N supply and the known function of LRR receptor like kinases a model was hypothesized to elucidate the function of LRR in

regulating root hair length: LRR perceives a ligand, an up to now unknown stimulus produced by changes in external Pi and N concentrations. Ligand binding at the extracellular LRR domain leads to activation of the intracellular kinase domain and subsequent transduction to downstream changes of gene transcription in the nucleus. This regulated transcription level initiates an early step in signal transduction leading to longer root hairs. To our knowledge no association between LRR receptor like protein kinases and nutrient signaling was up to now reported. Likewise, the ligands that bind to the LRR domains are very rarely clarified. So far known ligands were classified to peptides, proteins, carbohydrates, or small molecules such as steroids (reviewed in Morillo and Tax, 2006). In N signaling it was discussed that nitrate itself can act as the signal for downstream components. There is some evidence of a dual function of nutrient sensors and nutrient transporters but details of the signaling pathway are unknown (Zhang et al. 2007). Recently, a LRR-RLK of *Arabidopsis*, named SCRAMBLED (SCM), also known as STRUBBELIG, was shown to be responsible for position-dependent formation of root hair cells (Kwak et al. 2005). In *scm* mutant plants the arrangement of hair bearing and non hair cells is disturbed leading to the assumption that SCM acts as a receptor that can sense and mediate so far unknown positional cues determining cell fate (Kwak et al. 2005; Kwak and Schiefelbein, 2007). Although Pi and N starvation has an influence on root hair number no association of SCM to Pi or N response is up to now established.

It will be of special interest to identify the potential signaling pathway in nutrient sensing and to characterize the role, if any, for LRR. Furthermore, the identification of the unknown factor upregulating LRR transcription, the eliciting ligand and the determination whether there are common components in Pi and N signaling helps to understand nutrient signaling.

4.3. PRP and LTP are not required for root hair development

Pi deficiency induced a weak upregulation of PRP and LTP transcription compared to control, N and K starved roots (Fig. 3). Furthermore, Pi starvation affected PRP and LTP expression positively (Fig. 4B) while changing N supply showed no impact on gene

expression (Fig. 5B). The gene activity of both genes was restricted to the root (Fig. 6) and along the root most pronounced in the Pi starved root tip where both root hairs start to develop and the root is expanding (Fig. 7). The dynamics of gene expression suggested no relation to early steps of root hair growth since changes in gene expression occurred 48h after resupply or removal of Pi, whereas root hair phenotype was already different after 4h. Furthermore, an essential function in root hair development can be ruled out since PRP and LTP expression were not changed during N starvation leading also to increased root hair length (Fig. 5A, B). As a cell wall protein it is more likely that PRP functions in cell wall assembly in the growing root during Pi deficiency and other members of this family might fulfil this role in response to other stress factors. A participation in growth processes is strengthened by observations in hot pepper where the expression of a PRP (CaPRP1) was highly associated with elongation of cells in roots and leaves (Mang et al. 2004). Instead, Bernhardt and Tierney (2000) showed a coexpression of a root hair specific PRP (AtPRP3) with root hair formation in *Arabidopsis* and another study a predominant expression during the elongation stage in cotton fiber development showing the same tip modality as in root hairs (Tan et al. 2001; Feng et al. 2004). Non specific LTPs take part in the lipid metabolism of the plant membranes and were suggested to be involved in growth processes. In cotton fibers, an upregulation of nsLTPs was associated with fiber elongation (Feng et al. 2004). Observations for nsLTPs were often made in leaves and flowers but rarely in roots (Soufleri et al. 1996; Clark and Bohnert, 1999; Sohal et al. 1999; Arondel et al. 2000). However, in tobacco plants an increased transcription was observed in root epidermis and root hairs (Canevascini et al. 1996). Another aspect of LTP function was suggested by Nieuwland et al. (2005). In this study, LTP expression was mediating cell wall loosening in tobacco pistils. Recently, a role for nsLTPs in activation of polygalacturonases in pectin degradation was predicted (Tomassen et al. 2007). In summary, PRPs and LTPs are involved in extension processes in plant tissues.

4.4. Pi depletion induced IPS expression proposing a role in Pi signaling

IPS was highly upregulated by Pi depletion (Fig. 3B) with an induced transcription 24h after Pi withdrawal and a downregulation within 4h after readdition whereas N supply had no impact (Fig. 4B, 5B). IPS was expressed throughout the plant during Pi starvation showing no differential expression along the root (Fig. 6, 7). IPS could act as a Pi specific signal molecule with a function in early Pi signaling not directly related to root hair development. A Pi specific regulation of related molecules of IPS was also reported by Burleigh and Harrison (1999) in roots of *Arabidopsis*. However, analogues of IPS were reported to be induced after several days and involvement in maintaining Pi homeostasis was suggested (reviewed by Valdés-López and Hernández, 2008). Burleigh and Harrison (1999) demonstrated that the signal for controlling IPS expression is shoot-borne and that the regulation occurs systemically. The rapid induction (within hours) in the present study suggested an up to now unknown function for IPS in Pi signaling. Likewise, Müller et al. (2004) detected a decreasing transcript level of IPS 1h after Pi resupply in *Arabidopsis* roots but no function was specified. In *Arabidopsis*, IPS1 expression was shown to be controlled by PHR1, a key transcription factor mediating Pi response with no function in regulating root hair growth (Rubio et al. 2001). It might be possible that IPS1 triggers early downstream responses independent from PHR1 (Valdés-Lopez and Hernández, 2008).

4.5. The expression of MADS suggested no function in Pi response

The transcript level of MADS was weakly downregulated in response to all nutrient depletions (Fig. 3). However, no altered transcription level of MADS was determined by changing Pi and N supply (Fig. 4B; 5B). The expression was restricted to the root being most pronounced in the root tip (Fig. 6; 7). These observations suggested no correlation to root hair formation but rather to a general function in root growth irrespective of Pi nutrition. In contrast, in nitrate signaling a MADS box transcription factor was identified involved in the

localized morphological response of roots to nitrate (Zhang and Forde, 1998; Zhang et al. 2007).

4.6. Conclusion

Pi and N starvation both induced the formation of longer root hairs in *B. carinata* cv. Bale. Consistently, the increased expression of the receptor-like kinase LRR by Pi and N depletion suggested a function in a common signaling pathway inducing root hair elongation. This was strengthened by a high expression in the root tips where signals are perceived and root hairs start to initiate and elongate. For PRP, LTP and IPS a Pi specific regulation was observed with no obvious role in root hair development because of a delayed increase in expression after Pi removal (PRP, LTP) or no impact of N depletion (PRP, LTP, IPS). Whereas for PRP and LTP a role in root development was suggested because of high expression in the growing part of the root the function of IPS remains elusive because of a constant high expression in all plant organs exposed to -Pi. For MADS a general function in the root is likely with no correlation to root hair formation since no altered gene expression was observed during Pi and N starvation.

Chapter III

Transcriptomic analysis of differentially expressed genes in response to Pi starvation in *Brassica carinata* cultivars differing in root hair growth

Abstract

The efficiency of Pi in *Brassica carinata* cultivars was associated with the formation of longer root hairs in the Pi efficient cv. Bale compared to the Pi inefficient cv. Bacho. For the identification of genes responsible for root hair formation induced by Pi deprivation transcriptional profiling was performed by using *Arabidopsis thaliana* whole genome microarrays. Differentially expressed genes were evaluated by comparing the transcriptome of cv. Bale and cv. Bacho grown five days in Pi deprived conditions. Additionally, genes known to be involved in root hair development were included in further analysis. Candidate genes with a putative function in root hair growth during Pi starvation were (homologous genes to *Arabidopsis*): XTH (xyloglucan endotransglucosylase), MAPK (mitogen activated protein kinase), EXP (expansin-like), F-box family protein and LRX1 (leucine rich repeat/ extensin protein). Root hair phenotype and expression level of candidate genes were investigated in response to Pi, N and K starvation, after changing Pi supply and along the root by qRT-PCR.

Root hair length was enhanced besides Pi during N starvation. As well, root hair length was increased and reduced 4h and 2h after removal and resupply of Pi, respectively. XTH expression was upregulated in both cultivars during -Pi. Furthermore, expression was enhanced and reduced 48h and 2h after removal and resupply of Pi in Bale. Together with the differential expression along the root a function in stages of root hair and root development was suggested that were similar in both cultivars. LRX1 was converse regulated in Bale and Bacho in response to Pi starvation. Moreover, the delayed reduction in expression level after Pi resupply and a high differential expression in section 1-2 cm and 2-3 cm comparing +Pi and -Pi, respectively, suggested a function in root hair elongation that was different in Bale and Bacho. In contrast, F-box family protein was downregulated during -Pi in Bacho and upregulated in Bale in response to Pi and N deprivation both enhancing root hair growth. Meanwhile, the expression was ineffective to Pi removal and delayed after resupply. F-box could be involved in the regulation of target proteins by ubiquitin-mediated proteolyse. However, EXP and MAPK replied at no time to changes in Pi supply and the function of these genes remains elusive.

Keywords: root hair, Pi deficiency, microarray, Pi efficiency

1. Introduction

Phosphorus is an essential mineral nutrient for plant growth and development (Abel et al. 2002; Vance et al. 2003). Plants take up phosphorus in the form of orthophosphate (Pi) via the roots. Although Pi is relatively abundant in the soil, a significant amount of Pi is not readily available for plants because of binding to cations and organic compounds (Raghothama, 1999). Therefore, plants have evolved many morphological and physiological adaptations to cope with low Pi. An increased root to shoot ratio, enhanced lateral root growth and formation of root hairs resulted in the exploration of a greater soil volume (Lynch, 1995; Bates and Lynch, 1996; Jungk, 2001; Williamson et al. 2001). Moreover, the metabolism is changed by bypassing Pi requiring steps and reutilization of Pi (Duff et al. 1989; Essigmann et al. 1998). On the transcriptional level the expression of RNases (Bariola et al. 1994) and acid phosphatases (Duff et al. 1994; del Pozo et al. 1999; Haran et al. 2000) is enhanced during Pi starvation. Together with the exudation of organic acids this contributes to release Pi from internal and external Pi sources (Bariola et al. 1994; Duff et al. 1994; Raghothama, 1999; Lopez-Bucio et al. 2000). Additionally, upregulation of high affinity Pi transporters in the root enhances Pi uptake from the soil solution (Muchhal et al. 1996; Daram et al. 1999; Mudge et al. 2002).

It has been reported that many adaptive strategies in low Pi environment are regulated at the transcriptional level (Raghothama, 1999). Gene expression profiling in several plants has revealed genes with altered transcription during Pi starvation (Hammond et al. 2003; Wasaki et al. 2003a; Wu et al. 2003; Morcuende et al. 2007; Müller et al. 2007). However, the mechanisms that result in enhanced formation of root hairs during Pi deprivation are not well understood. As a global regulator for several Pi starvation responses PHR1 (PHOSPHATE STARVATION RESPONSE1) was identified (Rubio et al. 2001). PHR1 encodes for a Myb transcription factor and regulates the expression of target genes like acid phosphatases, phosphate transporters, a Pi depletion specific molecule (AtIPS1), and ribonucleases by binding to an imperfect palindromic sequence in their promoter (Martin et al. 2000; Rubio et al. 2001). But, PHR1 did not affect the number and length of root hairs under Pi depleted

conditions in *Arabidopsis* (Rubio et al. 2001; Nilsson et al. 2007) whereas overexpression of a PHR1 homologue in rice resulted in enhanced root elongation and enhanced root hair growth independent of Pi nutrition (Zhou et al. 2008). Nevertheless, two transcription factors have been described with a function in Pi starvation induced root hair elongation: BHLH32 and WRKY75 act as negative regulators of some Pi starvation induced processes including root hair formation (Chen et al. 2007; Devaiah et al. 2007). The molecular function of these genes is still unknown but these observations suggest that there exist many independent signaling pathways in regulating Pi starvation response.

Root hairs are protrusions from specialized epidermal cells, the trichoblasts. Genes involved in root hair development were reviewed by Bibikova and Gilroy (2003). The growth of root hairs is controlled by the genetic background and to a great part by environmental stimuli as Pi depletion. A putative function of particular genes in root hair growth during Pi deficiency was investigated by mutant analysis (Müller and Schmidt, 2004).

In this study, two Ethiopian mustard (*Brassica carinata*) cultivars differed in the ability to respond to low Pi with longer root hairs. Microarray experiments were performed comparing gene expression of both cultivars during Pi depletion in order to find differentially expressed genes related to root hair growth. A whole genome microarray of *Arabidopsis thaliana* was used since *Brassica* is not fully sequenced but both belong to the family of *Brassicaceae* and therefore high homologies were expected. Expression level of candidate genes was further examined in response to Pi, N and K starvation, after changing Pi supply and along the root axis to link a potential function in root hair growth.

2. Materials and Methods

2.1. Plant material and cultivation

Seeds of *Brassica carinata* cv. Bale and cv. Bacho were vernalized in the dark for three days at 4°C and then germinated for 5 days in the growth chamber with 16h light (20°C)/8h dark (15°C) photoperiod, a photosynthetically active radiation of 200 µmol photons $m^{-2}s^{-1}$ and a relative humidity of 70%. Uniform seedlings were transferred to a continuously aerated nutrient solution containing 1 mM Pi as KH_2PO_4 or free of Pi. The other nutrient composition in the solution was in mM: 2.25 $Ca(NO_3)_2$; 2.5 K_2SO_4; 1 $MgSO_4$; 0.25 KCl; and in µM 25 H_3BO_3; 1.5 $MnSO_4$; 1.5 $ZnSO_4$; 0.5 $CuSO_4$; 0.025 $(NH_4)_6Mo_7O_{24}$ and 35.8 Fe (Fe^{III}- EDTA) at pH 5.5. After five days in nutrient solution lateral root tips of 2 cm length were harvested 2h after the light period started, frozen immediately in liquid nitrogen and stored at -80°C before use. Seedlings were also transferred to nutrient solution lacking nitrogen by substituting $Ca(NO_3)_2$ by $CaCl_2$ and potassium by omitting K_2SO_4 and KCl and substituting KH_2PO_4 by NaH_2PO_4. For transfer experiments 10-days-old seedlings grown either in complete nutrient solution or free of Pi were transferred to -Pi or +Pi nutrient solution, respectively and root tips of 1.5 cm length were harvested after 0.5, 1, 2, 4, 8, 24, 48, 72 and 96h for RNA isolation. Root tips of plants remaining in original solution were harvested as control (0h, 24h). Additionally, sections of one cm (0-1 cm; 1-2 cm; 2-3 cm) beginning from the root tip of plants grown in +Pi and -Pi conditions were harvested after five days of cultivation for RNA isolation. Pictures of root phenotype were taken with the AxioCam MRc (Zeiss) under the Axioskop (Zeiss) after staining with 1% acid fuchsin (in A. dest).

2.2. RNA preparation and microarray experiment

Root samples were collected in Lysing Matrix D tubes (Bio 101® systems, Q-BIOgene, Montreal, Canada) and immediately frozen in liquid nitrogen. RNA extraction was performed with TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions with an initial homogenization step in a Fast Prep FP120 (BIO 101 Thermo Savant) for 45 sec at 6.0 speed. Yield and purity of the resulting RNA were quantified with the NanoDrop

Spectrometer (peqlab Biotechnology GmbH, Erlangen, Germany) and with an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany). First strand cDNA synthesis and labeling was performed in a total volume of 50 µl where 100 µg of purified total RNA of each treatment was reverse transcribed by incorporating 1 mM Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, USA), respectively, using the LabelStar Array kit (Qiagen, Hilden, Germany). After cDNA synthesis, remaining RNA was degraded by alkaline hydrolysis with 1 M NaOH at 65°C. Unlabeled primers and dyes were removed by using the Qiaquick PCR Purification Kit (Qiagen). The labeled samples were lyophilized in a SpeedVac followed by resolving in hybridization buffer (Ocimum Biosciences, IJesselstein, The Netherlands) and combination of the two labeled cDNA samples. The Cy3 and Cy5 labeled cDNAs were hybridized simultaneously in one experiment to the same array (dye swap-design). *Arabidopsis* oligonucleotide arrays (70mere, AROS V3.0, Operon) were provided and spotted by the lab of David Galbraith, University of Arizona. After over night hybridization at 42°C with gentle shaking (550 rpm) unbound and unspecifically fixed cDNA was removed from the array by stringent washings consecutively in 2x SSC + 0.1% SDS, 1x SSC and 0.5% SSC. Data from each array were collected with GenePix 6.0 (Axon Instruments Inc., Union City, CA). Therefore, the arrays were scanned different times with a 4000 B scanner (Axon Instruments). Images were quantified using GenePixPro 6.0 Software. The average pixel intensity within each spot was determined and a local background was computed for each spot. Net signal was determined by subtracting local background from the average intensity. Signals not consistently detectable (background corrected signal lower than two times of background standard deviation) were eliminated from further analysis. Following the primary analysis, data from different scans have to be summarized. Due to different laser power and PMT settings, the scans first have to be normalized by the sum of all corresponding spot-intensities. Afterwards, data from different scans for each individual spot can be averaged by the mean. Assuming, that the majority of genes on a whole genome microarray are not differently expressed, a locally weighted linear regression (*LOWESS*) has been employed as a normalization method in order to account for such intensity-dependent effects. The mean of

the data for differently labelled targets for each gene was taken (dye-swap). Assuming a normal distribution of the pre-processed data a standard two-state pooled-variance t-test (5% probability of error) was used in order to detect differentially expressed genes. The expression of many genes was too low (less than 1.0e-07) to perform data analysis and further, for higher reliability genes with more than 5-fold regulation were rated as differentially expressed. Genes meeting these requirements were checked for potential function in root hair development in publicly available databases (www.arabidopsis.org; www.genevestigator.com (Zimmermann et al. 2004)). However, for genes with a saturated signal of both spots no determination of differential expression was possible. For these genes, the same database search was undertaken and candidates were considered in quantitative realtime PCR (qRT-PCR) analysis. Additionally, a subset of genes with a reported function in root hair development of *Arabidopsis thaliana* was included in qRT-PCR analysis (Müller and Schmidt, 2004).

2.3. Quantitative real-time PCR (qRT-PCR) analysis

Results of microarray experiments were validated by qRT-PCR. Total RNA was isolated from roots with the NucleoSpin RNA Plant kit (Machery & Nagel, Düren, Germany). RNA was reverse transcribed using oligo(dT) primer with the RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) followed by an RNase H (10 U/µl, Epicentre, Madison, WI, USA) digestion for 20 min at 37°C. Real time PCR was performed on a CFX96 real time cycler (BioRad, München, Germany) using SYBR-Green I fluorescent dye (Invitrogen) and primers designed from *Arabidopsis*-specific gene sequences (www.arabidopsis.org) using the free Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm; Table 1). Standards for primer design were as followed: 20-22 bp, Tm 60°C, GC content 40-60% and an amplicon size in *Arabidopsis* of 100-180 bp. 100 ng of cDNA were used as template in a 25 µl PCR reaction: 14.74 µl H_2O, 2.5 µl 10x buffer, 3.6 µl $MgCl_2$ (25 mM), 0.5 µl dNTPs mix (10 mM each; Fermentas), 0.15 µl HotStart-Taq-Polymerase (5 U/µl; DNA cloning service, Hamburg, Germany), 0.25 µl 1:1000

diluted SYBR-Green I fluorescent dye, 0.63 µl forward and reverse primer (10 pM/µl each) and 2 µl cDNA (50 ng/µl). Primers for the ubiquitin (UBQ, forward primer

Table 1 Primer sequences designed from *Arabidopsis* used for qRT-PCR

Gene	*Forward primer*	*Reverse primer*
UBQ	5`AAACCCTAACGGGAAAGACG´3	5`GAGTTCTGCCATCCTCCAAC´3
PME2	5`TTGCCTTGACGGTTTCTCTTA´3	5`CATTGCTAATGCGTTGCTACA´3
XTH	5`AATAGCAACTCGTGGATGTGG´3	5`ACCTTGAGGGAACCTCTTGAA´3
EXP	5`ACACAACTTGGGGTTCTTGC´3	5`TATGGCCCTATGGCTACGAG´3
ROP	5`CGAGGATGAGAAGGAACCAG´3	5`TCTTCGTTTGATCGGTAGGC´3
MAPK	5`TCAGAGCGTGAACCAACAAC´3	5`TGATCACCACCACGAAGGTA´3
EXO	5`CTGCATCCTCTGGACATCCT´3	5`AAATGGTTCAGGCTGGTCAC´3
LRX	5`ACCGGTTTTGTGGGATCATA´3	5`ATCAGGCCAAGAGAGAACGA´3
LTP	5`GCTAACGTGTTGGATGTGGTTA´3	5`CAATGGTTTAGGACAACGTTCA´3
RHD1	5`AAAATCTCACCGTCCACCAG´3	5`ACGCTCTCACCAACTGCTTT´3
RHD2	5`TCAAGGCCTCAAAACTTCAAAT´3	5`GGTAATCATCTTGTGGTGCAGA´3
RHD3	5`TCGAAGAGACAGCAGCTTGA´3	5`TTGAACCAATCTTGGGAAGC´3
RHD4	5`AGCTTGATCAGTTCTTGCTTCC´3	5`CGTTTCTCCTAGTGCATCTCCT´3
RHL1	5`CCCACAGGGTCGTATGAAAC´3	5`GTCCCAATCCACCATGACTC´3
RHL2	5`TTAGGCCGAGTGATTTGGAC´3	5`GCCTGAATCTCAGCCTTTTG´3
RHL3	5`ACGACCCTAGTGGTGATTGTCT´3	5`CGTCTCTTCCACCCAAACTAAC´3
GL2	5`AAGCGCTATTCAAAGAGACACC´3	5`TTTTGGAACCAGAACTTGACCT´3
LRX1	5`GCTTCCGAGCTAGGTCTCCT´3	5`ACTTACCGACGAATCGGTTG´3
bHLH32	5`TAACGACGGTTTTGTCTCTCCT´3	5`ATTTTCCCATGATCTCACCATC´3
AP2	5`CGTTGATATTTCCTTCCAGAGG´3	5`AGACCTAGGACCTCTCCTGCTT´3
WD40	5`GCGATGTAATAACAGGGGAGAG´3	5`GACGTCGTTTATCTTCCACACA´3
LRX_{sat}	5`ACGCAGATATCGCTGGTTATTT´3	5`GGTTAAACCGGTGTGGTACAGT´3
LRX2	5`GTGCAGTGGATGATGAAGGTAA´3	5`CTTGGGTTTTCGAACTTGAGAC´3
WRKY57	5`GACTTCTTCGACCGAGACACTT´3	5`AGTGACGGTGGTAGGTTTGACT´3
WRKY36	5`TGCAAAGATGTGGAGAAGAAGA´3	5`GAAGAGGAGGAGCCAGATTGTA´3
Zincfinger	5`CAAAGTGTTCTCACCATTTCCA´3	5`CAGGATCAATGGCAACAAGTTA´3
JACKDAW	5`CAAGGTCTATCCGAGATGGTTC´3	5`AGGTGAGACTAGGGTTTGTGGA´3
F-box	5`ATGGCTTCCTCAAAGTGTTTGT´3	5`ATGAATCGTTCCGGTGAGTAGT´3
Protein kinase	5`GAAAGCAGAGCAAGTGGAGATT`3	5`ACTCACAGACCCAGGAACCTTA`3

5´-AAACCCTAACGGGAAAGACG-3´ and reverse primer 5´-GAGTTCTGCCATCCTCCAAC-3´) gene of *Arabidopsis* were used as reference gene and the relative expression levels of the genes were calculated with the $2^{-\Delta\Delta Ct}$ method of relative quantification (Livak and Schmittgen, 2001). Each real time run was performed with three biological and three technical repetitions. The following quantitative PCR program was used: 10 min at 95°C for 1 cycle followed by 15 sec at 95°C, 30 sec at 54°C, 30 sec at 72°C for 50 cycles. The threshold cycle (C_t) where the fluorescence signal raises above the background was automatically

determined and the results for each gene were normalized to the C_t level of the reference gene UBQ to calculate the relative gene expression. A melting curve (0.5°C steps from 65°C to 95°C) analysis was added after each PCR reaction to detect primer dimers or unspecific binding. The linearity and efficiency for each primer pair was checked by performing a qRT-PCR on 1:5 dilutions starting with 200 ng of first-strand cDNA samples of both cultivars independently (Table 2). Values minimum of 90% efficiency were aimed. Analysis and statistical evaluation was done with C_t values calculated by the BioRad CFX96 Manager program (BioRad) by determining the Confidence Interval (95%; $CI_{95\%}$ = [χ-1.96SE, χ+1.96SE]. The expression value of the calibrator was set at 1.0 and the relative expression levels were calculated according to this treatment. Calibrators were for validation of candidate genes Bacho +Pi, for nutrient element experiment Bale +, for transfer investigations Bale at start point 0h and for root zone experiment Bale +Pi (0-1 cm).

Table 2 Primer efficiency tests of used primers designed from *Arabidopsis thaliana* sequences in *B. carinata* cv. Bale and cv. Bacho for qRT-PCR. The desired value of this efficiency test is a minimum of 90% representing a doubling in PCR product with each cycle.

*** No primer pair could be designed according to chosen quality criteria**

Gene name	Primer efficiency (%)	
	Bale	Bacho
UBQ	107.7	101.2
PME2 At1g53830	90.7	103.6
XTH At5g57530	92.5	98.9
EXP At3g45960	102.8	103.6
ROP At3g23380	97.4	97.1
MAPK At1g18150	106.6	93.4
PME1 At3g17060	*	*
EXO At5g03540	108.2	97.9
LRX At4g33970	103.7	92.5
LTP At5g46890	104.8	*
RHD1 At1g64440	106.8	106.3
RHD3 At1g72960	99.7	96.7
RHL1 At1g48380	98.5	99.9
RHL2 At5g02820	110.2	100.6
RHL3 At3g20780	112.5	108.5
LRX1 At1g12040	100.8	97.2
RHD4 At3g51460	89.2	94.1
GL2 At1g79840	103.5	100.9
RHD2 At5g51060	99.7	91.3
bHLH32 At3g25710	181.9	187.8
WD40 At5g50120	101.4	107.0
LRX_{sat} At4g13340	98.3	*
LRX2 At1g62440	96.4	103.6
WRKY57 At1g69310	99.7	98.7
AP2 At5g60120	100.2	108.8
WRKY36 At1g69810	98.4	94.9
Zincfinger At5g15790	97.2	93.1
JACKDAW At5g03150	100.5	99.7
F-box family protein At3g08750	103.5	96.1
Protein kinase At4g25390	91.0	104.7

3. Results

3.1. Phenotypic characterization of B. carinata cultivars during Pi deficiency

Seedlings of *Brassica carinata* cultivars Bale (Pi efficient) and Bacho (Pi inefficient) were grown in complete nutrient solution or deprived of Pi for five days. Under Pi starvation root hairs of Bale were considerably more and longer than in Bacho whereas root hair length was similar in Pi sufficient conditions (Fig. 1). This time point was selected for microarray and quantitative real time RT-PCR (qRT-PCR) analysis.

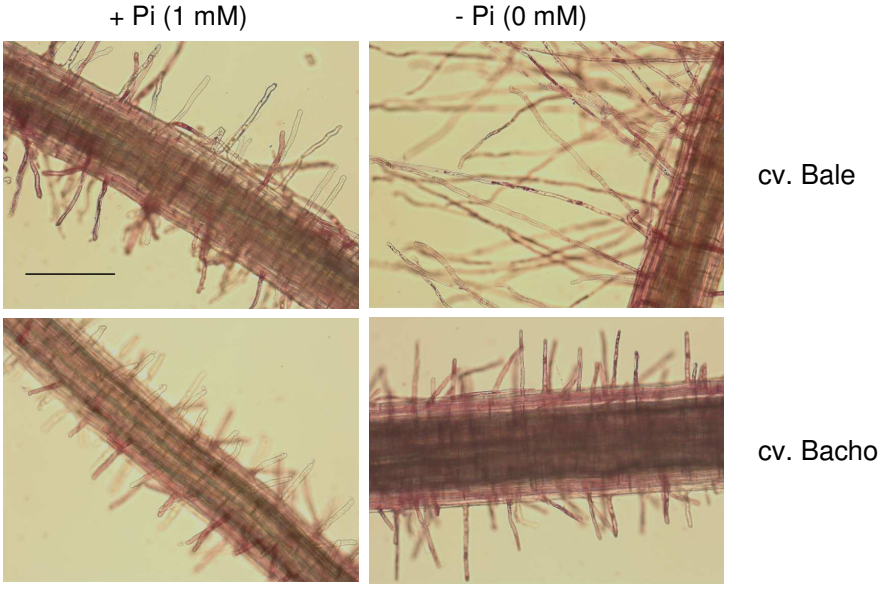


Fig. 1 Root hair phenotype of *B. carinata* cv. Bale and cv. Bacho in Pi sufficient and Pi deficient conditions. Bar = 300 µm

3.2. Identification of differentially expressed genes during Pi depletion by microarray analysis

To study changes in the *Brassica* root transcriptome during Pi deficiency, RNA of cultivars Bale and Bacho grown in Pi deficient nutrient solution was analysed using *Arabidopsis thaliana* oligonucleotide microarrays representing 29.953 elements (MWG Operon).

More than 220 genes were upregulated (more than 5-fold) in cultivar Bale compared to Bacho in response to Pi withdrawal representing approximately 1% of the *Arabidopsis* genome and more than 880 genes were higher expressed in cultivar Bacho (deposited in

GEO database http://www.ncbi.nlm.nih.gov/geo/: account GSE22677). For the majority of genes represented on the whole genome *Arabidopsis* microarray the expression level appeared unchanged or regulation was low (2-5-fold). Genes with a putative function in root hair growth according to databases were selected for evaluation by qRT-PCR. Candidate genes being upregulated in cv. Bale compared to cv. Bacho were (with the corresponding gene ID in *Arabidopsis)*: pectinmethylesterase (PME2, At1g53830), xyloglucan endotransglucosylase (XTH, At5g57530), MAP kinase (MAPK, At1g18150) and lipid transfer protein (LTP, At5g46890; Fig. 2). Candidate genes that were downregulated in the Pi efficient cv. Bale compared to cv. Bacho were: expansin like protein (EXP, At3g45960), ROP binding protein (ROP, At3g23380), another pectinmethylesterase (PME1, At3g17060), exocyst subunit (EXO, At5g03540) and leucine rich repeat family/ extensin family protein (LRX, At4g33970; Fig. 2).

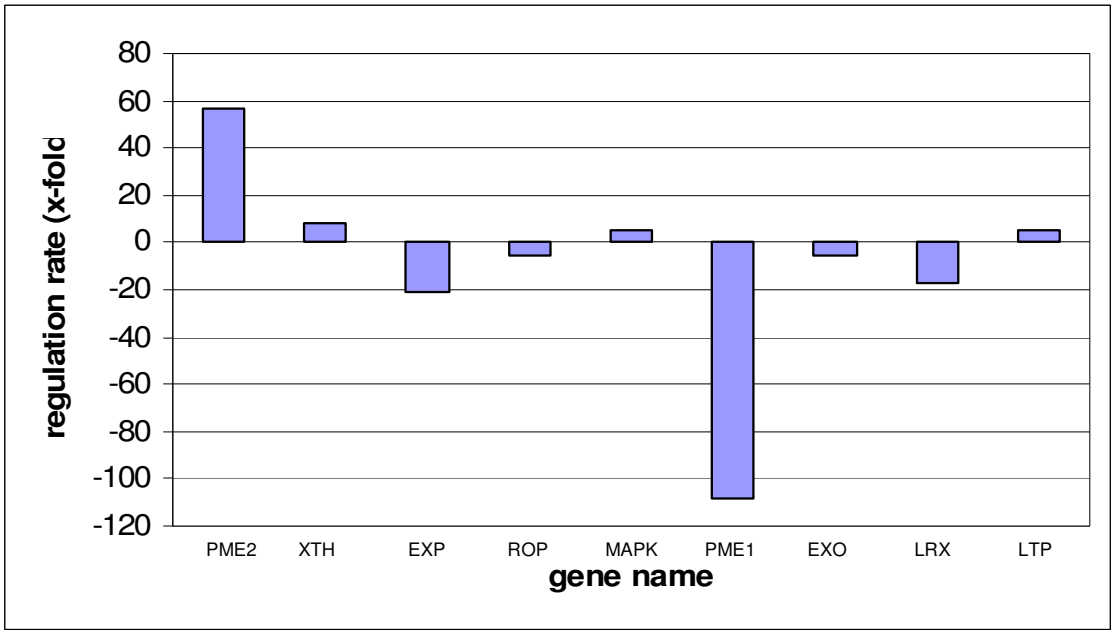


Fig. 2 Candidate genes selected from microarray analysis with a putative function in root hair development. Positive regulation rate is equivalent to an upregulation in Bale -Pi compared to Bacho -Pi while negative regulation rate is equivalent to a downregulation in Bale -Pi.
PME *pectinmethylesterase,* **XTH** *xyloglucan endotransglucosylase,* **EXP** *expansin-like protein,* **ROP** *ROP binding protein,* **MAPK** *mitogen-activated protein kinase,* **EXO** *exocyst subunit,* **LRX** *leucine rich repeat/extensin protein,* **LTP** *lipid transfer protein*

Furthermore, for approximately 400 genes a determination of differential expression was not possible due to a saturated signal of both spots (hybridized with cDNA from Bale and Bacho, respectively) on the microarray. Genes were checked for potential relevance in Pi starvation responsive root hair growth in databases and 10 genes were considered for qRT-PCR analysis.

3.3. Validation of candidate genes by quantitative RT-PCR (qRT-PCR)

Quantitative real time PCR (qRT-PCR) was used for confirmation of the microarray results. For PME1 no primer pair could be designed may be due to mismatch bases in the homologous gene in *Arabidopsis*.

In complete nutrient solution a higher expression in Bale than in Bacho was determined for all examined genes (Table 3). In Bacho, Pi starvation induced expression of PME2, LTP, ROP, EXO and XTH while MAPK was downregulated whereas transcript level for LRX and EXP was not changed. In Bale, Pi depletion resulted in a higher expression for XTH and EXP while a downregulation was visible for EXO, LRX and MAPK. No significant regulation induced by Pi stress was determined for PME2, LTP and ROP in Bale (Table 3). Comparing gene expression in Bacho and Bale during Pi deprivation MAPK and EXP were upregulated in Bale while all other genes were not differentially expressed (Table 3). Upregulation of XTH and EXP and downregulation of MAPK expression during Pi starvation in Bale together with a differential expression of MAPK and EXP in Pi stressed roots of both cultivars suggested a function in Pi starvation induced root hair growth.

QRT-PCR confirmed microarray results with regard to an increase of transcript level for MAPK in Bale compared to Bacho under Pi starvation. However, for all other genes no differential expression or even an opposite regulation was observed (Table 3).

Table 3 Relative transcript level of genes putatively involved in root hair development in roots of *B. carinata* cultivars as affected by Pi supply. Band intensities were normalized to UBQ band intensity and expressed as relative transcript level to Bacho +Pi that was defined as 1.0. Means followed by same letters are not significantly different (Confidence Interval 95%)

Gene	*Bacho +Pi*	*Bale +Pi*	*Bacho -Pi*	*Bale -Pi*
PME2	1^{b}	76^{a}	123^{a}	76^{a}
LTP	1^{b}	123^{a}	78^{a}	101^{a}
ROP	1^{b}	162^{a}	161^{a}	90^{a}
EXO	1^{c}	108^{a}	13^{b}	12^{b}
LRX	1^{b}	11^{a}	1.6^{b}	1.5^{b}
XTH	1^{c}	15^{b}	89^{a}	160^{a}
MAPK	1^{b}	32^{a}	0.1^{c}	1.3^{b}
EXP	1^{c}	8^{b}	1^{c}	57^{a}

Additionally, selected genes with a saturated signal of both spots in microarray analysis were evaluated by qRT-PCR analysis. In Pi sufficient conditions in Bale a higher transcript level was observed compared to Bacho except for F-box similar to previously examined genes (Table 4). In Bacho, Pi deficiency induced the expression of WD40, LRX_{sat} and LRX2 but decreased expression of AP2, JACKDAW and F-box family protein and was ineffective for all other genes. In Bale, the transcription in response to -Pi was enhanced for WD40 and F-box family protein while it was not significantly different for all other candidate genes (Table 4). No effect of low Pi on transcription level comparing both cultivars was detectable for LRX_{sat} and LRX2 while for all other genes a higher expression in Bale was observed. The converse regulation of an F-box family protein in response to -Pi in Bale and Bacho and the differential expression comparing Pi deficiency induced gene expression in Bale and Bacho suggested a putative function of F-box in Pi starvation response.

Table 4 Relative transcript level of genes with a regulatory function as affected by Pi supply. The relative amount of mRNA in Bacho +Pi was defined as 1.0. Means followed by same letters are not significantly different (Confidence Interval 95%)

Gene	*Bacho +Pi*	*Bale +Pi*	*Bacho -Pi*	*Bale -Pi*
WD40	1^{d}	70^{c}	197^{b}	353^{a}
LRX_{sat}	1^{b}	32^{a}	76^{a}	56^{a}
LRX2	1^{b}	228^{a}	89^{a}	91^{a}
WRKY36	1^{b}	36^{a}	0.71^{b}	23^{a}
WRKY57	1^{b}	96^{a}	0.48^{b}	92^{a}
AP2	1^{b}	17^{a}	0.37^{c}	20^{a}
Zincfinger	1^{b}	20^{a}	0.46^{b}	25^{a}
JACKDAW	1^{b}	21^{a}	0.24^{c}	25^{a}
F-box	1^{b}	0.8^{b}	0.005^{c}	3.1^{a}
Protein kinase	1^{b}	44^{a}	0.74^{b}	34^{a}

Furthermore, genes with a known function in root hair growth were investigated by qRT-PCR (Table 5). In Pi sufficient conditions, expression of all genes was higher in Bale than in Bacho except for GL2 that showed no differential activity (Table 6). Pi starvation induced in Bacho expression of all examined genes excluding LRX1 which was downregulated and bHLH32 that was not regulated. In Bale, Pi starvation upregulated GL2 and LRX1 and downregulated RHD1, RHD3 and RHD4 transcription while it was not effective for all other genes (Table 6). By comparing transcript level in Bacho and Bale during Pi depletion expression was increased for RHD2, RHD3, LRX1 and bHLH32 and was unchanged for all other genes. The gene LRX1 was conversely regulated in response to Pi stress in Bale and Bacho and was taken into account for a function in Pi depletion induced root hair formation. No function in root hair growth during Pi limitations was attributed to RHD2 and bHLH32 because of no differential expression in Bale. Likewise, the gene RHD3 fitted to the selection criteria but the reported positive function in root hair development was not congruent to a downregulation in Pi starved roots of Bale and therefore not explainable.

Table 5 Function of investigated genes in root hair development

Gene name	Function in root hair growth	Reference
GLABRA2 (GL2)	formation of non hair cells	Rerie et al. 1994; Di Cristina et al. 1996
ROOT HAIRLESS GENES (RHL1, RHL2, RHL3)	root hair initiation	Schneider et al. 1997; 1998
ROOT HAIR DEFECTIVE (RHD1)	restricting root hair swelling size	Schiefelbein and Somerville, 1990
ROOT HAIR DEFECTIVE (RHD2, RHD3, RHD4)	tip elongation	Galway et al. 1997; Wang et al. 1997, 2002a; Jones et al. 2002
LEUCINE RICH REPEAT/EXTENSIN1 (LRX1)	root hair morphogenesis	Baumberger et al. 2001; 2003
bHLH32	Root hair growth induced by -Pi	Chen et al. 2007

Table 6 Relative transcript level of genes involved in root hair growth in roots of *B. carinata* cultivars as affected by Pi supply. The relative amount of mRNA in Bacho +Pi was defined as 1.0. Means followed by same letters are not significantly different (Confidence Interval 95%)

Gene	*Bacho +Pi*	*Bale +Pi*	*Bacho -Pi*	*Bale -Pi*
GL2	1^{b}	1^{b}	32^{a}	33^{a}
RHL1	1^{b}	49^{a}	32^{a}	52^{a}
RHL2	1^{b}	32^{a}	30^{a}	32^{a}
RHL3	1^{b}	85^{a}	45^{a}	118^{a}
RHD1	1^{c}	53^{a}	16^{b}	26^{b}
RHD2	1^{c}	137^{a}	70^{b}	100^{a}
RHD3	1^{d}	416^{a}	31^{c}	151^{b}
RHD4	1^{c}	89^{a}	75^{ab}	44^{b}
LRX1	1^{c}	28^{b}	0.4^{d}	751^{a}
bHLH32	1^{b}	230^{a}	2.5^{b}	220^{a}

3.4. Effect of nutrient supply on root hair length and gene expression

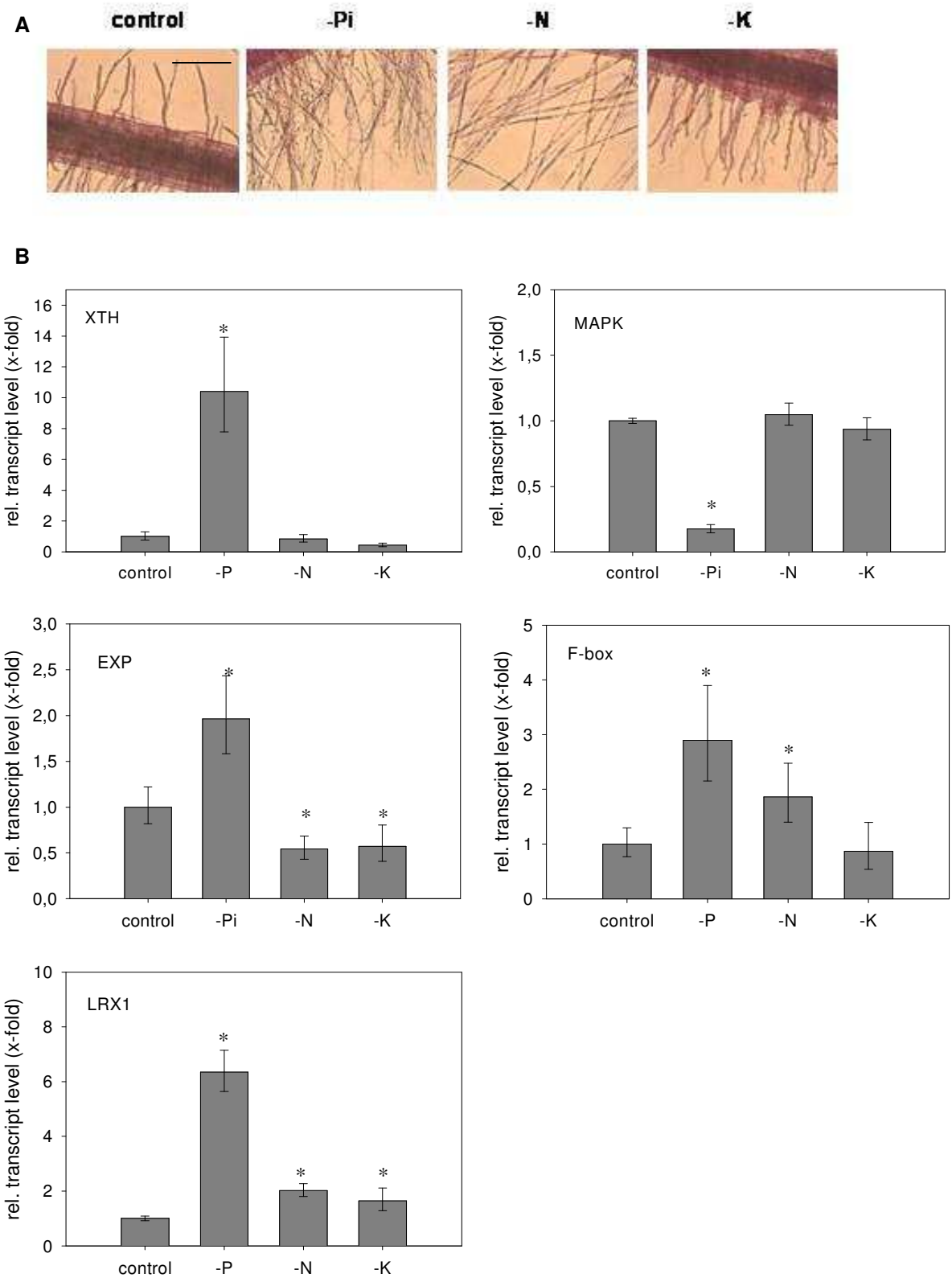
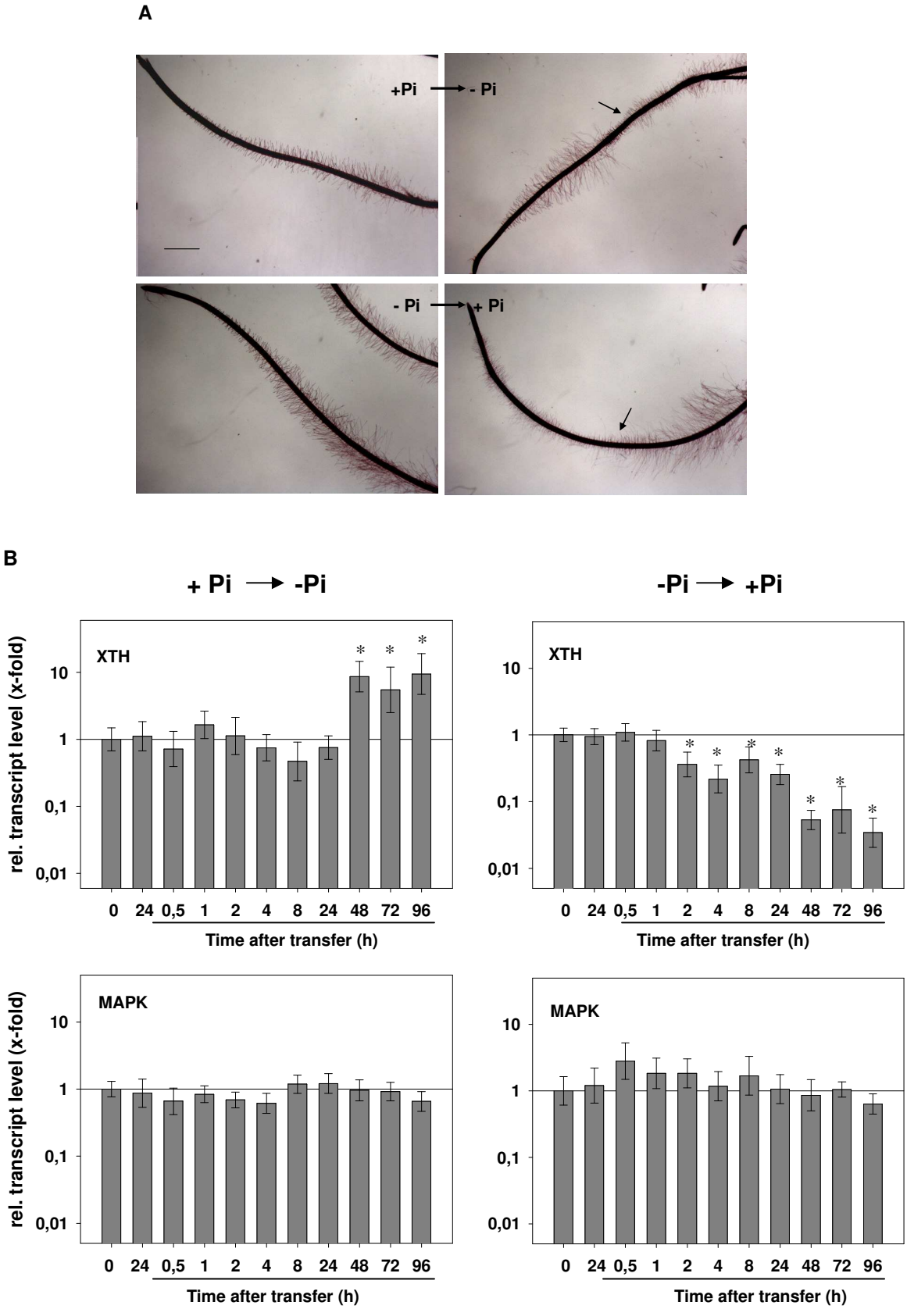


Fig. 3 Influence of Pi, N and K starvation for five days on root hair phenotype (A) and gene expression (B) in roots of *B. carinata* cv. Bale. The relative amount of mRNA in Bale + was defined as 1.0. Error bars indicate standard deviation. * significantly different to Bale + (Confidence Interval 95%). Bar = 400 µm

Effects of Pi, N and K stress on root hair phenotype and transcription of candidate genes were investigated. Deficiency of Pi and N induced the formation of longer root hairs in Bale while K starvation had no impact compared to control conditions (Fig. 3A). Root hair length was unaffected in Bacho at all (data not shown). Correspondingly, in Bale common signaling cascades induced by Pi and N stress could contribute to root hair formation. During Pi starvation an upregulation was confirmed for XTH, EXP, F-box and LRX1 as well a downregulation for MAPK compared to control conditions (Fig. 3B). Furthermore, starvation of N also increased expression of F-box family protein and LRX1 but EXP was downregulated (Fig. 3B). During K deficiency transcript level of LRX1 was enhanced whereas that of EXP was reduced (Fig. 3B). This suggests that these two genes are not involved in control of root hair development since P and N deficiency affected root hair growth but not K.

To investigate the temporal response of root hair growth and expression of candidate genes in Bale, roots were harvested in intervals after changing Pi supply. Withdrawal or resupply of Pi resulted in Bale in enhanced or reduced root hair length after 4h and 2h, respectively assuming constant growth rates (Fig. 4A). Putative genes responsible for root hair formation indicate a function by altering their expression before visible changes in root hair length occur. Removal of Pi induced expression of XTH after 48h whereas no significant changes were detected for MAPK, EXP, F-box and LRX1 (Fig. 4B). Resupply of Pi started to reduce transcript level of XTH and LRX1 after 2h and 48h, respectively (Fig. 4B). Expression of F-box decreased slightly 72h after re-addition of Pi (Fig. 4B). Instead, the expression of MAPK and EXP was not changed after resupply of Pi (Fig. 4B). It is noteworthy that resupply of Pi resulted in a more rapid response in root hair phenotype and expression pattern of investigated genes than removal of Pi.


A
+Pi
- Pi
- Pi
+ Pi
B
+ Pi → -Pi
-Pi → +Pi
XTH
MAPK
rel. transcript level (x-fold)
10
1
0,1
0,01
0 24 0,5 1 2 4 8 24 48 72 96
Time after transfer (h)

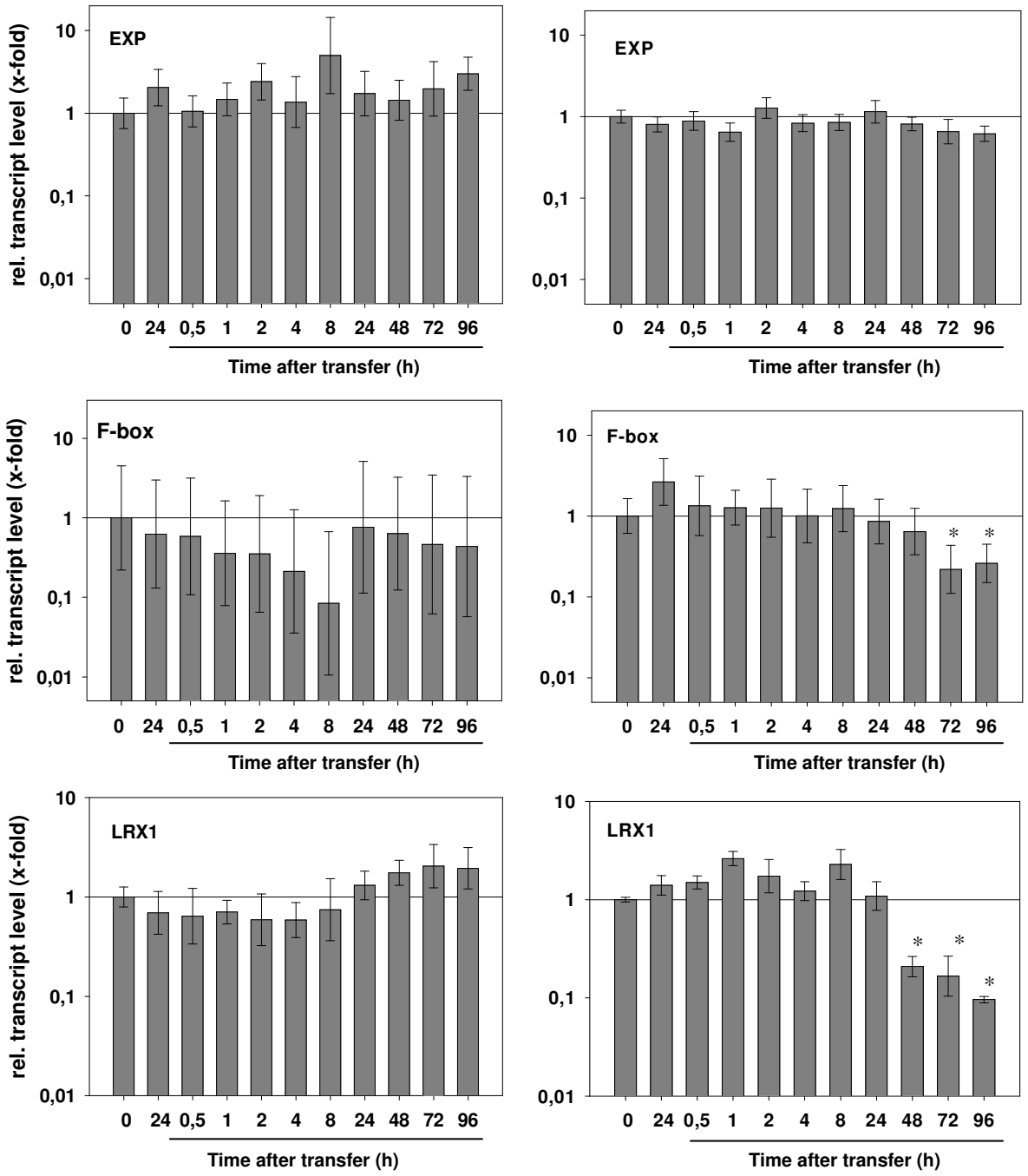


Fig. 4 Effect of changing Pi supply on root hair phenotype (A) and gene expression of XTH, MAPK, EXP, F-box and LRX1 (B). Arrow in A indicates time point of transfer. Growth rates were 1 cm/day for both transfers. Relative expression level was evaluated by setting time point 0 on 1.0. * significantly different to time point 0h (Confidence Interval 95%). Bar = 1.6 mm

3.5. Gene expression pattern along the root

For all examined genes expression was highest in the root tip and declined to older root parts irrespective of Pi nutrition (Fig. 5). Transcript level of XTH, F-box family protein and LRX1 was higher in the sections during Pi depletion compared to control conditions while for MAPK

expression no differences in response to Pi nutrition were observed in sections 0-1 cm and 1-2 cm (Fig. 5). In section 1-2 cm transcript abundance of EXP during -Pi was higher than in +Pi while in sections 0-1 cm and 2-3 cm the differences were negligible (Fig. 5).

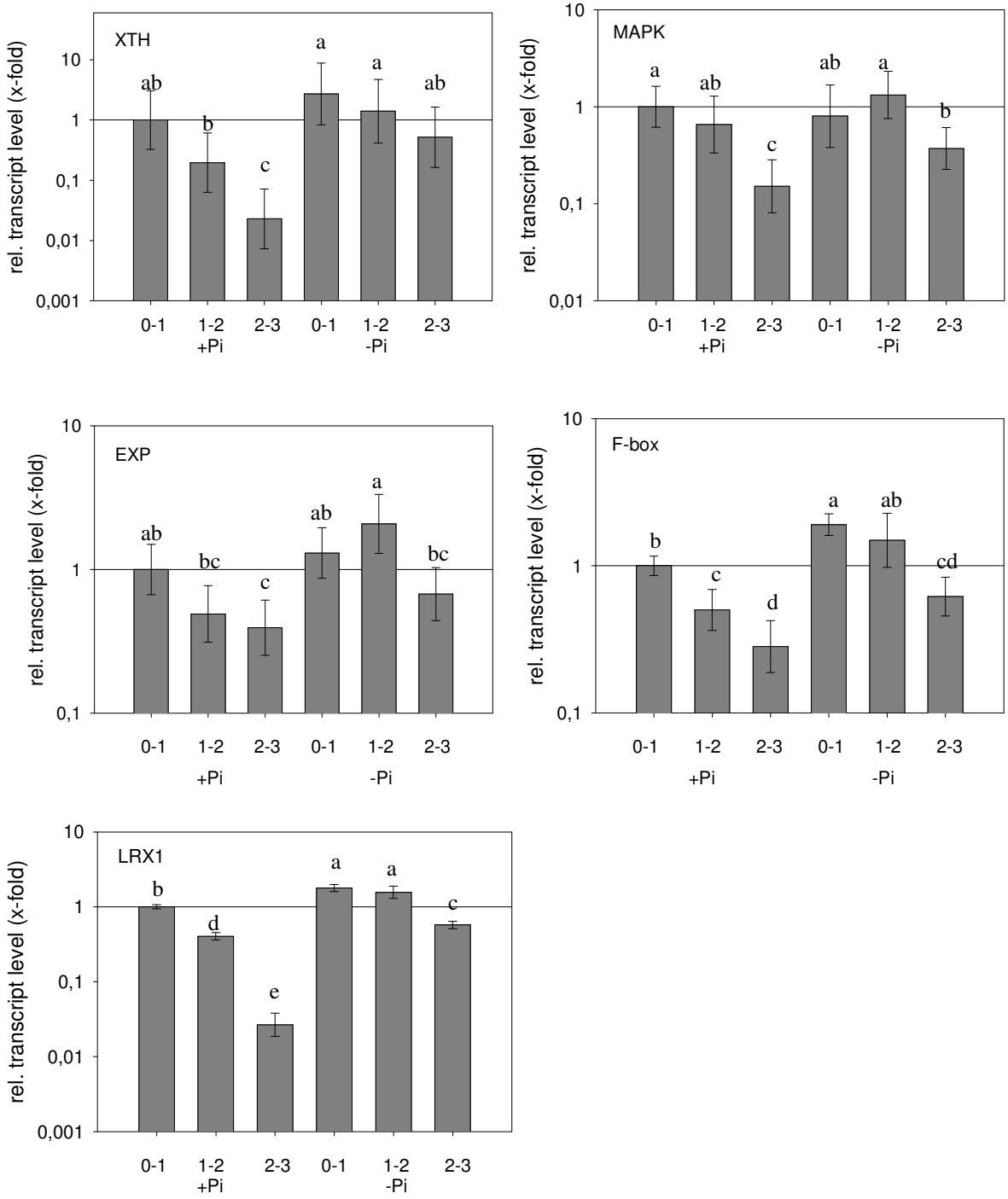


Fig. 5 Gene expression profile of candidate genes in root sections as affected by Pi supply in cv. Bale. Relative expression level was determined by qRT-PCR and evaluated by setting Bale +Pi 0-1 cm on 1.0. Means followed by same letters are not significantly different (Confidence Interval 95%)

4. Discussion

Root hair length was increased during Pi starvation in *B. carinata* cv. Bale (Pi efficient) compared to cv. Bacho (Pi inefficient) whereas no differences were observed in Pi sufficient conditions (Fig. 1). This response to -Pi contributes to the exploration of a greater soil volume for more efficient Pi uptake (Eticha and Schenk, 2001, Wissuwa and Ae, 2001; Gahoonia and Nielsen, 2004).

Confirmation of microarray result was carried out by qRT-PCR. However, only few genes were validated as differentially expressed. The reason for this discrepancy could be low specificity because of cross hybridization to an *Arabidopsis* microarray. However, also qRT-PCR primers were designed from *Arabidopsis* sequences but checked for optimal efficiency before use. Additionally, signal intensity of some spots was too low or too high in both cultivars to determine a potential differential expression. But, high expression could be correlated with high relevance in root hair development during Pi deficiency and consequently those genes with a possible role in root hair development or Pi signaling were included in qRT-PCR analysis. By microarray, also no differential expression was observed for a number of genes known to be involved in root hair development and were analysed by qRT-PCR.

Generally, qRT-PCR revealed in sufficient Pi supply a higher transcript level of *Brassica* homologues to XTH, MAPK, EXP and LRX1 in roots of Bale compared to Bacho indicating a genotypic difference (Table 3, 4, 6). However, the extent of differential transcript levels varied during Pi starvation and could contribute to a Pi response. Furthermore, exposure to -Pi enhanced in Bale transcription of XTH, EXP, F-box and LRX1 whereas there was a decline of MAPK transcript level suggesting either a positive or a negative function of these genes in root hair formation. Moreover, F-box and LRX1 were conversely regulated in Bale and Bacho during Pi starvation strengthening a potential function in -Pi induced root hair development (Table 3, 4, 6).

4.1. Root hair phenotype in response to nutrient stress

Root hair length was enhanced during Pi and N deficiency but not during K depletion in Bale (Fig. 3A). That was also shown by Bates and Lynch in *Arabidopsis* (1996). Further, root hair length was calculated to be increased 4h after removal and reduced 2h after resupply of Pi, respectively assuming constant growth rates (Fig. 4A). Similarly, alteration in root hair length was observed 10h after change in Pi supply in *Arabidopsis* (Bates and Lynch, 1996). The delayed response of root hair growth after removal of Pi compared to the more rapid response after resupply was attributed to a dilution effect in the root tissue.

4.2. XTH

Starvation of Pi resulted in an upregulation of XTH (homologous to At5g57530) in Bale and Bacho (Table 3). Transcript level was not responsive to N depletion resulting also in longer root hairs (Fig. 3 A,B). Resupply of Pi downregulated XTH and reduced root hair length at the same interval (2h) whereas Pi removal delayed upregulation of XTH (48h) compared to the increase in root hair growth after 4h (Fig. 4 A,B). Moreover, transcription during Pi depletion tended to be higher in the root tip but was highest differentially expressed in section 2-3 cm (Fig. 5). These observations suggested a function in all stages of root hair development during Pi deficiency. Further, an upregulation of XTH was detected in both cultivars during -Pi. This indicated that probably not root hair elongation was affected by the activity of this gene since both cultivars differed in root hair length during -Pi. XTHs catalyze the cleavage and rejoining of xyloglucans in the cellulose microfibril framework and play therefore a principal role in construction and remodelling of the cell wall allowing cell growth (Fry et al. 1992). The function of XTH in roots was attributed to initiation and elongation of root hairs (Vissenberg et al. 2001). As well, a higher expression of XTH related genes (At4g14130; At4g25810; At1g65310) 4h and 6h after beginning of Pi depletion was reported by Hammond et al. (2003) and Wu et al. (2003).

However, XTH was not upregulated during N depletion although root hair length was increased suggesting genetic redundancy of different members of a multigene family (Becnel et al. 2006).

4.3. LRX1

The transcript level of LRX1 (homologous to At1g12040) during Pi starvation was higher in Bale than in Bacho. However, Pi deficiency induced in Bale an upregulation of LRX1 and in Bacho a downregulation (Table 6). Expression was also increased during N and K deprivation in Bale but to a lesser extent than in -Pi but only N and not K depletion enhanced root hair length (Fig. 3). Resupply of Pi decreased transcript level after 48h while root hair length was reduced already after 2h (Fig. 4). Along the root LRX1 expression was significantly higher in all sections of Pi starved roots but most pronounced in section 2-3 cm where root hairs end with elongation and mature (Fig. 5). The converse regulation of LRX1 during -Pi in both cultivars suggested a function in root hair growth since in Bale but not in Bacho root hair length was enhanced. The delayed downregulation after Pi resupply suggested an involvement in a later stage of root hair growth. A function of LRX1 in the final stage of root hair elongation was supposed when calculating the time needed for full length root hair elongation lasts up to 40h. Also Bacho had root hairs but shorter ones during -Pi and consequently an earlier stage in root hair elongation seems not to be affected. The expression of LRX1 is necessary for proper cell wall structure during root hair enlargement (Baumberger et al. 2001). The structure of LRX1 suggested a function by perceiving signals derived from the Pi starvation event by the extracellular LRR domain and transmit this signal to the anchored extensin domain in the cell wall (Baumberger et al. 2001). To our knowledge, an altered expression of LRX1 in response to Pi starvation was not yet reported but a function for LRX1 in root hair growth during low Pi was suggested since in a mutant lrx1 background fewer root hairs than in wild type plants were formed during -Pi (Müller and Schmidt, 2004). However, Pi and more weaker N starvation enhanced expression of LRX1

whereas both induced root hair length. It is possible that in *Brassica* related genes act in response to N stress.

4.4. F-box

During Pi starvation an F-box protein (homologous to At3g08750) was higher expressed in Bale than in Bacho (Table 4). Both Pi and N deprivation enhanced F-box transcription in Bale corresponding to an increase in root hair length (Fig. 3). However, a retarded downregulation 72h after readdition of Pi and no regulation after Pi removal were not correlated to changes in root hair growth (Fig. 4). Furthermore, expression level tended to be higher in all stages of root hair development during Pi depletion (Fig. 5). The expression pattern indicated a common positive function during Pi and N starvation but the kinetics suggested no control in early root hair growth (Fig. 3B, 4B). However, expression along the root hinted at a function in root or root hair growth during -Pi. The F-box proteins contain often WD40 or leucine rich repeats (LRR) for binding to their targets with subsequent regulated protein degradation by the ubiquitin- proteasome pathway (Hershko and Ciechanover, 1998; del Pozo and Estelle, 2000; Kipreos and Pagano, 2000). It was reported that Pi starvation upregulated an F-box protein in *Lolium* roots (Venkatachalam et al. 2009) but downregulated two F-box proteins in *Arabidopsis* (At1g23390 and At3g59940; Morcuende et al. 2007). F-box could be involved in regulation of Pi starvation responses since phosphorylation and subsequent ubiquitination is a rapid mechanism to regulate activity of proteins (Kipreos and Pagano, 2000).

4.5. MAPK and EXP

MAPK (homologous to At1g18150) and EXP (homologous to At3g45960) were higher expressed in response to Pi deficiency in Bale compared to Bacho. However, the primary results could not be confirmed (Table 3). Removal and resupply of Pi had no effect on transcript level of MAPK and EXP (Fig. 4B). Moreover, MAPK expression was higher in root tips irrespective of Pi supply while EXP transcript abundance was responsive to low Pi only

in section 1-2 cm (Fig. 5). For both genes a role in early root hair growth induced by Pi depletion can be excluded.

4.6. Conclusion

The expression of XTH was induced in both cultivars during Pi deprivation. However, the higher transcript level in Bale may not sufficiently explain longer root hairs. The rapid downregulation after Pi resupply suggested a role in early control of root hair development. It is conceivable that Pi deprivation enhanced the initiation of root hairs in both cultivars but later on the elongation in Bacho was slower. F-box could be of interest as a transcriptional regulator of a number of unknown target genes in root hair development by the ubiquitin proteasome pathway. A hint was the upregulation in Bale in response to Pi and N depletion both inducing root hair growth but a downregulation in Bacho during Pi deficiency. Further, a function in early stages of root hair development was excluded because of a delayed response after resupply of Pi. LRX1 could be involved in root hair elongation. The converse regulation in Bale and Bacho resulted in different transcript levels and might be responsible for the root hair phenotypes in both cultivars. LRX1 is required for proper cell wall structure during root hair elongation and the delayed differences in expression level after Pi resupply and a high differential expression in sections 1-2 cm and 2-3 cm comparing +Pi and –Pi, respectively, suggested a function in root hair elongation. Instead, MAPK and EXP were not related to root hair growth because of no response to Pi supply and no differential expression in root tips.

General Discussion

Plants cope with limited Pi supply through adaptive strategies to enhance Pi availability in the soil and to increase its uptake. Improved Pi acquisition efficiency in plants is a result of morphological and physiological changes following a signal transduction that senses the Pi starvation signal and alters the expression of many genes.

In this study, low Pi availability enhanced root hair length and density in *Brassica carinata* cv. Bale (Pi efficient) to explore a greater soil volume while there was no response in cv. Bacho (Pi inefficient). Previously, longer and denser root hairs in *B. carinata* cultivars were suggested to be associated with Pi efficiency since no other differences in e.g. organic acid exudation or root induced pH changes were detected (Eticha and Schenk, 2001). The formation of longer root hairs was also observed in barley (*Hordeum vulgare*) and peanut (*Arachis hypogaea*) genotypes that enhanced Pi uptake by this response compared to genotypes with shorter root hairs (Wissuwa and Ae, 2001; Gahoonia and Nielsen, 2004). While the formation of longer root hairs is a well documented response to low Pi the molecular mechanisms underlying this adaptive response is not fully understood.

The present work aimed at the identification of genes responsible for Pi starvation induced root hair growth. Firstly, this was achieved by the construction of SSH libraries accumulating differentially expressed genes in the two cultivars Bale and Bacho during Pi starvation (Chapter I). Secondly, a SSH library was generated for the Pi efficient cultivar Bale comparing gene expression in control and Pi deficient conditions (Chapter II). Thirdly, global change in gene expression in both cultivars during Pi depletion was investigated by using a whole genome microarray of *Arabidopsis thaliana* (Chapter III). With all approaches, candidate genes were selected according to a putative role in root hair development and/ or Pi signaling. The presumed function of candidate genes in root hair growth induced by Pi depletion was estimated.

1. Root hair phenotype and gene expression in response to nutrient availability

Deprivation of Pi and N increased root hair length in the Pi efficient cv. Bale while the Pi inefficient cv. Bacho responded to none of the examined nutrient stresses. The formation of longer root hairs during Pi and N deficiency was well documented (Foehse and Jungk, 1983; Bates and Lynch, 1996; Williamson et al. 2001; López-Bucio et al. 2002). Comparison of the molecular events in Bale during Pi and N limitations could therefore share some common features in inducing root hair growth. A potential cross talk between nutrient signal transduction pathways was previously suggested (Coello and Polacco, 1999; Wang et al. 2002b). Furthermore, in Bale removal of Pi and N increased root hair length after 4h and reduced it 2h and 6.5h after resupply of Pi and N, respectively. Similarly, Bates and Lynch (1996) observed a change in root hair length 10h after withdrawal and resupply of Pi in *Arabidopsis*. The formation of longer root hairs after Pi removal was presumably not the result of belated elongation of already existing root hairs since Bates and Lynch (1996) demonstrated that only new developing root hairs responded to -Pi.

Removal of Pi supply showed delayed changes in root hair length compared to a more rapid response after Pi resupply. Additionally, alterations in transcript levels of investigated genes were often slower after removal of Pi compared to a more rapid response after resupply. This was presumably the result of a delayed sensing of the Pi deficit due to a gradual Pi dilution in plant tissue. Changes in root hair length during N depletion were also expected to respond similar to that of Pi depletion. However, adaptations of root hair length to altered N availability were faster by removal (4h) than by resupply (6.5h). This might be explained by a 2.6 times higher growth rate after transfer of +N plants to -N making a more rapid response feasible.

In the following, genes will be discussed in the temporal sequence of expression after resupply of Pi where a potential function in root hair development was suggested. This direction of transfer experiment was selected because of a more direct response to Pi availability irrespective of the dilution effect in the plant after removing Pi.

2. Response 0.5h – 4h after resupply of Pi

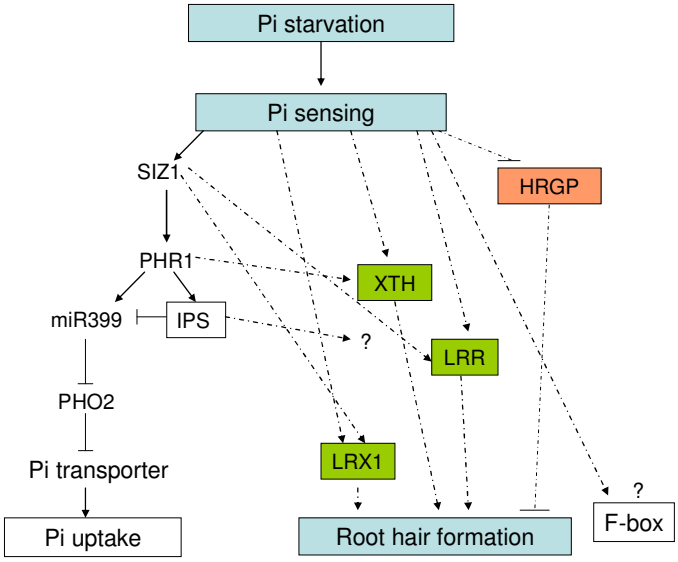
Root hair length was calculated to be reduced after 2h suggesting an immediate sensing of Pi by the root tip and a following signal transduction cascade to shorten root hair growth. The first responsive genes after resupply of Pi were XTH (2h; Chapter III, Fig. 4B), HRGP (0.5h; Chapter I, Fig. 6B) and LRR (4h; Chapter II, Fig. 4B). The function of these genes could be attributed to an early role in Pi signaling and/ or root hair initiation.

The rapid reduction in **XTH** transcript level and the higher expression during -Pi also in Bacho having shorter root hairs, suggested for XTH rather a contribution to early events like root hair initiation similar in Bale and Bacho than to root hair elongation differing in both cultivars (Chapter III). XTHs play a principal role in the construction and restructuring of the cell wall allowing growth (Fry et al. 1992). A putative function of XTH in root hair development beginning from bulge formation was previously reported (Vissenberg et al. 2001). The induction of different isoforms in response to different types of stress was shown and could explain the absence of XTH during N depletion (Becnel et al. 2006). Furthermore, an XTH (At1g65310) was the direct target of GLABRA2 (GL2) which is necessary for specifying the non-hair cell fate in the root epidermis (Tominaga-Wada et al. 2009). Promoter analysis of the homologous gene in *Arabidopsis* (At5g57530) revealed one root hair specific domain (PLACE ID: S000512; Plant *cis*-acting regulatory DNA elements (PLACE) database, Higo et al. 1999) and a *cis*-element that was found in many phosphate starvation induced (PSI) genes (NIT-2; Mukatira et al. 2001; Kim et al. 2006). Moreover, a P1BS (PHR1 binding site) motif was found in the promoter indicating a regulation by PHR1 (PHOSPHATE STARVATION RESPONSE1; PLACE database, PLACE ID: S000459; Rubio et al. 2001; Fig. 1). However, PHR1 regulated many Pi starvation induced responses but was up to now not found to be related to root hair growth (Martin et al. 2000; Rubio et al. 2001). Nevertheless, these observations in the promoter could only be a hint for the spatial-temporal expression pattern and the responsiveness to different stress factors and can not replace experimental analysis. Additionally, promoter information in *Brassica* is missing and there is no need for having the same domain structure as in homologous genes in *Arabidopsis.*

Resupply of Pi and N induced **HRGP** expression in Bale corresponding to a reduction in root hair length (Chapter I, Fig. 6B, 7B). Transcript level was also higher in Bacho forming only short root hairs in response to all nutrient deficiencies and along the root expression was higher in the mature parts (Chapter I, Fig. 5, 9). This suggested for HRGP a negative correlation to root hair formation with a common regulatory pattern during Pi and N starvation. HRGP encodes for a putative cell wall protein and could strengthen the cell wall by crosslinking (Bradley et al. 1992; De Cnodder et al. 2005; Roberts and Shirsat, 2006). However, the rapid response after resupply of Pi and N (0.5h) suggested a function in signaling since cell wall proteins were regulated by long term Pi depletion (Misson et al. 2005). Computer analysis of the promoter region in the homologous *Arabidopsis* gene (At1g21310) identified a root hair specific domain (PLACE ID: S000512) and manually three NIT-2 elements often found in PSI genes indicating at a possible relation to Pi starvation induced root hair growth (Fig. 1; Mukatira et al. 2001; Kim et al. 2006).

The expression of **LRR** was reduced by resupplying Pi and N after 4h and this correlated with changes in root hair length (Chapter II, Fig. 4B, 5B). Further, expression was highest in the root tip in Pi deficient conditions (Chapter II, Fig. 7). These observations suggested a function in early stages of root hair growth induced by Pi and N stress (Fig. 1). The rapid repression of LRR after resuppling Pi and N indicated a direct regulation by nutrient availability and no involvement in a secondary response (Morcuende et al. 2007). Since LRR encodes for a putative receptor molecule it is conceivable that a shared ligand produced during Pi and N starvation is perceived at the extracellular LRR domain and transmitted to the cytoplasmic protein kinase regulating downstream the activity of unknown genes to induce root hair growth. It will be of special interest to identify the target genes and the ligand of LRR besides the unknown factor upregulating LRR transcription. Recently, two root hair specific LRR-receptor-like kinases were identified in *Arabidopsis* (Won et al. 2009). Overexpression resulted in branched and shortened root hairs and had therefore a converse effect as here hypothesized. However, it could be a hint of a regulation mechanism due to internal or external stimuli to modify root hair development (Won et al. 2009). The

homologous gene of LRR in *Arabidopsis* (At2g28970) displayed *cis*-regulatory elements in the promoter related to Pi stress response (NIT-2; Mukatira et al. 2001) and was highest expressed in the root hair zone (www.genevestigator.com). Further, sumoylation motifs in the cytoplasmatic C-terminal as well in the extracellular LRR domain suggested a control for LRR by posttranslational modifications (SUMOsp2.0 database; Miura et al. 2005). Sumoylation regulates protein-protein or protein-DNA interactions and protects proteins against ubiquitin-mediated degradation (Yuan and Liu, 2008).



adapted from Gojon et al. 2009

Fig. 1 Scheme of possible interactions of candidate genes in root hair growth induced by Pi starvation. Dashed lines with arrows and blunt dashed lines represent possible interactions to induce and repress root hair growth, respectively. Solid lines with arrows and solid blunt lines symbolize positive and negative effects, respectively, as known by literature (Martin et al. 2000; Rubio et al. 2001; Franco-Zorilla et al. 2007).

XTH, LRR and LRX1 (green boxes) have a putative positive effect on root hair growth while HRGP (red box) acts as a negative regulator of root hair growth during Pi starvation. The functions of IPS and F-box are not fully understood. IPS is not related to root hair formation but could act as signal for genes in inducing root hair growth while F-box may be involved in ubiquitin-mediated degradation of target proteins. Sumoylation of the *Arabidopsis* homologous genes LRR and LRX1 might occur by SIZ1, a small ubiquitin-like modifier (SUMO) E3 ligase, upregulated by Pi starvation. Also PHR1 (PHOSPHATE STARVATION RESPONSE 1) has a sumoylation site. Genes regulated by PHR1 are the microRNA miR399 and IPS in *Arabidopsis*. As well, the *Arabidopsis* homologue of XTH has a PHR1 binding site and suggests a regulation by PHR1. A target of miR399 is PHO2 (PHOSPHATE 2) negatively regulating Pi transporters and therefore affecting Pi uptake. IPS can regulate miR399 by a mechanism called target mimicry and can contribute to Pi uptake (Franco-Zorilla et al. 2007).

Additionally, in the promoter region of the corresponding genes to XTH, HRGP and LRR in *Arabidopsis* several W-box elements (PLACE ID:S000390) were found by the PLACE database suggesting a regulation by WRKY transcription factors. However, with all approaches no differential expression of WRKY transcription factors during -Pi was identified maybe due to transient expression. WRKY transcription factors are involved in response to different stresses (Eulgem et al. 2000). The expression of WRKYs is often rapid and transient indicating a function in primary signal transduction (Eulgem et al. 2000). Activation of a LRR receptor-like kinase by WRKY transcription factors was previously reported (Asai et al. 2002). However, only one WRKY transcription factors, WRKY75, was found to be regulated by Pi stress (Devaiah et al. 2007).

3. Response 8h after resupply of Pi

After discussing the role of early responsive genes in root hair development the next gene downregulated 8h after resupply of Pi was IPS (Chapter II, Fig. 4B). A Pi specific response for IPS throughout the plant was observed and along the root no different transcript level was observed (Chapter II, Fig. 6, 7). These observations suggest a function with no direct relation to root hair growth. The function of IPS is unknown but a participation in Pi homeostasis was previously suggested (Burleigh and Harrison, 1999; Martin et al. 2000; Shin et al. 2006a). The Pi starvation inducible molecules from the IPS family were shown to be regulated by the Pi status of the shoot and late responsive (Burleigh and Harrison, 1999; Shin et al. 2006a, Valdes-Lopez and Hernandez, 2008). In contrast, in rice the transcript level of OsIPS1 was increased 4h after removal of Pi corresponding to a decrease in total Pi concentration in roots while there was no decline in shoots. This early response may be important for root hair initiation or elongation that were also responsive to local Pi status of the root (Fig. 1; Bates and Lynch, 1996; Martin et al. 2000; Hou et al. 2005).

4. Response 48h – 72h after resupply of Pi

Genes that were downregulated after the calculated reduction in root hair length in response to Pi resupply were LRX1 (48h) and F-box family protein (72h). Both genes are discussed in detail because LRX1 is required for proper root hair enlargement in *Arabidopsis* (Baumberger et al. 2001) and F-box is known for a regulation of target proteins by ubiquitin-mediated proteolysis (del Pozo and Estelle, 2000; Kipreos and Pagano, 2000). Furthermore, considering growth rates of 1 cm/day after transfer to +Pi the harvested root tip was in the elongation process between 12h and 48h approximately.

LRX1 was upregulated in Bale but downregulated in Bacho (Chapter III, Table 6). The downregulation after Pi resupply in Bale was delayed compared to the reduction in root hair length (Chapter III, Fig. 4 A, B). Furthermore, the differences in transcript level between +Pi and -Pi were highest in section 2-3 cm where root hair elongation ends (Chapter III, Fig. 5). This suggests for LRX1 no contribution to initial mechanisms in root hair growth rather to root hair elongation since Bale but not Bacho enhanced root hair length during -Pi. LRX1 encodes for a chimeric leucine-rich repeat/ extensin protein and was obviously required for correct tip growth of root hairs during Pi deficiency (Müller and Schmidt, 2004). In the sequence of action LRX1 is arranged after genes required for root hair initiation and early elongation in the morphogenesis of the growing cell wall (Baumberger et al. 2001; Müller and Schmidt, 2004) and these particular genes are as well unaffected in this study (Chapter III, Table 6). By promoter analysis of LRX1 in *Arabidopsis* (At1g12040) two Pi starvation elements were found manually and a root hair specific element (PLACE database; PLACE ID: S000512; Mukatira et al. 2001; Kim et al. 2006). Posttranslational modification is suggested because of the existence of a sumoylation site in the protein (SUMOsp2.0 database).

An **F-box** family protein was downregulated in Bacho and upregulated in Bale in Pi depleted roots (Chapter III, Table 4). F-box responded to Pi and N deprivation with an upregulation in agreement with the formation of longer root hairs but after resupply of Pi downregulation was delayed and not significant after Pi removal (Chapter III, Fig. 3, 4). Further, transcript abundance was highest in the root tip and more abundant in root sections subjected to Pi

starvation (Chapter III, Fig. 5). The converse regulation in Bale and Bacho indicated a function in root hair growth. This positive role for F-box family protein was supported by an upregulation during Pi and N starvation but kinetics excluded a role in early root hair growth. F-box could be involved in later Pi starvation response due to protein degradation of target proteins. For binding to their targets F-box family proteins often contain WD40 and LRR domains as recognition motives (Hershko and Ciechanover, 1998; del Pozo and Estelle, 2000; Kipreos and Pagano, 2000). However, in the F-box protein (At3g08750) sequence in *Arabidopsis* no LRR domain was found as a target recognition motif by PFAM and PROSITE database. Moreover, in the homologous *Arabidopsis* sequences of XTH, HRGP, LRR and LRX1 no F-box protein motives were detected making an interaction disputable.

However, the fact that enhanced root hair length was elicited by Pi and N starvation does not necessarily imply the involvement of the same signaling cascades. Also, it might be possible that the plant senses the initial Pi and N signal differently but more downstream the signal transduction pathway they use combined mechanisms to induce root hair growth. A hint for a common regulation could be conserved *cis*-regulatory elements in the promoter region of LRR and HRGP both regulated during Pi and N starvation. Up to now the signaling cascades of perceiving the nutrient signal and the subsequent induction of elongating root hairs is not known.

Changes in root hair length after Pi removal and resupply were calculated after 24h and determination of gene expression started within hours. It was assumed that the time needed for changes in root hair length corresponded to changes in gene expression. However, the period of root hair elongation began probably after 12-14h and lasted up to 48h, approximately (Chapter I, Fig. 1). In Chapter I and II genes with a delayed expression after resupply were not attributed to a function in root hair growth but in Chapter III LRX1 was taken into account. It might be possible that also XTR (Chapter I, Fig. 6B), PRP and LTP (Chapter II, Fig. 4B) that reduced expression 24h after Pi resupply have a function in root hair elongation and even a role in early hair elongation could be ruled out.

5. The methodic approach

The relatedness of *Brassica carinata* to the fully sequenced plant *Arabidopsis thaliana* make the use of a global transcriptomic analysis by microarray reasonable. However, the results of this cross hybridization give only a hint to homologues or gene families involved. A disadvantage by using microarrays is the relatively low sensitivity for detection of small differences in transcription level and genes with low expression intensity often failed for reliable evaluation (Morcuende et al. 2007). Furthermore, genes with high expression leading to saturated binding sites on microarray are difficult to detect. For example, some cell wall proteins and transcription factors (TF) were expressed at a high level making a differential expression determination impossible. But, TF are key signal proteins regulating expression of downstream target genes in response to many stimuli (Riechmann, 2002; Czechowski et al. 2004) and may contribute to elucidation of signaling and regulatory pathways in response to Pi depletion. Therefore, the qRT-PCR technology with enhanced sensitivity and efficiency is an improved approach to quantify differential expression of high and less transcribed genes or genes with low differences in expression level compared to microarray analysis.

The Suppression Subtractive Hybridization (SSH) method enriches differentially expressed genes in a PCR based step (Diatchenko et al. 1996). A great advantage compared to microarray analysis is no need for knowing the sequence of the examined organism and has been successfully applied on various plant species in response to a number of abiotic and biotic stresses (Tian et al. 2007; Hernandez et al. 2007; Venkatachalam et al. 2009). In the present study, the relatedness to *Arabidopsis thaliana* was helpful to identify the resulting ESTs (expressed sequenced tags). Surprisingly, the number of bacterial clones of the resultant subtractive library was comparatively low. A total of 511 individual bacterial colonies of three SSH libraries and 127 resulting genes with homology to described or unknown proteins in *Arabidopsis thaliana* were identified. From pre-selected genes only a small number was confirmed as differentially expressed. Guo et al. (2008) got 8,000 vector containing colonies and Wang et al. (2001) even 14,000 clones from which 215 and 716 cDNA ESTs, respectively were differentially expressed. This great discrepancy reveals a

high percentage of false positive clones and makes a verification step indispensable. A possible explanation for such a large number of false positives could be that differences in expression level are not that significant for less sensitive methods like semi-quantitative PCR. Likewise, a greater number of individual colonies after cloning was expected so that the quality of competent cell is questionable even though they has been ordered directly from a company. On the other side, it is conceivable that the quantity of differentially expressed genes of two genotypes under the same growing conditions is not higher due to similar responses. Another disputable aspect of the SSH method is the preference of a defined fragment size for ligation in the cloning vector. This fulfils no need of completeness of all resulting ESTs.

Surprisingly, the present SSH approach failed to evaluate typical PSI genes e.g. Pi transporter (Muchhal et al. 1996), RNases (Bariola et al. 1994), PHR1 (Rubio et al. 2001) or, as expected, genes associated with root hair elongation. It is possible that these formerly reported Pi starvation induced genes are expressed in both cabbage cultivars during the Pi response and for this reason were eliminated during the hybridization process. Similarly, Hernandez et al. (2007) found no Pi transporters in their Pi stressed cDNA root library, but the time point of sample taking (21 day old plants) was interpreted as too late for such highly but transient inducible genes. In the present study, the selected time point of harvest (5 days) determined mainly long term Pi specific responses leading to adaptive changes in morphology and physiology (Ma et al. 2003), i.e. some stress responsive/ signaling molecules involved in early responses that could be regulated transiently may be missed with this experimental design. Nevertheless, all stages of root hair development are represented along the root and they develop permanent longer root hairs by sensing Pi starvation independent of the harvest time point.

6. Outlook

To gain more information about the biological role of the here characterized candidate genes, especially LRR, HRGP and LRX1 are of interest and reverse genetic approaches has to be developed. Since sequence information in *Brassica carinata* is missing it is feasible to follow two different strategies: Firstly, characterization of candidate genes in *Brassica:*

- RNAi mechanisms can efficiently knock down target genes in the root and the particular root hair phenotype can be observed
- With *in situ* hybridization it is possible to localize gene activity in the root tissue and to relate this with a function in root hair growth during -Pi
- The sequencing of the complete genes including the promoter region

Secondly, a further investigation of homologous genes in *Arabidopsis:*

- With mutant analysis and overexpressor lines it should be possible to relate the gene function with a root hair phenotype induced by Pi deficiency. Having transgenic plants a combined large-scale gene expression profiling by microarray could further connect genes regulated by the same signaling cascades
- Promoter analysis by using the GUS reporter gene. The activity of the glucuronidase gene under the control of the promoter of the particular gene could give a hint at regulation mechanisms during -Pi

The identification of components of the signaling pathways and the knowledge of the molecular regulation of Pi specific responses can contribute to breeding programs to obtain more Pi efficient germplasms for a number of plants. Targeting a key regulatory molecule in the Pi starvation response inducing root hair growth represents a useful approach for molecular breeding of plants towards more efficient Pi uptake and may reduce the demand for Pi fertilizers (Vance, 2001).

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Supplementary Material

Chapter I: EST sequences from SSH, forward run

Clone	Sequence
BcBal-1 At4g12550 4.3e-16	ACCTCACTCAAGGTTAACATTCTTGGCATCAACCTTAACCTTC CCATCTATGTCAACATACTCCTCAATAACTGTGGCCGTATAAA TCCGGCATACTACCCATGCGCCTAAGTTGAATACTTATATGAC ATATAAAGACATACCTAAAAGACAAACAGATCGTATGGTTTC AAATGTTTGAAATTATTGCCTTATTTTCTTGTTTCTTTTAATCA GTGATTGCTTTTGTGTTTGTATTTTTCATGGATTGAATAAAAT GATGAACGTCATGTTCTCATGCAAAAAGGGATTATGGGTTTG TGTGTGTATCGGCAAGTTCATTCCCCTTTTGCTTCACTGTTCCT CCTTATCTTCGTTGTAAGCATTTCTTTTGAATTTGATTAATAA AACTATCAATCGATGTTGTTGTCCA
BcBal-2 At3g59970 2.1e-19	TCTCGAGATATATGTGTTGAGATAGAAAGATAAAAGAACACC CCACAGGGGAAACAGATGTCTATAGGGGGCACATCACATCAC ACAAATATCTTTAAAAGGGTCACAACAAATCGAGAAAATCGA GTGTCTCCTCCCCCCACCGGGGGGGGTCTCTCAGATATCACG CAAACACGGAGAATATCTCCCCGGAGATGTAATCGGTGCCCA CTAAGCTCCCCAAATAATATGTGTTCTTCACCCCCTCGAGCAA CTCTCTAAAGGGGTCATCTTCTGGGTACCTGCCCGGGGGGGC GCTCAAAGGGGCG
BcBal-3 At4g25820 8.9e-12	GTACAACTTTTTATTTTCTCAGGAACTTTATATATGCGTGTAT TATCGCATCTCAGAAACACTTGAAAACAAAAGAGAGAATAA ATCAAAGTAACATCACAAGAATCAAATAACACCAAAGGGAA ATAAAAAAACATTATTGAGGAATATGCTCGAAAATTCTCATA GTATGAGGATCAACCAAAGCTCAAAGGTAACACTCCTTGGCA AAACCCTGAGGGAACCTCTTGTAATCGGTACCTC
BcBal-4 At1g01050 1.4e-103	AGCGTGGTCGCGGCCGAGGTACTGAATAGCTTCAACAGCAGG ACCATTCGGTAGAAAATCATTTACAGCAACTTCCTTGTTCTCA TTCTTCTTGTAATCTTCAAAGAATCGCCGAATTTCAGTAAGAC GATGAGGAGGAAGTTCTTTGATGTCAGTGTAATGCTTATACT CAGGATCATCAACACATACCGCAATGATCTTGTCATCTTTTTC TCCCTGATCAATCATGGGCATTAATCCAATAGCTCGGGCGCG CAGAAAACAACCTGGAAGCACCGGCTCCTGCATGATGACTAA TACATCAATAGGGTCATTGTCTTCACAAAGTGTGCGAGGGAC GAAACCATAGTTGTGAGGGTAAACAACTGATGAGTAGAGAA TACGATCCACCTTGATGAGTCCAGTCTTTTTGTCAAGTTCATA TTTGACCTTGCTTCCCTTTGTGATCTCAACCACCACGTTGAAA ATCTGTGGAGCTCCAGGGCCAATCTCAAGATCGTGCCAGGGA TGAGCAGCTACGGAGCGTCTAGACAAGGAAGAGAGGATCCT CTCATTAAGACGAGGAGCAGGTCGCTGGTTCTCCTTCGTTTCC TCAGTCATCTTTGGAGTCGAAAAGGGAGAAACTTTGGGAG
BcBal-5 At3g17210 4.3e-13	ACGCGGGGTTGCTCATCCTATTCACGTTGAGTTCGCCAACATG TTCCTTGGCAGCTTGGATAAAGTGTTGGTCATAGACTACAAG CCTACCTCTGTTTAAAATTCATCATCTTTGTGTGACCTTTCTTT GTTATAAAGTATGTTCCTTTTGATGTAAGGCCTTTGTTATGAT GTTTCTGTCAATCTCTTTTTTTTTTTTTGTTGTTGTATGAGACC AAGTGTGTAAAACGATCATGAGTGTTTGCTTGTTTT
BcBal-6	TTTTTCGTATAATAAGAGTGTTCAAGGAATAGATTATTACACG

At1g20260 7.6e-54	AGAAATACATGGTTCACCAGTTTGGAAGACAAAAACACAAC AAGGCTTATAAATAGCGTTTGTGTTACTAGGCTTTTCCTACAC AAAGCCTTTATTATACAAATGAAAAACAAAAGAATAATCCCA AATCACAATTCTCTGAGTTTTTTTTCACGCAATCTTGTTTTTTC TTCTCGGGACATAATTTAAGTTCCAAACTTCCAAATAAGAAT GGAGCAACATAGAGGTTGCTTAAGAAGGCTTATCTTGTGGAG TCGCGGCTGTAGAATTGATCAAGTGTTTTGGCAGGAATACGG TGAAGAAGCTCACGAGGGAAGATACGGAGCAGTGTCCATGC CAAGTCAAGTGACTGGAAGATGTTTCTTGTATCCCCGCGTAC CTGCCCGGGCGGCCGACCGGGCAGGACGTGCAGTTACCTCCT GATGGAGTCCATGTTGA
BcBal-7 At3g12600 9.5e-56	TATTGAAAATAACTGAACTTTTTAAAACTTTGAAACTCCTATG TTTCGTAGTCACAAGCTTCTCCATGCATATAGACTAAATAAA AAAGAAGTACCAACAAAATCGAAAGGAAAGAAACCAACTTC TGAGTTTATATGCACACTTAAAACTTCAATCATCATCTGCTAT ATCCTCTCCTTTCCCCACATTCTCCTTATGCCATTTACAAAATC CTTCAACCAACGCATCCTTCATCCAAGCATGCCTGCAATTCTC TACAGCTTCTTCGATCGTCAGCCATGTCCTTGTTCTAGTCTTCT GTTCAGGCCACGTCTCTAGCTCTTCCTTCACATACAAGGCGTA CATTGCAGCTTTACATAATCCTTCCGGGCAAAACTCGTCTTGG TGAGTCTTGCTCTTGAACTCGTATTCTCCCAACAAATCCTGCA AGTTCGAAATCTACAGGTTCAGTCCAGCTTCCTACTAACAATT ACAGAGCCTATATGTAGCTAGTACCTGCCCGGGCGGCCGCCC GGGCAGGTACTTTT
BcBal-8 At1g23410 3.0e-52	AGCGTGGTCGCGGCCGAGGTACCCTCGCCGACTACAACATCC AGAAGGAGTCGACGCTTCATCTCGTCCTCCGTCTCCGTGGTG GTGCCAAGAAGAGGAAGAAGAAGACGTACACGAAGCCCAAG AAGATCAAGCACAAGCACAAGAAAGTGAAGCTCGCCGTTCT GCAGTTTTACAAGGTCGATGGGTCAGGGAAGGTTCAGAGGCT GAGGAAAGAGTGTCCTTCGGTCAGTTGTGGTCCGGGAACTTT CATGGCGAGTCATTTCGATAGGCATTACTGTGGCAAGTGTGG AACCACTTATGTCTTCAAGAAGCCTGATGAAGAGTGATTAGG TGTTCTGTTTTTTTCTTTCTTGCCTGTCGTTTTTAATTGAATTAT CGTATTGGCATCGGAATATTGGTTCTGTTCTATTGAGGTTTAA ACTTGTTGCGAAAA
BcBal-9 At4g34050 7.7e-23	ACCGTGATCGCGGCCGAGGTACGTTCGTTACTACAGAGACTT TGTTCTTGAGCTTAACAAGGCTCTTGCTGCAGACCCTAGGATT GAGATCCGCATGCTTCCTGTTGGTGATGGAATCACTATCTGCC GTCGGATCAATTGATTGATACCCCACTTTTGAGTGTGGGTTGA TTATTTTCATTTCTCTCTACCTTTCATTCACCAACCATCTTTAT GTATTTTGTTATGTAATATCTCTGTTATCCTTTTTTTTTACTTG
BcBal-10 At1g23720 4.0e-19	CGCGNGGGTTGGCTACAAATCGCCACCACCACCATATGTCTA CACATCTCCACCACCACCAGCATACTCTCCATCTCCAAAGGC CGAATACAAATCTCCTCCTCCACCTTCGTATTATTAAACTCCT TCTATATAATTCATAAATAAGATACCAAGTCTCATGTGATGTT TTGTCTTTTTATTTTTCTTGTGCTAGTCCTTTTTAATTTTCAAG GTTGGAATGGATTCTTTGGCTATTGA
BcBal-11 At3g05890 9.8e-29	ACGCGGGGATCTTGACGCTGTTTGGTTATCTTCCCGGGATCCT TTACGCTCTTTATATCATCACCAAGGACTGATTTTCATATCTC ATCTGTTCTTTCCTCTCGGTCTCCTTGAGGGAACAGCTGTTCG TCGTCGTGATCTCCCATCGGGACTGCTTCATCATTTGTTTTAT

	CTGTTCTGAGTAATTAATTGTCATTGTTGTATTTGTTTATCATA TACTTTATGCTGATTTATATAAAGAAGAATTCGTTGGTTTAAA
BcBal-12 At1g14820 3.5e-34	ACGCGGGCATCTTGCATATGCCTGGATTCTTCTTGACCGTTTG GAAATTCATCTGCCGTTTTCTAGAAGAAGCTACTCAGGAAAA GATTGTGATAATAACGGACGAAGAAGAAGAGAGAAAGTTCA AGGAGGAGATCGGAGTAGATGTCTTGCCAGAAGAATATGGT GGTAAAGCTAAGCTCACACTAATCCAAGATGTTCTTCTTCCTC AGACGTCTCCATAAATGTGAACAACAAACAATAACAATGTTT GACTATTGTCACACTAAACCCTGCATTAATCCCTACTAACAA AACTCTCGTTATAGAAGCAATGAAACACATAAGAAAGTGTGC GAAATAAATCTTGTGAACATCTGTTTTGCTTCCCATTTTATTA AAACATTGTGAAGTTTATTGTTTCGAG
BcBal-13 At2g22250 2.7e-09	ACCACTGTCTCCGTTTAATGGTTCGGATCGTTTATATGATGTT GTAAACTATATATCCTCTTTGTCTCCAATCTAATAAAAGAACT GGTCCGTAAGGCTCTGTTTTTTCAAATTCATGTAACCATAAAC TATTGGGAGTTGCGACTCATTTATTAAGATTAAAGTTTAT
BcBal-14 At5g42980 2.1e-12	TGAATATATCGTTTCTTCTTTTACCTATTTGCTAATATGTTTGG TGTTTTACTAATAATCCACTGTTGCGTTTCGCTTGAGTCATCTT CAATGGAGCTTACATGCCTTGAGTGTGTGAATAAATGTTTGC AAAACTTTATGGCTCGCATCTATGCTCATTTTCATGTTAGTTG TTTTTTAACTCTTTGAGACTATTGTGTTATGTCAGATATCAAT ATTATGTTTACTTGATGAA
BcBal-15 At1g63840 4.3e-12	TTACAAATTTTATAATAACTCAAATTTTAGTTTCCCTTTCCTG AATAGATGATGAGATGATACACGTTTCTTCTTCTATTTTAACT AGCCTGTAACCACTAACTATTTTTTAATTTACATCCATCAGCC ACCAAACACAATACTAACAACAAAGCACTAACAAAGAAAAT AACCTTTTATATTACCATGTCAAGCGAGAGGAAATAAACTAT GTCCTAAAATGATGATTGAGCAAGAGGTTCATAA
BcBal-16 At4g40090 1.4e-14	GCCCGGGCAGGTACGCCTTCGTTTTGGCTTAGGAGAGCCTTG AGTATATTACCCTCTTTTCTCTATCCTTTGCCCTTTTCTTTGTT CTTGTCATGATATGTTTGAAGACTCTCTATCTTTTCTCTAATAC ATTACGTGTTTCTTCCTCTTTGTTGGTGTTTATGTTTTCGTTTTT TAATTCTTTATAAGTTTGATGGACTATGG
BcBal-17 At4g33530 1.9e-19	ATTTTCATGATGTCGTCGAAGGACACGAAACAACACAAACCA ACTCGGGTGAGATATTTTTTTCTTTTACACATTGTTTCATAAA TGTTTTTGTCATAAAAGTATAGGAGAAACAAGATGATTTTTTG TTTTGAAGGTTGAGATATGAATATGTGCTTGTTCGTTATTGAT TGATTTGTCCTGACTCTTTCCAGACTCAGTATTGTATTGGAGT TGTATAATAAAAGGCTTGTTTTGGC
BcBal-18 At4g35350 3.0e-45	GAAGAGCATTTCAGAGGAGTCTCAGTTAGGAGAACTCTGACA ATATTATTTTAAAATCTCAAATTCATCAATACAATATAATGTG CTTTAAACAGGTACAATAGAAATGCATTGAAAGAAAAGAAA AACATAGGATGCAGTAGCAGAAGCAGAAGATAGATGTGTGT CTTAGTTGGACTTGGTAGGATATGAAGCCATCTTGTTGATTCC GCAGATTCCCTCCGGTTTACCGGTGTTTCTCTTCATCCTAATA AACCCTTTCTCTCCCCATCTTGGTCCCCATGAGTTCTTCACAA TAACATAGTCAGATCCCTTTGATGAACCGTACCCAACTGCAG CTACACCATGGTCCAGGTCTGTTCCACACTTCCCATTGAACAC TCCCCCTTTGTAGAACTGGAAGCCCCCGCGTAC
BcBal-19 At1g74840	TTATGTATTTGGCTGAAGAGACCTGTGTCAAAAGAAGAATAA TAGACAGAGGAGTTTGGAGAATAGTTTCTAAGTTTGGTTTTTA

1.4e-14	GGGTTTCATCATCATCATCATCTTTTTTTCCTTTTTTTTTTT TTTCCTTCCATGCTTGGAAGTTGTTGTTGCAATACTACTTACC TGGTCATTCGTGTGGATCTATATCAAAACTTATAAAAACTAT
BcBal-20 At1g70880 4.0e-24	TTTTCACAAAAACATAGGGCTTTTATTATTATCAAACATTTCA TACATAAGTATCGAGTAGCCTAAGCAACAACTTCCATACAAT CATAACACACAACGCTTTCAGAAACTCGGACAAGGGGGCTCC TTAGTCCTTATCTTACAGCACTTTTATTAAAGACTGAACTTAA AGGCAAACACACAAATATAAACATCGATATATAGTATGATTG ACACAAGAGATATTTCATCTATTCCCTGGAAAAGAGATGTTC ATCGATCGTTTTGGATACCTCGACGGCAAATTGGAGGAGAGA CTCAGGGTCAGCCACCTTCTTGTTGTTTTTCACGTATTTAAAG TGCCAATGTGCAACACTTCCAACATGTCCCTCCTTTGAGGTCA CTTGTAAGGTGATCAAGAAATGAGTGTACCTCGGCCGCGACC ACGCTAAGGGCGAATTCTGGAAATATGCAGGACG
BcBal-21 At5g47450 1.2e-08	ACCGTGGTCGCGGCCGAGGTACACCAAAGCAAAGGTCACAA TGATCTCCATCACGATGGCTTCGACCGCTCCTAAACCGGCTCC AACTCCGTGGGTCGGTA
BcBal-22 At2g03150 8.0e-52	ACGCGGGGAGAAGGGTTCTATTGTGGAGAAGACTGATTCCAA ATCTGCTGAGATCAAACCCAAGTCGGAGTCAAGCAAGCACGG AAAGCAAGAGGAAGGAACATCTGATGCACCAAAAAGAGAAG AAACGGTTGATAAAGAGTTGTTGCAGGCTTTCAGATTCTTTG ACCGTAATCAAACTGGCTATGTTAGGGTTGATGATTTGAGAA CAACTATGCACAGCTTGGGAAAGTTTCTATCTCACCGAGAAG TCAAGGAACTTGTGCTGAGTGCTTTGTTAGAGAGCAACACCG GAAGAGATGATCGTATTTTATACAATAAGCTTGTTAGGCTGT CTTTATAGAGAGATGATATTTAATAATGGCTGTATTGTTTAGT GATTTACTCTTTGGGAACTTCCCAAGAAATGTTTTTTTCCTTC AATTTATTTCGACTATTGTACAATAATATCTGCTGAGGCCAGA ATTT
BcBal-23 At1g73010 8.9e-15	ACTTGTTAAAAAAAAAATCACAATCATACAACTTCATCGTCG CATCTTATCTTACTTAGGATAAAAGTCCAATATGTTTTGGTAT ACTTTGACAAGGATAGAGTGATCACAGATAATATTACTCTTC CTCCGTTATATTAAACCATCTAATTAACCGGACTGAGAAACT CGAAGAGCACGGGGCATTATTGGCTCATTATGAACATTAATC CCGAGAGAGATATTCTGAATCTTGCAGTTATTCTCTGCACTAG
BcBal-24 At3g52730 1.4e-19	AGCGTGGTCGCGGCCGAGGTACGAAGACATCTCTGTGCTGGG TCAAAGACCGATGGAAGAATGAGATTTCTCCTTTTTGAATCTT CTCAGACCATAAGAAAGAAATGGAACTCTCCATGTTGTTTTG CACTCCAAGTCTCTTCCTTTTGTTGAATAAATCGTTGGGATGA TTTTTATATTATATCACCTAAAGCGCTTTAATTGAATTTTTGCT TTGTGAACTATGATAACATTCATCATGATACGTGGTTTTGTGC CT
BcBal-25 At5g06530 7.4e-02	ACTGTATCGTCTTTTCCCACTCTATTCCTGTTTCTTACCTTTTC CCTCTGTTTGTATGTTTGTTGGGTTGTTTATGTTCAATAAAAC AACAGAGTTTATGTAAATGTTAAACTAAACAGTAAACTCGCT AA
BcBal-26 At4g26570 3.1e-18	ACCCTTTAAAGCGGCCGAGGTACTTTGGCATCCTTCGCTTCTG ACCTTTATGACTCTTCAGTATCTCAAGGATATCACAACTACGT TTCCGAGCTTTGTGTTTCATTCGCAAGTGGAAGATACCTGAGA AAAACATTATTAGAGCAGGCAATTTGGCAAGTGTGTTTGTGT TTCTGAATGCCTCA

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BcBal-29 At2g06925 6.4e-03	GATATGAATCAAAAGATTGTTACGGATCATGAAAACAGAGAT AAAAACTAGTTAATAATGGAGCCCTTTAGAAAACACATTGAT CTGTTACAAAAAAAAAATTCTTAATACAAACCAGAAATTGGT ATTTGGTATCTCCTTTGGTTTACATCCGGTTTAATACTGATCA TTGATATTTGAATTGTTTTGGGCAGAGCAAAGAATGTAGCCG GAAATGTGAATCAGAGTTCTGTTCAGATGAACAATTTCTAAA CTAAATAAATAATCCTAGTAAAATTCACACACGTGTCATTTTC TGTATTATGGAAAATAATAATATAATGCTTAATAATCTGGAC AGTGCCTCCACTTTTGAGGTATGGAAAGTACCTCGGCCGCGA CCACGCT
BcBal-30 At4g40040 3.3.e-09	TTAATCAGGAGACATCAACATACTAATAGTTTTAGAAATGAG ATTAAACAACGAAAAGTTCACAAATACACAAAAAGCAAAAC ATAATAATACACGACCCAAACGAGCAACACAACACTTAAGC ACGCTCTCCTCTGATCCTAATAGTTTTAGAAATGAGATTAAAC AACGAAAAGTTCACAAATACACAAAAAGCAAATCATACTTG GACAAGAAAATAAGAAAAAACCAACAAAGCATCGTTCATCA TTGTTTTAGAGTCTTACTAATACGAAAAGCCTTCTTAAAAGAC CATTATCTAAGATCCAGCTACGTCTATTTAGAGAACAACAAA CCCCCCGCGTACCTGCCCGGGCGGCCGCCCGGGCAGGTACTT T
BcBal-31 At1g78300 2.8e-12	GTACTTTGATCATGCAGCTACTACGTAACAACCTCACTCTCTG GACATCCGACATGCAGGATGAATCTGCAGATGAGATAAAGG AAGCAACAAAGCCAACAGAAGAGCAGAAATGATGAATCTTC TTCGTCAATACTATGTCTAATCAAAAGAGATGTCGCTCTCTTT GTTTCTTTCCATGTTGAAGTTAACACTCTTCTTTCTCCCTTT
BcBal-32 At5g64260 7.6e-46	GCAGGTACGCGGGAAGGAAACGTCACTCTCAACCTCGTCTGG TACGGGAAGTTCACCTCGATCCAACGGTCGATCATCGTCGAC TTCATCCGCTCTCTCAGCTCCTCCTCCGCCGCCGTCGCAGCGA AAGGTCCGTCCGTCGCGTCGTGGTGGAAGACGACGGAGAAGT ACAAAGGCGGCTCGTCGAACATCGTCGTCGGGAAACAGCTCC TCCTCGAGAGCTACCCTCTCGGAAAATCCCTGAAAAATCCTT ACCTCCGAGCTCTGTCGGGGAAGCTCAACGGAGGCGGCGGCG TTCGGTCGATAACCGTCGTTCTAACGGCGAAAGACGTCGCCG TCGAGGGGTTCTGCATGAACCGGTGCGGGACTCACGGCGCGA AGGCCCGTTCCGTTAACAGCGGAGCTTACGTCTGGGTGGG
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BcBal-34	ACGCGGGTGATGTATGAATGTTTGATCTTTTGGGACCAACTG

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BcBal-36 At3g12610 1.3e-05	ACGCGGGGTTTTGAATTTGAGCCGAAATGCGTTGGAAGGATC CATACCCGATGTTTTCGGATCCACAACCTATTTCATGG
BcBal-37 AtCg00140 8.5e-13	ACGCGGGGTTTGGCTTTTATGGAAGCTTTAACAATTTATGGCC TGGTTGTAGCATTAGCGCTTTTATTTGCGAATCCTTTTGTTTA ATCCTAGAAATAAGAAAATTA
BcBal-38 At4g37220 3.5e-42	ACGCGGGGATTGGTTGGAGATTCCAGCAGCTTTGATCCTTCTT CTGGTGGTTGCACCAAGCCTCATAGCTGGGACAGTAAGAGAA AGTTGGGCTGGGGCAGTGATATGTCTTATCATAGCATGTTAC CTTTTCCATGAACACATTAAAGCTTCTGATGGATTCAGAAAC GCTTTAACTCAGAAGCATGGACTCTCCAATACTATTGGGATC GTTGCGCTCCTCGTTTACCCAGTTTGGACCATCTTTTTTAACA TCTTCTAAGTCGTACCTACCCATTGGCCCGGAAACACCTTCAA CAATCT
BcBal-39 At2g14890 1.7e-31	GGAAGAAAGCAATTTAGTACACTAATAATCAAATAGAAGCC ACAAAATATCTCCAAACGAAATATCCTGATATCCCCCATCTC AACTCTCAATAATATCCTCTCCAAAAGACAATAATCCAAAAG AATAAGGTTCCGAAAGTAATAACAATGGAACAAACAAACAT CAACATGAAAATAAATGCAAAGGTAACACCGGGCGAAAGGC TACCCGCGCGCTTAGATCATAAACCAAACGAGAACAGATCCA AGTACCAAGCTTGAAACTGTCTTGCTTGCTCCATTCTGGTCAT TCGAATCTGTGGAAGGCCCTGGAGAGATCCCATCGGTGCTCG GTCCAGGAGCATCAGTGGTCGGGGCAGGCGGGCTAGCCAAC GGAGATGTAGATGGAGCGTCTGGCT
BcBal-40 At4g12510 1.6e-19	GTACTGCCTTAAAGGCAAATGTCCTAGGAATTAAGCTTAATG TTCCTGTCTCTCTCAGTCTTCTTCCCAATGCTTGTGGCAGGAA GACCCCACGTGGATTCATATGCGCTTGATCAACCTATCAATG CTTCGATTGGAGGATATATATCCTCTCTTTTAATGTTCATCTA TCTTTATATCATTCTTTGAAATTACCTGTATTCAGTTTTAATCG AATAAATGTTACTTCTACTTCTGTTATTGTTTTTTTTTGTTACA AAAATTAAATTGTTCCCACTTCTGTT

EST sequences from SSH, reverse run

Clone	Sequence
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BcBac-2 At1g76930 1.6e-46	CCGAGGTACAAATCTCCTCCTCCTCCGGTTTACCAGTCTCCTC CCCCTCCGGTTTACCACTCTCCGCCACCACCAAAGAAACACT ACGAATACAAATCGCCGCCACCTCCCGTCTATTCTCCTTCTCC ACCGGTTCACTACTCACCTCCTCACCACCCCTACCTTTACAAA TCTCCACCTCCTCCATACCACTACTAGATAAACTGATCGTCAG ACTCATGGAGTTGACGACCCTACACAAGAAAACAAATAATCT TTTGGTGATATAATAAAAGATACAAGATCATTAAGAGATAAT ATCGAAACGGATATATTTTCTAAGAGAAGTTTCCCATTTAATG TGTTTTTTTCTCCCATAAAATAAGTTGTTGGAATAAACTTCAA ACATGTTTGATGAGTTGTCCCTTCATGCATGCAAATTGGTCAT ATGTTTGTAAGTTCAAGTCGTGTTTTCCAAATTAAAGTTTCAA AGTTTGATTGTTTACTTAAATAAAAGTATAAGTTCATTTT
BcBac-3 At4g32020 7.0e-19	GCCGAGGTACGCGGGATAACAGGTCTTAGTTTCTCTTCTTAG GGGTTTAGTGTTTGTGTAGCAAGTATTTGTTTTTTTTAGGCTTT TGTAATTCTAAGTAATCTGTGTCTTGTCTTTGGGTTTTTAATTC TGTTTTAGTCCTCTGATGCTCTGGTAAATTATAGGTTCGCTTT GTGTTTTCTTTCCTTATTATCGAGTCTAGTATTATTCCTAAGGG ACATAGAGGTGTATCTACTGGTAACTACCCTTGCGTTGTGTAT GCCCAGTATTACGATTCTATGTGTTTAATAGTAGTTGTTTTAA GGTCAGTCGAGTTTAATTTGTAATATCAACTTATGTTTCGATG TAAGCATGTATTACAGTTCTCTATCTATATAATATTAATCGTT TTT
BcBac-4 At3g01120 1.0e-40	ACGATAAAGTTTGTGGACTCTCTCAAGATTCCTTACATCGCAC CATCTTTTGGTGGCTGCGAAAGCATTGTGGGCCAACCGGCCA TCATGTCTTACTGGGATCTGACGCAGGAGGAGAGGCTAAAGT ATGGAATGAAAGACAATTTGGTTCGTTTCAGCTTTGGAGTTG AAGACTTTGAAGATGTCAAAGCTGACGTTCTTCAAGCTCTCG AAGCCATCTGAAATTCCAATTATTTCACCCATTAGAAATGTTG TCGTTTAAGTGATGCTTTGTCTTCTCTGTTTCCTTGCTTT
BcBac-5 At2g44670 4.7e-07	CGGCCGAGGTACGGACAGGAACTCTCGTGGTGCCGTAGTAGA CGTGTTTTTATTACATAAGAAGATCAAAAAGCAGATAGTTTTT CTTTTCTATATATGCAATGTTTTGCTCTGTTTCTATCTTATCTT TGTTCCGACGTCGGAATCTGAGAAAGTGTAGATCTGGTTTTTA TGTAAATGAATGTGGAGTTTGGGG
BcBac-6 At5g40690 1.8e-01	GCCGAGGTACGCGGGGGAAGAATATGGGTCCCTTGTTGCTGC TGCTGATGATGATGATGGAAGGATGAGTTTTTGTGATGTGGG GGTGATGATGATGATGATGGATCATGTGGAAGAATTTGATGA AGAAGGTT
BcBac-7 At5g13490 6.0e-04	ACCTTTATAGCGGCCGAGGTACACTACCGAACCTTCAAATAT ACGAAACCGATTGTTTCAACTTTCATATAATAATGTTACAGAC ACGAAGATATCCTCAAAATATTAAAAAAAAAAAAAACGGTTA AACTTAATAGAACAAAAGTCACAATCGAAAATAGGTTCAAA GGAGGAAGTCAAACTCCATCCCCATACAAAATTTATTCAATA AGAAAAATTCCCCTTGCCTCCTCTCTAGTTTTTATAGCAAAAG ATTTCATCAACATCTTGACCTTTTTTAAAAAAAGTCTGAAAAA ACTGCAAATTAAATCGAGAAAAATAAAAATAAAGCCGAAGC TTTTTA
BcBac-8 AtCg00130 5.0e-26	TTTTTTTATAGTTTAGCTACAAGAGGAGATTATATGAAAAATT TAACCGATTCTTTCGTTTACTTGGGTCACTGGCCATCCGCCGG GAGTTTCGGGTTTAATACCGATATTTTACCAACAAATCTAATA AATCTAAGTGTACTCTTCGGTGTATTGATCTTTTTTGGAAAGG

	GAGTGTGTGTGAGTTGTTCATTTCAAGAATAGGCTGGATTCGT CCAGTGGCTCTATA
BcBac-9 At3g16760 9.3e-21	AGGTTTGATCAATGAATCGATCAATCACCCAGCCATTTTGGTT AGGCGGTGAACGGTGCTTCTAGCGATTCTATTACCAGGATCG ATCTTCAAAACCATCCTTAGATCTTCAGCTCCTAGTTTGTATT TCTCCATGCTCTCGTATAAGAGTGCTCGTTG
BcBac-10 At1g21310 1.6e-50	AAAAACAAACTTATATTAATTGGACAAAGCAAACTTGCACAT GAAAAATAATCAAAAAGGAACAAGGCGTAAACTTACAAACG TTTATATGAATACTGATTACGTGCATATTGATATGACCATTAG TTGATCAACACACACATATTTCAAATTTATTGCAATAAAACTT TAAAAATACGTTGGAAAAGGTGAAAAAGCTTTCAATGCTTCG ATTCTCTAGTTCAGTCTAGGTTTCTCTTATTACAATCACCAAT AACGGTTTCCATCTTGTCTTTGTCTTCTTGTGCACATCGGGTTC TCACCGTGACAAAGAATTCTAATAGTGGTATGGAGGAGGAGG AGATTTGTAAAGGTAAGGATGGTGTGG
BcBac-11 At1g78370 2.1e-10	CCGAGGTACGCCGCTGAGTTTAGGAAGAATAATCTCTGATCT GAATAAAACCTTGTTTGTGCCTTTTGTTTCTGATGATGCTGTG GTCTGTTTTTAACTTTGGTTTTTGTGTGTTTCCTAATAATGCGT GAGGGCAAGCGTCGGCCTTTGTGTGGTGGTGTTGTATGGATC CTGAGTAATGCATTTTTAAATAAAATATATTTTCAAATAAA
BcBac-12 AtCg01130 8.9e-84	TCCAGTGACCGCCCGGGCAGGTACTAATAATGGGGTTCAACT ATCCGAAACAGAATTTCCACGAAACTGGTTAACGGATGGTAT TCAGATAAAAATCCTATTTCCGTTTTATCTTAAACCTTGGCAT AAATATAAATTTCAATCGTCTCAGAAGGCTCGACTAAAAAAA ACAAAAGGAGAAAAAAATGATTTTCGTTTTTTAACAGTTTGG GGGCTGGAAACTGACCTACCTTTTGGTTCTACCAAACCAAAG CCTTCTTTTTTTAAACCTATTTTTAAAGAATTAAAAAAAAGAA TCAAAAAATCCAAAACGAAGTCTTTTCCGGTTTTAAGGATTTT CAAAGAAAGAGCAACAATTTTCCTAAAAGTCCAAAAAGAAA TTAAAAACTGGATTATGAAAAACTTGCTTTTTCTAAAAGAAA AAAAAAAAACCTTTCAAAACGAAATAGAATTCCATTAGTTGG CCCGAGAGAAATATATGAATTAAATGAAACTAAAAAAGATTC AATAATGAGTAATCAGATGATTCATGAACTATCTGTTCAAAA AAAATCGACGGAGTGGCCAAATTCTTCACTCGGCGAAAACAA AATAAAAAATGTGATTGATAAAATAAAGACAATCAGAAATC AAACG
BcBac-13 At3g20630 1.2e-21	AGCGTGGTCGCGTCCGAGGTACGCGGGACTATAGCACTGCTC TGAAGGGAAAGACAACAGCTATCAAGACAACTGGTTTGACAT CTTTCCCAGATTACTTGGTCTTGCACATGCGGAAGTTTGTTAT GGATGCAGGCTGGGTGCCAAAGAAGCTTGATGTGTACC
BcBac-14 At3g47540 7.0e-20	GGTACTGAGCCAAGGATTTGGGGCCACCATTAGAGCCATCAA TGGAAAAGTAGAATGTGACGGTGCGAGTCCAGATAAGGTTA ATTCAAGGATTAGGTATTATAGAGAGTATTGTGAGCAGCTTG GTGTGGACCCTGGTTCTAACCTTAGCTGCTAAAAAGCTCTCTA AAGACTATTGTTGGACGGTCGCCTCTATGTTGAAATAATAAA ATGATTTC
BcBac-15 At1g66280 8.7e-54	GCCGAGGTACAGCTGACCATAACAGGAAATATTATATTCAGA GGCATCTTTTGAGCCTGAATGAAGCTATTTGCATCGACAAGG TGAATGTTACTGGATACTTTGTATGGTCATTGATGGATAACTT TGAGTGGCAAGATGGTTACAAGAACAGATTTGGACTTTATTA CGTTGACTTCAAGAATAACCTCGCACGACATGTTAAAGAATC

	TGGCAAGTATTACAAAGAGTTCCTAAGCCAAGGTGTTCGTCC ATCCATGATCAAGAAAGATGAGCTTTAATTGAGATGTATTCT GAGGATTTGGTTGTCTGTTTCAAGGTTTCTTTCTTATGTTTTCA TTTGTGATTTGACCAAGATCAATGAAGTCTTGGTTATAATAAG AATCTTTTTCAATTAAAAAAA
BcBac-16 At5g11520 7.9e-20	CGCACCTTGCAGACGCTATCCATGCCGTTGTCACCAAAGCCC TCTGAGACATCTCACACCACCACCACGGTTACATGGTGATAA TAAAGAATCTTACCTTCTTTCAGTATTTCCGGCAGTGACAAGT TAATTGTCACTAATATTATTGTTATTTCATCTCAGTGGTTAAT AAAGTAGGCAAGAGAGTTTTGTGCCTCTTCTACTATT
BcBac-17 At2g36530 2.0e-15	ACCCTGATACGGCCGAGGTACTAGACAAGTGCCAGCTCTTAC CCTCGAAGTGCTCTCCTTTGTGACGAGAAACAGATCTGAGTT GTGTTTGGTCCATTTTGCTTTAATAAAAACATACGTTTGCTTT TTTTTTCTCCCTTCGTTGGTTTATTTTCCGAACCGCCCTTGTTG GGAGTAAGTTGCTTATTTTTGTAAGAAAAAAACCTGAGATAG CTCTGCCTTTAAAGAGGACAGCAGCTCTTCTTTTATAATCTAA TTTCGGCTTCTCTTA
BcBac-18 At1g69880 6.1e-10	TTGTGGGTGTGAAGTTTGATGAATTACAGAGGAAACTCCACA AATACGCACAATCCTTCTTTTGAAAAATATGAAGCTTTTACTT GCATGCATCATGCATGATGACCACCTTATGCCAAGAATAGTA TGCTACTATCTAAATAAATGTAGCTCCTCGTGTATGATCACCA
BcBac-19 At5g61340 6.5e-27	ACGGAGAAGTTAGCTCTTATTACAAAGGAGACAGAGACATCA CCTCCATAGCTCTCGAAGGAACGTTCATAGCTTACTTATACGC TCTCTTCTTGGTTCTTGACACCATAGTCAACTTTTTATTTTACC AAAGCTGCGTCAAGAACGATGAGGATCAGAAGATAGGTAGA GAAGACGAGTACC
BcBac-20 At3g11780 5.4e-17	ACCTCATACGCGGCCGAGGTACTGACTACACAAAGAATATCC ATTTCTTTCTTCATTTTTCAGTTTCCAAAAGACTGTTTTCGAAA CACTCGCGGAAAAGTTGTTTCCTTGCGTTTTTTTTGTTGAATA AATTTGTTTATTTTGTAACAATGCCCTGAAGGTTTGATCTCCA GAATGGCAATTTAGTTTGTTGAATAAAGTTTCTGAAAGACAA
BcBac-21 At1g25570 2.1e-19	AAACAGGAACAAACAAATGTGGAGTATGGTACATACAAAAC AAACACAGTGAAGGGAGTAAGTAAAAATGTTTTTCTGTCATT AAAAATAATTTATCCTCCCTAACATATCTACTAAAACCCCCA AAATGAAATTACAGTGAAACAAGTTCTATCTTACTCTTGATG CAATGCCATTCTTCAAAGTTTACTTGGGGTCTTCTTGAGGAAA AATGCTTTGTATACTAATTACACAAAAAATCCTCAAATCTCAT CTCCTTGCTCTCTGCCCAAAGTCAAAACTCTCTCTTTCTCGGC TATTGCAAGTTCAAGGGAA
BcBac-23 At3g60600 1.2e-10	ACCCTGTAAACGGCCGAGGTACGTTCTTCTGTTTGGACTAATT CGTCTGATTTTGGGATACATTATGAAGAGCACATAACCACCA CCTCGAGTTCTTAGTCCCACACACCGCCACAGACTTATTTTTA ACTCTTTCTCGGGTCAGTCATTTTCTCTCTCTCTCTCTGTATTT GACAATGTCACCTAAAAAAAAGGTGCCTCTAAATGATTTGTA TTCCGGTAGTAAAAAATTCATAACTCATAA
BcBac-25 AtCg00670 1.1e-07	AGCGTGGTCCGTTAAAGGTACCTTTTCGAAGTCCTGGAGAAG GAGATACATCTTGGGTTGACATATAGTGCGACTTGTCAGATA TATTGGGCTATATGG
BcBac-26 At1g13180 1.7e-58	TCGAGCGGCCGCCCGGGCAGGTACGTGCGTTATTAGCAAGAA CACGGGCATCAACAATCTTTTTGAGATCCCTTTGTAGCCTTCT TCCGAAATCTTTGAACATAGTTGAGCCTCCGGACAATACTAT

	GTTCTTATATAAAGCTCGTCGTGTGTCAATTGGTGCAGACTGG ATACATTTGTCTATTACAGCCGGTAAAGAAGTTGTAAAATCA TTGCTGTATATCTCTGGATTAAAGAAGACCTCAGGTCCAAGG AATCGTTCGTATCCAACGTCACAAGTGTATGGTGCACCAGTC TTTGGCTTAACACCTTTCCATTGTTTAATATACTTTCCTGGT
BcBac-27 At4g04860 2.0e-17	AGCAACTCATTAACTTTCATGATGGTGTATGTGTGGAGCAAA CAAAATCCTTATATTCACATGAGTTTCCTGGGTCTGTTCACCT TCACAGCTGCTTACGTACCATGGGT
BcBac-28 At2g34690 6.3e-11	CCAAAGCGGTTCATGAAATGATCCAGCTTTTGGAGGCCGTAA TGTCATTACATGCATAGATTGATAAAAGAGAGAAACTTTTAA GAGTGATAATACTTGCAAAATAACCAGGGCCCATGCTAGTTT TCAGAGTCTTCACCAAGTCGATACCAAGGTTCTTGGAGAGGA AGAGTTTGTCGAGATATGCAATTACTGGAGTCGATGCAGTAA CATAGCTTTGCAGATGTACCTGCCCGGGCGCCGCTCAAAA
BcBac-29 At2g39330 3.5e-05	AGCCTGTTCGCGGCCGAGGTACTCGTTTGAGGAGAAAGGCCA CAAGATCACAGGGTTCCATGGACGAGCTACCACCGATGTCAT CTACAGTATCGAAGCCATTTCCAGGCCATTCTAGCTGATAATC TGCTAGTCACATCGACTAAATTTCAGTAAGAGATTGGTGTGT GTTTCTCTTTATGAATAATAATATTACTTTCAATATACTGGTG TGTAAACTCTTTTATAATTTTGCTATTCTTGTTTCTTTTGAGAC CCTTCAAATATAGTTTACCTATCTTTATCGACTT
BcBac-30 At4g12520 1.9e-22	TACCGCCTTAAAGGCAAATGTTCTAGGAATTAAGCTTAATGT TCCCGTCTCTCTCAGTCTTCTTCTTAATGCTTGTGGCAGGAAG ATCCCTCGTGGATTCATATGCGCTTGATCTACTATCAATGCTT CGATTGGAGAATATATATCCTCTCTTTTTCATATTCATCTCTCT TTATATCGTTCTTTAAAATTACCTATTTTCAGTTTTTAATCCAA TAAATGCTACTTCTATTTCTGTTATTGTATTCGCTTTAAAAAG TGTGCCCATGCATAATAAAAGTAATT
BcBac-31 At5g52530 1.4e-01	CTGTTGTTCTCGTCGTGTAAGCTTTTGTTAGTTCTGCTTCTCTT GGTATTTTTGGTTTAAATTAATGTTTCCATTCTTCAGTATCCTG TAGCTGTAGGGGTGACAACCGAAGATATATGATGTCCTCTTT TGTGAAGAAATCAAGTTGTAATAATTTTGAGTTGTTTTTGTGG
BcBac-32 At5g04950 8.0e-19	CGTGGCTGCATGTTCATGCCTTGAAACTGCTCTAAGGTCCATG CGATTATGAACAATCGTTGCAAGAAGATGATGATCGAGGAGT TTAGTGCCATCGAGTAACTAAAGCAAGTAATTACTGAACACA TCTCATCTCTTGAGACTATATGTGTTTTAATGTTTTTGTCTTGT ATTAATTTCTCTTTTTAATTTCATGTGATCTTGTGTGTTTTCTG ACGTGTGTGTGGCTCGTATAAGTTGGTGAATTTTGAGTTCTTT GACCTTCGTTATGAACTGTGAAGATACAGAAGAACTCTTTCTT TGTTCCCTTTTTTTTCATCAATAAAAAATAATATAAATAATGT TTTAAAA
BcBac-33 At4g25630 2.1e-05	ACTTTTAAAGCGGCCGAGGTACCTACCGTGCCCCTAAGAAAC CCTAGGCTACTACTGCTGCTTAGAAAAATGCTTGTGTTTCTAC TGTCTTGTTTTTTTCTCAGTTTCTGCTTCTGTCTTGTGTTCTTTT TTTANAATCTGTAGTGCTTTANAATTTTGTTAACGAGAGACCT TGAACTCATTACCAGATTTTGCTCGTTGAGAAAGTTTTAATAG
BcBac-34 At2g19590 1.3e-37	GTACACCGTGTAATGACATTGACGGATGGATGTAGACTGTCG TTAGCAACATTTTACAATCCAGCTGGTGATGCCGTAATATCTC CAGCTCCAGAGCTTTTGTACCCAAGTGGCTACCAGTTTCAAG ATTACCTTAAGCTTTACTCAACCACTAAGTTTGGAGATAAAG GCTCCAGATTTCATACCATGAAGAAAATGGAGAATGGTGATT

	CCGTCTAGGATGCATCTTTAATGTAACAATTCTGTATTGCAAC ATGAAACTCTCTTTTTATTAAACTTTGTCTTCTTCTT
BcBac-35 At1g26250 8.3e-16	ACCTATGACGCGGCCGAGGTACGTGTACAGTTCTCCTCCACC ACCATCTTACATCTACAGCTCTCCACCACCTCCAAGCTATAGT TATAGCTATAGCTCACCCCCTCCACCAATATACTAACCATATA TACATAATACAAAGAAGGTCTTACTAATAAGTTACGACCATG GTATCCACGGTTTTACAATATTTCCCTTTCGAGACTTCAATAT AGACGAAATGT
BcBac-36 At3g04120 5.6e-07	GGCCGCCTAAGTCGATGAAGATCTCGAATGATGTAATTGGTG TTTTTAAATTTGTCGTTTTCCGAATAAGTTTTCTTGGGTTTTGA AACCTTTATGGTTTTGGCGAATTCTCTACTTTCACGTGACGTG ATAAGAAGTTTGTAGACCGGTTGTTT
BcBac-37 At5g23910 7.0e-15	TCACATTGGACAGATTCAGGATACAATCCTTCAAACTCTTTGT AGAAAACATGCATCAATATACTTCAGCTGTGACCATATATAA TATCTTCAGCTACTGAAAAATATCACCAATCTCCTTCCTTAGC AATCCCTTAATCTGTTTTTCTGAGAGTCCGATATCTTTCAAGT CATCAAGTTCCTTAAATGGTTCGGGAGATTCTTGGCGGAGCT CTACTATGTAAGTAGCTCTCTTCTC
BcBac-38 At5g43830 1.7e-03	TGTTTGGGTTAAAATATCTCTGATAATTTCCTGATTACTTTAA CGATGTTGTTTCTCTGTTTGCGTTTCTCGGATTCTAGTACAAA ATAAGATTTTTTCGATC
BcBac-39 At4g37520 6.5e-10	AACCGGTCAAAATGGCAATATCCGCCGTGATTGTGGAGCCTT CAATTGAATTTAAATACTCATCGATTTCTTCTATTTTTATCCA ATTTCAGTTGTTGTTTAGGATGGTTTCGTGATGCTGGTTCCGC TCCAGTATAATAAGTCTCAATTTTGGGTTTCAGTTTGCTAAAA GTAAAAATG
BcBac-40 At5g28900 7.8e-08	AGATGCATCCAATGGAAGTGCCGAGGCTTGGGATGAGTCCCC GGAGTCTCCCTTCTGAGTTCATAACAGAACAGAAGATACAAA GTCATTGCCTCAATGGGAAGAATCTTTGGAGATCATCCTCTAT GTTGAAACTTGAAAACCATAGTTTTGATTTTCTATAATTTCTA TCTAAAACTATACATAGTTCTCTATTTATTAATAATCCTTTAG CTCATGGGGTTT
BcBac-41 AtCg00140 2.8e-16	CCCATCAGGGCGGCCGAGGTACTTTATTGCTTAGTTTGGCTTC TATGGAAGCTTTAACAATTTATGGCCTAGTTGTAGCATTAGCG CTTTTATTTGCGAATCCTTTTGTTTAATCCTAGAAATAATAAA ATTAGGAA
BcBac-42 At5g54500 1.9e-19	AGGTACAAATCATTACATATCTATACGTAAAAACCAAGACAA GAATTATTATTACTATGAAATAATACCAAGTAGTTGATAGAT AATGAAACCCAAACAAATCACAAAAGAGAGAAAGAATCTAT GATTGTATATATAAAACAGCAGAACCAAACCGGTCATGTTTT CTTTTCCTTCTTGATATGTATTCTTTTTAAAAAGCTCTAAACA GTGGTAGATCCCTTGAGC
BcBac-43 At3g19200 2.0e-26	AACTAGGTCTAAGTAATCCCAAGAAAATTCTCTCCAAGAACA ACAACAACAACTTCCACCTTCCCCACAAATCTCAGAATTGTCT TGCGAGTATATTGGAATCTGATGACTGAACGATGGGCTCCTA AAACATTTAGCTAATTACGACGAACTAGACTTCTAATGCTAA TGACAAAATAATTTATTTCCGATTTTTTATTGTTCTTGTTTTTC TTTGAGTTCTCGACTTATCTCTTATTATAATGTAGTTTCCCCGT TATTCTGACCTGTGATCGTGACTTCATTATAAATAAGCAATTC GTATGCAATTGGTTTAGT
BcBac-44	TCCCTCGGCCGCAACCACCCTAAGGGCAATTTCGGCAAATAT

At4g38520 4.6e-02	CCTTCACATGGGGGGCCGCTCAAGCTTGCTTCTAAAGGGCCC ATTTCCCCCTAAAGGGAGTCGAATTACATTTCCCGGGCCGTCT TTTTACAACGTCGGGACGGGAAAAACCCTGGGGTTACCCAAT TTAATCCCTTTGAAGCACATCCCCTTTTCCCCAGTTGGGGAAA AACCAAAAAGGCCCGCACCAATCCCCCTTCCAAAAATTTGCC CACCCGGAAGGGCAAAGGGACCCCCCCTGTACCGGCCAATTA AGCGCGGGGGGGGGG
BcBac-45 At4g12545 3.3e-16	AAGACAAACATCCCGTATGGTTTCAAATGCTTGAATTTATTGC CTTATTTTCTTGTTTCTGTTAATCATTGATGATTGCTTTTGTGT TTGTAACTTTCTTGGATTGAATAAAATGATGAACGCACGTTCT CATGCAACAAGGGATTATGGGTTTGTGTGTATAACCGGCAAG TTTGTTCCCCTTTAACTTCATTGTTCCTCCTTATCTTCGTTGTA AACGTTTCTTTTGAATTTGATTAATAATACTATCAAACGGTGT TGTTG
BcBac-46 At5g13420 2.5e-41	TTCATTCAATGGCTGGCTAATTTGAGAGTGTTGGCCTTGTCTT GTAGTGTACCAAGAAGACTCTCAAAACTCTTTTTGAAAGAGT CAACACCTTCTTCTTCCAACTGTTCTCCCACCTTGTTCCAGTCT ATTCCCAGCTTCTCTAGTGCACTGTAAATCCCTTCTGCTTCCG ACACATTTGCATCTATAGTCCTCTTCACGGGTCCATGATCTAT GAATGCTTCCAGCGCTTGATCCGGCATGGTTG
BcBac-47 AtCg01110 1.4e-47	TTGTAAATTGAAAACACCCGACGGACGGATTTTCCATCACCA AGGAAAACCACTTTGATCTCCTATGACAAAAATTGCCAATTA CCCTTTTGGAGCTTCAACACTTACGAAAGGTTCTTGTTTCGAT AATTCAAAAGTAGGGTAAGGTTTTTTACTAATGAATCGATAT TCAAAATCATTCCACTCTGGATTCCTTTTTTTATCATAGCCTCT GCTTTCAAAATTTTCATAGGGACCCCCCGGAAGTCCTTCCACA GCCTGTTGAATAATTTTGATGGATTCTGT
BcBac-48 2.3e-01 At5g15210	TAGATGGGGTATGCAAATTTTCAAAACCAACACATCAATGAC TAAATATTTTAAAATAAACTTAAACTAGTGGATACAATAAAA GATAGTCACACAAGCAATAAAGAAGAAGCAGCTTGTTGACCC GTTGGACTACTACGTCCATGTAGTTCACTTGTGGATGGGCTCA CTATTTATGGATGCAGC
BcBac-49 At3g52300 1.9e-22	GACTTTGAGAAGACCCAGAACTCAAGAAGAAGTTTGATGACG AGATCCGTAACGACTACTGGGGATACTGATCATCATATGTTC TGTATCTCCGGTCTGGAAATGAAACTCTCTCTTCTCTTTTTTCT TTTGATGTGATTTTGTGAGAGTCAATCATAACATAATAACGA CCACTCCATTTACGTAAGCAGTGTGTTGAGATATTTCATTGGA GATCCAATGAGACG
BcBac-50 At1g79750 3.5e-09	GCCGAGGTACAGCCCTTCCTATCATAGCTACCTCTAAGGGGA TAAGCTTAAACTATCTTCCCATTCTTTTCACCATCTTAATATAT GTGTTTGTTATCATCACTATTCGAAATAATCTACTAAAACTGC TTCTCTAGAGTTTAGACTCTTGTGTTTGTAGTCATGTTATGTTG TTTAGCATCTTCGTATACACATATATGCAACTGATTTCATATG GTTTTCCTTTTAGGAAATTTTCATGATAATCCTTTTTTTGTT
BcBac-51 At4g34720 1.8e-16	TCGAGCGGCCGCCCGGGCAGGTACGCGGGGGCATTGTTCCTT TCCAACACTTTCAGAGAGAGAGAATGGCGACGGCGATTGTAC GTTCAGCTCTAACCCGAGCAGCAGCGATCAGGGCAGCTCCGA GGACATCATCTGCCCCGAAGCGAAGATTTTCATCTTCTGCCG GCCGTGACGATGCTTATGAAGCTCAAAAGTGGGATAGTATAA C
BcBac-52	AGGGCTCGAGCGGCCGCCCGGGCAGGTACGTTCTGTTCATAG

At1g63120 4.7e-24	TTGCAGTCCTTCTACTTGTGGTTTGTTTAACTGTTGGATCAGT GATGCTTTTCAAAGGAGAGAATGGTAACAAACATTGCAAATG GTGTCATCGCCTCAGCTGTTTCCCCACTTCGAAATGGACCTGT TAATCATCATCATCATCATCATCATCATTATCCTCTAAAAATT CTTATATGTGTTCTTCTTGTAGCAGATAATTTGTTTGTTCTTTT CGCCTTTGACCT

Supplementary material Chapter II: ESTs from SSH

Clone	Sequence
BcBal-1 At3g19560 2.5e-01	AAAGAACAATTCTTTCAGTCTCTTTTTTTCCATTATGTTTCTCA ACTAGAGAAAGAGGAAGTTGATCATCTGAAAGTCTACAAATA ATTTGAGTAACATAAACATCACGATAAAGAGGAACAATTAGT CACGGGTCAAATTTTTTTAAAAACAAAACAGAGTTAAAATCT TTGTGTTAACTCTAATGCTTCCAGGATTTTTCGATAGGTAGAG GCGTGATATGCATCTAAAGAGTTAGTGATTTGCAGCTCCCCT AGTAAAGTTACAACATACGTTATTTCAAATAGAAAAATCTAT
BcBal-2 At1g23720 6.3e-55	ACAAATCTCCACCACCACCATATGTTTACACTTCTCCACCCCC ACCACCATATTACTCTCCTTCTCCAAAGGTTGACTACAAATCT CCTCCACCTCCATATGTCTACAGTTCACCACCTCCACCACCAT ATTATTCTCCTTCTCCAAAGATTGAATACAAATCTCCACCACC ACCATATGTATACAGTTCTCCACCCCCACCACCATATTACTCT CCTTCTCCAAAGATTGAATACAAATCTCCTCCACCTCCCTATG TCTACTGTTCACCACCTCCACCACCATATTATTCTCCTTCTCCC AAGGTTGAATACAAATCTACACCACCACCACATGTATACAGT TCTCCACCCCCA
BcBal-3 At1g55340 2.7e-26	AGCGTGGTCGCGGCCGAGGTACTTGGTTATCAGATCTTTGCA AGGAGAGGTATGAAGTAAGAGAGAAGAAGACTTCAAAGAAG CGACCAAGAGGACTGAAGGCAATGGGGAGCATGGAAAGCGA TTCAGAGTAAAGATCCAACGAAAGGAAACATTCAAGATTTTG GTGAGAGCTAGAAGGGAGTAGACAAGAATCTGTTTTGTTTCT TTCATTACATTTTGATACGCCCTCTCTTCATTACAAGGTCATC
BcBal-4 At3g28550 9.3e-28	ACAAATCTCCACCACCCCCATATGTCTACAGCTCTCCACCACC ACCATACTATTCACCATCTCCTAAAGTAGACTACAAATCCCCT CCACCACCATACGTCTACAGTTCTCCACCACCACCATACGTCT CACCTTCTCCAAAAGTTGAATACAAATCTCCACCACCAGCTTC GCATTACTGAAAACCAGTAAACGCGGGTCTTAACTAAGCTTT CCAGCATTATTTGATTTCAAATATTTTCTTCTTGTTACATGTGT GCAATCAATTTCAATTTTCTTGAGAGTGTGGACTAATAACATG GCTACAATACCTTTATAT
BcBal-5 At4g29480 5.4e-18	TCGAGCGGCCGCCCGGGCAGGTACTGCGCAGGCGAAATCGCT GGCAGAGGATTCACCTTCACCGGCTACTACCCTTGAAAGAGA GGAACAATAACTTGAACAAGATTGTGTAATCTGAGTCATTTT TTTTCGAAGTTTTCCCCTGAAACGGAAACAGAGTATTTGGATC TGAGAAATTTCCATTTTGCTAAACTATCTTTGAGATTAATAAA GAACAAAAAATTTCCTCTCTAAAAAA
BcBal-6 At1g29850 1.5e-10	AGCGTGGTCGCGGCCGAGGTACTTAAACGTTACTGTGAAATA TGTTTATTGTGGTAAATCTTTGATGATCTTCCAAATGCTTGGG ATGAGATTATAAGAGTAATCTGAAACTGAAACTCT
BcBal-7 At4g37940 2.2e-26	TCGAGCGGCCGCCCGGGCAGGTACAAAGGATTCATCAAGAA AATGTGGAGCTCTACAAGAAGGCTTATACAACAAACGGGTTT GCGCACCGTGAACTAGTTGTCGCGGATGATGAATCACACACT CAGATTCGGCTGCAGCTAAGCCAACCGGATCATTCCGATTAT GAGACCCCACCAAGAGGAAGCGAATAACAGCATATTGAAGT TCGAAGCTCGAAGATACCATGATGTTGAAGAACAAACCAAA GTTTTGGTTTGAATAATTGGAAACAAAGAAGAAACCTCAAGA AACCAAGCATGTTCCTTCTCATCAATTATATATATTTTTGTCT CATTCTCATGTGCATTTGAATTATA

BcBal-8 At4g12545 4.1e-16	ACTCCTCAATAATTGTGGCCGAATAACTCCGACATATTATCCA TGTGTCTAAGTTGAATACTTATGACATATAAAGACATGCCTA AAAGACAAACATATCGTATGGATTCAAATGCTTGAATTTATT GCCTTATTTTCTTGTTTCTGTTAATCATTGATGATTGCTTTTGT GTTTGTAACTTTCTTGGATTGAATAAAATGTTGAACGCACGAT TCTCATGCAACAAGGGATTATGGGTTTGTGTGTATAACCGGC AAGTTTGTTCCCCTTTAGCTTCATTGTTCCTCCTTATCTTCGAT GTAAACGTTTCTTTTGAATTTGATTAATAATACTATCTCTGAA GCATATATAATACAAAAAAAATACGTGCCTCGCCCGCTACCA CACTGAGGGCCACTTCCGCAGAT
BcBal-9 At2g45960 2.0e-69	TACATTCACAAAAGGAAAAACCAAACTAAGAGGAACAAAAA AGCCAAGTTTTTAAAAGAGTAACTCAAATCAGCTTCTGGACT TGAATGGGATGGCTCTGATGACGATCACGTGGTAAAGGGCAG CAAGTGCAGCACCGATGAAGGGTCCAACCCAAAAGACCCAA TGGTCATCCCAAGCGTTGTCCTTGTTGAAGATGATTGCAGCTC CGAGACTTCTTGCTGGGTTGATTCCAGTTCCAGTGATGGGGAT GGTCGCCAAGTGGACCAAGAACACAGCGAATCCAATAGGAA GAGGTGCAAGAATGGGAACGTGAGAGTCACGAGCGTTTCTCT TGGCATCAGTAGCGGAGAAGACGGTGTAAACAAGAACAAAG GTTCCAATAATCTCAGCTCCGAGACCACTTCCTTTAGTGTACC TGCCCGGGCGGCCGCCCGGGCAGGTACCTGCTGCTGCTTCTT
BcBal-10 At3g13782 2.7e-59	TGAAGTCTTCTCTCTAAAGTTACTTCTTCATATGTGTGAGAGA TTCTTGGAACTCTTGGTGTATCTTCTCTTCTCGTTAGTCATCAT TCTCATCATCATTATATTCATCTTCATCAACAAGAGCCTCTCC AGTAAACCATGAAACTGCGTGAGGGATCAGTTTGTCTCGGAT TGTCACAGCTATGTCATAGTCTTGATCCGTCAGGTTTTGGAGT TCTTCATCAAATAGTCTAACCATGGTTGTATAACATTCATCAA CTTCATCAATCTCAGGAGGCTTGAAGAAGTTAAAGAAACTCT CGCAGTTTTCGGTTTTAGTCATGGGGATGTTGTTCACTTTCTTT GAACCCTTCTTTGGCCTCTTCTTGACAACAACCTTATGAGTCA AACACTTACCTGGACACCATTCTATGTCCGTTCCAATCACTTT ATCAAGAACAGGACCATCATCCCCGCGTACATGTTGTATTTTT TTTTTGGTTCACCAACTACATGTTGGTGAACAACTGCATGTTG TAACTATGGGAGTAAAATAATAGATGTA
BcBal-11 At5g04740 2.1e-119	ACCGTGGTCGCGGCCGAGGTACTTGGTTCCAAAGCCAATGGT TTTGATAGATCAGGATGCTGACCCTGAAGCTACTATTGTTCAA CTCAGTTTTGGTGACCGTCTCGGGGCTCTCATCGACACAATGA AGGCACTAAAAGATTTGGGATTGGATGTAATAAAAGGAACTG TCACAACCGAAGGATCCGTTAAGCAGACTAGATTTTCTATTA CAAAACTAGACACTGGTCGGAAAGTGGAAGATCCTGACTCAT TGGAGCAAATTCGGTTAACAATCATCAACAATCTCTTGAAAT ATCATCCGGAATGCAGTGAGCAACTTGCAATGGGTGAGACTT TTGGAATCAAGGCTCCTGAAAAGAAGATTGATGTCGACATCG GGACTCGCATACATGTGAAAGAAGATGGACCAAAGAGGAGC ATGCTATGCATAGAGACAGCAGACAGGCCAGGTCTGGTTGTA GAGATGATAAAAATGATGGCAGATATCAATATAGAAGTGGA ATCCGCAGAGATAGACACTGAAGGGCTGGTTGCGAAGGACA AGTTTCACGTAAGCTACCAAGGGCAAGCGCTAAACAGTTCCT TGTCACAGGAGTTAGTGAACTGTCTGCGTTACTTCCTGAGAA GGCCTGAAACTGACATCGACAGCTATTAAAAAAAAATGCTCT TTCTCCTTTTCGAAATCGCATTTGCGATCTCTATCAAAGTCTT

	CTATTTTGAATATTATGTGGAAACTCGTAATACCATGCATAAT ATTTCTGTCTGGTAACTAAACCGTTGGG
BcBal-12 At5g05780 2.0e-45	TCGAGCGGCCGCCCGGGCAGGTACCAACCTACAACATGCTCC TTAGCATTAATTCTCTTGAACACGTGGAACATCGACTCATGGT AGTTATGATCAAGAAACCATATACTCGGATCCTTGTCATCCTC CTCGAACGGCACTGCGTAGCTGTTGGTGACGTCGACGACGCC GCGTGAGCTGCTCCCGAGGAGAACGCCGACGACGCGCTTGCG CGAGTCTTTGGCGACGCGGTTGTAGTGGTCGGCGATGCTGAG CAAGACGAGTGGGTGAACGACGACTTTCTCGATCGTCCTTGC TGATATCTGCTG
BcBal-13 At4g13940 9.2e-19	GTACAACNGGTGGTCACAACACAAAAGCAAATCAAGAGGGA ACTCATAACCAAAAGCTAATTTAAGTTTCAAGAAAAAGAAAA ACAACAAGCACAACCTTCAAAGCCAATAACAATTATTCAGAA GGATATTTGGTCAACTTCATCAGCGAACCAATATCTTCCACTC TTCCCCCGCGTC
BcBal-14 At4g02270 3.5e-22	CGGGCAGGTACGCGGGGATGTGAACAAGGGTGTGAGAGGAA TGTCATTGAACAAATACCGTGTTCTTGAAGACAAGAGCTTCA AACTTTACTGGGCTGGTCCTTTCTTCTACACCTCTGAACCTAC TTACTACTAAACAAAGCTTCTTTTGATTGACGTGAAATGTAAC TTTGAGAGTCCTTTTGTTTGTTTGATTCGTTGTCTGAAAATGA GCCGAGACTTTTTATGTTTTTTATATTGTCTTTCTTGGAAACGT TTTAACATATATATAAACCGTGTTGTGAAGTANAAAA
BcBal-15 At3g46580 6.2e-04	AGCGTGTTCGCGGCCGAGGTACATCACCTCGGTCCTTGACCG GAACTTACGATTTGTAACAGGTTCGTAGTAGAACTATATTAC AAAAAAAAAAGGAAAT
BcBal-16 At5g44020 2.1e-28	CCCCTTAGCGTGGTCGCGGCCGAGGTACTCTTTGCGAGAAGA AACCAAAAAGATCTTCAAACCCCTCTCTCTGATCTCATGGAA CAGCTTCACCATGTTTGGAACAGCTGGTGCCTTTCCCCAACTC TGCCATTCCTCGAATTTGGCGATGTTTAGTTGCTCACCACCGA AACAGCCGTTGCTCTTGTGGTAAGGAATGGTGGA
BcBal-17 At2g28970 7.6e-53	GAAATACAACAGAACAAATCTTCTGAACAAGAGAAAGGATG CAATACATGTAAGAGTCTAGCGTGCATCAGGGGTCATTTGGG TTCCAGAATTATCAGTCAATTCTAAGGAGTTGTTCGATATATC ATCTTGAATCTGTCCTCCACTCCTCGAATTCTCATAGATCAAA CACTCTTTAAGAACGTTAACAACTTTCGACATGTTTGGTCTAT CACATGAAGAATGACTAACGCATGACATTGCTAGTTCAAGAA TCTTCCAGACAGTATTAGAGTCATAATCTCTACCAAGGTTTGA GTCAACGATTTTCTCAATATCTCCTCTGCTTATAAGAATCTTA ACCCATTCTGCTATATGAGCTTTTTCTTAGTTAACTCAATCAC AAATCGGTTTGTGATTATTTCTATTAGCACAATACCGAAACTG TAGACATCACTCTTCTCATTTAACCAATTTGTTAGGTAATATT CGGGGTCAAGATATCCAGGTGTTCCTGCAACTTCTGTTGCTAC TTGAGTTTCAGATCCGACGGAAAATGATCTTGAAAGTCCAAA ATCCGCAAGTTTAGCCTCAAAACGGTTGTCCAATAGGATCCC GCGTACCTCGGCCGCGACCACGC
BcBal-18 At3g04120 4.4e-13	AGCGTGGTCGCGGCCGAGGTACCCGTGTGGTCGACTTGATCG TTCACATGTCAAAGGCCTAAGCACAACAAGACCTCGAATGGT GTTTGGAGGATGAAGTGTCGTCTTTCGTCCCTGCTAATGGTTT AAATTTGTCGTTTTTTGAATAAAATTTCTTGGGATTTAAATCT CTTTTTTTTTCAGTTTAAGCGAATCTCCTTCAGAGTTTGTGAA GAGGCTCTTTATGTATGGCTTTTGATATATTGAGTTGTGTTTT

	ATCAGGATTCTAAAACGTTTCGTATGATCTATCATAACAGCGT AAAATGTTTTAGAACAAGAAGAAGTTTATTTCTA
BcBal-19 At3g54590 2.8e-58	AGCGTGGTCGCGGCCGAGGTACATTACAAGTCTCCTCCACCA CCATATGTCTACAGCTCTCCACCACCACCATACTACTCACCAT CCCCTAAAGTTCATTACAAATCTCCACCACACCCACGTGTATG TGTATGCCCACCACCACCTCCATGTTATGCTCCTTCACCAAAA GTGACATACAAATCTCCTCCACCACCATATGTTTACAGTTCTC CACCACCACCATACTACTCACCTTCCCCTAAGGTGTATTACAA GTCTCCACCACCACCATATGTTTACAGTTCTCCACCACCACCA CCATACTACTCACCATCTCCTAAGGTTGAATACAAGTCTCCTC CACCACCATATGTTTACAGCTCACCACCACCACCATACTACTC ACCTTCCCCTAAGGTGTACCTGCCCGGGCGGCCGCTCG
BcBal-20 At4g12550 6.1e-18	TGAGCGGCCGCCCGGGCAGGTACCGCACTCAAGGTTAACATT CTTGGCATCAACCTTAACCTTCCCATCTATGTCAACATACTCC TCAATAACTGTGGCCGTATAAATCCGGCATACTACCCATGCG CCTAAGTTGAATACTTATATGACATATAAAGACATACCTAAA AGACAAACAGATCGTATGGTTTCAAATGTTTGAAATTATTGC CTTATTTTCTTGTTTCTTTTAATCAGTGATTGCTTTTGTGTTTG TAATTTTCATGGATTGAATAAAATGATGAACGTCATGTTCTCA TGCAAAAAGGGATTATGGGTTTGTGTATGTATCGGCAAGTTC ATTCCCCTTTTGCTTCATTGTTCCTCCTTATCTTCGTTGTAAAC ATTTCTTTTGAATTTGATTAATAAAACTATCAATCGATGTTGT TGTCAAAAAAAAAAAAAAAAAAAAAGTACCTCGGCCGCGAC CACGCT
BcBal-21 At4g40090 4.2e-09	AACAAGAAGAATGAGTGGAGTTGTTTCATTGTCCATCAGAGT TATAAAGAATCAAAAGACGAGAAAAAAAGAAACATCAACAA AGTGGAAGTGAACACAAAATGTATTTAGAGAAAAGTAGAAA ACAGAGAGATTCGTCAAACAAATCATGATAAGAAAGAACAG AGGAGAGAGATAGAGAGTGTAATTTACTCAAGGCTATCTTAA GCCAAAACGACGGAGTACCTGCCCGGGGGGCCGCTCG
BcBal-22 At3g60330 3.1e-51	TGAAGGCAAGTATGATTTTTTTCCATCAAATAAGAGTAACAT CGTTACAAGAATCAAATGAAGGATAACAACAACATTAGATAT CTTCCACCAAGATATCAGAATGAATGTCAATTTCAAGAAGAA TAGTAGTAATGCATATAACAAAAAACATTTTAAAAAATGAAT TTAAGCAGAGACTCTCAGATTGTGTAGTTGTTGTTGTTAGCAT CTTCAAGATCAATTCCTTTCAGCTTTGCAGCTGATTCAACTTT CCCTTTCAATGTTTGAAGCTCTCTCATTCTTGCAATTTCTGCAC GTCTCTTAGCTTCATCAGCCAAACTGTTAAGTTCCGTTGCACC ATTTCTCTCATACATTGGCTTTTGACCTGTCTCTAAACCGTGC TGTGTACCTGCCCGGGCGGCCGCTCG
BcBal-23 At1g11840 5.2e-09	ACGCGGGCACAGCTAAAACGTAGCCATAATAATAAAATATGT GATCTTATGAAGTAGAGGGGAAGCCTTTGATACTTGATCTAA ATAAAGTGTTGTTGGTTCGGTTTGTGTATCGTGTGCTATGTGT TCGTTCCTTTTGTCTTTGAGCTAAACTCTGTTGCGTGCCTGAA TAAAATA
BcBal-24 At5g42980 4.7e-11	TACATGAAGGGTGAAGAGAAGCTCGACAAGGTGGTTGGTGCT TCGAAAGACAAAATCGAAGTCAAGCTTGTGAAGCACACTCAA GTTTCCGCGGCTTGATTATATCGTTCTTCTCCTTTACCTATTTA CTAGTATGTATGGTGTTTTAATAAAAATCCATGTTGCGTTTCG CTTGAGTCATCTTTGTGGTGCTTACACGCCTTGGGTGTGTGAA TAAAAA

BcBal-25 At5g16150 8.5e-50	TCGAGCGGCCGCCCGGNGCAGGTACCGGCTCTTCTTCTTCCA GAGATATTCGCATCTCGGATCAGAGCAAAAGCCGTCGCTCTT TCTCTCGGCATGCACTGGATCTCAAACTTTGTGATTGGACTCT ACTTCTTGAGTGTTGTGACTAAATTCGGAATCAGCAGTGTCTA CTTGGGTTTCGCGGCAGTATGTGTCCTTGCAGTCATCTACATT GCAGGAAACGTGGTCGAGACTAAAGGACGGTCACTTGAGGA AATAGAGCTTGCTCTTAACTCCGGAGCTTGAAATTTAGAAAA AAACAAAAACCCCCACTTTGTTTCATCATTGATCTTGTGAGTC TCTCTTTCGTTCATGCTCTTTGAATAACTTGTACCTCGGCCGC GACCACGCT
BcBal-26 At2g31480 1.3e-36	AGATGTTGTTATAGTCTCGGGTTTTCGAACCTGTAAAGCCCTT TTCTTTTTCATAGAGCATCTTCAAACAAGAAACCATCAGTGTT GAAGTCTGAATCAATGCCTTCTCTTCCCCTGAACTCGCAAAGC CCCACCGCATTTTCTATAGAGAAATCATTCATCAAGGCAAAG TCATCTTCTATGAAAGTCCTGGACCTGCGTGTCATGATAAGCT TCTTAGGTGTTTTTCTGCACATCTCTACACTCTCTTCAGCAGCT TGAACAAGTACCTGCCCGGGCGGCCGCTCG
BcBal-27 At1g17710 9.8e-54	ACAAAATAATCTACGTATCAACCCTTCAATGTTAGACATCTTA TACATTCTTTCTCGGGTGCATGAAGAATATAATACTATATTCG AAACAAAAAGATCTCAGATTCTTTGTTCTCTTCATAAATCACT TAACGAGATTAAGGGGAACTTGAAGAGCAAGAGGCACCAAA GGCTCGTGAACAATCCCAACCGATATCTTGCAATGATCAGAG CTCAACATCTTCTCCATCTCCTCTTCATCTGATGATATAATCTC GTTGATTATTCCCATTAGTATTCTCTCCATAGCTTCACCATCG GTCCAATCTCTAACCGTGGCCTTGACCAAGGTCCGGTTTTGAC TGATCAAATCCCAAACCGGGAAATTATTCCTTGGCATCATGT AATCTTCAGCTTTGAGTCTAAGACTCGGACAGTAATCACCAG CACCATCTCCCAGATAGATCATCTTCATCTTGTTTCCTTCTTTG GGAAAAGAAGCTTGAATCCTCTCAATTATCAAACCC
BcBal-28 At3g24160 4.4e-23	ACATGCCTTTGACGAAGGACACACTCCTATATTCAAACGTTA AGCTCGATTAATACAAGATACAGCCGTGTTTCTCAACTGGCC ACATTTGAAACCAGTAGTCTTTATTTGAATATTTTAAGAGGAG GACACTATTTTTTGTGGCCAGACCAAGTGGGATGTGTTTATAT TACCATAAGGTTGCATTTTTCGATTTTATAATTTTTTTTCTTAC ATATATGTGGGTCTCAATCCTAATAATAATAAAGAACAATTT GGACATCTTCCTGTAATCTTCTGTTTCTTGTAAGAGCGTTTGA TCATGAAATATGAACAGATTATTGCAGCGTCTTAATGTTCAA GA
BcBal-29 At3g14280 7.2e-72	ACATTCTTTATTCAAGAGGCCTTTGTGAAGTGAACAGATTTTG ATTTATGAGAGAGAATTTAGAGGAAGTCATAGTTGCTCCATT TGATCATTGCTAACTCTTGTTGTGAAGCAATTTCCTGTCTTCG ACCATCACCCTTGTATGCAACTCTCAGTTCCTGATAATTTATT TGCAGAAGATCAAGAATCTCCACCTCTCTTGAGTAAGAAGCT ACCATTGAGTTGTCTGGTGGACAACGAGGACCTTCATCATAC CCTTCACATATAACCTCTCCGTTATCATCAGTGTAACATATGA TCCCTTGCACTGGATCTTCATATACAGTGTTCTTGGTGGAGTA TCTTATTGGCTGAGGAACTGTGACTTGCTTGACTTTAACTTGA ATCTGACTCTGTTGAAGCATCAAAGTCTTTGATCTTACTGTCT TTGGAACAAAACATTGTGTTTGAAGAGGAAGACAAAGCTTAG TTGAATATGCCATAGCCCCTTTGTGTTTTCTTTCAGATGAAAA TTTGATCTCTCCCCGCGTACCTGCCCGGGCGGCCGCCCGGGC

	AGGTACAAATCTCCACCACCCCCATATGTCTACAGCTCTCCAC CACCACCA
BcBal-30 At3g18845 3.8e-23	CCCGTAAGCGCGGCCGAGGTACTCTTGCAAGCCCTATGTGCA GAGTAAGAACCCAGTGACCGCCGCGATTGATCCCAAGGGCCC TTGCTGCACTGCCCTGTCTAAGGCTGACTTCCAGTGTCTCTGC AAGCAGAAAACCAAAACCAATCCTTTCCTCAGTTCGATCGAC CTCGACCTCGCTTCTAAACTCCCGGAGAAGTGTGGATTATCTG GTGCAACGTGCTAAGACAGGTTTCCTTTTCGAAGG
BcBal-31 At3g01130 2.3e-40	CCATTGACGCCCGGGCAGGTACCAGCACTCTTGCTCTGGTGG CCCGTGCTTCAGCTTTCGGGCTGGGTCTCATCTACGGGAACGT CAAGCTCAAGGCTTTGAAGATCAAGAAGAACTCACAGATTAA AGCAGAAGCCAAGGCTCATCACTAAGCACCAAACATCTTTCT CTCCATTCAAAATAAGAAGATGAAGCCCAAGTGACTCTCCTC TCCAAATGTTTGTTGTTTCTTCATAAGCTATATCTCCTTCAGTG ACATTTTTCGGCTTTTTTTTTATCGAGGAACATAACATTTTGTC TGCAAAACAATCATAATACCATTTTGGTTTGAAGCCGGAGTC CCTTTTTGGTGCTTTGCTATTTTAATAAACACCATTTTAACAA GATTGGTTTTATATAACCTATTGTGCTTTGCTATTAAA
BcBal-32 At3g09922 7.5e-30	ACGCGGGGAAACCTTCTCTTAATCTGGCACAACACCACAAAT CAAAAGAGAATGGCCATCCCCTAGGGCGAAGAAGAGAAAAG CCTCTGATTCATCTAGAGGTTTATCCGTAGGGGATGGCCTAG ACACATAAAAGGAGATTACTATTATTGTCTAGCTAAGCGGGT TTGTGTTCCTGGAAAGTGTTTTAAATATATGGAGCAATGAAG ATTGCAGAAGGCTGATGATGAAGAGGCAGCTGGTTTAGACAG CGAGTGTTCTTTATCTCCCTCTATAAATTGGGCAACTTCTATC CTTTGGCAAGCTTCGGTTCCCCTCGGAATCAGCAGATTATGA ATTTACTTTGTAATACTCTCTTTCTCTATGTTTTGTTTACTTCA TCTTGATCATGTTTGTATTGTATCCAGTTCCATGGTAAGATCT TAGTGTGAGGAATAAGAAAATATTCGGATTTGAGAGATTAAA A
BcBal-33 At1g79210 3.4e-18	CGCGGCCGAGGTACGAAACATTCTGAACATTGTCTAAACGAG TTAAAGGAGAACAGATTCTAAACAGCGTCTCGAGTTTTAACA AGTTCCAAGAGTTTAACATAAGCAAGAACCAGACACAAAAG CAAAAGGTAGTATTGAGATTCGAATCCGGGCTTGTTGCGCAG CCAAGTGAAAGTTTACTCGACCTCAGCCAAGTAATCGTCGAT CTCTGCTGGTGTTAGTA
BcBal-34 At3g06400 1.4e-48	TCCAGCGGCCGCCCGGGCAGGTACAGCTTCCCTTTGTTCTGAC CATACTGAATCTTCATTTCCAGCCAAGGGTTTCTGTAGCGATC CAGTTTCTTCCCTAAGGCTTTCATGATTTCATCTTTCCTAGAG ATTCTTGCCTCTCCCCTCTCAATGTTCTTAATGATTCTATCGTA ATCGTTCAGCTCCTTGTATCGCTCTTTGAATACTTGGGCATAT CTTTCAACTTCTTCCTCTGTTTTACCTTCCATCTCAGAGGCAAT GCTTTTGATGTCGTTGCGGCCGTACCTCGGCCGCGACCACGCT
BcBal-35 At4g02620 2.5e-15	TCCAGCGGCCGCCCGGGCAGGTACCTCTTCTCTGCTGAATCTG TGTCACAGCGTTAAACATATATACATCCTGCAAGCTCGCTGA ACCTTTATATCATTTGTTTGTTTTTGATATTCTTGTTTTTTTTCC TTTCAAACTCAGAAACTTTCAATAAATGTATCTCTTGATTTTT ATTTGGAGATGATTGTTATATTATTAAGAAGCAATGGTAACA CCAAAAAA
BcBal-36 At3g26810	CCACTGAAAGCATGAAGATAGGCATTTGATAGATAAGAATGT AAGAGTAGCCAAGAAAACAAAACAATAACATGTTTGTGTCAC

1.9e-01	TTCTTCAAGATGATAATCAAAAGAGGCAGTGTCTATGTCAAA GCCTTATATGATCTTGAAACACACAACAACATTAATAGATAA TAAACAAAACAAACCAAGTTTCATGGGTTTATTTATGTCGAC ATCCCGACAGCAGAACAAAGCTGCAAATATTAGATAGAAGG AAATCAAAACCCCAAACTTCGATTTCCTAACAGAATCACAAA AGCAAAGCAAAGCAAAATCAAAGAGTAGAAGCAGTTGGATC AGATACTCATAACCCTCCTGGTGCTGCTGACAGCTTTCATAGT ACCTCGGCCG

Supplementary material: General discussion

Promoter sequences from http://ppdb.gene.nagoya-u-ac.jp/cgi-bin/index.cgi
Protein sequences from http://www.arabidopsis.org/
PLACE http://www.dna.affrc.go.jp/PLACE/signalscan.html
PFAM http://pfam.sanger.ac.uk/
ScanPROSITE http://expasy.org/tools/scanprosite/
SUMOsp2.0 http://sumpsp.biocuckoo.org/online.php

At5g57530 XTH

Promoter sequence

ATGATGTGTATGGATGAATGCAAAATAACAACGAAACGCAAGAAGACAGAAACAGTAGAGATAGATGTGAATGCA
TGCATGGGAATGGACAAGATGGGAAGAACAAAGAAGCTTGTGCTTTCCTGTTGTGGTTGAAAGTTTTTGTTGTAG
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AACCAAAAAAAAAACATGGAAGGTAGGTATCTCTGATATATGTTTAGACCCTAATTTGAAGCTGAAGAAATGTATG
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TAAAATCGCCTAGTGTAACACGACCATGTCGATGATGGAACTCCACGAACCTCTCTAATGCACTCACTATTAAAA
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Protein sequence

MAAFATKQSPLLLASLLILIGVATGSFYDSFDITWGAGRANIFESGQLLTCTLDKTSGSGFQSKKEYLFGKIDMK
IKLVPGNSAGTVTAYYLSSKGETWDEIDFEFLGNVTGQPYVIHTNVFTGGKGNREMQFYLWFDPTADFHTYTVLW
NPLNIIFLVDGIPIRVFKNNEANGVAYPKSQPMKIYSSLWEADDWATQGGKVKTDWTNAPFSASYRSFNDVDCCS
RTSIWNWVTCNANSNSWMWTTLNSNQLGQLKWVQKDYMIYNYCTDFKRFPQGLPTECNLN

At1g21310 HRGP

Promoter sequence

TTCTCAAAATGAATATTGCAAATGCTTTTTCTAAAAGTGTCAAAGGCAATGTTATTCGTTTCCCATTATTCATAA
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GATGCATGTAACCAAAAATAGAAAGATAAAAGCGGACTAAAATTTAGTAACTTTGTTAAGTAAAATATAAGATAA
ATAAATTCCAAATTAATAATTTGGACCATGTTATGACATTACATTCGCTGGTTATAGTTCGACTTACACATATTA
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CGTAGTCAGAAATGATTTTCTCATGTTTCCCCTTTTAACTTAGAGGCCATATATATCTTCTTTACCGGAAAAAGA
AAAACTCAAGAGGCCATCAACATATTTTCTCTTACAACGTTCTACAATTACAATTACTACTTTCTTAGCTTTTGG
GACATTGTATTATTGTTCTAAAGTGAATTTTCACACACAAAAAAAAGTGAGTATTATCAAAAGGGACATAACAAA
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GAACATGGAGAACATGTCAATCATCGATGGCTGAAGAAAAAAGTTTAATAATGAGAGTCGGAGAGACCAACAAAC
ATCACTTCTTTCTATATACTAATATTTATATATGTTTGGTGTACATAATTGCATGTACTTTTTACATGACGTAAG
TTACCATTTTACAACGAGTGTGAAAATAGTGAAACTGAAAAAGCTATTTAAAGGGTGTGAAGGGAAGGCACTAAA
TCACAAACCAAAAACAAAACAACCAA

Protein sequence

MGSPMASLVATLLVLTISLTFVSQSTANYFYSSPPPPVKHYTPPVKHYSPPPVYHSPPPPKKHYEYKSPPPPVKH
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KSPPPPVKHYSPPPVYHSPPPPKKHYVYKSPPPPVKHYSPPPVYHSPPPPKKHYVYKSPPPPVKHYSPPPVYHSP
PPPKKHYVYKSPPPPVKHYSPPPVYHSPPPPKKHYVYKSPPPPVKHYSPPPVYHSPPPPKKHYVYKSPPPPVKHY
SPPPVYHSPPPPKKHYVYKSPPPPVKHYSPPPVYHSPPPPKKHYVYKSPPPPVKHYSPPPVYHSPPPPKKHYVYK
SPPPPVKHYSPPPVYHSPPPPKEKYVYKSPPPPPVHHYSPPHHPYLYKSPPPPYHY

At1g12040 LRX1

Promoter sequence

TATTGGACATTTCTTGCTCTGTTATAGGGCCTATATATACATCCCCTTGGATCAATCTCCAGAAAACGAAAATTA
CTTGAACATGAGCTTTGCTTATTTAACTACCCGGCAACATAGCTTAAAAGTGAGGTATTTAGGTCATTTTTATGT
GGATCTACGCGTATCGCTTGATTTTCTTTAAACTTTGTTTGATCAAACGAATGGACCAATTTATCCTATGATATT
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Protein sequence:

MLFPPLRSLFLFTLLLSSVCFLQIKADHDDESDLGSDIKVDKRLKFENPKLRQAYIALQSWKKAIFSDPFNFTAN
WNGSDVCSYNGIYCAPSPSYPKTRVVAGIDLNHADMAGYLASELGLLSDLALFHINSNRFCGEVPLTFNRMKLLY
ELDLSNNRFVGKFPKVVLSLPSLKFLDLRYNEFEGKIPSKLFDRELDAIFLNHNRFRFGIPKNMGNSPVSALVLA
DNNLGGCIPGSIGQMGKTLNELILSNDNLTGCLPPQIGNLKKVTVFDITSNRLQGPLPSSVGNMKSLEELHVANN
AFTGVIPPSICQLSNLENFTYSSNYFSGRPPICAASLLADIVVNGTMNCITGLARQRSDKQCSSLLARPVDCSKF
GCYNIFSPPPPTFKMSPEVRTLPPPIYVYSSPPPPPSSKMSPTVRAYSPPPPPSSKMSPSVRAYSPPPPPYSKMS
PSVRAYPPPPPPSPSPPPPYVYSSPPPPYVYSSPPPPPYVYSSPPPPPYVYSSPPPPYVYSSPPPPYVYSSPPPP
PPSPPPPCPESSPPPPVVYYAPVTQSPPPPSPVYYPPVTQSPPPPSPVYYPPVTNSPPPPSPVYYPPVTYSPPPP
SPVYYPQVTPSPPPPSPLYYPPVTPSPPPPSPVYYPPVTPSPPPPSPVYYPPVTPSPPPPSPVYYPSETQSPPPP
TEYYYSPSQSPPPTKACKEGHPPQATPSYEPPPEYSYSSSPPPPSPTSYFPPPMPSVSYDASPPPPPSYY

At3g09922 IPS1

Promoter sequence

ATCTTGTATATTTCAGTAAAAAGACTTTCACAGAGTAATTATTTATATAAAACAGTTTGCTTTTTCATGAGTAAT
TTGCATTTGTATTTTACTAAAACAAATGTACGTTCCTGACAGCTCAAGGCATTATACCTGACTAATTAAGTATTG
AATTAAAAATCAACTCAACTTTCTGTGTATGTCATCAGATGAGTGTGTATATGTCTGTGTAAACTGAAAAGGCAA
TTTTGGTAACGGCATATTCCATCGGATGATCCAAAATTCTCAAAAGCATATTCACGTAAGACTCCCCTTGTTTCC
ATCATCTGCTTCCACTTATAAACATTTATATTTTTAAATTACTTGCATACTCTCTTTACACCATATATATATTTA
TTCCCCTTTACACCAATAATATATACTCTCTTCTTTTTCCTTTGTGTTTCTTCTTTCCAGAATTTTAATATCAAG
ATAAACGTGTACATTGGTGAATTTTCGTATCTTTATTTTGTATGTTACGTGTTTATGATACCAAAATATAAATAT
CATATACGATCTATTAACTATAAAAGAAAAGAAATCATTTATAAAGAACCTTAACAAACATGAAATATATTGGTT
GATTGTTACAAAAAGTTTAGTTATTTTTTTCTAATGAAAAAGAAATGAGGAAAGGTGGAAAGAACAATATAGCTT
ATGTAATATATATATGGGGTTTCAGTGTATCTTTTTAATGTCTTCATGACCAAAGACAACAACAACAATAAAACG
CAAAAAAGAAAGAAATATGCTATAAAACCCCCCTAGAGCCTCCATTGTTGAACCCTCTCTTAACTTGGCAAACAC
CACAAAAACAAAAGAAAAATGGCCATCCCCTAGCTAGGTGAAGAAGAATGAAAACCTCTAATTTATCTAGAGGTT
ATTCATCTTTTAGGGGATGGCCTAAATACAAAATGAAAACTCTCTAGTTAAGTGGTTTTGTGTTCATGTAAGGAA
AGCGTTTTAAGATATGGAGCAATGA

Fbox At3g08750

Protein sequence

MASSKCLLLPSLPFELIEEILYKIPAESLIRFKSTCKKWYNLITEKRFMYNHLDHYSPERFIRTYDQQIIDPVTE
ILSDALIPDEFRDLYPIYSMVHCDGLMLCTCRKWDNSLAVWNPVLREIKWIKPSVCYLHTDYVGIGYDDNVSRDN
YKILKLLGRLPKDDDSDPNCEIYEFKSDSWKTLVAKFDWDIDIRCNNGVSVKGKMYWIAKKKEDFTIIRFDFSTE
TFKEICVCPYTLVTRLGCFDGDRLSLLLQGEESQGIEVWMTNKLSDKVVSFSQYFNVTTPDLPALHLQSCMACPG
YSIGKRRNIRVWCEGSVDVDDKSYVIITFYEIDEGGVIKQIETASYGQFEYDDPFICCYVYVPSLIPIQ

LRR At2g28970

Promoter sequence

ATAGCTCATATTCTCAGTATTACTATACATTGTCAACCAAGAAATTCTACATCAACCTTTGTTAATATCCCTTGT
TAGGTGGGATTTAGTGGGAAAAGAGATCAAACCTTATTTTGGAAAGATCATTTCCAAACGCTACTGGACCATTAT
CCACTTGGTTCACAATTTGCTTTTTTAATGGAGCTTTGACAATCGGAAACTGTATGTGTTAACTATATAAAAATA
TTAATTATGTGATAATTCTTATTTTTTATTGTTGCTTTTTTAACAAGTTTGTAATAGTTAAAGTGTGGTTGTAAA
TATATTTTTAAAATTTAGTACTGGCTACCTTGATGCACATTATTTGTATAAATACCTGAGTTTGTAAGAAGCAAA

TCATTGAATCAAACAAAATGATCTTTATCTTTATTTTTTCCTGTCCATGTTGTAATATACGAATTCTCTCTTTTA
CATAATTATTCTACAACAAGTTACATAAGTTACGTCTATAATTATTGTGGATCAGACGTAATGTTTCTTTATGTG
AATCAGACGTAATGTTTCAAATAGGTTGTAATATGTAATTGTTTTTCATATGTAGTAAATCCTTTTTTGTAACGA
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CTTATGATCATAACCTATTATTTTAAACACAAGTTGGGGTTGGAACATGTCAAAGAAATTCACACAATAAACCAA
AAATAAAGCAATTCTCGCAATTATTCAATCTTCATGTCTGGCTTAAGTTGACTTTCTTTAATGTATTATTCCTTC
CAATCTGTGTATGTGAGGTATTTCTCATTTACGTAGCTCCTTGTTTTCACTAAGCAAGTCTTTTTTATGTTCTTG
ATTTCCATATTAGCTTTCCAAGAAG

Protein sequence:

MMSHLLLAIIGTFAVIVGAQKQEGFISLDCGFPIEESPYSDPSTGLTFTSDSTFIQTGESGRVDKELNKIFRKPY
LTLRYFPEGKRNCSLRNSFRVHCSTSDSEIRYDDDSYDRVWYPFFSSSFSYITTSLNINNSDTFEIPKAALKSAA
TPKNASAPLIITWKPRPSNAEVYFYLHFAEIQTLAANETREFDIVFKGNFNYSAFSPTKLELLTFFTSGPVQCDS
DGCNLQLVRTPNSTLPPLINALEAYTIIEFPQLETSLSDVNAIKNIKATYRLSKTSWQGDPCLPQELSWENLRCS
YTNSSTPPKIISLNLSASGLTGSLPSVFQNLTQIQELDLSNNSLTGLVPSFLANIKSLSLLDLSGNNFTGSVPQT
LLDREKEGLVLKLEGNPELCKFSSCNPKKKKGLLVPVIASISSVLIVIVVVALFFVLRKKKMPSDAQAPPSLPVE
DVGQAKHSESSFVSKKIRFAYFEVQEMTNNFQRVLGEGGFGVVYHGCVNGTQQVAVKLLSQSSSQGYKHFKAEVE
LLMRVHHKNLVSLVGYCDEGDHLALIYEYMPNGDLKQHLSGKRGGFVLSWESRLRVAVDAALGLEYLHTGCKPPM
VHRDIKSTNILLDERFQAKLADFGLSRSFPTENETHVSTVVAGTPGYLDPEYYQTNWLTEKSDVYSFGIVLLEII
TNRPIIQQSREKPHLVEWVGFIVRTGDIGNIVDPNLHGAYDVGSVWKAIELAMSCVNISSARRPSMSQVVSDLKE
CVISENSRTGESREMNSMSSIEFSMGIDTEVIPKAR

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Bremer M, Schenk MK (2009) The expression of P responsive genes is related to root hair growth, IPNC Sacramento, The Proceedings of the International Plant Nutrition Colloquium XVI, Department of Plant Sciences, UC Davis. http://escholarship.org/uc/item/1rs3j45j

Eticha D, Zahn M, Bremer M, Yang Z, Rangel AF, Rao IM und Horst WJ (2010) Transcriptomic analysis reveals differential gene expression in response to aluminium in common bean (*Phaseolus vulgaris* L.) genotypes. Annals of Botany 105 (7) 1119-1128

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität

Für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, das ich meine Dissertation mit dem Titel

Molecular mechanisms of root hair growth induced by Pi deficiency in *Brassica carinata*

selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, d. 17.06.2010

Melanie Bremer