

**Molecular mechanisms of the
phosphate deficiency-induced root hair growth
in *Brassica carinata***

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades
Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation
von
Thomas Wolfgang Kirchner, M. Sc.

2017

Referent: Prof. Dr. rer. hort. Manfred K. Schenk
Korreferent: Prof. Dr. rer. nat. Helge Küster
Tag der Promotion: 26.09.2017

Zusammenfassung

Die Bildung langer Wurzelhaare unter limitierenden Phosphatbedingungen vergrößert die Verarmungszone um die Wurzel und erhöht infolgedessen die Aufnahme an anorganischem Phosphat (Pi). Die molekularen Mechanismen, welche der Regulation der Wurzelhaarlänge unter Pi-Mangel zugrunde liegen, sind noch nicht vollständig geklärt. Ziel dieser Arbeit war es daher, neue regulatorische Komponenten des Signalnetzwerks zu identifizieren, welches zur Pi-Mangel-induzierten Wurzelhaarelongation führt. Die Identifizierung potentieller Kandidatengene, welche für diese Komponenten kodieren, erfolgte durch den Vergleich von mit Hilfe von *massive analysis of cDNA ends* (MACE) erstellten Expressionsprofilen zweier *Brassica carinata*-Kulturen, welche sich in ihrem Wurzelhaarphänotyp als Reaktion auf Pi-Mangel unterscheiden: die Sorte Bale bildet lange Wurzelhaare unter Pi-Mangel, nicht aber die Sorte Bacho. Die in der MACE produzierten Sequenzabschnitte (*tags*) wurden zu *Brassica rapa* und *Brassica napus* annotiert und die erhaltene Genliste wurde nach potentiellen regulatorischen Genen gefiltert, welche in Bale, jedoch nicht in Bacho als Reaktion auf Pi-Mangel differentiell exprimiert waren. Nach Validierung der Kandidatengenexpression wurde ein *Split-root*-Versuch durchgeführt, um lokal und systemisch regulierte Gene zu unterscheiden. Des Weiteren wurden die Pflanzen Stickstoff (N)- bzw. Kalium (K)-Mangel ausgesetzt, um Gene zu identifizieren, die spezifisch auf Pi-Mangel reagieren. Die Genfunktion letzterer wurde mit Hilfe von CRISPR/Cas9 ausgeschaltet und die Wirkung auf die Wurzelhaarlänge in transgenen *hairy roots* bestimmt. Zusätzlich wurde ein System für die Bestimmung von *indel*-Größen mittels Polyacrylamid-Gelelektrophorese (PAGE) etabliert.

Zwischen 16 und 26 Millionen *tags* wurden in der MACE produziert, von denen 70-80 % zu *B. rapa* und 50-60 % zu *B. napus* annotiert werden konnten. Ca. 500 Gene waren unter Pi-Mangel in Bale differentiell exprimiert, ohne eine durch Pi-Mangel veränderte Expression in Bacho aufzuweisen. Von dieser Gruppe an Genen wurden 33 Kandidatengene mit einer potentiellen regulatorischen Funktion ausgewählt, von denen für 30 Gene die transkriptionelle Regulation in insgesamt vier unabhängigen Versuchen durch quantitative PCR (qPCR) bestätigt werden konnte. Nur fünf Gene schienen entweder ausschließlich lokal (zwei) oder systemisch (drei) reguliert zu sein, während 25 Gene sowohl in lokalen als auch in systemischen Signalwegen eine Rolle zu spielen schienen. K-Mangel beeinflusste weder die Wurzelhaarlänge noch die Expression der 30 Kandidatengene. Dagegen induzierten sowohl Pi- als auch N-

Mangel die Bildung längerer Wurzelhaare und in 26 Fällen führten beide zu veränderten Transkriptleveln. Die Expression von vier Genen wurde jedoch ausschließlich durch Pi-Mangel verändert.

Sechs Gene wurden für die weitere Funktionsbestimmung durch *knockout* mittels CRISPR/Cas9 ausgewählt: (i) die vier P-spezifisch reagierenden Gene, da sie Teil eines Signalwegs sein könnten, welcher das Pi-Mangel-Signal auf den Wurzelhaarelongation-vermittelnden Signalweg überträgt, (ii) *RAPID ALKALINIZATION FACTOR-LIKE 1 (BcRALFL1)* als eines der ausschließlich systemisch regulierten Kandidatengene mit einer mutmaßlichen Rolle in der Signalübertragung durch Ca^{2+} und (iii) *INORGANIC PHOSPHATE TRANSPORTER 1 (BcPHT1)*, welches unter Pi-Mangel in Bale stark hoch-, in Bacho jedoch herunterreguliert war, was eine Rolle von BcPHT1 als P-Sensor in Analogie zur Rolle des Nitrat-Transporters NRT1.1 als N-Sensor vermuten lässt. Da die MACE nur die Gensequenzen am 3'-Ende liefert, ein Gen-*knockout* jedoch wahrscheinlicher ist, wenn das Gen am 5'-Ende editiert wird, wurden die Gensequenzen zunächst mittels 5' *RNA ligase-mediated rapid amplification of cDNA ends* (5' RLM-RACE) erweitert. Die anschließende Editierung der Gene am 5'-Ende mittels CRISPR/Cas9 führte bei fünf Genen zu einer Reduzierung des Transkriptlevels, was jedoch nur im Falle von *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1 (BcFLA1)* zur Bildung kürzerer Wurzelhaare unter Pi-Mangel führte. Die Komplementation der Genfunktion von *BcFLA1* durch zeitgleiche Überexpression eines synthetischen *BcFLA1*-Gens (*BcFLA1_{amut}*), welches gegen die Editierung durch die Cas9/*guide* RNA-Kombination resistent war, stellte die Wildtyp-Wurzelhaarlänge unter Pi-Mangel wieder her. Ausgehend von diesen Ergebnissen ist eine Beteiligung von BcFLA1 an der Pi-Mangel-induzierten Wurzelhaarelongation in *B. carinata* wahrscheinlich. Unterstützt wird diese Annahme durch die Fähigkeit von FLAs, umweltbedingte Signale in die Zelle zu übertragen, da sie eine physische Verbindung zwischen der Zelle und der extrazellulären Matrix herstellen können.

Die Auftrennung der Amplikons der mutmaßlich editierten *BcFLA1*-Genregion durch PAGE machte es unter Zuhilfenahme einer DNA-Leiter mit 1 bp-Abständen möglich, die exakten *indel*-Größen zu bestimmen. Folglich stellt diese Technik eine gute Möglichkeit dar, *knockout*-induzierende *indels* zu identifizieren, und somit die Herunterregulation eines Gens in einem chimären Gewebe wie *hairy roots* nach Wirkung des CRISPR/Cas9-Mechanismus abzuschätzen.

Abstract

Development of longer root hairs under limiting phosphate (P) conditions increases the depletion zone around the root and thus the inorganic P (Pi) uptake. However, the molecular mechanisms which are responsible for the regulation of the root hair length under Pi starvation are still not completely understood. Therefore, this study aimed at the identification of new regulatory components participating in the signaling network leading to the Pi deficiency-induced root hair elongation. Potential candidate genes encoding for these components were identified by comparison of massive analysis of cDNA ends (MACE)-provided expression profiles of two *Brassica carinata* (Ethiopian mustard) cultivars differing in their root hair response to Pi deficiency: cultivar (cv.) Bale develops longer root hairs under Pi deficiency but not cv. Bacho. The tags produced in the MACE were annotated to *Brassica rapa* and *Brassica napus* and the list of genes was filtered for potential regulatory genes, which were differentially expressed in Bale but not in Bacho in response to Pi deficiency. After validation of the candidate gene expression, a split-root experiment was conducted for the differentiation between locally and systemically regulated genes. Furthermore, plants were exposed to nitrogen and potassium deficiency to identify P-specific reacting genes. The latter were knocked out by CRISPR/Cas9 and the effect on the root hair length was determined in transgenic hairy roots. Additionally, a system for the detection of the indel sizes was established via polyacrylamide gel electrophoresis (PAGE).

Between 16 and 26 million tags were produced in the MACE, from which between 70 % and 80 % could be annotated to *B. rapa* and between 50 % and 60 % could be annotated to *B. napus*. About 500 genes were differentially expressed under Pi deficiency in cv. Bale, while these genes did not respond to the low P supply in cv. Bacho. Out of this group 33 candidate genes with a potential regulatory role were selected and the transcriptional regulation of 30 genes was confirmed by quantitative PCR (qPCR) in altogether four independent experiments. Only five genes seemed to be either exclusively regulated locally (two) or systemically (three), whereas 25 genes seemed to be involved in both local and systemic signaling pathways. Potassium deficiency did affect neither the root hair length nor the expression of the 30 candidate genes. In contrast, both P and nitrogen deficiency increased the root hair length, and in 26 cases both affected the transcript levels. However, four genes specifically reacted to Pi starvation.

Six genes were selected for further functional characterization by knockout using CRISPR/Cas9: (i) the four P-specific reacting genes, since they may act in a pathway which transduces the low Pi signal to the root hair elongation pathway, (ii) *RAPID ALKALINIZATION FACTOR-LIKE 1 (BcRALFL1)*, because it was one of the three exclusively systemically regulated candidate genes with a potential involvement in Ca²⁺ signaling, and (iii) *INORGANIC PHOSPHATE TRANSPORTER 1 (BcPHT1)*, as it was strongly upregulated in Bale but downregulated in Bacho under Pi deficiency, suggesting a role in P sensing in analogy to nitrate sensing by nitrate transporter NRT1.1. Since the MACE provides only the 3' ends of the gene sequences though a gene knockout is more likely by targeting the 5' end, the gene sequences were extended by 5' RNA ligase-mediated rapid amplification of cDNA ends (5' RLM-RACE). The transcript levels of five genes were clearly decreased after targeting by CRISPR/Cas9, but only the downregulation of *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1 (BcFLA1)* reduced the root hair length in the transgenic hairy roots under Pi-deficient conditions. Complementation of the *BcFLA1* gene function by simultaneously overexpressing a synthetic *BcFLA1* gene (*BcFLA1_{mut}*), which was resistant to the indel induction by the Cas9/guide RNA combination, recovered the wildtype root hair length under Pi starvation. Therefore, the results indicate the involvement of *BcFLA1* in the Pi deficiency-induced root hair elongation in *B. carinata*. This is supported by the ability of FLAs to transmit environmental signals to the cell by physically connecting the cell with the extracellular matrix.

Additionally, separation of the amplicons of the potentially edited *BcFLA1* gene region by PAGE enabled the determination of the exact indel sizes by using a ladder with 1 bp-gaps. This technique could be a tool for the detection of knockout-producing gene editing events and therefore the estimation of the overall downregulation of a gene in a chimeric tissue like hairy roots after targeting by CRISPR/Cas9.

Schlüsselwörter: Pi-Mangel; Wurzelhaar; CRISPR/Cas9

Keywords: Pi deficiency; root hair; CRISPR/Cas9

Contents

Zusammenfassung	i
Abstract	iii
Contents	v
Abbreviations	ix
Gene Abbreviations	xii
List of Figures	xiv
List of Tables	xvii
General Introduction	1
1. State of the knowledge.....	1
1.1 Regulation of Pi homeostasis.....	2
1.2 Root hair formation.....	2
1.2.1 Cell fate determination.....	3
1.2.2 Root hair initiation.....	3
1.2.3 Root hair elongation.....	4
1.2.4 Pi deficiency-induced root hair elongation.....	5
1.3 Preliminary studies.....	6
2. Hypotheses.....	6
3. Central methods.....	7
3.1 Massive analysis of cDNA ends (MACE).....	8
3.2 CRISPR/Cas9.....	8
CHAPTER I: Identification and functional characterization of potential candidate genes with a regulatory function in Pi deficiency-induced root hair growth in <i>Brassica carinata</i>	10
Abstract	11
1. Introduction	12

Contents

2. Materials and Methods	15
2.1. Plant material and growth conditions.....	15
2.2. Determination of the nutrient concentrations	16
2.3. Root hair length measurement	17
2.4. RNA isolation.....	17
2.5. Massive analysis of cDNA ends (MACE).....	18
2.6. Candidate gene selection	18
2.7. Expression analysis.....	19
2.8. Knockout of the candidate genes by CRISPR/Cas9.....	19
2.9. Genomic DNA isolation	20
3. Results	21
3.1. Characterization of the plant material used for MACE regarding the P deficiency and root hair response.....	21
3.2. Analysis of the MACE data.....	22
3.3. Validation of the candidate gene regulation.....	24
3.4. Split-root experiment	25
3.5. Investigation of the nutrient specificity of the candidate genes.....	30
3.6. Knockout of candidate genes by CRISPR/Cas9.....	32
4. Discussion	39
4.1. Identification of regulatory candidate genes for Pi deficiency-induced root hair elongation.....	39
4.2. Investigation of the candidate gene characteristics regarding their role in signaling and their nutrient specificity	43
4.3. Functional characterization of the candidate genes by knockout via CRISPR/Cas9	46
5. Conclusion	50

CHAPTER II: Efficient generation of mutations mediated by CRISPR/Cas9 in the hairy root transformation system of <i>Brassica carinata</i>	51
Abstract	51
Introduction	52
Materials and Methods	53
Obtainment of <i>BcFLA1</i>	53
Construct preparation	53
Guide RNA (gRNA) design	53
Vectors.....	54
Plant material, cultivation and hairy root transformation	54
RNA & gDNA isolation.....	55
Expression analysis.....	55
Verification of the gene editing	55
Root hair length measurement	56
Results	56
Proof of concept study with <i>BcFLA1</i>	56
Screening of the transgenic roots.....	57
Detection of deletions and insertions with PAGE	57
Validation of deletions and insertions with Sanger sequencing	58
Expression of <i>BcFLA1</i> in roots carrying the CRISPR construct.....	60
Root hair length	61
Discussion.....	61
An optimized CRISPR construct is required for efficient selection of roots with a disrupted gene function	61
The relaxed specificity of a gRNA might be compensated by using a complementation construct in parallel	64
The size of deletions caused by CRISPR in hairy roots is highly variable	64
Sensitive detection of indels with fragment analysis via PAGE	65

Contents

Effect of deletions/insertions in <i>BcFLA1</i> on root hair length	66
Conclusion	66
Acknowledgments	67
Author Contributions	67
References	68
General Discussion	71
1. Functioning and position of BcFLA1 in the Pi deficiency signaling network..	72
2. Role of the other candidate genes in the root hair elongation pathway	74
3. Potential and limits of the applied methods	79
4. Outlook	81
References	83
Supplementary Material	102
1. Chapter 1	102
2. Chapter 2	144
3. General Discussion	152
Danksagung	153
Lebenslauf	155
Publikationsliste	156

Abbreviations

A.	<i>Arabidopsis</i>
ADP	adenosine diphosphate
AFLP	amplified fragment-length polymorphism
AG	arabinogalactan
ARF	ADP-ribosylation factor
ATP	adenosine triphosphate
B.	<i>Brassica</i>
bHLH	basic helix-loop-helix
BnGI	<i>Brassica napus</i> gene index
bp	base pair
Ca	calcium
CAPS	cleaved amplified polymorphic sequences
Cas	CRISPR-associated
cDNA	copy DNA
CDS	coding sequence
compl	complementation
CRISPR	clustered regularly interspaced short palindromic repeats
Ct	cycle threshold
cv.	cultivar
DEPC	diethylpyrocarbonate
dm	dry matter
DNA	deoxyribonucleic acid
DSB	double strand break
E.	<i>Escherichia</i>
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
exp.	experiment
FAS	fasciclin
GAP	GTPase-activating protein
gDNA	genomic DNA
GFP	green fluorescence protein
GPDP	glycerophosphoryl diesterphosphodiesterase

Abbreviations

GPI	glycosylphosphatidylinositol
gRNA	guide RNA
GTP	guanosine triphosphate
ICP-MS	inductively coupled plasma - mass spectrometry
IRD	infrared dye
indels	insertions and deletions
K	potassium
LRR	leucine-rich repeat
MACE	massive analysis of cDNA ends
miRNA	microRNA
mRNA	messenger RNA
MYB	myeloblastosis
N	nitrogen
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
NHEJ	non-homologous end joining
NMD	nonsense-mediated mRNA decay
PAGE	polyacrylamide gel electrophoresis
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
P	phosphate
Pi	inorganic phosphate
PITP	phosphatidylinositol transfer protein
PSR	Pi starvation responses
qPCR	quantitative polymerase chain reaction
RHF	root hair-specific transcription factor
RhoGDI	Rho GTPase GDP dissociation inhibitor
Ri	root-inducing
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	ribonucleic acid
RNAi	RNA interference
ROP	Rho GTPase
ROS	reactive oxygen species
SE	standard error

Abbreviations

SSH	suppression subtractive hybridization
SUMO	small ubiquitin-like modifier
T-DNA	transfer DNA
T _a	annealing temperature
TAE	Tris-acetate-EDTA
TALEN	transcription activator-like effector nuclease
TBE	Tris-borate-EDTA
T _m	melting temperature
tRNA	transfer RNA
U	units
UTR	untranslated region
WT	wildtype
XET	xyloglucan endotransglycosylase

Gene Abbreviations

<i>ACT</i>	<i>ACTIN</i>
<i>AGD</i>	<i>ARF-GAP DOMAIN-CONTAINING PROTEIN</i>
<i>AGP</i>	<i>ARABINOGALACTAN PROTEIN</i>
<i>AKT</i>	<i>ARABIDOPSIS K⁺ TRANSPORTER</i>
<i>AL</i>	<i>ALFIN-LIKE</i>
<i>ATL</i>	<i>ARABIDOPSIS TÓXICOS EN LEVADURA</i>
<i>CaBP</i>	<i>CALCIUM-BINDING EF-HAND FAMILY PROTEIN</i>
<i>CML</i>	<i>CALMODULIN-LIKE</i>
<i>CNGB</i>	<i>CYCLIC NUCLEOTIDE-GATED CATION CHANNEL BETA</i>
<i>COW</i>	<i>CAN OF WORMS</i>
<i>CPC</i>	<i>CAPRICE</i>
<i>CSLB</i>	<i>CELLULOSE SYNTHASE-LIKE</i>
<i>DUF</i>	<i>DOMAIN OF UNKNOWN FUNCTION</i>
<i>EF-1-a</i>	<i>ELONGATION FACTOR 1 ALPHA</i>
<i>EIN</i>	<i>ETHYLENE INSENSITIVE</i>
<i>ETC</i>	<i>ENHANCER OF TRY AND CPC</i>
<i>EXO70L</i>	<i>EXOCYST COMPLEX COMPONENT EXO70-LIKE</i>
<i>EXP</i>	<i>EXPANSIN</i>
<i>FER</i>	<i>FERONIA</i>
<i>FLA</i>	<i>FASCICLIN-LIKE ARABINOGALACTAN</i>
<i>HRGP</i>	<i>HYDROXYPROLINE-RICH-GLYCOPROTEIN</i>
<i>HRS</i>	<i>HYPERSENSITIVE TO LOW PI-ELICITED PRIMARY ROOT SHORTENING</i>
<i>IPS</i>	<i>INDUCED BY PI STARVATION</i>
<i>IRE</i>	<i>INCOMPLETE ROOT HAIR ELONGATION</i>
<i>KJK</i>	<i>KOJAK</i>
<i>LPR</i>	<i>LOW PHOSPHATE ROOT</i>
<i>LRL</i>	<i>LOTUS JAPONICUS ROTHAIRLESS1-LIKE</i>
<i>LRR-RLK</i>	<i>LRR RECEPTOR-LIKE SERINE/THREONINE PROTEIN KINASE</i>
<i>LRX</i>	<i>LRR/EXTENSION PROTEIN</i>
<i>MRH</i>	<i>MORPHOGENESIS OF ROOT HAIR</i>
<i>MRI</i>	<i>MARIS</i>

Gene Abbreviations

<i>PDR</i>	<i>PHOSPHATE DEFICIENCY RESPONSE</i>
<i>PHF</i>	<i>PHOSPHATE TRAFFIC FACILITATOR</i>
<i>PHL</i>	<i>PHR-LIKE</i>
<i>PHO</i>	<i>PHOSPHATE</i>
<i>PHR</i>	<i>PHOSPHATE STARVATION RESPONSE</i>
<i>PHT</i>	<i>INORGANIC PHOSPHATE TRANSPORTER</i>
<i>PIP5K</i>	<i>PHOSPHATIDYLINOSITOL PHOSPHATE 5-KINASE</i>
<i>PP</i>	<i>PROTEIN PHOSPHATASE</i>
<i>RALF</i>	<i>RAPID ALKALINISATION FACTOR</i>
<i>RHD</i>	<i>ROOT HAIR DEFECTIVE</i>
<i>RHS</i>	<i>ROOT HAIR SPECIFIC</i>
<i>RLCK</i>	<i>RECEPTOR-LIKE CYTOPLASMIC KINASE</i>
<i>ROPGEF</i>	<i>ROP GUANINE NUCLEOTIDE-EXCHANGE FACTOR</i>
<i>RSL</i>	<i>ROOT HAIR DEFECTIVE 6-LIKE</i>
<i>SCN</i>	<i>SUPERCENTIPEDE</i>
<i>SHV</i>	<i>SHAVEN</i>
<i>TIP</i>	<i>TIP GROWTH DEFECTIVE</i>
<i>TRH</i>	<i>TINY ROOT HAIR</i>
<i>TRY</i>	<i>TRYPTICHON</i>
<i>UBC</i>	<i>UBIQUITIN CONJUGATING ENZYME</i>
<i>UNK</i>	<i>UNKNOWN</i>
<i>WRKY</i>	<i>WRKY TRANSCRIPTION FACTOR</i>
<i>ZC3H</i>	<i>ZINC FINGER CCCH DOMAIN-CONTAINING PROTEIN</i>
<i>ZFP</i>	<i>ZINC FINGER PROTEIN</i>

List of Figures

CHAPTER I

Fig. 1: Exemplarily measurement of the root hair length of cv. Bale grown under P deficient conditions	17
Fig. 2: Shoot dm, shoot P concentration, <i>AtIPS2</i> expression, root hair length and representative root hairs of <i>B. carinata</i> cv. Bale and cv. Bacho as affected by P supply in the 1 st experiment (MACE experiment).....	21
Fig. 3: Relative number of normalized tags assigned to the selected candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by P supply determined by MACE.....	23
Fig. 4: Relative expression of selected candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by P supply determined by qPCR in samples obtained from the MACE (1 st) experiment (exp.) and the independently conducted 2 nd exp	24
Fig. 5: Shoot and root dry matter of <i>B. carinata</i> cv. Bale as affected by P supply in a split-root system	26
Fig. 6: P _{total} concentration in shoot and root as well as P _{inorganic} concentration in shoot and root of <i>B. carinata</i> cv. Bale as affected by P supply in a split-root system.....	27
Fig. 7: Root hair length of <i>B. carinata</i> cv. Bale as affected by P supply in a split-root system	28
Fig. 8: Relative expression of candidate genes for increased root hair length during Pi deficiency in <i>B. carinata</i> cv. Bale as affected by P supply in a split-root system	29
Fig. 9: Shoot dry matter and root hair length as affected by nutrient deficiency.....	30
Fig. 10: Relative expression of candidate genes for increased root hair length during Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR	31
Fig. 11: Relative candidate gene expression and root hair length of the transgenic hairy roots after targeting the respective candidate genes by CRISPR/Cas9	36
Fig. 12: CRISPR/Cas9-induced deletions and insertions in <i>BcFLA1</i>	36
Fig. 13: Validation of the CRISPR/Cas9-induced gene editing of <i>BcFLA1</i> by fragment analysis by PAGE.....	37
Fig. 14: Relative expression of candidate genes for Pi deficiency-induced root hair growth in <i>bcfla1</i> roots grown under Pi-deficient conditions.....	38

CHAPTER II

Fig 1: Screening and harvesting of the transgenic root material **58**

Fig 2: Validation of the CRISPR/Cas9-induced gene editing by polyacrylamide gel electrophoresis **59**

Fig 3: CRISPR/Cas9-induced deletions in *BcFLA1*..... **60**

Fig 4: Expression of *BcFLA1* as affected by CRISPR/Cas9-induced gene editing and complementation, respectively **62**

Fig 5: Root hair length of *B. carinata* cv. 'Bale' as affected by downregulation and complementation of *BcFLA1*, respectively..... **63**

CHAPTER I (Supplementary Figures)

Fig. S1: Exemplary 2-cm root tip of *B. carinata* cv. Bale grown under Pi-deficient conditions. **102**

Fig. S2: Produced tags during MACE in million and relative number of tags annotated to *Brassica rapa* and *Brassica napus* **116**

Fig. S3: Number of normalized tags annotated to the different genes depending on cultivar, treatment and annotation **117**

Fig. S4: Shoot dry matter shoot P concentration and root hair length of *B. carinata* cv. Bale and cv. Bacho as affected by P supply in the 2nd experiment..... **120**

Fig. S5: *B. carinata* cv. Bale plants after an eight-day-cultivation in a split-root system containing 1 mM Pi on both sides, 1 mM Pi on one side and 0 mM Pi on the other side and 0 mM Pi on both sides **123**

Fig. S6: P, N and K concentration in the shoot of *B. carinata* cv. Bale and cv. Bacho as affected by P supply **125**

Fig. S7: Representative root hairs of *B. carinata* cv. Bale and cv. Bacho as affected by different nutrient deficiencies..... **125**

Fig. S8: Relative expression of selected candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by nutrient deficiency..... **128**

Fig. S9: Expression of the 7 candidate genes for Pi deficiency-induced root hair elongation as affected by CRISPR/Cas9-induced gene editing..... **141**

Fig. S10: Root hair length of the transgenic 1st and 2nd order lateral hairy roots after targeting the respective candidate genes for Pi deficiency-induced root hair elongation by CRISPR/Cas9..... **142**

CHAPTER II (Supplementary Figures)

S1 Fig: Expression cassette and *BcFLA1* guide RNAs..... **145**
S2 Fig: CRISPR/Cas9-induced deletions and insertions in *BcFLA1* **149**
S3 Fig: *BcFLA1* primers are specific for the mutant and the wildtype version of the gene, respectively..... **149**
S4 Fig: Pretest of the qPCR primers confirming the primer specificity and revealing a successful downregulation and complementation **150**
S5 Fig: Expression of *BcFLA1* as affected by CRISPR/Cas9-induced gene editing and complementation, respectively **151**

General Discussion

Fig. 1: Scheme showing the potential location of BcFLA1 and the other candidate gene products in the root hair signaling network **73**
Fig. S1: Topology prediction of BcFLA1 **152**

List of Tables

CHAPTER I

Table 1: <i>B. carinata</i> cv. Bale gene sequences of candidate genes for increased root hair length during Pi deficiency obtained by 5' RLM-RACE	32
---	-----------

CHAPTER I (Supplementary Tables)

Table S1: Number of genes in the different annotations of the MACE tags according to the filter steps	102
Table S2: Partial cDNA sequences of candidate genes for Pi deficiency-induced root hair growth obtained by MACE	103
Table S3: qPCR primers	112
Table S4: Primers used for 5' RLM-RACE	114
Table S5: Primers used for amplification of the Bale gDNA sequences.....	115
Table S6: Primers used for construct preparation.....	116
Table S7: Selected candidate genes for the Pi deficiency-induced root hair growth in <i>Brassica carinata</i>	118
Table S8: Relative expression of candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by P supply determined by qPCR in samples obtained from the MACE (1 st) experiment	121
Table S9: Relative expression of candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by P supply determined by qPCR in samples obtained from the 2 nd experiment.....	122
Table S10: Relative expression of candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale as affected by P supply in a split-root system determined by qPCR	124
Table S11: Relative expression of candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR (<i>AtUBC9</i>).....	126
Table S12: Relative expression of candidate genes for increased root hair length under Pi deficiency in <i>B. carinata</i> cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR (<i>BcEF-1-a1</i>).....	127
Table S13: Bale gene sequences obtained by 5' RLM-RACE (candidate genes, which were not further investigated by knockout).....	129

List of Tables

Table S14: Bale gDNA sequences of candidate genes for Pi deficiency-induced root hair growth..... **137**

Table S15: Amino acid sequences encoded by the two *BcFLA1* alleles..... **143**

CHAPTER II (Supplementary Tables)

S1 Table: Primers used for construct preparation..... **144**

S2 Table: qPCR primers **148**

S3 Table: Primer pairs for the verification of gene editing..... **148**

General Discussion

Table S1: Amino acid sequence of BcAGP1, BcAGP2 and BcRHS10L **152**

General Introduction

1. State of the knowledge

Phosphate (P) deficiency is one of the most important factors limiting plant growth. It is noticeable that not the overall P content in the soil, but rather the low concentration of plant available P restricts P nutrition of plants. The main part of P in soils is stable or labile bound. Depending on the pH, P forms insoluble complexes with aluminum, iron and calcium (Bielecki, 1973; Vance *et al.*, 2003). P is taken up by the plant as inorganic phosphate (Pi) either as HPO_4^{2-} or H_2PO_4^- . Because of the low concentration of solute Pi in soils, the uptake is an active process against a strong concentration gradient (Sakano *et al.*, 1992).

Phosphorus fulfills many important functions in the plant. It has a role as structural element (phospholipids, nucleic acids), it participates in the energy transfer (adenosine triphosphate, ATP) and it is a part of important biological processes like photosynthesis (Marschner, 2012).

The problem of the limited content of plant available Pi in the soil can be counteracted in two approaches: (I) the fertilization of soils with P and (II) the use of P efficient plants. Rock phosphate, the source of P fertilizers, is limited, so that P fertilizers will become more and more expensive over the years (Cordell *et al.*, 2009). Hence, beside the minimization of the loss of P and the development of effective P recycling systems (Kodera *et al.*, 2013), the breeding of P-efficient plants becomes more and more important. Plants can be more efficient in the utilization of Pi (Pi utilization efficiency) by using the P taken up and / or they can be more efficient in the acquisition of Pi (Pi uptake efficiency; Raghothama (2000)). The latter comprises the increase of the spatial availability of P by an altered root morphology including a higher root to shoot ratio, a smaller root radius and longer root hairs as well as the mobilization of adsorbed P by e.g. organic anions, protons and phosphatases, and a higher expression of high affinity Pi transporters, whereby the latter rather play a tangential role in increasing the Pi uptake under low Pi conditions, since diffusion coefficients for Pi in soils are usually very low, so that the Pi reaching the root surface is the key limiting factor (Duff *et al.*, 1991; Bates and Lynch, 1996; Raghothama, 2000; Gilroy and Jones, 2000; Vance *et al.*, 2003; Rengel and Marschner, 2005; Lambers *et al.*, 2006). In order to develop P-

efficient crop plants, it is important to understand the mechanisms leading to a higher P efficiency on the molecular level.

1.1 Regulation of Pi homeostasis

Both local and systemic signaling play a role in Pi responses in Arabidopsis. Whereas changes in the root system architecture seem to be mainly controlled by local responses, the Pi homeostasis appears to be regulated rather systemically (Thibaud *et al.*, 2010). A key role in the regulation of Pi starvation responses is played by the R2R3 myeloblastosis (MYB) transcription factor PHOSPHATE STARVATION RESPONSE 1 (PHR1), together with the partially redundant PHR1-LIKE (PHL) transcription factor. Both are able to repress and activate many genes in response to Pi starvation. Whereas the transcriptional repression of genes by PHR1/PHL is mostly indirect, genes containing the *cis*-regulatory element P1BS (GNATATNC) can be activated directly by PHR1/PHL (Bustos *et al.*, 2010). Another important player in the Pi signaling networks are microRNAs, especially miR399, which can move from the shoot to the root and enhance the uptake and translocation of Pi by the inhibition of the expression of *PHOSPHATE 2 (PHO2)* encoding a Pi transporter-degrading ubiquitin-conjugating E2 enzyme (Lin *et al.*, 2008; Kumar *et al.*, 2017). However, besides micro RNAs, other types of molecules like hormones, sugars, proteins and Pi itself are predicted to be involved in the systemic signaling during Pi starvation (Chiou and Lin, 2011; Scheible and Rojas-Triana, 2015). Also sumoylation both negatively and positively controls different Pi starvation responses. For example, PHR1 is sumoylated by the small ubiquitin-like modifier (SUMO) E3 ligase SIZ1 (Miura *et al.*, 2005). Furthermore, *INDUCED BY PHOSPHATE STARVATION 1 (IPS1)* exhibits sequence similarity to miR399, so that it can bind to it, thus inhibiting its activity. This mechanism, called target mimicry, enables a fine-tuning of the Pi deficiency responses and homeostasis (Franco-Zorrilla *et al.*, 2007).

1.2 Root hair formation

In *Brassicaceae*, the development of longer root hairs under Pi deprivation is especially important, because a symbiosis with mycorrhizal fungi is not possible. Root hairs can grow out of epidermal cells and increase the depletion zone around the root and thus the acquisition capability (Jungk, 2001). Their development can be divided into cell fate determination, root hair initiation and the elongation of the root hairs, ending with the termination of the growth (Datta *et al.*, 2011).

1.2.1 Cell fate determination

In some plant species like in rice any epidermal cell can develop a root hair, named type 1 development (Datta *et al.*, 2011). However, in many plant species a specific cell pattern determines, if an epidermal cell develops a root hair or not. Thus, type 2 development is characterized by alternating root hair (trichoblasts) and no root hair (atrachoblasts) developing cells within a cell file. *Arabidopsis* epidermal cells, which lie across two cortical cells, develop a root hair, whereas epidermal cells, which are only connected to one cortical cell develop no root hair (type 3 development; Galway *et al.*, 1994; Dolan *et al.*, 1994). The mechanisms underlying this specific cell fate in *Arabidopsis* were studied extensively in the past and a complex network, existing of several feedback loops, was uncovered. However, major differences were observed regarding the expression and structure of genes involved in the cell fate determination of other plant species compared to *Arabidopsis* (Huang *et al.*, 2017). Hence, the mechanisms underlying the root hair patterning in *Arabidopsis* cannot easily transferred to *Brassica carinata*. Furthermore, under Pi deficiency the normal root hair pattern is abolished as the root hairs grow ectopically (Dolan *et al.*, 1994). For these reasons, the description of the root hair development is focused on the root hair initiation and especially the elongation in the subsequent sections.

1.2.2 Root hair initiation

In root hair developing cells a row of basic helix-loop-helix (bHLH) transcription factors becomes active like ROOT HAIR DEFECTIVE 6 (RHD6), which is involved in the determination of the root hair initiation site just as ACTIN 2 (ACT2; Masucci and Schiefelbein, 1994, 1996; Ringli *et al.*, 2002). At the beginning of root hair initiation, the cell wall is acidified at the initiation site and this acidification is maintained until the transition from the initiation to the elongation phase (Bibikova *et al.*, 1998). The acidification is required for the expansin mediated cell wall loosening and leads to a turgor-driven formation of a bulge out of the initiation site, whose size is restricted by TIP GROWTH DEFECTIVE 1 (TIP1) in cooperation with RHD1 (Cosgrove, 1997; Bibikova *et al.*, 1998; Parker *et al.*, 2000; Baluska *et al.*, 2000). Expansins are able to disrupt the non-covalent bonds between cell wall polymers (McQueen-Mason and Cosgrove, 1995). Expansin genes with a relation to the root hair initiation are for example the *EXP7* and *EXP18* genes in *Arabidopsis* and the *HvEXPB1* gene in barley (Cho and Cosgrove, 2002; Kwasniewski and Szarejko, 2006). The Rho GTPase (ROP) GDP dissociation inhibitor (RhoGDI) SUPERCENTIPEDE 1 (SCN1) is also involved in

the root hair initiation by regulating the activity of plant ROPs, which are required for the restriction of the root hair initiation site (Jones *et al.*, 2002; Carol *et al.*, 2005). Recently, a C2H2-type ZINC FINGER PROTEIN 5 (ZFP5) was defined as a key regulator of both root hair initiation and root hair elongation (An *et al.*, 2012). Furthermore, Kwasniewski *et al.* (2013) proposed a model, in which a local and coordinated production of reactive oxygen species (ROS) plays an important role in the non-enzymatic cell wall loosening at the initiation side of root hairs.

1.2.3 Root hair elongation

After root hair initiation, the nucleus moves into the growing root hair and maintains a constant distance to the tip (Ketelaar *et al.*, 2002). Secretory vesicles accumulate in the root hair tip, which contain material for the new expanding cell wall (Galway *et al.*, 1997; Carol and Dolan, 2002). In this regard, Qi *et al.* (2016) revealed, that a fine endoplasmic reticulum (ER) structure in the subapical region, thought to be formed by microtubules and a ROP2-guided atlastin GTPase, RHD3, is required for the apical secretion and hence the polarized root hair growth. Furthermore, a tip-focused Ca^{2+} gradient, maintained by microtubules, is formed by an increased activity of Ca^{2+} influx channels in the root hair tip, which is required for the determination of the growth site in elongating root hairs (Wymer *et al.*, 1997; Bibikova *et al.*, 1997; Bibikova *et al.*, 1999). ROS produced by a NADPH oxidase encoded by *ROOT HAIR DEFECTIVE 2* (*RHD2*) can increase the activity of these Ca^{2+} channels (Foreman *et al.*, 2003). In addition to Ca^{2+} , also K^{+} plays an important role in the root hair tip growth. There are two K^{+} transporters, *ARABIDOPSIS K⁺ TRANSPORTER* (*AKT1*) and *TINY ROOT HAIR 1* (*TRH1*), which are essential for the root hair tip growth, maybe to establish a high turgor pressure (Rigas *et al.*, 2001; Desbrosses *et al.*, 2003; Ishida *et al.*, 2008). Moreover, *TRH1* is proposed to integrate the developmental root hair pathway with environmental/hormonal pathways since it can modulate the auxin homeostasis (Daras *et al.*, 2015). Additionally, a receptor-like kinase, *FERONIA* (*FER*), can interact with ROP GUANINE NUCLEOTIDE-EXCHANGE FACTORS (ROPGEFs) thus regulating ROP GTPase signaling and therefore root hair development (Duan *et al.*, 2010). Furthermore, several cell wall synthesizing and modifying enzymes are likely to be involved in the root hair tip growth such as xyloglucan endotransglycosylases (XETs), extensins, a cellulose synthase like protein encoded by *KOJAK* (*KJK*), and a leucine-rich repeat/extension protein (*LRX1*; Datta *et al.*, 2011). In addition, several bHLH transcription factors are involved in the root hair elongation, its regulation or

maintenance (Bruex *et al.*, 2012). RHD6 positively regulates the expression of *RHD6-LIKE 4 (RSL4)*, which in turn positively regulates genes encoding for endomembrane and cell wall modifying proteins, and controls genes involved in cell signaling and secretion (Yi *et al.*, 2010; Vijayakumar *et al.*, 2016). Constitutively expressed *RSL4* leads to continuous root hair elongation until cell death (Yi *et al.*, 2010). Auxin increases the *RSL4* expression, thus also having a positive influence on the root hair elongation (Pitts *et al.*, 1998; Yi *et al.*, 2010). Besides auxin, also ethylene was shown to positively affect root hair elongation (Pitts *et al.*, 1998). Furthermore, LOTUS JAPONICUS ROOTHAIRLESS1-LIKE 3 (AtLRL3), a functional paralog of RSL4, was shown to be necessary for a proper root hair elongation in Arabidopsis (Bruex *et al.*, 2012; Salazar-Henao *et al.*, 2016).

1.2.4 Pi deficiency-induced root hair elongation

Low phosphate supply modified root hair growth through a modulated RSL4 activity (Yi *et al.*, 2010). Pi deficiency is known to upregulate the R3 MYB proteins ENHANCER OF TRYPTICHON (TRY) AND CAPRICE (CPC) 1 (ETC1) and ETC3 as well as to increase the auxin responsiveness, both positively affecting RSL4 (Salazar-Henao and Schmidt, 2016). Hence, Pi starvation increases the duration of RSL4 synthesis, so that RSL4 is present in the root hair for a longer time, leading to an extended growth duration and therefore an enhanced final root hair size (Datta *et al.*, 2015). However, Bates and Lynch (1996) observed, that not only the duration, but also the rate of the root hair elongation is enhanced under low phosphorus conditions in *A. thaliana*. Interestingly, a key transcription factor in the ethylene signaling pathway encoded by *ETHYLENE INSENSITIVE 3 (EIN3)*, which is upregulated by Pi deficiency, can bind to the promoters of RSL4 target genes, thus additionally enhancing their transcription and therefore increasing the root hair length (Song *et al.*, 2016). Also Lin *et al.* (2011) identified several genes potentially involved in the Pi deficiency-induced root hair elongation in Arabidopsis. A reduced transcript level of an oxygenase, a calcium-binding EF-hand family protein (CALMODULIN-LIKE 25, CML25), which may be involved in calcium signaling, and *ROPGEF4*, which plays an important role in the activation of ROPs, led to the development of longer root hairs under Pi deficiency but not under sufficient Pi conditions. Conversely, a reduced expression of a cysteine proteinase, a gene with similarity to *LRX1*, and *CELLULOSE SYNTHASE LIKE 5 (CSLB5)* led to shorter root hairs exclusively under Pi deficient conditions (Lin *et al.*, 2011). *ALFIN-LIKE 6 (AL6)*, encoding a homeodomain transcription factor, putatively

controls the transcription of several genes involved in root hair elongation under Pi deficiency, encoding ETC1, an UDP-glycosyltransferase, a nonspecific phospholipase and a Pi pyrophosphate-specific phosphatase (Chandrika et al., 2013). Moreover, Lan *et al.* (2013) presented a series of protein kinases with a potential role in the Pi starvation-induced root hair remodeling. Furthermore, phosphatidylinositol phosphate 5-kinases (PIP5Ks) were identified to promote root hair elongation in response to Pi starvation (Wada *et al.*, 2015). PIP5Ks produce a signaling peptide, phosphatidylinositol 4,5-bisphosphate, and the authors concluded, that, at least at young seedling stages, the *PIP5K* genes are responsible for transferring the Pi deficiency signal to the root hair elongation pathway. However, the authors predicted signaling pathways independent of the *PIP5K* genes playing a role during prolonged Pi starvation.

1.3 Preliminary studies

Preliminary studies revealed two cultivars (cv.) of *Brassica carinata* (Ethiopian mustard) with a difference in their Pi uptake efficiency (Eticha and Schenk, 2001). Cv. Bale develops long root hairs under Pi deficiency, but not cv. Bacho, while both cultivars develop short root hairs under sufficient Pi cultivation. Differentially expressed genes could be identified by microarray and suppression subtractive hybridization (SSH) analysis. A time course experiment as well as tissue specific expression patterns confirmed the participation of three genes in root hair development: *LEUCINE-RICH RECEPTOR-LIKE PROTEIN KINASE*, *HYDROXYPROLIN-RICH GLYCOPROTEIN (HRGP)* and *LRX1* (Bremer, 2010).

2. Hypotheses

The aim of the present study was to increase the knowledge of molecular mechanisms of the phosphate deficiency-induced root hair elongation. Several genes have already been identified with a contribution in the Pi starvation induced root hair growth, but the total network of mechanisms leading to this response is still not clear. Especially regulatory genes, which may act at the beginning of the signal transduction cascade leading to the longer root hairs under Pi deficiency, were not completely identified. However, massive analysis of cDNA ends (MACE) offered the opportunity to find new candidate genes for the Pi deficiency-induced root hair growth, because in this method one single cDNA fragment is analyzed per transcript molecule, so that MACE provides a high resolution. Therefore, also medium and low level transcripts can be detected

(Kahl *et al.*, 2012), so that it was presumable, that this method would catch more genes in comparison to the SSH and the microarray approaches used in the preliminary studies.

We used the MACE to get whole expression profiles of the *B. carinata* cultivars Bale and Bacho under sufficient and deficient Pi conditions, thus identifying new regulatory candidate genes for the Pi deficiency induced root hair growth. Then, the function of the candidate genes was investigated with regard to local and/or systemic signaling and nutrient specificity. P-specific reacting candidate genes were further functionally characterized by knockout using clustered regularly interspaced short palindromic repeats / CRISPR-associated 9 (CRISPR/Cas9) in a hairy root system. Consequently, the hypotheses of this work were the following:

1. Comparison of the expression profiles of Bale and Bacho under high and low Pi conditions, generated by MACE, enables the detection of candidate genes for the Pi deficiency-induced root hair elongation.
2. Annotation of the MACE data to related *Brassica* species enables the limitation of the candidate genes to those with a potential regulatory function.
3. A split-root experiment gives hints about the involvement of the candidate genes in rather local or systemic signaling pathways.
4. The expression of the candidate genes identified by MACE / quantitative PCR (qPCR) is nutrient-specific. Investigation of their expression under different nutrient deficiencies will uncover their P-specificity.
5. The downregulation of the candidate gene expression as measured by qPCR leads to an altered root hair phenotype. The wildtype (WT) root hair phenotype will be recovered by complementation of the candidate gene function.
6. The downregulation of the candidate gene expression has effects on the transcriptome. These changes give hints for the position and function of the candidate genes in the pathways leading to the Pi deficiency-induced root hair development.

3. Central methods

Starting point for the identification of candidate genes for the Pi deficiency-induced root hair formation was the MACE, whereas the functional characterization of these genes was mainly based on CRISPR/Cas9. In the following the principle and advantages of both methods are described.

3.1 Massive analysis of cDNA ends (MACE)

The MACE is a deep sequencing method, which allows the comparison of genome-wide expression profiles. In MACE, total RNA is isolated and then reverse transcribed into cDNA using biotinylated primers. In the next step, the cDNAs are bound to a streptavidin matrix via the 3'-biotin ends. Then the cDNAs are shredded to 50-500 bp fragments and unbound fragments are discarded. The bound fragments are then sequenced by next generation sequencing (NGS), thus producing 50-100 bp fragments, called 'tags'. Therefore, the MACE provides mainly the 3' ends of the cDNAs. However, the 3' untranslated region (3' UTR) is expected to contain the most variable regions in related sequences (Kahl *et al.*, 2012; Vaughn *et al.*, 2012), which enables the opportunity for differentiation between isoforms. In the last step, the tags can be annotated to database entries, whereby the assembly of tags into contigs can account for a successful annotation (Kahl *et al.*, 2012).

3.2 CRISPR/Cas9

Manipulation of the gene expression is a suitable method for investigating the function of genes. An appropriate way to do this is the knockdown of the gene expression by RNA interference (RNAi) as reviewed by Shan (2010). However, with CRISPR/Cas9 a novel method for gene editing was reported (Jinek *et al.*, 2012). Compared to RNAi this method has the advantage to completely knockout target genes and to have a lower off-target rate (Barrangou *et al.*, 2015). In recent years, the CRISPR/Cas9 system grew into a simple, well-established and efficient method for performing gene editing events in plants (Belhaj *et al.*, 2015; Zhang *et al.*, 2016). In this system, the Cas9 nuclease is targeted to specific genomic sequences, where it induces double strand breaks (DSBs) (Zhang *et al.*, 2017). In somatic plant cells these are predominantly repaired by non-homologous end joining (NHEJ), which leads to errors during the repair, so that insertions, deletions (indels) or replacements occur (Knoll *et al.*, 2014). The specificity of the CRISPR/Cas9 system, which was shown to be especially high in plants (Zhang *et al.*, 2014; Feng *et al.*, 2014), is ensured by a 20 bp protospacer sequence belonging to the so-called guide RNA (gRNA), which guides the Cas9 nuclease to the corresponding site in the genomic DNA (gDNA) (Zhang *et al.*, 2017). The protospacer sequence has to be complementary to the target sequence, which must be followed by the protospacer adjacent motif (PAM), consisting of a 'NGG', so that the Cas9 can bind and cut the DNA double strand. However, base pairing of defined bases near the PAM, known as 'seed sequence', is essential and

therefore decisive for the specificity (Semenova *et al.*, 2011; Jinek *et al.*, 2012; Jiang *et al.*, 2013; Cong *et al.*, 2013) (for a review, see for example Bortesi and Fischer (2015) and Ding *et al.* (2016)). A multiplex editing system was developed, which takes advantage of the endogenous transfer RNA (tRNA) processing system, enabling to guide Cas9 to more than one site in the same gene or to targets in numerous genes by several gRNAs using only one construct (Xie *et al.*, 2015). Therefore, also large fragments can be cut out of a gene by inducing two DSBs in one gene at the same time, thus increasing the probability of the gene knockout (Zhou *et al.*, 2014).

The knockout of genes is unlikely, if the gene sequence is edited at the 3' end, because possible essential upstream gene regions, which are sufficient for the functionality may not be affected. Therefore, extension of the known sequence at the 3' end to the 5' end of the gene was necessary, which was done by 5' RNA ligase-mediated rapid amplification of cDNA ends (5' RLM-RACE). In short, an adapter sequence is added to only intact, full length messenger RNA (mRNA), following reverse transcription and amplification by nested PCR using primers binding to the adapter sequence and in the known region at the 3' end (Schaefer, 1995).

CHAPTER I

Identification and functional characterization of potential candidate genes with a regulatory function in Pi deficiency-induced root hair growth in *Brassica carinata*

Contributor Roles:

Under supervision of Thomas Kirchner

- the split-root experiment was done in the course of the Bachelor thesis of Kim L. Rössig
- the nutrient specificity experiment was done in the course of the Bachelor thesis of Timo Lauterbach
- the CRISPR experiments were done in the course of the Master thesis of Markus Niehaus

Abstract

Formation of longer root hairs under limiting phosphate (P) conditions can increase the inorganic P (Pi) uptake. Here, regulatory candidate genes for Pi deficiency-induced root hair growth were identified by comparison of massive analysis of cDNA ends (MACE)-provided expression profiles of two *Brassica carinata* cultivars differing in their root hair response to Pi deficiency: cultivar Bale develops longer root hairs under Pi deficiency but not cultivar Bacho. A split-root experiment was conducted for the differentiation between locally and systemically regulated genes. Furthermore, plants were exposed to nitrogen and potassium deficiency to identify P-specific reacting genes. The latter were knocked out by CRISPR/Cas9 and the effect on the root hair length was determined.

About 500 genes were differentially expressed under Pi deficiency in cultivar Bale, while these genes did not respond to the low P supply in cultivar Bacho. 33 candidate genes with a potential regulatory role were selected and the transcriptional regulation of 30 genes was confirmed by quantitative PCR (qPCR). Only five candidate genes seemed to be either exclusively regulated locally (two) or systemically (three), whereas 25 genes seemed to be involved in both local and systemic signaling pathways. Potassium deficiency did affect neither the root hair length nor the expression of the 30 candidate genes. In contrast, both P and nitrogen deficiency increased the root hair length, and in 26 cases both affected the transcript levels. However, four genes specifically reacted to Pi starvation. These genes and additionally *RAPID ALKALINIZATION FACTOR-LIKE 1 (BcRALFL1)* and *INORGANIC PHOSPHATE TRANSPORTER 1 (BcPHT1)* were targeted by CRISPR/Cas9. However, even if the transcript levels of five of these genes were clearly decreased, *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1 (BcFLA1)*, was the only gene whose downregulation reduced the root hair length in transgenic hairy roots under Pi-deficient conditions. To our knowledge, this is the first study describing a fasciclin-like arabinogalactan protein with a predicted role in the Pi deficiency-induced root hair elongation.

Keywords: CRISPR/Cas9; hairy root; PAGE; fasciclin-like arabinogalactan protein; FLA; local and systemic response; N deficiency; K deficiency; MACE; split-root

1. Introduction

Since plant available phosphate (P) in the soil is often limited, plants rely on mechanisms increasing the inorganic P (Pi) uptake (Vance *et al.*, 2003). One of these is the development of longer root hairs under Pi deficiency, which increase the depletion zone around the root and thus the acquisition capability (Jungk, 2001). Among the root hair development steps, the root hair elongation, during which the root hair grows by tip growth, is decisive for the final root hair size (Datta *et al.*, 2011). The general root hair elongation mechanisms are, at least in *Arabidopsis thaliana*, relatively well known, but the regulating mechanisms underlying the phosphate deficiency-induced root hair elongation are still not completely understood.

Recent studies in *Arabidopsis* revealed, that transcription factors play an important role in the root hair elongation under Pi starvation. The bHLH transcription factor ROOT HAIR DEFECTIVE 6 (RHD6) induces the expression of another bHLH transcription factor, RHD6-LIKE 4 (RSL4), which in turn regulates genes needed for cell growth (Yi *et al.*, 2010; Vijayakumar *et al.*, 2016). Interestingly, under Pi deficiency RSL4 synthesis and therefore its presence in the root hair was prolonged, so that the root hair growth duration and accordingly the final root hair size was enhanced (Datta *et al.*, 2015). Moreover, some of the target genes of RSL4 can be upregulated by ETHYLENE INSENSITIVE 3 (EIN3), which is a key transcription factor in the ethylene signaling pathway that is upregulated by Pi deficiency, thus additionally increasing the root hair length (Song *et al.*, 2016). In addition, there is a functional paralog of *RSL4*, *LOTUS JAPONICUS ROOTHAIRLESS1-LIKE 3 (LRL3)*, which was upregulated in response to Pi deficiency and also shown to be necessary for a proper root hair elongation (Bruex *et al.*, 2012; Salazar-Henao and Schmidt, 2016; Salazar-Henao *et al.*, 2016). Furthermore, the homeodomain transcription factor *ALFIN-LIKE 6 (AL6)* controls the transcription of several genes involved in root hair elongation under Pi deficiency (Chandrika *et al.*, 2013).

Besides transcription factors, also protein kinases seem to have key roles during root hair elongation under Pi deficiency, since many protein kinases, which were regulated in response to Pi starvation, were associated with root hair growth in a co-expression analysis (Lan *et al.*, 2013). Phosphatidylinositol phosphate 5-kinases (*PIP5Ks*) were identified to promote root hair elongation in response to Pi starvation (Wada *et al.*, 2015). *PIP5Ks* produce a signaling peptide, phosphatidylinositol 4,5-bisphosphate,

and the authors concluded, that, at least at young seedling stages, the *PIP5K* genes are responsible for transferring the Pi deficiency signal to the root hair elongation pathway. However, the authors predicted signaling pathways independent of the *PIP5K* genes playing a role during prolonged Pi starvation.

Moreover, calcium signaling and plant RHO GTPases (ROPs) seem to be involved in the Pi deficiency-induced root hair elongation as a calcium-binding EF-hand family protein (CALMODULIN-LIKE 25, CML25) and ROP GUANINE NUCLEOTIDE-EXCHANGE FACTOR 4 (ROPGEF4), which plays in cooperation with the receptor-like kinase FERONIA (FER) a role in the activation of ROPs (Duan *et al.*, 2010), seem to negatively regulate the Pi deficiency-induced root hair elongation in Arabidopsis (Lin *et al.*, 2011).

In spite of the identification of several players involved in the Pi deficiency-induced root hair elongation, especially the network of regulatory genes, which may act at the beginning of the signal transduction cascade leading to the longer root hairs under Pi deficiency, still exhibits major knowledge gaps. Therefore, the aim of the present study was to identify and functionally characterize new candidate genes for the regulation of the Pi deficiency-induced root hair elongation.

Preliminary studies revealed two cultivars (cv.) of *Brassica carinata* (Ethiopian mustard) with a difference in their Pi uptake efficiency (Eticha and Schenk, 2001). Cv. Bale develops longer root hairs under Pi deficiency compared to sufficient Pi conditions, whereas cv. Bacho develops short root hairs under both conditions. We used massive analysis of cDNA ends (MACE) to compare expression profiles of the *B. carinata* cultivars under both sufficient and deficient Pi conditions. Since one single cDNA fragment is analyzed per transcript molecule, the MACE provides a high resolution, so that also medium and low level transcripts can be detected (Kahl *et al.*, 2012). Before starting the MACE, Pi deficiency was confirmed by a reduced shoot dry matter (dm) yield, a low P concentration in the shoot dm as well as an enhanced expression of a marker gene for Pi deficiency, *INDUCED BY Pi STARVATION 2* (*AtIPS2*) (Shin *et al.*, 2006), and the root hair length was increased in Bale. Indeed, we identified around 500 genes in two different annotations with a regulation that indicated a role in the root hair elongation under Pi deficiency. More than 30 of these were further investigated because of a potential regulatory function. The regulation observed in the MACE could be validated by quantitative PCR (qPCR) for nearly all genes. A split-root

experiment showed, that most of these genes may be involved in both local and systemic signaling pathways, whereas only a few genes were exclusively locally or systemically regulated. Furthermore, most of the genes were upregulated under both Pi and nitrogen (N)-deficiency, but the expression of just a few genes was enhanced exclusively under Pi deficiency, whereas none of the genes responded to potassium (K) deficiency. Lack of K was not reflected in root hair growth whereas under both N and Pi deficiency root hair length was increased in cv. Bale. One of these genes exclusively regulated under Pi deficiency was *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1 (BcFLA1)*, whose downregulation by CRISPR/Cas9 in a hairy root system led to a reduced root hair length compared to the wildtype. The role of *BcFLA1* in Pi deficiency signaling is discussed.

2. Materials and Methods

2.1. Plant material and growth conditions

For the MACE experiment, seeds of *B. carinata* cv. Bale and cv. Bacho were surface sterilized for 1 min in 1 % sodium hypochlorite, put on 0.7 % (w/v) phyto agar (pH 5.8; Duchefa Biochemie, Haarlem, Netherlands) and then stratified for three days in the dark at 4°C. Afterwards, the seeds were germinated *in-vitro* for 5 (Bale) and 6 days (Bacho), respectively, with a day/night interval of 16/8 h at 23°C. Then the plants were transferred into a climate chamber (photoperiod, 16/8 h light/dark; temperature, 20/15°C day/night; relative humidity, 75 %; light intensity, 220 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and cultivated in nutrient solutions containing (mM) 2.25 $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$, 2.5 K_2SO_4 , 1 $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 0.25 KCl and exclusively in the control treatment 1 KH_2PO_4 . Furthermore, the nutrient solutions contained the following micronutrient concentrations (μM): 25 H_3BO_3 , 1.5 MnSO_4 , 1.5 ZnSO_4 , 0.5 CuSO_4 , 0.025 $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ and 35.8 Fe (Fe^{III} -EDTA). The pH was adjusted to 5.3 and readjusted during the cultivation as needed. After eight days, root tips with a length of two centimeter (cm) (Fig. S1) were cut for later MACE and qPCR analysis, directly frozen in liquid nitrogen and then stored at -85°C. For each treatment, the root tips of 42 plants (two root tips per plant) were pooled. Additionally, six replications, each consisting of seven plants (four root tips per plant), were harvested from the same plants. Before cutting the root tips, the roots were washed in diethylpyrocarbonate (DEPC) two times, to minimize the contamination with RNAses. Furthermore, total shoots for dm and P determination were harvested and dried for three days at 60°C (four replications á four shoots). Eight total roots per treatment were incubated for 1 hour in 70 % (v/v) EtOH and then stored in tap water at 4°C for later root hair length measurement.

The independently replicated experiment was identical to the MACE experiment, but 30 root tips were harvested per replication (six plants á five root tips) for the expression analysis. Furthermore, six replications á three shoots (for dm and P determination) and 12 total roots (for root hair length measurement) were harvested per treatment.

For the split-root experiment, *B. carinata* cv. Bale plants were germinated as before and then hydroponically grown under the above conditions and in the above nutrient solution containing 0.05 mM KH_2PO_4 for five days. Then the main root was cut and one day later the plants were transferred into a split-root system consisting of nutrient solutions with (+ +) or without (- -) 1 mM Pi on both sides and without Pi only on one

side and sufficient (1 mM) Pi on the other side (+ -). After the appearance of P deficiency symptoms at the shoot and a distinct increase of the root hair length in the [-]-plants, root tips (three replications á ten plants á two root tips for expression analysis) and 30 total roots (+ + and - -) and root halves (+ -), respectively (for root hair length measurement), were harvested and treated as before. Additionally, three replications á 21 shoots and three replications á five total roots (+ + and - -) and root halves (+ -), respectively, were harvested for dm and P determination.

For the nutrient specificity experiment, seeds of *B. carinata* cv. Bale and Bacho were germinated between filter papers under the same climate chamber conditions as before. For the control and Pi deficiency treatment the composition of the nutrient solutions was the same as before, but in the N deficiency treatment $\text{Ca}(\text{NO}_3)_2 \times 4 \text{H}_2\text{O}$ was substituted by $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$ and in the K deficiency treatment K_2SO_4 and KCl were omitted and KH_2PO_4 was substituted by $\text{NaH}_2\text{PO}_4 \times 2 \text{H}_2\text{O}$. After eight days, root tips (six replications á six plants á five root tips for expression analysis), shoots (six replications á three shoots for dm and P determination) and 12 total roots (for root hair length measurement) were harvested and treated as before.

For the CRISPR experiments, plant cultivation and hairy root transformation were done as described in Chapter II page 54-55.

2.2. Determination of the nutrient concentrations

For the extraction of total P and K, 50 mg dm were dry ashed overnight at 480°C, dissolved in 1 mL 1:3 diluted HNO_3 , diluted with 9 mL H_2O and filtrated (Rotilabo®-round filters, type 15A).

Pi was extracted according to Grunwald *et al.* (2009) using 10 mg (shoot) and 5 mg (root) dm.

Both total P and Pi concentration were photometrically determined according to Gericke and Kurmies (1952).

In the nutrient specificity experiment, shoot P and K concentration were determined by inductively coupled plasma - mass spectrometry (ICP-MS) (Agilent 7500 cx, Agilent Technologies, Santa Clara, CA, USA). Shoot N concentration was determined by CHNOS elemental analyzer (Vario EL III, Elementar, Langenselbold, Germany).

2.3. Root hair length measurement

Roots were stained in 0.05 % (w/v) toluidine blue for one to two hours. Then, two 2-cm root tips were cut per plant, put into tap water and examined by the MZ10 F (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Root tips were pulled over 0.3 % (w/v) agar-agar, until the root hairs were straight. Pictures were taken from the

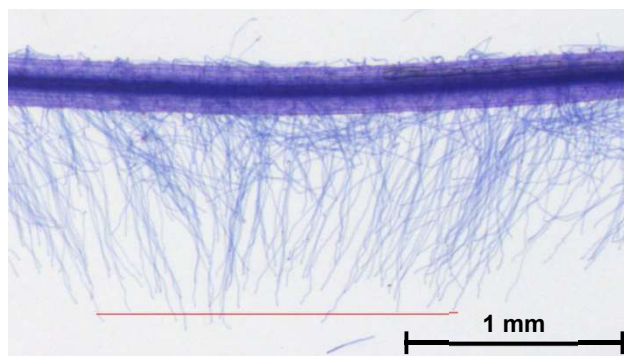


Fig. 1: Exemplarily measurement of the root hair length of cv. Bale grown under P deficient conditions.

root sections with fully elongated root hairs and the root hair length was measured (Fig. 1). For the nutrient specificity and CRISPR experiments, pictures were taken using the 'SMZ25' (Nikon, Düsseldorf, Germany) and root hair length was measured using the corresponding software 'NIS-Elements' instead. Root tips of the transgenic first and second order lateral roots were cut for the CRISPR experiments.

2.4. RNA isolation

Before starting the RNA isolation, frozen **pool samples** were homogenized by mortar and pestle. Because of the important role of micro RNAs (miRNAs) in the Pi signaling pathways (for example Bari *et al.*, 2006), the small and large RNAs of the pool samples were isolated in separate fractions using the NucleoSpin miRNA kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. The small RNA was stored for later analyses and the large RNA served as starting material for the MACE.

The frozen samples of the **biological replications** were homogenized in a swing mill at 28 s^{-1} for 2 min (MM 400, Retsch GmbH, Haan, Germany). RNA was isolated using the GENEzol™ reagent (Geneaid Biotech Ltd., New Taipei City, Taiwan) (MACE, 2nd experiment and split-root experiment) and the peqGOLD TriFast™ reagent (VWR International GmbH, Darmstadt) (nutrient specificity and CRISPR experiments), respectively, according to the manufacturer's instructions.

RNA Quality was electrophoretically tested on a 1 % (w/v) non-denaturing agarose gel containing 0.004 % (v/v) Midori Green Advance (Nippon Genetics Europe, Düren, Germany) and a runtime of 35 min at 6.9 to 7.6 V cm^{-1} in Tris-acetate-EDTA (TAE)

buffer. RNA quantity was determined using the NanoPhotometer® P-Class P 300 (Implen, Munich, Germany).

2.5. Massive analysis of cDNA ends (MACE)

For each treatment, one isolated RNA pool sample with a total amount of 6 to 12.7 µg and a concentration between 280 and 550 ng µL⁻¹ was sent to GenXPro (Frankfurt am Main, Germany), where the MACE was performed according to Kahl *et al.* (2012).

The tags of the MACE were annotated to two related plant species. The first annotation process was done by GenXPro in which the tags were annotated against the genome and transcriptome of *Brassica rapa* (Ensembl, annotation release 20, Kersey *et al.* (2016)), whereby non-annotatable tags were *de-novo* assembled using TrinityRNASeq (version: trinityrnaseq_r20140717) and CAP3 ("version date: 12/21/07). Consensus sequences were generated against the reference sequences (*B. rapa* genome and transcriptome and *de-novo* assembly) using NovoAlign (V3.00.05). The second annotation process was performed with CLC Genomics Workbench (version 7.5.1; CLC Bio, Aarhus, Denmark). First, the tags were trimmed in two steps using a 50 and afterwards a 25 bp poly-A adapter as template. Simultaneously, tags were trimmed to a quality limit of maximal 0.05 and to contain less than two ambiguous bases. Tags shorter than 50 bp were discarded. Then the tags were annotated against *Brassica napus* (*Brassica napus* gene index (BnGI) release 5) using the RNAseq tool (settings: similarity fraction = 0.8, length fraction = 0.8, mismatch cost = 2, insertion cost = 3, deletion cost = 3) and consensus sequences were generated from the annotated tags.

2.6. Candidate gene selection

The list of annotated genes was filtered automatically using the criteria 2-fold regulation in Bale (I), at least three normalized tags in Bale (II), a regulation in Bacho between 0.7 and 1.5 (III) and the number of normalized tags in the Pi deficiency treatment had to be at least 1.5-fold higher (upregulated genes) or lower (downregulated genes) in Bale than in Bacho (IV) (Table S1). From the resulting list only genes with a potential regulatory function were chosen for the subsequent analyses. Additionally, *BcPIP5K1* was selected from the MACE dataset, because Wada *et al.* (2015) uncovered a role in the Pi-deficiency-induced root hair elongation for its counterpart in *Arabidopsis thaliana*, *AtPIP5K3*.

2.7. Expression analysis

cDNA synthesis and qPCR were performed as described in Chapter II S2 text. Gene-specific primer pairs were designed on the consensus sequence of each candidate gene obtained by MACE in regions with high similarity between the four treatments (Table S2, Table S3). *LOTUS JAPONICUS ROOTHAIRLESS1-LIKE 3-LIKE* (*BcLRL3L*) primers were designed to bind both *BcLRL3L1* and 2, whereby there was one mismatch in the reverse primer for *BcLRL3L2* at position 16. Primers for *AtIPS2* were designed on the corresponding Arabidopsis sequence (AT5G03545).

Primers with an efficiency between 85 % and 115 % were used for expression analysis, if the discrepancy in the efficiency of the two cultivars was 10 % at the most.

UBIQUITIN CONJUGATING ENZYME 9 (*AtUBC9*; AT4G27960) was used as endogenous control in all the experiments because of its invariant expression under nutrient stress conditions (Czechowski *et al.*, 2005). In the nutrient specificity experiment, *ELONGATION FACTOR 1 ALPHA 1* (*BcEF-1-a1*) was used as additional control and evaluated separately.

In all qPCR runs the annealing temperature (T_a) was consistently 60°C.

Statistics were performed according to Steibel *et al.* (2009) using the R package 'qpcrmix'.

2.8. Knockout of the candidate genes by CRISPR/Cas9

The construct preparation was done as described in Chapter II page 53-54. In short, the gene sequence of selected candidate genes obtained by MACE was extended by 5' RNA ligase-mediated rapid amplification of cDNA ends (5' RLM-RACE). A list of the gene-specific primers used for the RACE can be found in Table S4. Based on the MACE and RACE sequences, primers were designed (Table S5) for amplification and sequencing of the gDNA sequences of the candidate genes. cDNA and gDNA sequences were aligned to identify introns and exons within the genes. For *INORGANIC PHOSPHATE TRANSPORTER 1* (*BcPHT1*) introns and exons were identified based on the annotated gDNA sequence from *B. rapa* instead. Then, two guide RNAs (gRNAs) were designed in an exon near the 5' end of each gene with a distance of (bp) 69 (*BcFLA1*), 34 (*RAPID ALKALINIZATION FACTOR-LIKE 1*, *BcRALFL1*), 56 (*BcPHT1*), 98 (*MARIS-LIKE*, *BcMRIL*), 26 (*ROP GUANINE NUCLEOTIDE EXCHANGE FACTOR 1*, *BcROPGEF1*), 43 (*BcLRL3L1*) and 66

(*BcLRL3L2*), and cloned into pB-CRISPR+35S::GFP. The additionally used primers for the construct preparation for the different genes are shown in Table S6.

Gene editing of *BcFLA1* was verified by the amplification of the potentially edited gene region ($T_a = 60^\circ\text{C}$) and its separation by agarose gel electrophoresis (primers 'TTCAAACGCAATGGCAACCA' and 'ACCCAAATCAAACGACGAGTTTC'). For it, PCR was performed with the KAPA Hifi HotStart PCR Kit (KAPA Biosystems, Wilmington, MA, USA) together with the supplied GC buffer and a primer concentration of 0.3 μM according to the manufacturer's instructions. Resulting products were purified from the agarose gel and sequenced. Additionally, indels were detected by polyacrylamide gel electrophoresis (PAGE) (primers 'GTA AACGACGGCCAGTTTCAAACGCAATGGCAACCA' and 'TTCCGACTCGTGTTGTGTTT') as described in Chapter II page 55-56. The primers were designed to bind two different alleles of *BcFLA1* (*BcFLA1a* and *BcFLA1b*), whereby there was one mismatch for *BcFLA1b* in the reverse primer used for the validation by PAGE (base position 9).

2.9. Genomic DNA isolation

For genomic DNA (gDNA) isolation, the remaining transgenic roots were used after cutting the 2-cm root tips. gDNA was isolated using a modified method from Oppermann (2004). All centrifugation steps were performed at 15000 U min^{-1} (4°C). Frozen samples were homogenized similarly to the RNA isolation and resuspended in 1 mL buffer, containing 0.2 M Tris-HCl (pH 9), 0.4 M LiCl, 25 mM EDTA and 1 % (w/v) SDS. After addition of 50 μL RNase A (10 mg mL^{-1}), samples were incubated for 10 min at 60°C followed by a centrifugation step for 10 min. 1 mL of the supernatant was mixed for 10 sec with 900 μL phenol/chlorophorm/isoamyl alcohol mix (25:24:1) (Roth, Karlsruhe, Germany). Samples were cooled on ice for 5-10 min and centrifuged for 10 min. 900 μL of the supernatant were mixed for 10 sec with 1 mL phenol mix (Roth, Karlsruhe, Germany) and centrifuged for 5 min. Then, 900 μL of the supernatant were given into 1 mL cold isopropanol and the resulting precipitate was pelleted by a centrifugation step of 10 min. The pellet was washed in 1 mL 80 % (v/v) EtOH by a 20-min centrifugation. After drying, the pellet was resuspended in 50 μL TE buffer (pH 9), containing 10 mM Tris and 1 mM EDTA. gDNA quality and quantity was tested as described for RNA.

3. Results

3.1. Characterization of the plant material used for MACE regarding the P deficiency and root hair response

To characterize the P deficiency in the 0 mM H₂PO₄ (-P) treatment, shoot dry matter, shoot P concentration and expression of a marker gene for P deficiency, *AtIPS2*, were determined. In *Bacho* the -P treatment resulted in a decrease of the shoot dry matter yield compared to the control (Fig. 2A). In tendency, this effect also occurred in *Bale*. However, in both cultivars shoot P concentration was about 2 mg (g dm)⁻¹ in the -P treatment and thus only one-fifth compared to the shoot P concentration in the 1 mM H₂PO₄ (+P) treatment (Fig. 2B). Furthermore, the *AtIPS2* expression was strongly enhanced during Pi starvation (Fig. 2C). *Bale* exhibited a clear increase in the root hair length in the -P treatment compared to the +P treatment, whereas *Bacho* developed short root hairs under both P conditions (Fig. 2D,E).

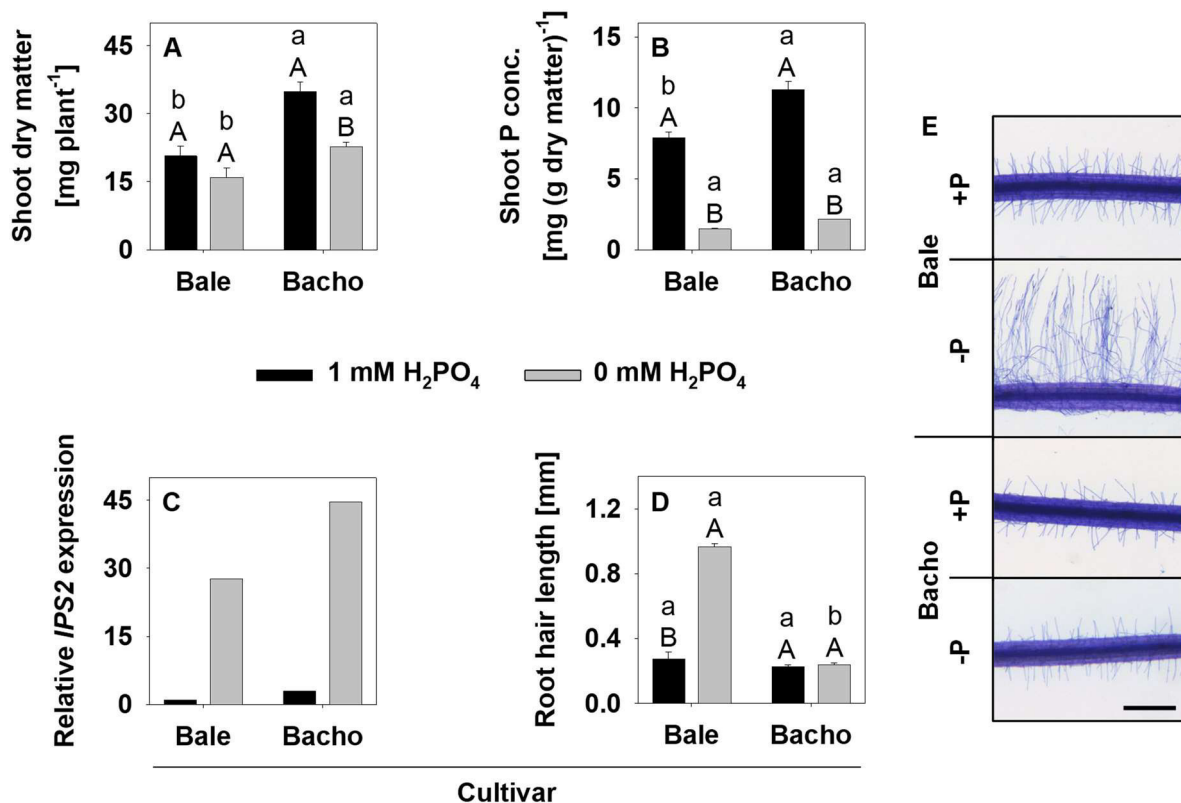


Fig. 2: Shoot dm (A), shoot P concentration (B), *AtIPS2* expression (C), root hair length (D) and representative root hairs (E, scale = 0.5 mm) of *B. carinata* cv. *Bale* and cv. *Bacho* as affected by P supply in the 1st experiment (MACE experiment). Small letters denote significant differences at $P < 0.05$ between cultivars at the same P-level; capital letters between P-treatments of the respective cultivar (Tukey test). Columns represent means and bars indicate SE; $n=4$ (A, B) and 8 (D) biological replications, whereas 3 technical replications of pooled samples were realized for C.

3.2. Analysis of the MACE data

Between 16 and 26 million tags were produced during the MACE (Fig. S2A). Around four million more tags were produced in the 0 mM P treatment of both cultivars and in both treatments of Bacho six million more tags were produced compared to Bale. Around 70 % to 80 % of the total number of tags could be annotated to *B. rapa* (Fig. S2B), whereas only about 50 % to 60 % of the tags could be annotated to *B. napus* (Fig. S2C). About 10 % more tags could be annotated to cultivar Bacho compared to Bale in both annotations (Fig. S2B,C).

To check, if the MACE was successfully performed and if there were any displacements in the annotations between the two cultivars, scatterplots were created showing the number of normalized tags in Bale and Bacho, annotated to the different genes (Fig. S3). As expected, under both sufficient and deficient P conditions and in both annotations the majority of the dots occurred along the bisecting line, so that it was assumed, that the dataset was reliable (Fig. S3A-D). When comparing the treatments within the cultivars it was striking, that in Bacho many genes exhibited relatively more assigned tags and therefore a higher expression under Pi deficiency compared to the sufficient Pi condition (Fig. S3G,H). However, since there was no root hair effect in Bacho, these genes may be upregulated as part of the general Pi deficiency responses.

Criteria for the selection of candidate genes with a possible participation in the Pi deficiency-induced root hair elongation were, based on a representative number of tags: (i) significant up- or downregulation under Pi-deficient conditions compared to sufficient conditions in Bale, and (ii) no regulation in Bacho. (iii) Furthermore, the expression level in Bale should be clearly higher (upregulated genes) or lower (downregulated genes) compared to Bacho in the -P treatment. Filtering the dataset according to these criteria resulted in about 200-300 remaining genes in each filter process and annotation (Table S1), from which 33 candidate genes with a potential regulatory role were selected encoding for arabinogalactan proteins, calcium-related proteins, proteins involved in phosphate transport, protein kinases, GTPase-related proteins, transcription factors, zinc finger proteins, a protein phosphatase, proteins with unknown function and other proteins with a known root hair affecting role (Table S7). The protein phosphatase was the only selected downregulated gene in Bale under Pi deficiency. Based on the results of the split-root and nutrient specificity experiment,

seven candidate genes were selected for the functional analysis by knockout and further described. Information on the others is given in tables S7-12. According to the MACE, most of the for the knockout selected candidate genes exhibited a two to three-times higher expression under deficient P conditions in Bale compared to sufficient P conditions, whereas *BcPHT1* was even 15-fold upregulated (Fig. 3). In Bacho, the expression of these genes was nearly the same under both P conditions.

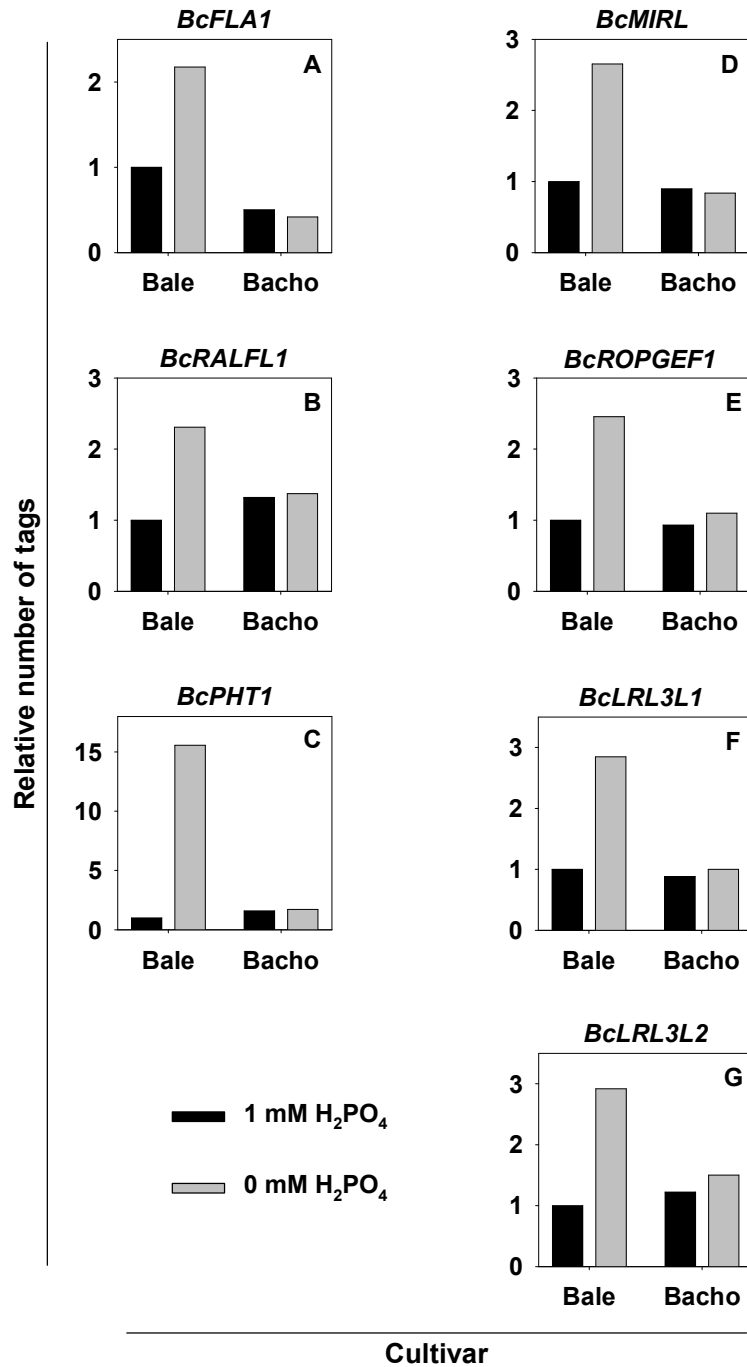


Fig. 3: Relative number of normalized tags assigned to the selected candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by P supply determined by MACE.

3.3. Validation of the candidate gene regulation

In the next step, relative gene expression was tested by qPCR in three retained biological replicates originating of the same plant material utilized for the MACE (1st experiment) as well as in samples obtained from an independently conducted 2nd experiment with an equivalent design as the MACE experiment. For the replicated experiment, P deficiency was confirmed by decreased shoot dry matter and shoot P concentration (Fig. S4A,B), and root hair length was enhanced in the -P treatment in Bale similarly to the MACE experiment (Fig. S4C).

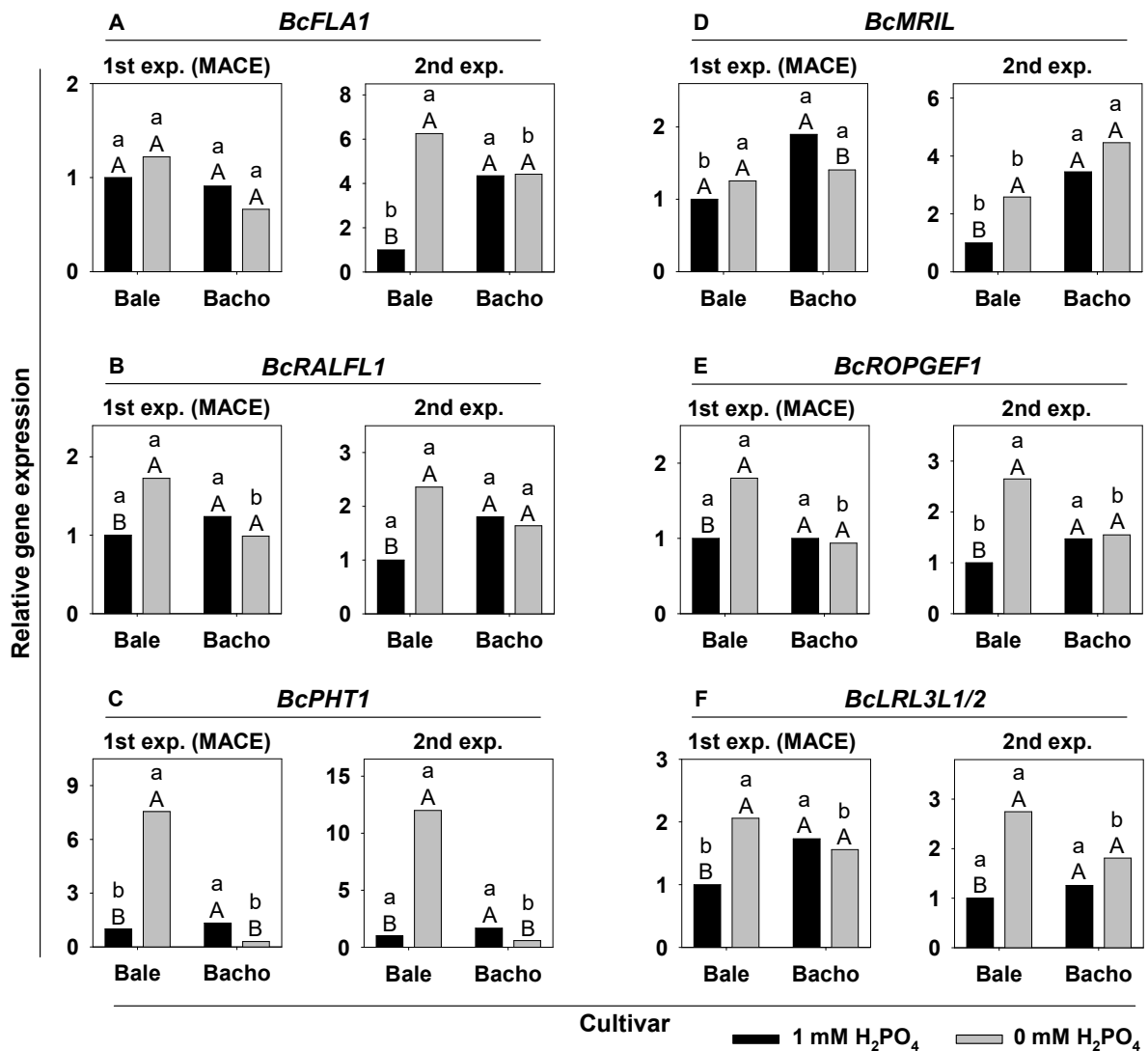


Fig. 4: Relative expression of selected candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by P supply determined by qPCR in samples obtained from the MACE (1st) experiment (exp.) and the independently conducted 2nd exp. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Small letters denote significant differences at $P < 0.05$ between cultivars at the same P-level; capital letters between P-treatments of the respective cultivar. Columns represent means; $n=3$.

The up- or downregulated gene expression in Bale under P deficient conditions was confirmed for 22 and 29 candidate genes in the samples from the MACE (1st) experiment and the 2nd experiment, respectively (Table S8; Table S9). Overall, the P deficiency effect on the gene regulation in Bale was stronger in the 2nd experiment. Every gene exhibited the expected regulation in Bale in at least one of both experiments. However, *PHOSPHATE TRAFFIC FACILITATOR 1 (BcPHF1)*, *DOMAIN OF UNKNOWN FUNCTION 620 (BcDUF620)* and *PROTEIN PHOSPHATASE 1 (BcPP1)* were similarly regulated in Bacho like in Bale in both experiments, so that they were excluded from the following experiments.

The upregulation of the six genes, which were selected for knockout, was confirmed in Bale under Pi deficiency (Fig. 4). However, for *BcFLA1* and *BcMRIL* this upregulation was significant only in the 2nd experiment (Fig. 4A and D). As predicted by MACE, *BcPHT1* exhibited the strongest upregulation and interestingly, in Bacho a contrary regulation was observed (Fig. 4C).

3.4. Split-root experiment

To test whether the candidate genes are involved in rather local or systemic signaling pathways, Bale seedlings were grown in a split-root system in nutrient solutions with (+ +) or without (- -) Pi on both sides and with Pi only on one side and without Pi on the other side (+ -).

After eight days in the split-root system, shoot growth of the [+ +]- and [+ -]-treatment was similar (Fig. S5A and B), but was clearly less in the [- -]-plants, which had small dark green leaves (Fig. S5C), typical P deficiency symptoms. Accordingly, the shoot dry matter yield of the [- -]-plants was about 50 % lower compared to the other treatments (Fig. 5A). However, the root dry matter yield was not significantly affected by any of the treatments (Fig. 5B).

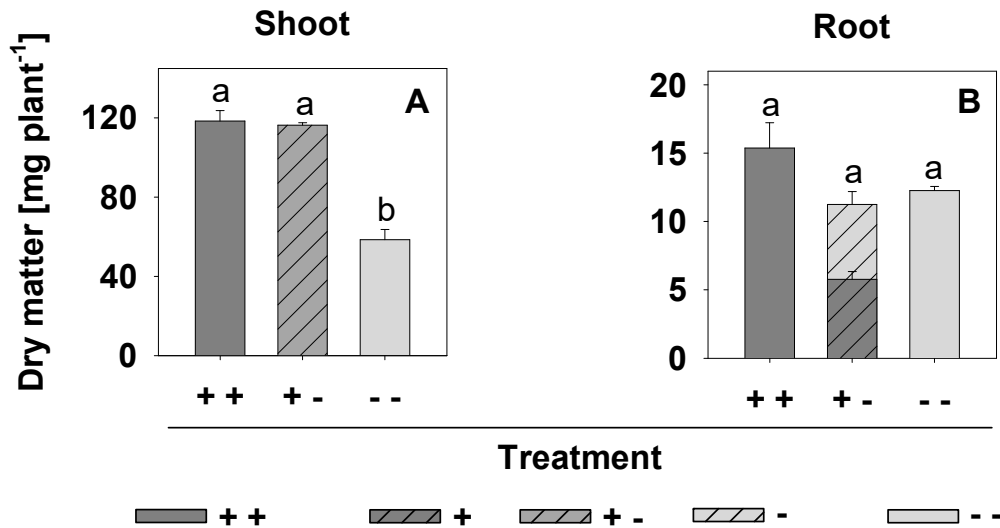


Fig. 5: Shoot (A) and root (B) dry matter of *B. carinata* cv. Bale as affected by P supply in a split-root system. Different letters denote significant differences at $P < 0.05$ (Tukey test). Columns represent means and bars indicate SE; $n=3$.

The similar shoot dry matter yield of the [+ -]-plants and [+ +]-plants indicated, that the overall P status was sufficient in the [+ -]-plants, which was in line with a high P_{total} concentration in shoot and root dry matter in both treatments (Fig. 6AB). In contrast, the P_{total} concentration in shoot and root dry matter of the [- -]-plants was reduced to about 1/3. The $P_{\text{inorganic}}$ concentration was steepest reduced in shoots and roots of the [- -]-plants (Fig. 6CD). However, also the shoots of the [+ -]-plants and the related root halves grown on the P deficient side ([-]-roots) exhibited a decrease of the P_i concentration compared to the [+ +]-plants.

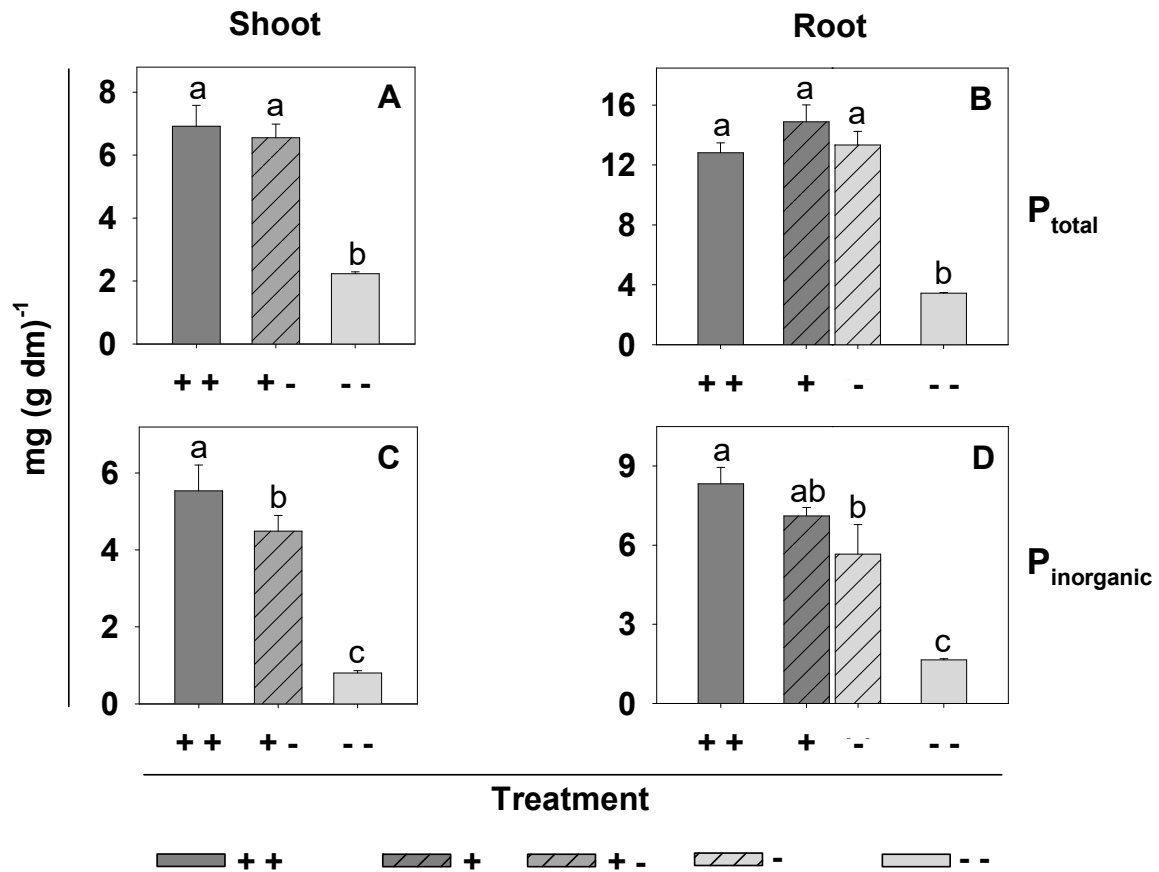


Fig. 6: P_{total} concentration in shoot (A) and root (B) as well as $P_{\text{inorganic}}$ concentration in shoot (C) and root (D) of *B. carinata* cv. Bale as affected by P supply in a split-root system. Different letters denote significant differences at $P < 0.05$ (Tukey test). Columns represent means and bars indicate SE; $n=3$.

Root hair length of the [- -]-plants was enhanced by a factor of 2.5 compared to the [+ +]-plants (Fig. 7). In the [+ -]-treatment root hair length was increased by 30 % in the - P side, but not affected in the +P side compared to the [+ +]-treatment.

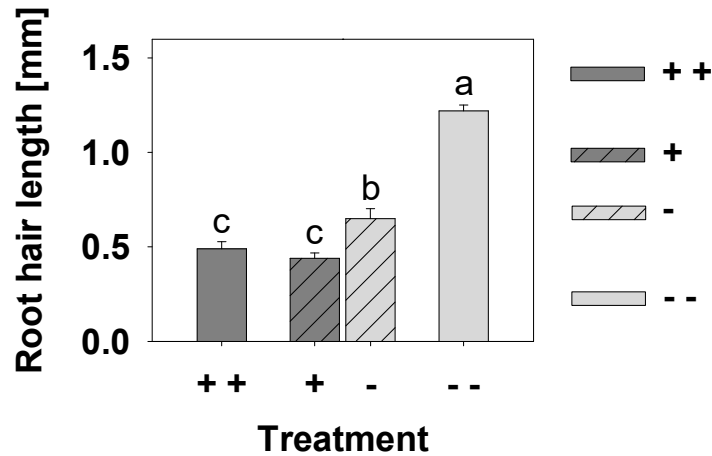


Fig. 7: Root hair length of *B. carinata* cv. Bale as affected by P supply in a split-root system. Different letters denote significant differences at $P < 0.05$ (Tukey test). Columns represent means and bars indicate SE; $n=30$.

The upregulation of the candidate genes by Pi deficiency ([- -]-treatment) was confirmed (Table S10; Fig. 8). In the [+ -]-treatment 25 of the 30 candidate genes were upregulated in the -P side. However, in most of the cases this upregulation was not as high as in the [- -]-treatment. Three candidate genes, namely *ARABINOGALACTAN PROTEIN 2* (*BcAGP2*), *BcRALFL1* and *SHAVEN3-LIKE* (*BcSHV3L*) were not affected by the P supply in the split-root system (Table S10; Fig. 8B). Conversely, a few genes, most of all *CALCIUM-BINDING EF-HAND FAMILY PROTEIN 1* (*BcCaBP1*) and *EXOCYST COMPLEX COMPONENT EXO70-LIKE* (*BcEXO70L*) were in the roots of the -P side nearly as strongly upregulated as in the [- -]-treatment (Table S10). *BcPHT1* exhibited the strongest upregulation in the [- -]-treatment, whereas in the split-root variant the upregulation was only 2-4-fold in the [+ -]- and [-]-side, respectively (Fig. 8C). The expression level of *BcFLA1*, *BcMRIL*, *BcROPGEF1* and *BcLRL3L1/2* in the [-]-side of the [+ -]-treatment was higher than in the sufficiently supplied roots but lower compared to the [- -] treatment (Fig. 8A,D-F).

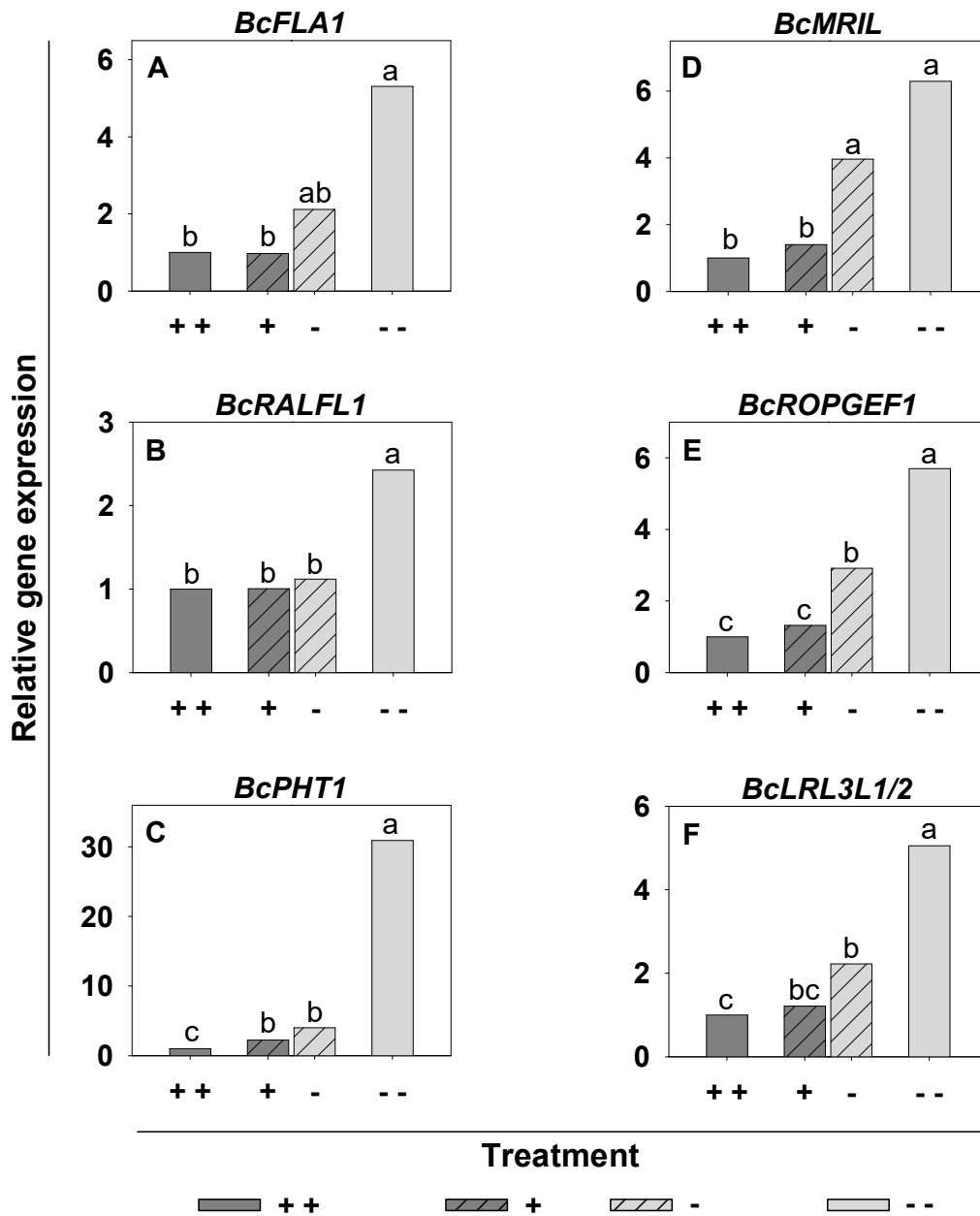


Fig. 8: Relative expression of candidate genes for increased root hair length during Pi deficiency in *B. carinata* cv. Bale as affected by P supply in a split-root system. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Different letters denote significant differences at $P < 0.05$. Columns represent means; $n=3$.

3.5. Investigation of the nutrient specificity of the candidate genes

To check, if the successfully validated candidate genes are regulated specifically during root hair growth under Pi starvation, Bale and Bacho were also grown in nutrient solutions lacking nitrogen and potassium, respectively.

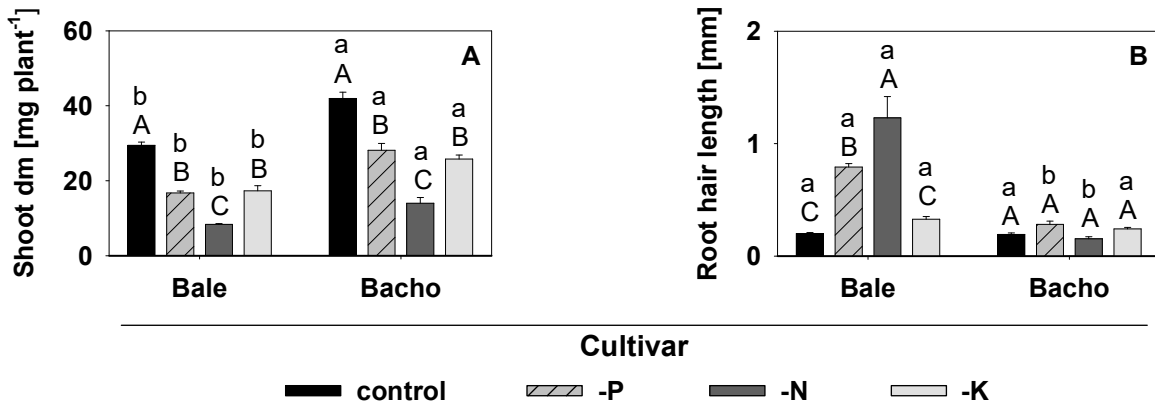


Fig. 9: Shoot dry matter (A) and root hair length (B) as affected by nutrient deficiency. Significant differences at $P < 0.05$ are denoted by small letters between cultivars for the same nutrient treatment and capital letters between nutrient treatments for the same cultivar (Tukey test); columns represent means and bars indicate SE; $n=6$ (A) and $n=12$ (B), respectively.

The lower shoot dry matter yield (Fig. 9A) and nutrient concentration in the shoot dry matter (Fig. S6) in the deficiency variants compared to the control with sufficient nutrient supply indicated deficiency for all three nutrients in both Bale and Bacho. Root hair length was increased under P and N deficiency in Bale, but not in Bacho (Fig. 9B; Fig. S7) as it was expected from previous studies (Bremer, 2010). N deficiency even led to a higher root hair length than P deficiency in Bale. K deficiency did not affect the root hair length in both cultivars.

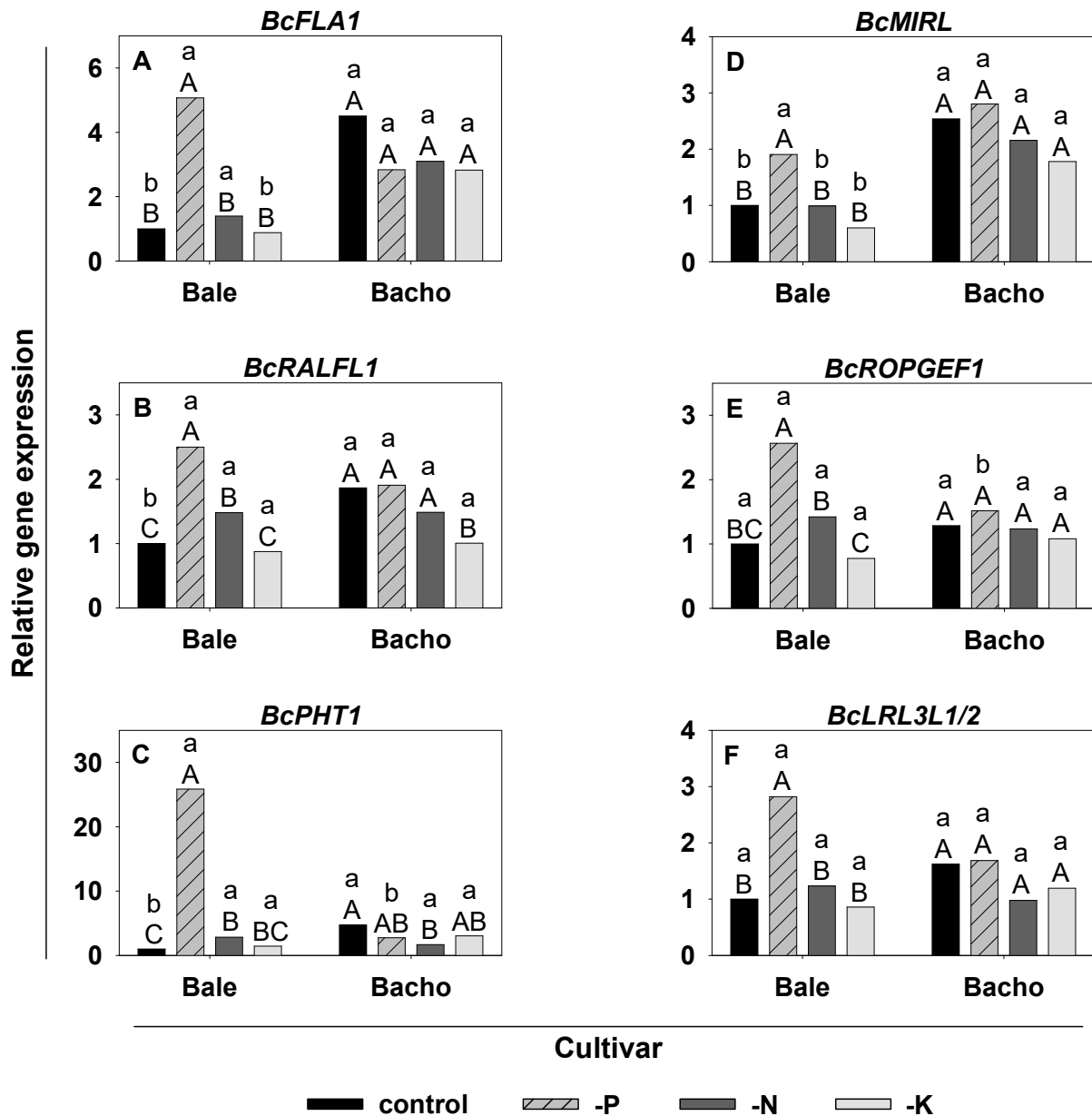


Fig. 10: Relative expression of candidate genes for increased root hair length during Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Significant differences at $P < 0.05$ are denoted by small letters between cultivars for the same nutrient treatment and capital letters between nutrient treatments for the same cultivar. Columns represent means; $n=3$.

Using *AtUBC9* as endogenous control, most of the 30 candidate genes for Pi deficiency-induced root hair growth were upregulated in Bale by both Pi and N deficiency compared to sufficient conditions (Table S11, Fig. 10). However, in most of the cases, the increase of the expression was at least in tendency higher under Pi deficiency compared to N deficiency. Interestingly, four genes (*BcFLA1*, *BcMIRL*, *BcROPGEF1* and *BcLRL3L1/2*) were specifically upregulated by Pi deficiency (Fig.

10A,D-F) and also the upregulation of *BcPHT1* under Pi deficiency was many times over that during N deficiency (Fig. 10C). In contrast, K deficiency did not affect the expression of the candidate genes. In Bacho no upregulation was observed for all genes and nutrient deficiencies compared to sufficient conditions. Using *EF-1-a1* as endogenous control resulted in similar but less strong effects of the nutrient deficiency (Table S12; Fig. S8), so that most of the genes were significantly upregulated exclusively under Pi deficiency in Bale.

3.6. Knockout of candidate genes by CRISPR/Cas9

According to the prior experiments, six candidate genes were selected for further functional investigation by knockout using CRISPR/Cas9. These included the four P-specific reacting genes *BcFLA1*, *BcMRIL*, *BcROPGEF1* and *BcLRL3L1/2* (Fig. 10A,D-F). Furthermore, *BcPHT1* was selected because of its very strong upregulation under Pi deficiency in Bale, while it was downregulated in Bacho (Fig. 4C). Additionally, *BcRALFL1* was chosen, hence it was one of the few genes, which did not respond in the -P side of the [+ -]-treatment in the split-root experiment (Fig. 8B).

Table 1: *B. carinata* cv. Bale gene sequences of candidate genes for increased root hair length during Pi deficiency obtained by 5' RLM-RACE. Guide RNA binding sites with PAM (underlined) and RACE inner primer (bold). Since, according to the corresponding Arabidopsis sequence, we did not receive about 400 bp at the 5' end of *BcPHT1* by RACE, a degenerated primer (ATGGCYGAMCARCARCTAGGAGTG) was designed considering differing bases in five related sequences (*Arabidopsis thaliana*, *Camelina sativa*, *Eutrema salsugineum*, *B. napus* and *B. rapa*) and used to amplify the missing sequence (red) together with a reverse primer binding in the already known sequence (CAACACCTTGCATGGCGAAA).

	Bale sequence (5' → 3')
BcFLA1a	AAAAAACTCAAACCTTCAAACGCAATGGCAACCATTCATCTAACTCTAGCTCCTCTCCTCATCC TTGCCGCTGTCTTCTCTCCACGGAGATAACTGCTCAGCCCGCTGCTCCAGCTCCTGGTCCTGC CGCTCCGACCAACATCACGGCGATCCTTGAAAAAGGCGGTCAATTCGCTACTTTTATCCATCTT CTAAACACAACACGAGTCGGAAACCAATCAACATCCAAGTCAACAGCTCATCAGAAGGCATGA CCGTGTTTGCACCAACAGACAACGCTTTCCAAAACCTTAAACCAGGAACCCCTAAACAACTAAG CTACGACGACCAAGTCAAACCTCATTCTTTACCATGTTAGTCCCAAGTTCTACACATTGGATGAT CTTCTCTCCGTAAGTAACCCGGTAAGGACTCAAGCTTCAGGACGAGACAGCGCGGTATATGGAC TTAATTTACCAGTCAAGGGAACCAAGTCAACGTATCAACCGGTTTCGTTGAAACACGTGTCAG TAATGCATTAAGAAAAGAACGTCTCTTGCGGTTTACGTGGTGGACATGGTATTGTTGCCGGAG GAGATGTTTGGAGAAAACAAGGTCTCACCAGCGGCACCTGGTCCAAAATCAAATTTCTCCTGATG TCTCCGATGACCAAGAGAAAGCAGGGGCTCCATCGGATAAGTCTGGCTCCGGTGAGATGAAGGC TGGATTAGGGTTGGCTCTTGGACTTGTTTTCTTATGTTTGAA ACTCGTTCGTTTGATTTGGGT

CHAPTER I

BcRALFL1	<p>AGTCCTAAGCTGTGTACATAATCAAGTTTCAGACAGAGAGAGAGAGAAACAAATTCACGAAAGA CGACTAAAGAAATCTCAAAAAGCCTCTGATTCAGCCAGAAGGTTTTGGTCAAAGAAAAAGATGA ACTCTCGCGGATCTACGCCGTCATCGCGCTCCTGGCGCTCGTAATCTCTGCCGTCACGCAAC CGGAGGCTTCGGAGACTCGCTCGATTTTCGTACGGAGCGGATCTTCGTCGCTCTTCTCTGGATGC GAAGGCTCGATCGCTGAGTGTATAGCCGAGGAAGAGGAGATGGAGTTCGATTCAGAGATCAGCC GGCGCATTCTAGCGCAGAAGAAGTACGTTAGCTACGGTGCGATGAGGAAGAACAGTGTGCCTTG CTCGCGGCGTGGAGCTTCGTATTACAACCTGCCAGCGTGGCGCTCAGGCGAATCCTTACAGCCGT GGATGCAGCACCATTACCAGGTGCAGGCGTTGAAGAG</p>
BcPHT1	<p>AACANCARCTAGGAGTGCTGAAGGCACTCGATGTTGCGAAAACGCAACTTTACCATTTACGGC GATTGTCATCGCCGGTATGGGTTTTCTTTACAGATGCTTATGATCTGTTTTGCGTCTCCTTGGTG ACCAAGCTTCTTGGCCGCCTCTACTACTTCAATCCGTTATCAGAAAAGCCTGGTTCACTTCCCC CTCATGTTGCGGCGGCGTCAACGGTGTGGCCCTTTGTGGAACCCTTGGTTCAGCTTTTCTT CGGATGTTAGGTGACAAGCTCGGAAGGAAAAAGTGTATGGTATCACTTTGATCATGATGATC GTGTGCTCCGTGGCTTCCGGTCTATCTTTGCGTAACAAAGCCAAGGGTGTATGACCACCCTTT GCTTCTTCAGGTTTTGGCTGGGATTTGGTATTGGAGGTGATTACCTTCTTTCTGCTACCATTAT GTCTGAATACGCTAACAAGAAGACTCGTGGTGTCTTTCATCGCTGCCGTTTTCGCCATGCAAGGT GTTGGTATCTTAGCCGGAGGTTTCGTGGCACTTGCCGTCTCTTCCATTTTTGACAAAAAGTTC CAGCTCCAACCTATTTAGTCGACAGGGCTCTCTCAACGCCTCCACAAGCTGACTATATTTGGAG AATCATCGTCATGTTTGGTGTCTTACCCGCAGCCTTAACTTACTACTGGCGTATGAAGATGCCT GAAACTGCTCGTTACACCGCTTTAGTTGCAAAGAACATCAAGCAGGCCACACAAGACATGTCTA AAGTCTTACAAGTGGAGCTTGAGGTGGAGGAAAGAGCAGAGGATGTCGTTCAAGACCCTAGGCT TAACTATGGATTGTTTTCCAAGGAGTTTGCCAAACGTCATGGTCTTCCCCTCCTCGGATGTACC TCCACTTGGTTCTTGCTTGACATTGCCTTTTACAGCCAAAACCTGTTCCAAAAGGATATCTTCT CGGCTATCGGATGGATCCCAAAGGCAGCAACCATGAACGGAATCCACGAGGTTTTCATGATTGC TAGGGCACAAACTCTTATCGCACTTTGCAGTACTGTCCCTGGTTACTGGTTCACGGTTGCGTTT ATTGATATCATGGGAAGGTTTTGCGATCCAACATAATGGGTTTTCTTCATGATGACCGTCTTTATGT TTGCCATTGCCTTCCCCTACGACCATTTGGATCAAACCAGACAACCGTATCGGTTTTCGTGATTAT GTACTCTTTACTTTCTTCTTCGCCAATTTTTGGACCAAACGCAACCACCTTCATCGTACCGGCT GAGATCTTCCCAGCTAGGCTAAGGTCCACATGCCATGGAATTTAGCTGCAACAGGTAAGGCTG GAGCCATCGTGGGAGCTTTTTGGGTTCTTATACGCAGCTCAGTCACAAGACAAGAACAAGACGGA TGCAGGATATCCACCGGCATCGGAGTCAAGAACTCTCTGATCATGCTTGGTGTCAATTAACCTT ATTGGTATGGCTCTTACCTTCTTGTCC</p>

CHAPTER I

BcMIRL	<p>AAAAGAAACAAAGAGACTTGCAAGAATTCATTTCTTTTTCTCTTTGTCAAACCTCCAAAACATT CCAAGATCGTTGATCTTTTTTCCAGAAACTGGACGTTGATTTTCTTCAAACCTCTCGTAAACAG ATCCTTTTGTGGTAATCTAAAGTATTCATCATCATCATTCTTGTGGTGTGATTTCGATGGCAG AATAAAGATAAAATAGAGATTGGCATAAAGAGAATCCAAAAACACACAAGAATAAGACAAAAAG ATCTGAAGATATGTTCTGTTGCGGAGGTGCGGATGAGGAACCCGCCGGTCCGCCAGCAAACCAG TATTCATCAGCGCCTCCTAACAAGGCCGAAATAACAATTTTGGCGGCGGTGGGAACAGAGGAG AGCCGAGGAATACAAACGCACCTAGATCAGGAGGTCCTGCAAAGGTTTTACCTATAGAGATCCC TTCTATTCCATTGGATGAGCTGAACAAAATATCAGGTAACCTCGGAAACAAGGCACTGATCGGT <u>GAAGGCTCTTATGGACGTGTCTTCCAAGCTAAGTATAATGGAGGCGATGTTGCTATTAAGAAGC</u> <u>TTGATGCTAGCTCTTCTGAAGAACCTGACTCCGATTTACCTCACAGTTATCGGTGGTGTACAG</u> GCTTAAAAATGAACATTTTGTGGAACCTGATGGGGTACTGCTTGAAGCAAACCTACCGCATTCTT GTCTACGAGTTCGCAACTAAAGGTTCCCTTACACGATGTGTTACATGGGAGAAAAGGGCGTGCAAG GAGCTGAGCCAGGACCGGTGCTGAACTGGAACCAAAGGGTCAAGATCGCATATGGAGCAGCCAA AGGGCTTGAGTTCCCTTCATGAGAAGGTCCAGCCACCAATAGTCCACAGGGACGTAAGGTGAGC AATGTCTTGTGTTTGTGATGACTTTGTGGCCAAAATGGCTGATTTCAACTTGACCAACGCATCTT CAGATACTGCCGCGAGGCTTCATTCTACTCGTGTATTGGGAACGTTTGGCTATCACGCTCCCGA GTATGCTATGACGGGACAGATAACGCAGAAGAGTGATGTGTATAGTTTTGGTGTGTGCTATTG GAGCTCTTAACAGGAAGAAAACCTGTAGATCATAACCATGCCTAAAGGCCAACAAAGTCTTGTTA CTTGGGCAACTCCGAGACTAAGTGAAGACAAAGTCAAACAATGCATAGATCCTAAGCTTAACAA TGAAGTCCCTCCCAAAGCAGTAGCGAAGTTGGCTGCCGTGGCGGCCTTGTGTGTTGAGTATGAG GCGGATTTCCGGCCTAACATGACCATTGTTGTCAAGGCACTTCAGCCTCTCCTTAACTCCAAAC CGGCCGGTCTGAGTCTTCTTCCCTGAATTACCAGTTAACCAATTCTCTTGGTTTTGTTTTTGT TTGTTCTGGCTCTCT</p>
BcROPGEF1	<p>AAAAACGAACATTGAAACTCGATCATTACACAGAGTCTCGATGGAGAGCTCACCGAGTTCCGAC CAAAACGAGGTATCTGCCTCTGAAACTCCGACAAGCTCCGTCTCTTACCCTACCGGAGAAGCT ACTCAGATATCTCCGGTTTATCTCACCGGTTTCGACGTTTCAGAGCTTCTATAACCGGCCGTCAA CACAAACGCCGTTGTCCATGAAGAAGATCTCTCCGAAGACGCCGTGGAACCAAAGATAACGTC GACGGCGATGGAGAAGATCATGACCGGGACAGTGACATTGACTCTGCAGAAGACGCAGAGCTGG AGATGATGAGGGAAAGATTTCGCGAAGCTGTTGCTCGGTGAAGATATGTCGGAAGTGGGAAAGG AGTTTGCAGTGTGTTACTGTCTCTAACTCCATAACTAATCTCTATGCGACTGTGTTTGGACAG AGTTTGAAGATTGCAACCATTGAGTACAGAGAAGAAGGATCTATGGAACCGTGAGATGAATTGTT TTATGTCTATATGTGATTACATTGTTGAAGTTGTTCCAAGATCTCTTGGTAATAATGTTGAGAT AACGGAAACAAAACCTAAGATCCGACATTCTCATGAACCTCCCTGCTTTGAGAAAACCTCGATAAC ATGCTCATGGATATATTGGATGGTTTTACGGAGAATGAGTTCTGGTACATGGAGAGAGGAAGCT CATCGATGAACTCTAACAATGGTGGTAGAGATTCTGGATCGTTTCGGAAAGTTGTGGTTCAAAG GAAAGACGAGAAGTGGTGGCTTCCCTGTGCCATGTGTTCCCTGCTGAAGGTTTATCAGAAAATGAA CGAAAGAATTTGCGTCACAAACGTGACTGTGCTAATCAGATACATAAGGCTGCTTTGGCCATTA ACGACTCCACTCTAAATGACATGGATATTCCCTGACTCTTACCTCACCCTCTCCCAAAGAGTGG GAAAGCAAGTGTAGGAGACGTGATATACAAGCAACTGTGCACAGCTGAGAAGTTTTATCCAGAC CAGCTTCTTGATATCTTGAAGATTTTCATCGGAGCATGAAGCGCTTGAGCTAGCTGACAAAAGTTG AGGCTTCACTGGTAAACATGGAGGCGTAAGACCGGAGGGTTGGCTCACTCTAAATCCTCATGGGA CATGATGAAAGATATGGGCGGCGACGCTGGGAATGACAAGAACCACATTCTCGCAGCTCGAGCT AGGAGCTTACTGTTCTGTCTGAAACAGAGATTCCCTGAACTCTCTCAGACCT</p>

BcLRL3L1	<p>AAAAGAAGACAAGAAGAGATGGAGAAGGGGAATGGAGAAGGAAAGGGGGAGTTCATAAACCAAA ACAGTAACGGTTTCTTCCATGACTCCATGTCAATGCTTTTCTCTCTTCCCTCCCTGGGGCTC ATCTCTTCCCCCTTCTCCTCCGCCGATTCACTCCTCCACGCTCTCACCGTCCCCGACAAGTTC CATCAACCTCAGGAGTCTGGTGGTCCAACAACGGGTAGCCAAGATGGGTTGCAGCATGCACAAG GGACAGTGTGACCACCACGAGCCCACCTGTGGCCCGCCAAAAGCCGAGGGTGCGAGCCAGGAG AGGCCAAGCCACCGATCCTCACAGCATTGCTGAGCGGTTGAGAAGGGAACGCATTGCAGAGAGG ATGAAGTCTCTCCAAGAAGTGGTTCCCAACACCAACAAGACGGACAAGGCATCAATGCTGGACG AGATCATAGAGTATGTTAGATTCCCTCAGCTTCAAGTCAAAGTACTAAGCATGAGCAGATTGGG AGCTGCAGGGGGAATTCGTCCACGCCTCAATGGTCTCACCTCTGATGTAGGAGGACGGCTCAAC GCACTCTCAGTACCGTCCAATGGCATAAGTAAACGGGAATGGAAACGCACCAGGATCATCCAACG AAAGCCTAAGGACAACGGAACAGAGGGTGGCGAAACTGATGGAAGAAGACATGGGATCAGCAAT GCAGTATCTTCAAGGGAAGGGGCTTTGTCTAATGCCATCTCTCTCGCAACTGCTATGTCATCT TCATCTGCTCACTCTCGTGGAGCTCTCTTCAACCCGACCTCAAATGTCGTAGCAACAGAGGAGG CAAATGTAGTAGCAGCAGCTGCACCTGAAGCCTCCTCTCCTATGGATGATGTCTCCGCCTCCAA CAAGGCCTAAACCATA</p>
BcLRL3L2	<p>AAAATAGAAGACAGAAGACAGAAGAGATGGGAAATGAGAATGGAGAAAGAAAGGGAGAGTTCAT AAACCAAAACAACAATGACTTCTTCCCTTGATTCAATGTCAATGCTCTCCTCTCTCCCTCCTTGC TGGGACTCTTCTTCCCCCTCCTCATCCTCCTCCTCCGCACGCTCTGACCGTCCGACGCTCTT TCCCCGACCAGTTCATCAACCTCAGGAGTCAGGTGGTCCAACAATAGGCAGCCAAGATGGGTT GCAGGGACAAGGGACAGTCTCGACCACGAGTGCACCTGTGGCTCGTCAAAGCCAAGGGTGCGA GCTAGGAGAGGTCAAGCAACCGATCCTCACAGCATCGCTGAGCGGTTGAGAAGAGAACGCATTG CAGAGCGTATGAAGTCTCTTCAAGAACTGGTTCCCAACACCAACAAGACGGACAAGGCATCGAT GCTGGACGAGATCATCGAGTATGTTAGATTCCCTCAGCTGCAAGTCAAGGTACTAAGCATGAGC AGATTGGGAGGTGCAGGTGCAGTTGGTCCACGCCTCAACGGTCTCACTTCCGAGGTAGGAGCAC GGCTCAACGCCCTCTCTGGACCGTGCATGGCTTAAACGGGAAAGGAAACGCAACAGGATCTTC CAACGAGAGCTTAAAGTCAACAGAAAATAGGGTGGCAAAGCTGATGGAAGAAGACATGGGATCT GCAATGCAGTACCTTCAAGGGAAAGGGCTTTGCTTAATGCCATCTCTCTCGCAACTGCAATGT CATCTACATCTAATCACTCTCGTGGAGCTCTCTTTAACCCCAAATCCAATGTGTTAGCAGCAGA GGAGAAAATGTAGCAGCAGTAGCTGCACCTGAAGACTCCTCTCCTATGGATGATGT</p>

5' RLM-RACE successfully extended the cDNA sequences to their 5' ends (Table 1). However, around 400 bp of the 5' end of *BcPHT1* were missing, which could be amplified using a degenerated primer designed on relative sequences in other plants. The successfully extended cDNA sequences of the rest of candidate genes are shown in Table S13 and the gDNA sequences of the for the knockout selected candidate genes are to be found in Table S14.

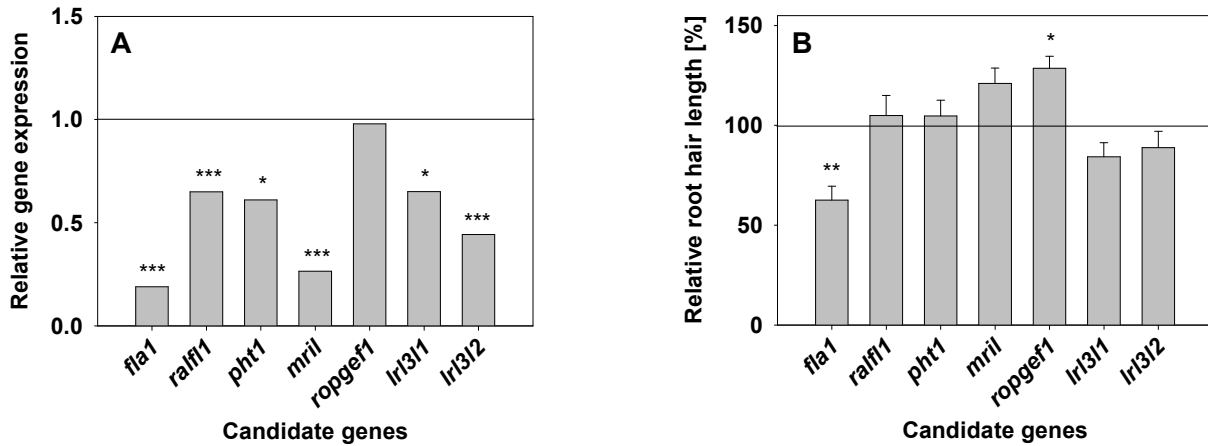


Fig. 11: Relative candidate gene expression (A) and relative root hair length of the transgenic hairy roots (B) after targeting the respective candidate genes for increased root hair length under Pi deficiency by CRISPR/Cas9. Horizontal lines represent the average WT expression and root hair length, respectively. Stars denote significant differences, determined according to Steibel *et al.* (2009) (A) and Tukey test (B) (significance codes: 0 ‘****’ 0.001 ‘***’ 0.01 ‘**’ 0.05). Columns represent means for n=4-8 (A) and 5-14 (B), respectively. Bars in B indicate SE.

The gene expression of all candidate genes except for *BcROPGEF1* was reduced in the transgenic CRISPR roots compared to the control (Fig. 11A). The steepest reduction was observed for *BcFLA1* (20 %), followed by *BcMRIL* (25 %) and *BcLRL3L2* (45 %). *BcRALFL1*, *BcPHT1* and *BcLRL3L1* exhibited a reduction to about 60 %. For *BcFLA1* (Fig. S9A), *BcRALFL1* (Fig. S9B), and both *BcLRL3L* isoforms (Fig. S9F,G) the reduction was relatively homogenous in the different transgenic roots, whereas the expression of the other genes exhibited strong fluctuations (Fig. S9C-E).

The root hair length was reduced only in the *bcfla1* roots (to about 60 % compared to the control) (Fig. 11B; Fig. S10). In contrast, the *bcropgef1* roots exhibited about 30 % longer root hairs.

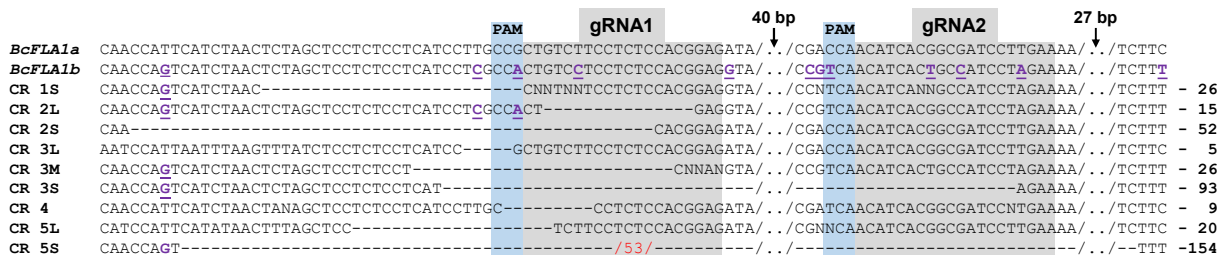


Fig. 12: CRISPR/Cas9-induced deletions and insertions in *BcFLA1*. gRNA regions (grey), PAM sequence (blue), number of inserted bp (red), number of deleted bp (on the right). Nucleotides used for differentiation of *BcFLA1b* from *BcFLA1a* (violet, underlined). CR = CRISPR, /./ = break.

CHAPTER I

Since the strong downregulation of *BcFLA1* was accompanied by a reduction in the root hair length, we took a closer look at the gene editing events caused by CRISPR/Cas9. Sanger sequencing detected deletions in *BcFLA1* in all replications reaching from 5 to 154 bp (Fig. 12). Both alleles of *BcFLA1* were affected. Up to three different deletions were observed per replication (transgenic root). However, only two deletions (CR 3S and CR 5S) spanned both gRNA regions. All other deletions exclusively occurred in the gRNA1 region. Furthermore, a 53 bp insertion occurred in CR 5S, the replication with the largest deletion of 154 bp. Often there was an overlapping of at least two sequences in the sequencing chromatograms starting at gRNA1, which indicates that several amplicons with different gene editing events were purified at once.

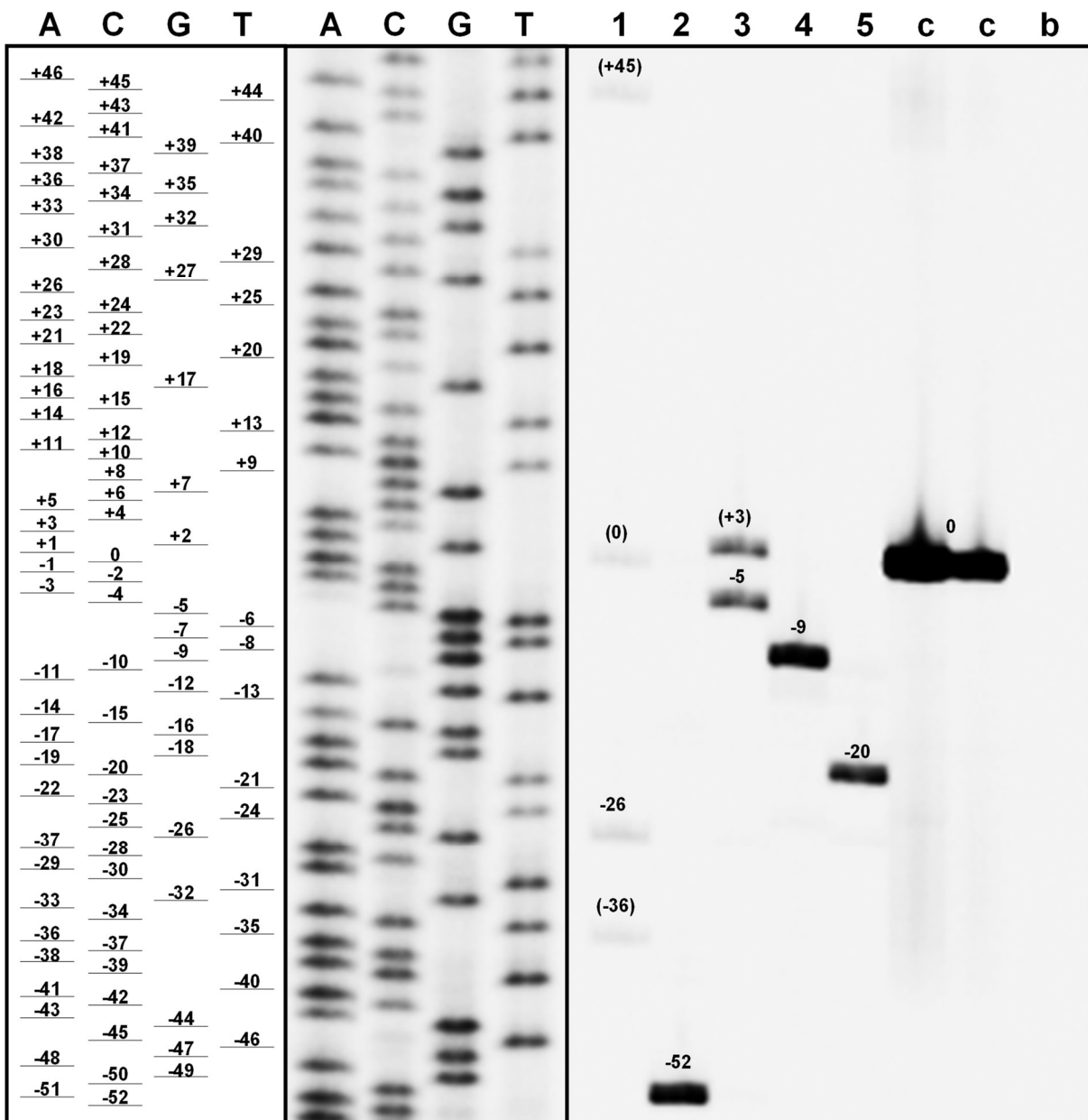


Fig. 13: Validation of the CRISPR/Cas9-induced gene editing of *BcFLA1* by fragment analysis by PAGE. A, C, G and T = Sanger sequencing reactions with the corresponding dideoxynucleotides; c = control; b = blank (no template control). Numbers indicate the deleted (-) and inserted (+) bp compared to the WT size (0). Brackets indicate indels with approximated size, which did not occur during sequencing.

The fragment analysis by PAGE uncovered indels reaching from -52 to +45 bp (Fig. 13). Three replications (2, 4 and 5) exhibited only one gene editing event, whereas in replication 3 one insertion and one deletion occurred. Replication 1 even exhibited two deletions and one insertion, whereby there was some of the wildtype sequence left. Within the replications, the intensity of the amplicon bands and therefore the frequency of the gene editing events was similar.

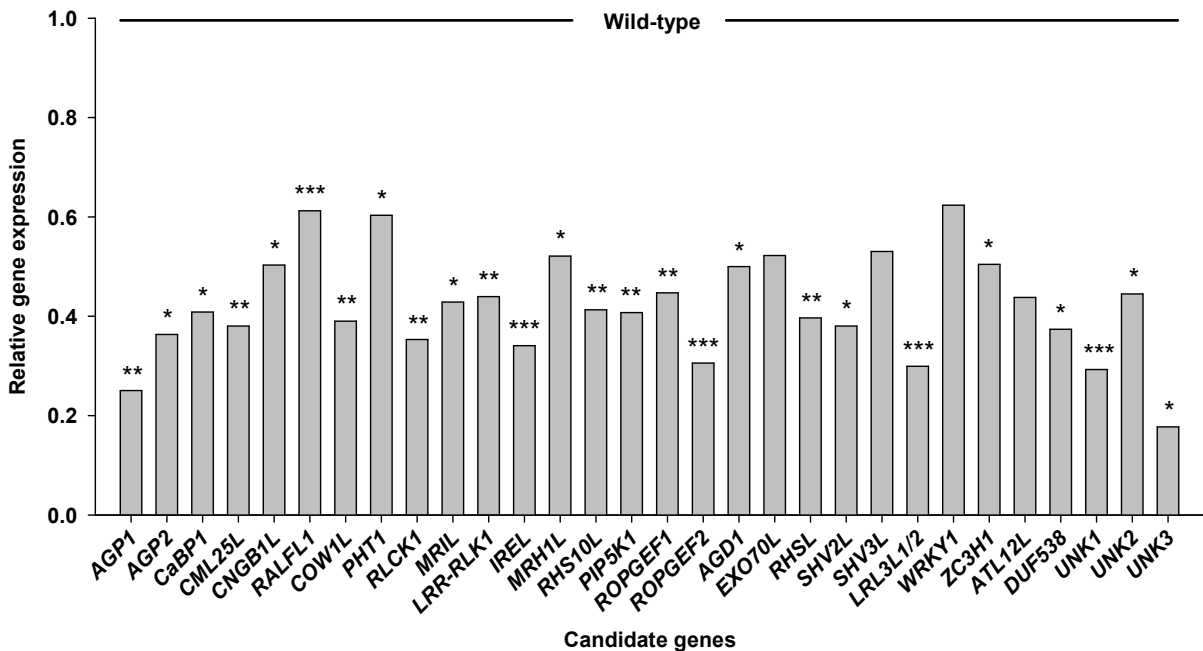


Fig. 14: Relative expression of candidate genes for Pi deficiency-induced root hair growth in *bcfla1* roots grown under Pi-deficient conditions. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009) (significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05). Columns represent means; n=3.

To investigate, where *BcFLA1* may be working in the signal transduction cascade leading to the Pi deficiency-induced root hair elongation, the expression of the other candidate genes was analyzed in the *bcfla1* roots. Most of them exhibited a clear decrease in their expression (Fig. 14), whereby the deepest reduction occurred for *UNKNOWN 3 (BcUNK3)* (to a level below 20 %).

4. Discussion

4.1. Identification of regulatory candidate genes for Pi deficiency-induced root hair elongation

The aim of the present study was to identify candidate genes for the Pi deficiency-induced root hair elongation in *B. carinata*. For it, we used two different cultivars of *B. carinata* differing in their root hair length under Pi starvation. Cv. Bale develops long root hairs in response to Pi deficiency, whereas cv. Bacho develops short root hairs under both sufficient and deficient Pi conditions (Eticha and Schenk, 2001). Genome-wide expression profiles of Bale and Bacho under low (0 mM) and high (1 mM) Pi conditions were received by MACE. For it, RNA was extracted from two-cm root tips, because this length ensures to receive root hairs with different developmental stages from the initiation to the termination of the root hair growth (Fig. S1), so that also the genes participating in the root hair elongation should be expressed in the harvested plant material. The reduced shoot dry matter and shoot P concentration in both Bale and Bacho as well as the upregulation of the marker gene expression (Fig. 2A-C) confirmed P starvation in the respective treatment of the MACE experiment. Additionally, longer root hairs were induced exclusively in Bale and not in Bacho under Pi deficiency (Fig. 2D) as it was expected from preliminary studies (Eticha and Schenk, 2001; Bremer, 2010), so that the plant material was defined as suitable for the subsequent MACE.

Since no database was available for *B. carinata*, the tags produced in the MACE were annotated to *B. rapa* and *B. napus*, which are, as *B. carinata*, a member of the Triangle of U (Li *et al.*, 2017). The latter predicts a higher similarity between *B. carinata* and *B. napus*. However, more tags could be annotated to *B. rapa* than *B. napus* (Fig. S2). This may be due to the different annotation methods and the degree of completeness of the databases. Nevertheless, after validation of their reliability (Fig. S3), we used both annotations to have a greater basis for the candidate gene selection.

According to the root hair response, we selected genes exhibiting a clear down- or upregulation under Pi deficiency in Bale and no regulation in Bacho. Furthermore, only genes with a potential regulatory function were chosen, because we were interested in genes regulating the root hair elongation according to the Pi availability rather than genes involved in the general root hair elongation process. According to their function

or structure, the 33 candidate genes were assigned to nine groups. Finally, six candidate genes were selected for functional analysis by knockout: (i) Since we were most interested in regulatory genes transmitting the signal of low P to the general root hair elongation pathway, we chose the four P-specific reacting candidate genes *BcFLA1*, *BcMRIL*, *BcROPGEF1* and *BcLRL3L1/2* (Fig. 10A,D-F). (ii) *BcPHT1* was selected, because of its very strong upregulation under Pi deficiency in Bale and the contrary regulation in Bacho (Fig. 4C), which may indicate a role in P sensing in analogy to nitrate sensing by nitrate transporter NRT1.1 (Krouk *et al.*, 2010). (iii) Furthermore, *BcRALFL1* was selected, because, in contrast to the other selected genes, it seemed to play a role rather in the systemic and not in the local regulation of responses to Pi deficiency (Fig. 8B). In the following paragraphs, these genes will be described in more detail.

Arabinogalactan proteins are extracellular glycoproteins consisting of a core protein, one or more arabinogalactan (AG) side chains and most often a glycosylphosphatidylinositol (GPI)-anchor. Among others, they may be involved in cell wall synthesis and remodeling. Their regulatory function could be based on their ability to act as modulators or coreceptors and to serve as a source of small signaling molecules for example by the cleavage of the GPI-anchor, thus producing lipid signals. Because of their amphiphilicity, they could mediate between the cytoplasm, the plasma membrane and the cell wall (Seifert and Roberts, 2007). Marzec *et al.* (2015) already showed the involvement of AGPs in root hair development in barley. However, the authors concluded, that they are involved rather in an earlier stage of root hair development than in the elongation stage. One of the three AGPs, *BcFLA1*, was annotated to *BrFLA27 (AtFLA13)* and belongs to a group of chimeric AGPs since it has a fasciclin (FAS) domain between two regions with AGP motifs (Table S15). Furthermore, it possesses a N-terminal signal peptide with a cleavage site between position 25 and 26 as well as a C-terminal GPI-anchor. FLAs are thought to play important roles during plant development and in abiotic stress responses and may be involved in interactions within the extracellular matrix (Johnson *et al.*, 2003). As they physically connect the cell with neighboring cells or the extracellular matrix, they can have a key function in transmitting environmental changes to the cell (Faik *et al.*, 2006). Via their FAS domains they may mediate protein-protein interactions by facilitating the adhesion of plasma membrane or cell wall-associated ligands (Tan *et al.*, 2012). Interestingly, for several FLAs a role in elongation processes was documented, for

example the involvement of GhFLA1 in the initiation and elongation of cotton fibers (Huang *et al.*, 2013a) and AtFLA4, which maintains cell expansion during salt stress (Tan *et al.*, 2012).

Calcium also has an important role in signaling and additionally a central role in root hair elongation forming a calcium gradient (Wymer *et al.*, 1997; Bibikova *et al.*, 1997; Bibikova *et al.*, 1999). *BcRALFL1* belongs to a family of small signaling peptides named Rapid Alkalinization Factor (RALF) (Murphy and De Smet, 2014). The family diverged into four major groups (Campbell and Turner, 2017) with *BcRALFL1* belonging to group I. RALF peptides can affect the pH of the extracellular matrix thru different pathways and therefore have influence on the cell elongation. Besides their ability to induce MITOGEN-ACTIVATED PROTEIN (MAP) KINASE activity, they can bind to the FER receptor, which leads to a subsequent inhibition of a H⁺-ATPase located in the plasma membrane, so that the apoplastic pH is increased and the cell elongation is inhibited (Murphy and De Smet, 2014; Haruta *et al.*, 2014). Because *AtRALFL22*, to which *BcRALFL1* was annotated, is known to be able to increase the cytosolic Ca²⁺ concentration, the effect on the pH could also be a secondary response via Ca²⁺ signaling (Murphy and De Smet, 2014; Morato do Canto *et al.*, 2014). Furthermore, members of the RALF family seem to be able to regulate the root hair pH and therefore elongation. Silencing of *NaRALF* in *Nicotiana attenuata* increased the apoplastic pH at the root hair tip, which led to shorter or even disrupted root hairs most probably because the cell expansion could not be controlled as it is necessary during the transition from the initiation to the elongation (Wu *et al.*, 2007).

Furthermore, two genes involved in **Pi transport** were selected, since Pi itself could serve as signaling molecule (Chiou and Lin, 2011). The regulation in the MACE could be validated only for *BcPHT1* encoding an inorganic phosphate transporter, whose relative in Arabidopsis (*AtPHT1;3*) is expressed in the epidermis and the pericycle of the main root as well as in trichoblasts of lateral roots (Mudge *et al.*, 2002; Młodzińska and Zboińska, 2016). *AtPHT1;3* is known to be induced by low Pi concentrations (Mudge *et al.*, 2002), and contributes to Pi uptake under these conditions, whereas under sufficient Pi conditions its contribution to Pi uptake is negligible (Ayadi *et al.*, 2015). Unexpectedly, in this work *BcPHT1* was upregulated under Pi deficiency only in Bale while it was downregulated in Bacho. This indicates, that *BcPHT1* may have another or an additional role apart from the Pi uptake in Bale. This is supported by the

existence of isoforms of *BcPHT1* in the MACE dataset, which exhibited an upregulation in both cultivars and therefore may fulfil the usual role in Pi uptake. Furthermore, it was shown, that even if *AtPHT1;3* contributed to the Pi uptake under Pi starvation, most of the Pi was taken up by another Pi transporter (*PHT1;4*), indicating that Pi uptake is not the main function of *PHT1;3*, or that there is at least an additional function. Thus, Młodzińska and Zboińska (2016) suggested an involvement of *AtPHT1;3* in the regulation of the Pi translocation into the xylem. Since Pi may function as signaling molecule in sensing Pi starvation (Chiou and Lin, 2011), the allocation of Pi may have a great impact, particularly as it is suggested, that systemic Pi sensing is caused by events downstream of *PHT1* (Ayadi *et al.*, 2015). In relation to this, overexpression of a Pi transporter in Arabidopsis (*AtPHT1;5*) and rice (*OsPHT1;1*) increased root hair number and length independent of the P supply and specifically under sufficient Pi conditions, respectively, which was thought to be a consequence of an altered Pi distribution or a disorder of signaling pathways (Młodzińska and Zboińska, 2016).

Protein kinases are another important group of regulators. They can regulate protein activity by transferring phosphate groups to proteins. Many protein kinases were specifically upregulated in Bale under Pi deficiency. For a selection, we used the paper of Lan *et al.* (2013) where the authors generated co-expression networks to identify protein kinases potentially involved in the Pi deficiency-induced root hair development. For the relative of *BcMRIL* in Arabidopsis a central role in this process was predicted, because it exhibited many connections to other protein kinases in the co-expression network (high edge count) (Lan *et al.*, 2013). Recently, *AtMRIL* was shown to positively regulate the root hair elongation by acting downstream of the FER receptor and controlling the cell wall integrity in tip growing cells (Boisson-Dernier *et al.*, 2015).

Also **ROH GTPases** control a variety of signaling pathways. They not only act as regulators of the actin cytoskeleton, but can amongst others also affect cell polarity, gene expression and vesicle transport (Etienne-Manneville and Hall, 2002; Scheffzek and Ahmadian, 2005). *BcROPGEF1* encodes for an activator of ROPs and for its relative in Arabidopsis, *AtROPGEF4*, it was shown that the activation was performed via the interaction with the FER receptor (Duan *et al.*, 2010). Furthermore, *AtROPGEF4* seems to negatively regulate root hair elongation (Won *et al.*, 2009; Lin *et al.*, 2011; Huang *et al.*, 2013b). However, while Lin *et al.* (2011) observed longer root hairs in a knockout mutant of *AtROPGEF4* only under deficient Pi conditions,

which indicates a specific role in the Pi deficiency-induced root hair elongation, Huang *et al.* (2013b) concluded that *AtROPGEF4* is exclusively important for the developmental and not the environmental root hair growth regulation.

Two isoforms annotated to *AtLRL3*, encoding a bHLH **transcription factor** were also investigated by knockout. *AtLRL3* is a functional paralog of *RSL4* and is necessary for a proper root hair elongation (Bruex *et al.*, 2012; Salazar-Henao *et al.*, 2016). Furthermore, *AtLRL3* was upregulated in response to Pi deficiency (Salazar-Henao and Schmidt, 2016).

Overall there was a good agreement between the regulation of the candidate genes observed in the MACE and in the qPCR (Table S7, Table S8). In the 2nd experiment even stronger upregulations were observed, so that it correlated actually better to the MACE than the biological replications from the MACE (1st) experiment itself (Fig. 4, Table S9). Furthermore, the regulation of the candidate genes was confirmed in the split-root and nutrient specificity experiments (Table S10, Table S11).

Additionally, to the candidate genes which were selected after filtering the MACE data, *BcPIP5K1* was investigated, because its counterpart in *A. thaliana* (*AtPIP5K3*) was predicted to have a key role in increasing the root hair elongation response to Pi starvation at least at young seedling stages. However, in this study *BcPIP5K1* was upregulated by Pi deficiency both in Bale and Bacho, indicating that it has another or an additional role in *B. carinata* or at least in Bacho.

4.2. Investigation of the candidate gene characteristics regarding their role in signaling and their nutrient specificity

Genes, whose regulation according to MACE was confirmed in at least one of the two independent experiments, were further investigated regarding to their role in signaling and nutrient specificity.

To investigate, if the effect of Pi deficiency on root hair growth in *B. carinata* cv. Bale is mediated locally or systemically, Bale seedlings were grown in a split-root system containing sufficient (+ +) or deficient (- -) Pi on both sides or sufficient Pi only on one and deficient Pi on the other side (+ -). Reduced shoot dry matter yield and low shoot P_{total} conc. indicated strong Pi deficiency in the [- -]-treatment (Fig. 5A, Fig. 6A), accompanied by clear Pi deficiency symptoms on these plants (Fig. S5). However, in the [+ -]-treatment shoot dry matter yield, P_{total} concentration and phenotype were

similar as in the [+ +]-treatment, indicating that the Pi uptake on the +P side was enough for an overall sufficient P status of the plants. The root dry matter yield was not affected by any of the treatments (Fig. 5B), although the P_{total} concentration in the roots of the [- -]-treatment was reduced (Fig. 6B), so that the root/shoot ratio of the [- -]-plants was enhanced as it is usual under Pi deficiency (Scheible and Rojas-Triana, 2015). The similar P_{total} concentration in both root halves of the [+ -]-treatment indicated that P was translocated from the roots on the +P side to the roots on the -P side. Besides the P_{total} concentration we measured the $P_{\text{inorganic}}$ concentrations in the shoots and roots, because Pi itself could serve as signaling molecule in transmitting the low Pi signal (Chiou and Lin, 2011). In contrast to P_{total} , $P_{\text{inorganic}}$ also decreased in the shoots and in the -P side roots of the [+ -]-treatment compared to the [+ +]-treatment (Fig. 6C,D). $P_{\text{inorganic}}$ represents the storage form contained in the vacuoles (Bielecki, 1973). In this storage pool, a lower P supply in the [+ -]-treatment compared to the [+ +]-treatment was reflected. However, the root halves of the [+ -]-treatment were not different, suggesting that the slightly enhanced root hair length (Fig. 7) and increased gene expression in the -P side roots of this treatment were not caused by $P_{\text{inorganic}}$ but local Pi deficiency in the medium. This fits with the results of Thibaud *et al.* (2010), who concluded, that local P deficiency responses are mediated by the external Pi.

However, systemic signaling also seems to have an influence, because the root hair length of the roots from the -P side was by far not as strongly increased as in the [- -]-treatment (Fig. 7). The assumption, that both local and systemic signaling plays a role in the Pi deficiency-induced root hair elongation was supported by the expression level of most of the candidate genes in the root tips from the -P side of the split-root variant, which was between the level in the root tips grown in the sufficient P solutions and that of the [- -]-treatment (Table S10). However, three candidate genes, among them *BcRALFL1* (Fig. 8B), did not exhibit any local P response in the split-root variant, so that they may be involved exclusively in systemic signaling pathways. The other way round, two genes (*BcCaBP1* and *BcEXO70L*) exhibited a strong local P deficiency response, which was nearly as high as in the [- -]-treatment, so that they may particularly play a role in local signaling pathways (Table S10). Therefore, both local and systemic signaling may play a role in the Pi deficiency-induced root hair elongation in *B. carinata*. Regarding to the local signaling, interaction of a P_5 -type ATPase, encoded by *PHOSPHATE DEFICIENCY RESPONSE 2 (PDR2)* and a multicopper oxidase, encoded by *LOW PHOSPHATE ROOT 1 (LPR1)* was necessary for a proper

response to low external Pi concentration in Arabidopsis (Ticconi *et al.*, 2009). The systemic signaling is predicted to be mediated by micro RNA miR399, but besides Pi also hormones, proteins, peptides and sugars may be involved (Plaxton and Tran, 2011). Thibaud *et al.* (2010) observed that locally regulated genes were mainly involved in root responses, whereas systemic signaling played rather a role in regulating the Pi homeostasis. However, we cannot exclude, that some of the candidate genes have functions apart from the root hair development, which would explain the partially systemic regulation. Furthermore, there may be a crosstalk of local and systemic signaling pathways during root hair elongation under Pi deficiency. Stetter *et al.* (2015) also identified candidate genes for an increased number or length of root hairs under local Pi deficiency in Arabidopsis, but they did not match one of the candidate genes of our study.

To uncover candidate genes, which are specifically reacting in response to Pi deficiency, Bale and Bacho were cultivated additionally under N- and K deficiency. After validation of the nutrient deficiencies by a reduced shoot dm (Fig. 9A) and nutrient concentration (Fig. S6), the expression of the candidate genes was determined (Table S11). Interestingly, none of the candidate genes reacted in response to K deficiency, which was the only deficient nutrient, that did not lead to an enhanced root hair length (Fig. 9B). This strongly indicates that the candidate genes indeed are directly or indirectly involved in the root hair elongation and not in general nutrient deficiency pathways. However, most of the candidate genes were upregulated by both P and N deficiency in Bale (Table S11), which indicates, that these genes are rather involved in the general root hair elongation pathway. In contrast, the four specifically under Pi deficiency upregulated candidate genes *BcFLA1*, *BcMRIL*, *BcROPGEF1* and *BcLRL3L1/2* (Fig. 10AD-F) may play a role in the regulation of the root hair elongation pathway dependent on the Pi status. From these genes, the relative of *BcROPGEF1* in Arabidopsis was already associated with the Pi deficiency-induced root hair elongation (Lin *et al.*, 2011), although Huang *et al.* (2013b) attributed rather a developmental and therefore general role than an environmental role for *AtROPGEF4*. A connection of *BcMRIL* to Pi deficiency signaling could be the FER receptor which interacts with *AtROPGEF4*, because *AtMRI* was shown to be active downstream of FER (Duan *et al.*, 2010; Boisson-Dernier *et al.*, 2015). Also for the relative of *BcLRL3L1/2* in Arabidopsis a role in the Pi deficiency-induced root hair elongation is likely, because (i) it is preferentially expressed in root hairs and upregulated in

response to Pi deficiency (Salazar-Henao and Schmidt, 2016), (ii) it is necessary for a proper root hair elongation (Bruex *et al.*, 2012) and (iii) it is a functional paralog of *RSL4* (Salazar-Henao *et al.*, 2016), whose role in root hair elongation under Pi deficiency was already verified (Datta *et al.*, 2015). FLAs have not been associated with Pi deficiency so far. However, it has been shown in several studies, that they have the ability to counteract abiotic stress by altering gene expression (Zang *et al.*, 2015).

4.3. Functional characterization of the candidate genes by knockout via CRISPR/Cas9

To further investigate the function of the most promising candidate genes, they were targeted by CRISPR/Cas9 to cause a knockout of the gene function, whose effects on the root hair length under Pi deficiency were investigated. For it, the genes were targeted by two gRNAs to cut out a relatively large fragment between the two DSBs thus enhancing the probability of a gene knockout.

BcFLA1 was the only gene, whose mean downregulation led to an on average reduced root hair length (Fig. 11). Even though the other candidate genes, except for *BcROPGEF1*, were also successfully downregulated, the root hair length was not affected. Since the single transgenic roots represent different transformation events, large differences in the downregulation and therefore the expected root hair length are possible. Indeed, the *BcFLA1* expression level and the root hair length in *bcfla1* was relatively homogenous, but the transgenic CRISPR roots of the other candidate genes exhibited partially strong fluctuations in the expression level of the corresponding genes and the root hair length (Fig. S9, Fig. S10). However, also the root hair length of the single transgenic roots could not be correlated to the corresponding expression levels of these genes in most of the cases. Therefore, these genes may not be involved in the Pi deficiency-induced root hair elongation in *B. carinata*. Another possibility is, that there was still a low activity of the gene function, which was enough for a normal root hair response. Furthermore, the gene function of the candidate genes could be substituted by an isoform (Bouché and Bouchez, 2001), which was not affected by the CRISPR mechanism, because of divergent sequences in the gRNA binding regions. Indeed, for the RALF family overlapping functions were assumed, since other small peptides with similar motifs were already shown to be functional redundant (Campbell and Turner, 2017). Also, Pi transporters of the PHT1 family in Arabidopsis are known to have overlapping functions, so that the absent activity of one single transporter can

be compensated (Ayadi *et al.*, 2015). Additionally, the two BcLRL3L isoforms may act redundantly, so that the gene function of one isoform could be substituted by the other one. Nevertheless, because of the reduced root hair length in *bcfla1*, there is a strong evidence for a role of *BcFLA1* in the Pi deficiency-induced root hair elongation in *B. carinata*.

Since the amount of a functional gene may be overestimated by qPCR, because of an ineffective nonsense-mediated mRNA decay (NMD) in case of an absent frameshift mutation, we further investigated the CRISPR/Cas9-induced gene editing events in *BcFLA1* by Sanger sequencing and fragment analysis by PAGE. As observed in Chapter II page 60, both alleles of *BcFLA1* were affected by CRISPR/Cas9 (Fig. 12), which was important to avoid the substitution of the gene function of one allele by the other one. Again, the gene editing events were mainly caused by gRNA1, which was explained by unfavored bases at position 20 and the variable base of the PAM sequence of gRNA2. The two larger, both gRNA regions spanning deletions were probably also caused only by gRNA1, because sequencing indicated, that these deletions occurred in *BcFLA1b*, to which the second gRNA could not bind due to an absent PAM sequence. This explanation is supported by the fact, that relatively large deletions were caused by gRNA1 also in most of the other samples (Fig. 12) as it was observed and discussed in Chapter II page 64-65. Therefore, we were not able to cut out the large fragment between the two gRNAs in most of the cases, but this circumstance was compensated by the large deletions caused by only one gRNA.

Since not all gene editing events can be separated by agarose gel electrophoresis (see Chapter II page 60 and 65), a PAGE was performed to separate the amplicons of the potentially edited gene region with a higher resolution and to estimate their frequency. However, not all the indels identified by Sanger sequencing were detected by the fragment analysis by PAGE (Fig. 13). As mentioned in Chapter II page 65-66, the DNA amount loaded on the gel has a great impact on the accuracy of the fragment analysis by PAGE. If a PCR with a higher template amount and more cycles was performed for the fragment analysis by PAGE, some more indels, including all the indels uncovered by Sanger sequencing, were visible (data not shown). However, this led to an overloading of the gel, therefore impeding the determination of the indel sizes, the separation of all amplicons and the estimation of the indel quantity, because the saturation phase was reached during the PCR. Therefore, we lowered the template

size and cycle numbers, so that we lost the indels occurring with a negligible amount, but were able to separate all amplicons and determine the indel sizes more precisely. Furthermore, the intensity of the amplicon bands allowed the estimation of the quantity of every indel. Even if the bands of the first replication were weak due to a low DNA amount, four bands of similar intensity were observed from which one may represent the wildtype sequence. In contrast, in the other replications the wildtype sequence was completely absent. Some of the indels may not have induced a frameshift mutation, but due to the large size of most of the indels it can be assumed, that the gene function was knocked out. This fits with the strong and relatively homogenous downregulation of the *BcFLA1* gene expression measured by qPCR (Fig. 11A, Fig. S9A), since a possible self-stimulation of the *BcFLA1* expression may be impeded due to a defective BcFLA1 protein. Additionally, as the small indels around gRNA1 affected the N-terminal signal peptide of BcFLA1 (Table S15), this may have caused a mislocalization of BcFLA1, so that it could not perform its normal function in the cell wall and/or the extracellular matrix.

The decreased transcript level of *BcFLA1* was accompanied by a reduced root hair length of the *bcfla1* roots (Fig. 11B, Fig. S10A). However, even if the specificity of the CRISPR/Cas9 mechanism is relatively high in plants (Zhang *et al.*, 2014; Feng *et al.*, 2014; Jacobs *et al.*, 2015), off-target effects cannot be excluded, especially if non-model plants without the full genome information are used as in our study. Therefore, we developed a complementation variant with an additional overexpressed version of *BcFLA1*, which was resistant to the CRISPR/Cas9 mechanism in combination with the two gRNAs used in this study (for details see Chapter II page 54). Indeed, the complementation of the *BcFLA1* gene function led to a significant increase of the root hair length compared to the CRISPR variant (Chapter II Fig 5), so that it was confirmed that the shorter root hairs of the transgenic CRISPR roots arose from the downregulation of *BcFLA1*.

In the last step, we wanted to test, if the downregulation of *BcFLA1* had an influence on the expression of the other candidate genes. Unexpectedly, nearly all candidate genes were strongly downregulated in the *bcfla1* root tips (Fig. 14). This may indicate, that *BcFLA1* indeed has a role at the beginning of the signal transduction cascade leading to a higher root hair length under Pi deficiency. However, this result can be falsified, because the number of root hairs may also be affected in *bcfla1*. A lower

number of root hairs would automatically lead to an overall reduced expression of root hair-related genes in the sample. Therefore, to confirm this result, the number of root hairs should be taken into account in further analyses.

Considering all results, we suggest a regulating role for *BcFLA1* in the Pi deficiency-induced root hair elongation. The specific upregulation of *BcFLA1* in response to Pi deficiency and the effect of its altered expression on the root hair length indicates that *BcFLA1* acts in a P-specific pathway which may have an impact on the general root hair elongation pathway. According to the properties of FLAs described in section 4.1 and 4.2, BcFLA1 is predestinated to transmit the low P signal into the cell. It may be the connector between the low P signal in the extracellular matrix and downstream signaling events in the cytoplasm. Furthermore, the GPI-anchor could be cleaved from BcFLA1 so that it is released from the plasma membrane possibly leading to further signaling events. Since it was proposed that RSL4 may coordinate endogenous and exogenous signals while it regulates the root hair elongation (Datta *et al.*, 2015), it could be the link between the general and P-specific root hair elongation pathway. Also the functional paralog of RSL4, LRL3 (Salazar-Henao *et al.*, 2016), may be a connecting point of both pathways. However, it is also possible, that the K⁺ transporter TRH1 is involved since it was proposed to be a convergence point between environmental and developmental root hair pathways as it is necessary for proper root hair elongation and able to affect the auxin homeostasis (Daras *et al.*, 2015). Further studies are necessary to obtain a more detailed picture of this network. Interestingly, another FLA was preferentially expressed in root hairs of Arabidopsis and was upregulated at the protein level in response to Pi starvation, which may indicate the involvement of several FLAs in the Pi deficiency-induced root hair growth (Salazar-Henao and Schmidt, 2016).

Taken together, the results of this study in combination with the proposed functional abilities of fasciclin-like AGPs strongly indicate a role of *BcFLA1* in the Pi deficiency-induced root hair elongation, even if the exact function and interaction partners remain to be elucidated. But, the other candidate genes should not be excluded from further investigation, since an overexpression of these genes would avoid a replacement of the gene function by gene isoforms, so that root hair effects may be visible. Especially *BcRALFL1*, *BcMRIL* and *BcROPGEF1* should be further investigated since they all are connected to the FER receptor. One can speculate that the interaction of all three gene

products is necessary in combination with the FER receptor for the Pi deficiency-induced root hair elongation in *B. carinata*. The interaction of a member of the RALF family and a receptor-like cytoplasmic kinase (RLCK) was already observed. In more detail, a RLCK was shown to be recruited to FER by a RALF peptide leading to the phosphorylation of both FER and the RLCK (Du *et al.*, 2016).

5. Conclusion

The aim of the present study was the identification of regulatory candidate genes for the Pi-deficiency-induced root hair elongation. Expression analyses as well as knockout and complementation experiments clearly indicated that a fasciclin-like arabinogalactan protein, BcFLA1, is involved in this process. Furthermore, the study revealed several other potential candidate genes, whose participation has to be examined in further investigations.

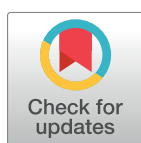
RESEARCH ARTICLE

Efficient generation of mutations mediated by CRISPR/Cas9 in the hairy root transformation system of *Brassica carinata*

Thomas W. Kirchner¹, Markus Niehaus¹, Thomas Debener², Manfred K. Schenk¹, Marco Herde^{1*}

1 Institute of Plant Nutrition, Leibniz Universitaet Hannover, Hannover, Germany, **2** Institute for Plant Genetics, Leibniz Universitaet Hannover, Hannover, Germany

* mherde@pflern.uni-hannover.de



Abstract

A protocol for the induction of site-directed deletions and insertions in the genome of *Brassica carinata* with CRISPR is described. The construct containing the Cas9 nuclease and the guide RNA (gRNA) was delivered by the hairy root transformation technique, and a successful transformation was monitored by GFP fluorescence. PAGE analysis of an amplified region, presumably containing the deletions and insertions, demonstrated up to seven different indels in one transgenic root and in all analyzed roots a wildtype allele of the modified gene was not detectable. Interestingly, many of these mutations consisted of relatively large indels with up to 112 bp. The exact size of the deletions was determined to allow an estimation whether the targeted gene was not functional due to a considerable deletion or a frame shift within the open reading frame. This allowed a direct phenotypic assessment of the previously characterized roots and, in fact, deletions in *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1 (BcFLA1)*—a gene with an expression pattern consistent with a role in root hair architecture—resulted in shorter root hairs compared to control roots ectopically expressing an allele of the gene that cannot be targeted by the gRNA in parallel to the CRISPR construct. As an additional line of evidence, we monitored *BcFLA1* expression with qPCR and detected a significant reduction of the transcript in roots with an active CRISPR construct compared to the control, although residual amounts of the transcript were detected, possibly due to inefficient nonsense-mediated mRNA decay. Additionally, the presence of deletions and insertions were verified by Sanger sequencing of the respective amplicons. In summary we demonstrate the successful application of CRISPR/Cas9 in hairy roots of *B. carinata*, the proof of its effectiveness and its effect on the root hair phenotype. This study paves the way for experimental strategies involving the phenotypic assessment of gene lesions by CRISPR which do not require germline transmission.

OPEN ACCESS

Citation: Kirchner TW, Niehaus M, Debener T, Schenk MK, Herde M (2017) Efficient generation of mutations mediated by CRISPR/Cas9 in the hairy root transformation system of *Brassica carinata*. PLoS ONE 12(9): e0185429. <https://doi.org/10.1371/journal.pone.0185429>

Editor: Xiu-Qing Li, Agriculture and Agri-Food Canada, CANADA

Received: May 31, 2017

Accepted: September 12, 2017

Published: September 22, 2017

Copyright: © 2017 Kirchner et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All gene sequences are available in the NCBI database (<https://www.ncbi.nlm.nih.gov>), accession numbers KY905141, KY905142, KY965932, KY965933, MF061227).

Funding: This work was funded by the German Research Foundation (DFG, <http://www.dfg.de>) as part of the Research Training Group (GRK1798) "Signaling at the Plant-Soil Interface".

Competing interests: The authors have declared that no competing interests exist.

Introduction

With the advent of next-generation sequencing, the scientific community was provided with a cost-effective approach of obtaining genome and transcriptome sequences of many non-model plants with unique features or high relevance for agriculture. Until recently, for a functional characterization of genes with a loss-of-function approach in non-model plants, that, for example, do not have a collection of insertional mutants, the available toolbox mainly consisted of the RNA interference (RNAi) technology [1].

The discovery of nucleases that induce double strand breaks at defined positions in the genome, resulting in site-directed deletions within a gene, was a major breakthrough, especially in plants where the targeted knockout of genes was challenging [2,3]. Notably, the clustered regularly interspaced short palindromic repeats (CRISPR) and the transcription activator-like effector nuclease (TALEN) technologies are easily available and applicable in all laboratories with standard equipment for molecular biology. In contrast to the previously used RNAi technique, the advantage of the TALEN/CRISPR approach is to completely abolish gene functionality and a lower off-target rate [4]. In recent years, the CRISPR/Cas9 system grew into a simple, well-established and efficient method for performing gene engineering in plants. In this system, the Cas9 nuclease is targeted to specific genomic sequences, where it induces double-strand breaks (DSBs). In somatic plant cells, these are predominantly repaired by non-homologous end joining (NHEJ), which leads to errors during the repair causing insertions, deletions (indels) or replacements [5]. The specificity of the CRISPR/Cas9 system, which was shown to be especially high in plants [6,7], is ensured by a 20 bp protospacer sequence belonging to the so-called guide RNA (gRNA), which guides the Cas9 nuclease to the corresponding site in the genomic DNA (gDNA). The protospacer sequence has to be complementary to the target sequence, which must be followed by the protospacer adjacent motif (PAM), consisting of e.g. 'NGG' so that the Cas9 can bind and cut the DNA double strand. However, base pairing of defined bases near the PAM, known as 'seed sequence', is essential and therefore decisive for the specificity [8–11] (for a review, see for example [12,13]).

However, a major bottleneck in the functional characterization of genes in non-model plants is the lack of fast and efficient transformation protocols for recalcitrant genotypes or whole species. Nonetheless, techniques such as *Agrobacterium* infiltration, protoplast transformation or hairy root transformation were developed which do not primarily aim at a transgenic event that enters the germline rather, they aim to transform a cell or organ whose features as a consequence of the transformed construct can be directly studied. These techniques are frequently used to evaluate the efficiency of the customized nuclease system such as the gRNAs for CRISPR [14] or the establishment of CRISPR in a certain plant species [15,16] and few studies used the hairy root technology in combination with CRISPR to directly gain insight into the gene function [17,18].

Since our research focus is on alteration of the root architecture in response to phosphate starvation, we used the *Agrobacterium rhizogenes*-mediated hairy root transformation technique [19] for delivery of the CRISPR construct. This approach offers the unique opportunity to swiftly examine the effects of a potential gene modification because these are directly visible in the hairy roots.

One of the challenges of such an approach is the necessity to assess the CRISPR-induced deletions quantitatively and qualitatively in every single root that is used for phenotyping, assuming that the deletions are heterogeneous amongst different transformation events. It must be ensured that the CRISPR mechanism was efficient in the actual tissue so that the wild-type allele is more or less completely absent. Furthermore, it is paramount to determine the

exact number of deleted or inserted bases, as small in-frame codon deletions may not lead to a completely abolished gene function.

In this study, we explored the possibility of correlating deletions in a gene induced by a CRISPR construct delivered by hairy root transformation with a root hair phenotype. As a proof of principle, we chose *FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 1* (*BcFLA1*), a gene in *Brassica carinata*, whose RNA-seq expression profile in a preliminary study suggested a role in the regulation of root hair elongation. The frequency of alleles with deletions in *BcFLA1* was sufficient to select roots that were presumably without a functional gene of interest and these roots were phenotypically altered compared to roots that were additionally complemented with a CRISPR resistant allele of the gene. We present a cost-effective, medium throughput approach to estimate the frequency of deletions, the absence of the wildtype allele as well as the deletion sizes in a single experiment. This approach, based on polyacrylamide gel electrophoresis, may complement the existing methods for the detection of genome indels and is uniquely suited for the evaluation of CRISPR-induced events in combination with the direct assessment of a putatively associated phenotype.

Materials and methods

Obtainment of *BcFLA1*

BcFLA1 belongs to a list of candidate genes for the Pi deficiency-induced root hair elongation, which was obtained by a genome-wide expression profile analysis by massive analysis of cDNA ends (MACE; GenXPro GmbH, Frankfurt Main, Germany) [20].

The *BcFLA1* sequence obtained by MACE was extended by 5' RNA ligase-mediated rapid amplification of cDNA ends (5' RLM-RACE) using the 'FirstChoice[®] RLM-RACE Kit' (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For both outer and inner PCR, the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Wilmington, MA, USA) was applied according to the manufacturer's specifications, in which 'CAATCCATGCATCATA TCCAAC' was used as the outer and 'ACCCAAATCAAACGACGAGT' as the inner reverse primer. The products were cloned into the pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by GATC Biotech AG (Konstanz, Germany) and SEQLAB (Göttingen, Germany), respectively. During validation of the CRISPR/Cas9-induced gene editing, we observed a second allele of *BcFLA1* with a similarity of 94%. The cDNA and gDNA sequences of both the first (*BcFLA1a*) and the second allele (*BcFLA1b*) were submitted to the NCBI database (<https://www.ncbi.nlm.nih.gov>) with the accession numbers KY905141 for *BcFLA1a* (cDNA) and KY905142 for *BcFLA1b* (cDNA) as well as KY965932 for *BcFLA1a* (gDNA) and KY965933 for *BcFLA1b* (gDNA).

Construct preparation

gRNA design. The *BcFLA1* gene sequence was scanned for PAM sequences (NGG) on both gDNA strands. A list of potential gRNAs was filtered in consideration of the following quality standards: GC content at least 50% [21], gRNAs had to be located within an exon, not more than six double-stranded bases in a row within the protospacer's secondary structure [21] predicted by 'The mfold Web Server' [22] and the gRNAs should be as near as possible at the 5' end of the *BcFLA1* coding sequence. Two different gRNAs were selected within a distance of 69 bp (gR1: CTGTCTTCCCTCCACGGAG, gR2: ACATCACGGCGATCCTTGAA). To enable the usage of both gRNAs within one construct, a polycistronic tRNA-gRNA gene was cloned as described previously [23] with the following changes: the restriction digestion of the tRNA-gRNA gene and the vector was done using Anza[™] BpiI (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol, following an insertion of the

tRNA-gRNA gene into pB-CRISPR+35S::GFP (V112) vector *via* the BpiI cutting site creating pB-CRISPR+35S::GFP+fla1-guides (H278) (S1A Fig), so that the primer sequences had to be adjusted (S1 Table). Furthermore, the PCR products were purified using the 'Fast Gene Gel/PCR Extraction Kit' (Nippon Genetics Europe, Dueren, Germany) and the PCR using the ligation product as template was done using the Phusion[®] High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) as in the first PCR.

Vectors. CRISPR+35S::GFP (V112) was constructed (S1 Text) to possess a GFP cassette for the selection of the transgenic hairy roots containing a GFP gene with introns for reduced silencing under control of a 35S promoter. Furthermore, it consisted of a CRISPR cassette containing the Cas9 gene driven by the ubiquitin4-2 promoter from parsley and the polycistronic gene carrying the two gRNAs under control of the U6-26 promoter (S1A Fig).

A complementation vector (H280) was constructed (S1 Text) from pB-CRISPR+35S::GFP+fla1-guides additionally possessing an overexpression cassette, containing the CDS of a mutated *BcFLA1* (*BcFLA1a_{mut}*) enclosed by the same artificial UTRs used for the Cas9 and under control of the ubiquitin4-2 promoter (S1B Fig). The mutation consisted of three base pairs (bp) in each of the gRNA target sequences, which were exchanged codon-optimized according to the codon usage in *Arabidopsis thaliana* without changing the amino acid sequence (S1C Fig). One of the exchanges destroyed the PAM sequence of both gRNAs so that the Cas9 was unable to bind and cut these sites. Therefore, *BcFLA1a_{mut}* was resistant against the CRISPR mechanism in combination with the two gRNAs used in this study.

As a control, pB-CRISPR+35S::GFP (V112) vector without gRNAs was introduced into the plants.

The completed plasmids were transformed into electrocompetent *E. coli* DH10B or DH5 α , confirmed by Sanger sequencing (GATC Biotech AG, Konstanz, Germany) and then transformed into electrocompetent *Agrobacterium tumefaciens* C58, containing a root-inducing (Ri) plasmid (ARqua1).

Plant material, cultivation and hairy root transformation

We chose *B. carinata*, because varieties of this species were shown to differ in their response to P and N deficiency regarding the root hair formation. Seeds of *B. carinata* cv. Bale were vernalized for three days in the dark at 4°C. and then germinated for 3 days (photoperiod, 16/8 h light/dark; temperature, 18/15°C day/night; relative humidity, 75%; light intensity, 220 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Hairy roots were induced by following a modified protocol from [24]. *Agrobacterium tumefaciens* C58 (ARqua1) was grown for two days on a YEB plate, scratched off the plate and resuspended in 6 mL PS buffer (0.7% Na₂HPO₄ (w/v), 0.3% KH₂PO₄ (w/v), 0.5% NaCl (w/v), 150 μM acetosyringone, pH 7). After an incubation time of at least 1.5 h, the *B. carinata* seedlings were dampened with the bacterial solution and wounded along the hypocotyl with an insulin syringe (U-40 Insulin, 0.3 mm x 12 mm). Additionally, the bacterial solution was injected into the plants. Then, the seedlings were further cultivated in clay granulate under the same conditions as during the germination, except for an incubation in the dark for 20 h directly after the transformation. After one week, the plants were fertilized with a nutrient solution (pH 5.3) containing (mM) 2.25 Ca(NO₃)₂ x 4 H₂O, 2.5 K₂SO₄, 1 MgSO₄ x 7 H₂O, 0.25 KCl, 1 KH₂PO₄ and (μM) 25 H₃BO₃, 1.5 MnSO₄, 1.5 ZnSO₄, 0.5 CuSO₄, 0.025 (NH₄)₆Mo₇O₂₄ and 35.8 Fe (Fe^{III}-EDTA). Two weeks after the transformation, clay granulate was earthed up to cover the grown hairy roots. One additional week later, the plants were screened for transgenic roots using GFP as fluorescent marker ('SMZ25', Nikon, Düsseldorf, Germany, or 'FastGene[®] BG-LED Flashlight', Nippon Genetics Europe, Dueren, Germany). The non-transgenic roots were cut off and plants with at least one transgenic root were further cultivated for eight days

under the same conditions as before and in the same nutrient solution which was used as fertilizer, but without KH_2PO_4 and pH was readjusted to 5.3 during the cultivation as needed. Then, 2-cm root tips were harvested for root hair length measurement as well as RNA and gDNA extraction.

RNA & gDNA isolation

RNA and gDNA were isolated using the peqGOLD TriFast™ reagent according to the manufacturer's instructions (VWR International GmbH, Darmstadt). For RACE-PCR, RNA was isolated using the 'NucleoSpin® miRNA' Kit (Machery-Nagel, Dueren, Germany) following the manufacturer's specifications (using the total RNA isolation procedure). RNA Quality was electrophoretically tested on a 1% (w/v) non-denaturing agarose gel containing 0.004% (v/v) Midori Green Advance (Nippon Genetics Europe, Dueren, Germany) for a runtime of 35 min at 7.6 V cm^{-1} in Tris-acetate-EDTA (TAE) buffer. Both RNA and gDNA were quantified using the NanoPhotometer® P-Class P 300 (Implen, Munich, Germany).

Expression analysis

Wildtype- and *BcFLA1a_{mut}*-specific primers were designed on the differing bases in the gRNA binding sites. Additionally, unspecific primers for the total *BcFLA1* expression were designed with binding sites near the 3' end of *BcFLA1*.

The geometric mean of the Ct values of two endogenous control genes was calculated according to Vandesompele *et al.* [25], which was then used to calculate the fold over reference of the different samples ($2^{-\text{delta Ct}}$).

For more detailed information regarding the cDNA synthesis, primer design, primer efficiency tests, endogenous controls and quantitative PCR (qPCR) conditions we refer to the [S2 Text](#).

Verification of the gene editing

For the verification of the gene editing, the gene region including the two gRNA regions was amplified. For it, *BcFLA1a_{mut}*-specific, wildtype-specific and unspecific primers were designed using Primer3Plus [26] in consideration of the following quality standards: amplicon size between 600 and 800 (large) or 200 and 260 (small) bp, primer size between 20 and 25 bp, primer T_m between 58 and 62°C and a GC content between 40 and 60% (S3 Table). The specificity of the primers was realized by placing at least one primer into the wildtype or artificial UTRs and tested in the same way as the qPCR primers, whereupon the large amplicons were separated on a 2.5% (w/v) agarose gel for 45 min at 6.3 V cm^{-1} .

The principle of the M13-SSR-PCR was described previously [27]. A M13-tail (GTAAAACGACGGCCAGT) was added to the 5' end of the forward primers and a nested PCR was performed with each 10 µL reaction containing: 1x B1 buffer, 2 pmol dNTPs, 0.25 pmol M13 tailed forward *BcFLA1* (wildtype-spec. small) primer, 1.25 pmol 700 infrared dye (IRD) labelled M13 forward primer, 2.5 pmol reverse *BcFLA1* (wildtype-spec. small) primer, 0.3 U 'DCS DNA HotStart Polymerase' (DNA Cloning Service e.K., Hamburg, Germany) and 20 ng template. The protocol consisted of an initial step of 95°C for 10 min, followed by 24 cycles of 95°C for 1 min, 60°C or 63°C for 1 min and 72°C for 1 min, as well as 8 cycles of 95°C for 1 min, 52°C for 45 sec and 72°C for 1 min, and a final step of 72°C for 10 min.

A 500 bp sequence was synthesized (Integrated DNA Technologies, Inc., Coralville, IA, USA) without the same nucleotides occurring consecutively, whereby the beginning and end of the sequence matched the *BcFLA1* (unspec. large) primers. The latter were then used in a M13-SSR-PCR as described before and the resulting fluorescently labelled amplicon served as

a template for a sequencing reaction by the “Thermo Sequenase Cycle Sequencing Kit” (Thermo Fisher Scientific, Waltham, MA, USA). The fluorescently labelled fragments were used as ladder with 1 bp intervals. The sequencing reaction was done according to the manufacturer’s instructions but, a formamide loading dye consisting of 98% formamide, 10 mM EDTA (pH 8) and 0.05% pararosaniline was used instead of the stop solution.

A 4% polyacrylamide gel with a thickness of 0.25 mm, containing 7.5 M urea, was prepared using the Rotiphorese[®] DNA Sequencing System PK 1 (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) according to the manufacturer’s instructions. Formamide loading dye was added to the samples following a denaturation step at 95°C for 6 min. The separation was performed using the 4300 DNA Analyzer (LI-COR, Lincoln, NE, USA) in Tris-borate-EDTA (TBE) buffer at 1500 V, 40 mA, 40 W, 45°C and a wavelength of 700 nm.

In addition to fragment analysis by polyacrylamide gel electrophoresis (PAGE), the amplicons were separated by agarose gel electrophoresis. For the first experiment, a nested PCR was performed as described for the PAGE analysis, except for the cycle numbers, which were increased to 26 and 10 respectively. For the replicated experiment, PCR was performed with *BcFLA1* (unspec. large) primers using the KAPA HiFi HotStart PCR Kit (KAPA Biosystems, Wilmington, MA, USA) together with the supplied GC buffer, 50 ng of template, a primer concentration of 0.3 μM and a cycle number of 35 according to the manufacturer’s instructions. The products were separated on a 2.5% (w/v) non-denaturing agarose gel containing 0.004% (v/v) Midori Green Advance (Nippon Genetics Europe, Dueren, Germany) for 3.75 h at 4.1 V cm⁻¹ in TAE buffer. All visible bands were cut out of the gel and DNA was purified using the ‘PCR clean-up Gel extraction’ kit (Machery-Nagel, Dueren, Germany) according to the manufacturer’s instructions. Purified DNA was additionally amplified (only replication 1) and sequenced by GATC Biotech AG (Konstanz, Germany) or SEQLAB (Göttingen, Germany), using the *BcFLA1* (wildtype-spec. small) (replication 1) and *BcFLA1* (unspec. large) (replication 2) reverse primer.

Root hair length measurement

2-cm root tips were fixed in 70% (v/v) EtOH overnight, stained in 0.05% (w/v) Toluidine Blue for 3 h and stored in tap water at 4°C. For root hair length measurement, root tips were pulled over 0.3% (w/v) agar-agar until the root hairs were straight. Pictures were taken using the ‘SMZ25’ (Nikon, Düsseldorf, Germany) and root hair length was measured using the corresponding software ‘NIS-Elements’. The measurement was done in a region with the longest and fully developed root hairs of 2-cm root tips cut from the transgenic first and second order lateral roots.

Results

Proof of concept study with *BcFLA1*

The background to this work was the study of the phosphate deficiency-induced root hair elongation in the non-model organism *Brassica carinata*, the Ethiopian mustard. In our previous studies, we observed that enhanced root hair length was paralleled by increased expression of *BcFLA1*, indicating a role in the regulation of the root hair elongation. *BcFLA1* is a member of the arabinogalactan protein (AGP) family that is *inter alia* thought to have regulative functions and AGPs possessing fasciclin (FAS) domains are predicted to be involved in protein-protein interactions [28]. Thus, we hypothesized root hair length in roots with a *BcFLA1* loss-of-function is reduced.

We customized a CRISPR vector with a strong ubiquitously active promoter (ubiquitin promoter from parsley) driving Cas9 expression and the U6-26 promoter from Arabidopsis

controlling the expression of the gRNA (S1A Fig). The vector can be easily modified in one step to target any gene of interest with target-specific gRNAs. For *BcFLA1* two gRNAs with target sites at a distance of 69 bp (S1C Fig) were expressed in parallel using a polycistronic gRNA as described previously [23].

Since we cannot exclude that the gRNA used to target *BcFLA1* is able to produce off-target effects, especially in a non-model plant without a complete genome sequence, we added a mutated allele of *BcFLA1* (*BcFLA1_{mut}*) to the CRISPR vector as a control construct (called *BcFLA1* compl.) (S1B Fig). The mutations in the *BcFLA1* gene abolished binding of the gRNA but the wildtype amino acid sequence was maintained, allowing the ectopic expression of *BcFLA1* in the presence of the nuclease and gRNA (S1C Fig).

Screening of the transgenic roots

Upon transformation of the CRISPR construct with the hairy root technique, roots emerging from the inoculation site were transgenic or non-transgenic and a fluorescent marker (GFP) encoded on the vector allowed the selection of transgenic roots by assessing the GFP fluorescence. The transformation efficiency was between two and four percent, whereby one to three transgenic roots were generated per plant. The GFP fluorescence was sufficiently strong to detect a signal with a blue-green LED flashlight within one week, allowing the evaluation of a transformation event early after inoculation without removing the plant from the potting medium (Fig 1A). The GFP signal was equally distributed, showing no indication of gene silencing probably due to the introns included in the GFP coding sequence [29]. Although non-transgenic roots were cut off before the phosphate depletion was started, some of them recovered until the harvest (after eight days of treatment) causing a reduced growth of the transgenic root (Fig 1C) compared to a transgenic root, which was grown alone (Fig 1D). Nevertheless, independent of the growth of the transgenic roots, we harvested a maximum of 2-cm root tips from 1st and 2nd order lateral roots (Fig 1B). In all roots with GFP fluorescence, we were later able to demonstrate evidence for the presence of indels in the genome, suggesting an efficient selection strategy. The position of the GFP gene in the vector close to the left border ensured a proper transfer of the Cas9 gene and the gRNA. As described previously [30], a strong fluorescence signal is putatively correlated with an insertion locus beneficial for transgene expression, suggesting that screening for strong GFP expression may facilitate the selection of roots with a high Cas9 and gRNA expression, resulting in a higher mutation rate.

Detection of deletions and insertions with PAGE

Initially, we attempted to induce the deletion of a larger fragment within *BcFLA1*, therefore we decided to use a multiplex system, through which it was possible to guide Cas9 to two different sides in the same gene by two gRNAs [23]. As long as the two gRNAs are active at the same time, this will lead to the loss of a large fragment between the two occurring DSBs, so that the knockout of the gene is very likely [31]. However, an initial fragment-analysis by agarose gel electrophoresis suggested that the expected deletion of the fragment between the gRNAs did not occur and Sanger sequencing of DNA amplicons with a size differing from the wildtype allele revealed that the second gRNA induced deletions only very inefficiently (S2 Fig). Nonetheless, although deletions of bigger fragments may ensure the abolishing of gene function, residual fragments resembling the size of the wildtype allele putatively have small indels that cannot be detected by agarose gel electrophoresis and therefore would be misinterpreted as wildtype. Thus, we decided to focus on the detection of small indels rather than producing big deletions, hypothesizing that a precise determination of the amplicon size will reveal a sufficient frequency of the mutational rate for subsequent phenotyping.

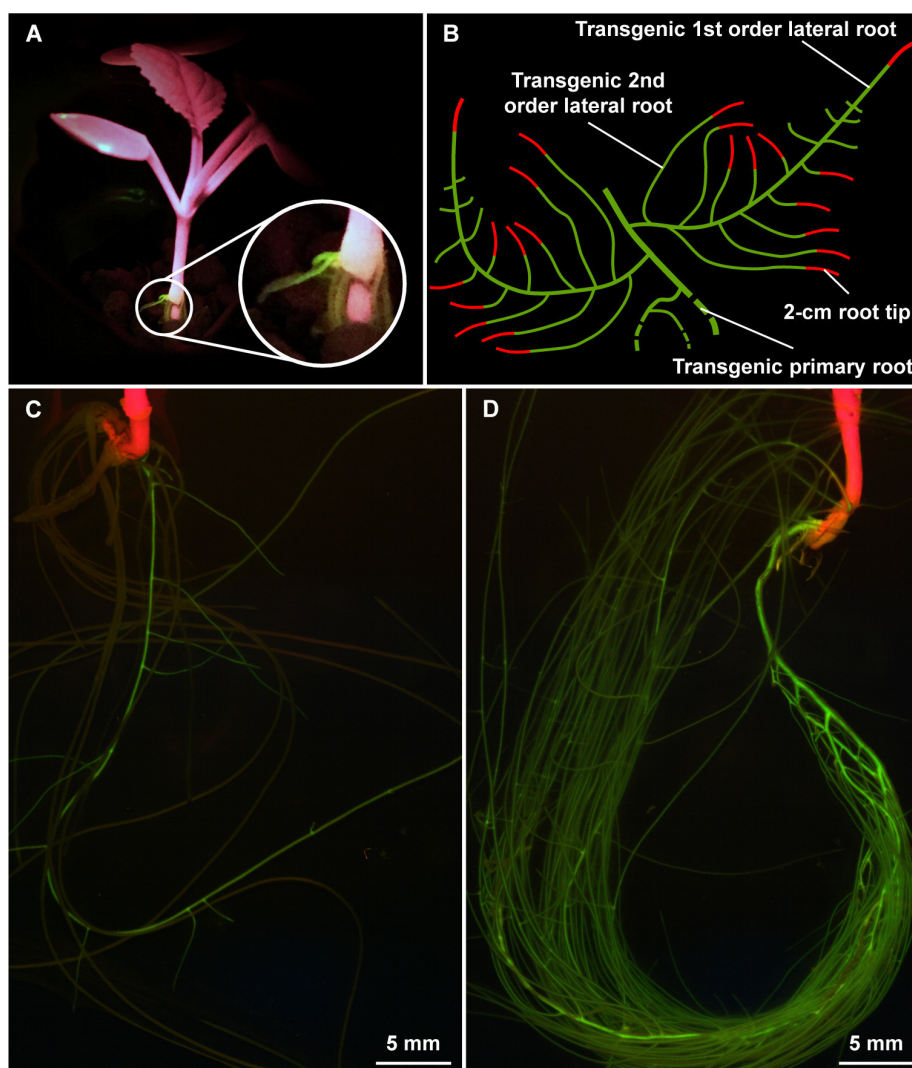


Fig 1. Screening and harvesting of the transgenic root material. Young transgenic root growing out of the inoculation side (A, picture was taken using a blue-green LED flashlight), scheme showing the harvested root material (B), discrimination of transgenic and non-transgenic roots (C) and example of a transgenic root (D) before the harvest (pictures were taken with a fluorescence stereomicroscope).

<https://doi.org/10.1371/journal.pone.0185429.g001>

In fact, fragment-analysis by PAGE allowed us to determine the fragment size with a resolution of one base pair (Fig 2) which eliminates the limitation on the size of detectable deletions previously discussed for AFLP [32]. We detected up to 7 fragments (average 4) in one transgenic root and no transgenic roots were found to have an amplicon with the wildtype size. The size of the deletion ranged from 1 to 49 bp and the size of the insertion from 1 to 19 bp. In 26 out of 39 cases a deletion or insertion resulted in a frame shift mutation.

Validation of deletions and insertions with Sanger sequencing

We validated six distinct deletions by sequencing reaching from 4 to 36 bp, whereby two of the samples (CRISPR 4L, and compl 7L) exhibited exactly the same deletion of 4 bp (Fig 3). Most

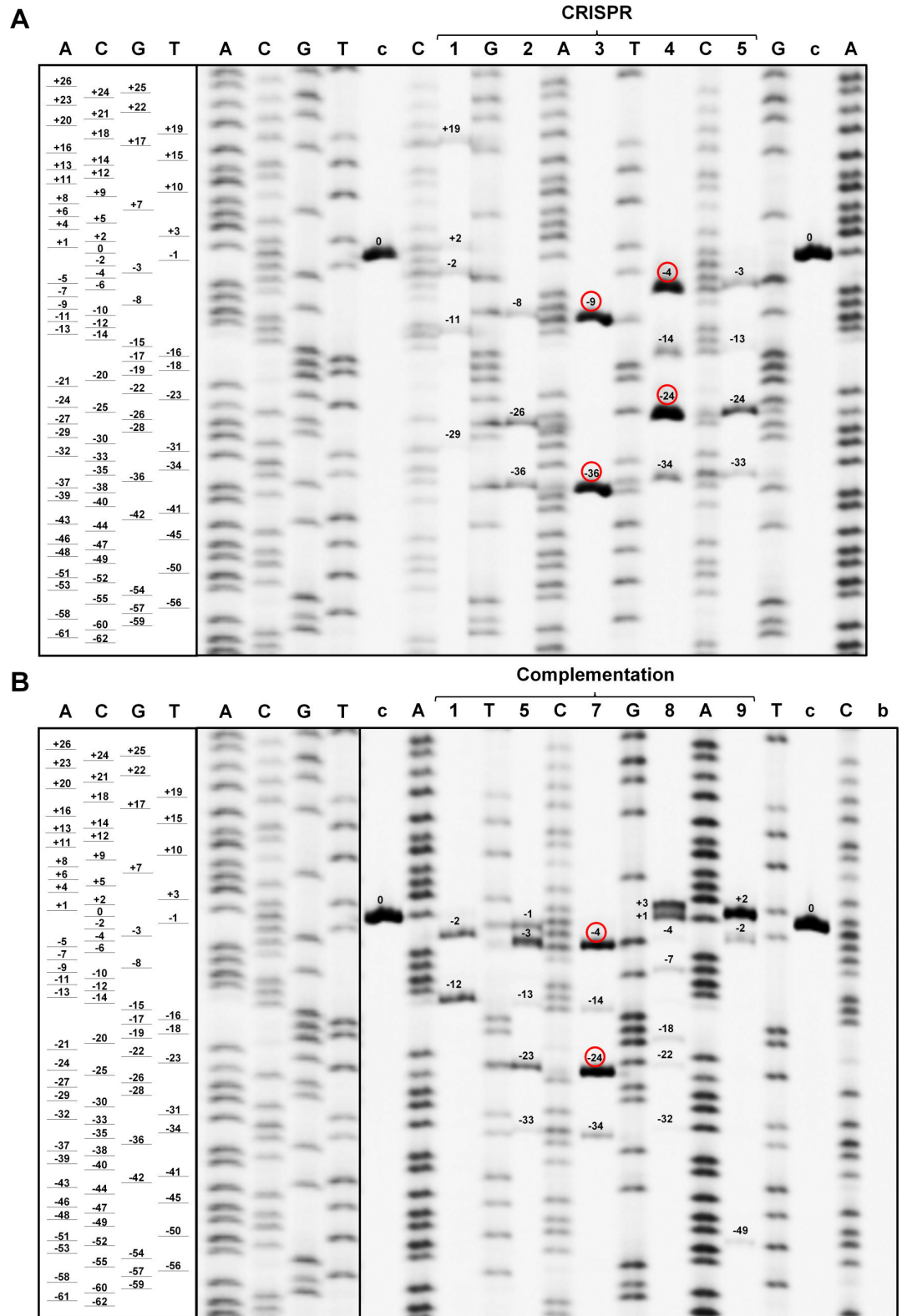


Fig 2. Validation of the CRISPR/Cas9-induced gene editing by polyacrylamide gel electrophoresis. CRISPR samples (A) and complementation samples (B). A, C, G and T = Sanger sequencing reactions with the corresponding dideoxynucleotides; c = control; b = blank (no template control). Lane numbers indicate the respective transgenic roots and are consistent with the following figures. Numbers close to the bands indicate the deleted (-) and inserted (+) bp compared to the wildtype size (0), red circles indicate deletions, which could be validated by Sanger sequencing.

<https://doi.org/10.1371/journal.pone.0185429.g002>

of the sequencing reactions resulted in overlapping of at least two sequences starting at the location of the first gRNA. A completely independent experiment replication revealed twelve further distinct deletions ranging from 3 to 112 bp and three insertions ranging from 6 to 68 bp (S2 Fig). In one case (CRISPR 7S), there were two distinct deletions, one around the first and one around the second gRNA target site. Furthermore, the largest deletion of 112 bp spanned both gRNAs, possibly indicative of two simultaneously active gRNAs. However, all other identified deletions were exclusively around the gRNA1 target site. Additionally, the sequencing revealed that *BcFLA1* had two naturally occurring alleles (a and b). However, although the sequence recognized by the protospacer of gRNA1 was different in one base for *BcFLA1b* (position 15 is A in *BcFLA1a* but G in *BcFLA1b*) (S1C Fig), we detected an efficient induction of deletions and insertions for both alleles (S2 Fig).

The sequence amplified by primers designed to amplify exclusively *BcFLA1a_{mut}* matched the mutagenic *BcFLA1* sequence without any deletions.

The separation of the most abundant amplicons of the potentially edited *BcFLA1* gene region, found by fragment analysis by PAGE, was confirmed by Sanger sequencing (Fig 2, Fig 3). However, with fragment analysis by PAGE, we detected weaker bands that could not be analyzed by Sanger sequencing.

Expression of *BcFLA1* in roots carrying the CRISPR construct

As an independent line of evidence, we monitored the *BcFLA1* expression with qPCR in transgenic roots expressing an inactive (without protospacer) gRNA (control), an active gRNA (CRISPR), and an active gRNA plus *BcFLA1a_{mut}* (compl.). We reasoned that indels in *BcFLA1* will result in a frame shift, causing the degradation of the transcript *via* nonsense-mediated mRNA decay (NMD). In the samples complemented with *BcFLA1a_{mut}*, no deletions and therefore no transcript reduction was expected. To discriminate between the wildtype *BcFLA1* and the *BcFLA1a_{mut}* transcript, we tested primer pairs specifically amplifying *BcFLA1* and *BcFLA1a_{mut}* (S3 Fig). The *BcFLA1a_{mut}*-specific primers produced an amplicon only with the complementation plasmid as template, whereas the wildtype-specific primers produced an amplicon exclusively with a wildtype version of *BcFLA1* as a template, confirming primer specificity. Additionally, a third primer pair amplifies the wildtype *BcFLA1* and *BcFLA1a_{mut}* equally well providing an estimate of the wildtype *BcFLA1* and *BcFLA1a_{mut}* transcript together.

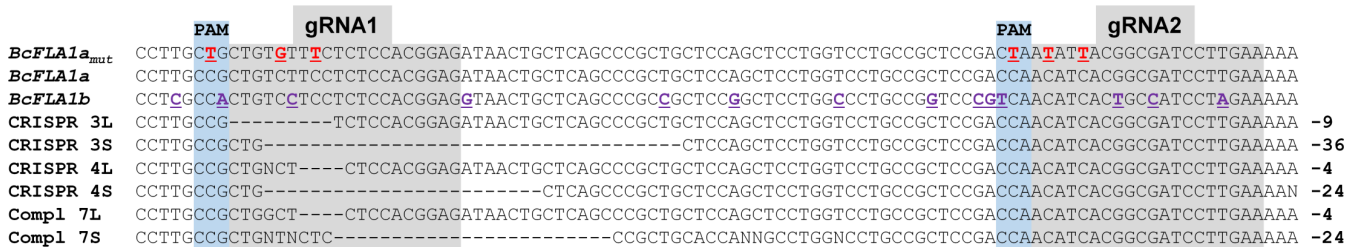


Fig 3. CRISPR/Cas9-induced deletions in *BcFLA1*. Grey background indicates gRNAs, blue background the PAM. Red letters indicate replaced bases in *BcFLA1a_{mut}*, violet letters differing bases in *BcFLA1b*; numbers on the right indicate deleted bp. There were no deletions around gRNA 2. Compl = complementation. Numbers on the left indicate the respective transgenic roots.

<https://doi.org/10.1371/journal.pone.0185429.g003>

A pretest assessing *BcFLA1* expression using semi-quantitative PCR resulted in a clearly lower expression of the wildtype *BcFLA1* in most of the CRISPR and complementation samples (S4C Fig), which was successfully complemented by the expression of *BcFLA1a_{mut}* (S4B and S4D Fig).

A detailed expression analysis by qPCR revealed an average total *BcFLA1* expression of ca. 15% in the CRISPR samples compared to the control (Fig 4A). The same can be observed for wildtype *BcFLA1* where the CRISPR samples had only 0.6% of the transcript compared to the control samples (Fig 4C). This result suggests that a successful induction of deletions and insertions of *BcFLA1* correlates well with the reduction of its transcript.

The complementation of *BcFLA1* by *BcFLA1a_{mut}* resulted in more than 40-times higher total *BcFLA1* expression than in the control (Fig 4A). The results show that ectopic expression of *BcFLA1a_{mut}* with the ubiquitin promoter (compl.) resulted in much higher transcript abundance than the expression of the wildtype *BcFLA1* with the native promoter (control). This was confirmed with the primer pair specific for *BcFLA1a_{mut}*, where the average fold over reference was around 0.7 in the complementation samples, which indicated that *BcFLA1a_{mut}* was not affected by NMD (Fig 4B). In control and CRISPR samples, the respective transcript was not detectable or negligibly small ($< 10^{-4}$) since *BcFLA1a_{mut}* was absent. One out of eight transgenic roots carrying the complementation construct (H280) showed much lower expression of *BcFLA1a_{mut}*, possibly due to gene silencing effects or a chromosomal position effect regarding the T-DNA insertion site caused by adjacent condensed chromatin [33].

Interestingly, the average expression of the wildtype *BcFLA1* transcript in the complementation samples was about only 20% of that found in the control (compared to 0.6% in the CRISPR samples) (Fig 4C). NMD, causing transcript reduction, seems to be less efficient in the roots carrying the complementation construct compared to the CRISPR construct alone.

An independent biological replication exhibited similar results regarding the expression of *BcFLA1* (S5 Fig).

Root hair length

In the final step, we tested the hypothesis that the deletions and insertions in *BcFLA1* not only affect the open reading frame and transcript expression, but also result in a phenotypic alteration of the root hairs. We scored the root hair length of root tips in the previously characterized transgenic roots and found that compared to the control, transgenic CRISPR roots exhibited a reduced root hair length by trend (Fig 5). In contrast, the complementation of *BcFLA1* led to an increased root hair length compared to the CRISPR roots. The root hairs in the roots carrying the complementation construct were slightly longer than the control roots, which is consistent with the enhanced expression of *BcFLA1* shown by qPCR (Fig 4, S5 Fig).

Discussion

An optimized CRISPR construct is required for efficient selection of roots with a disrupted gene function

In this study, we successfully induced site-directed deletions and insertions in the genome of the non-model plant *Brassica carinata* using the CRISPR technology in combination with a hairy root transformation protocol. The efficient generation of these mutations in *BcFLA1* gave us the confidence to directly attempt a phenotypic assessment of the obtained roots. For the success of such a strategy, it is mandatory to optimize the efficiency of the CRISPR construct allowing the medium-throughput selection of individual roots with a disrupted gene function. Primarily, the expression of the Cas9 nuclease, the gRNA, and the fluorescent marker

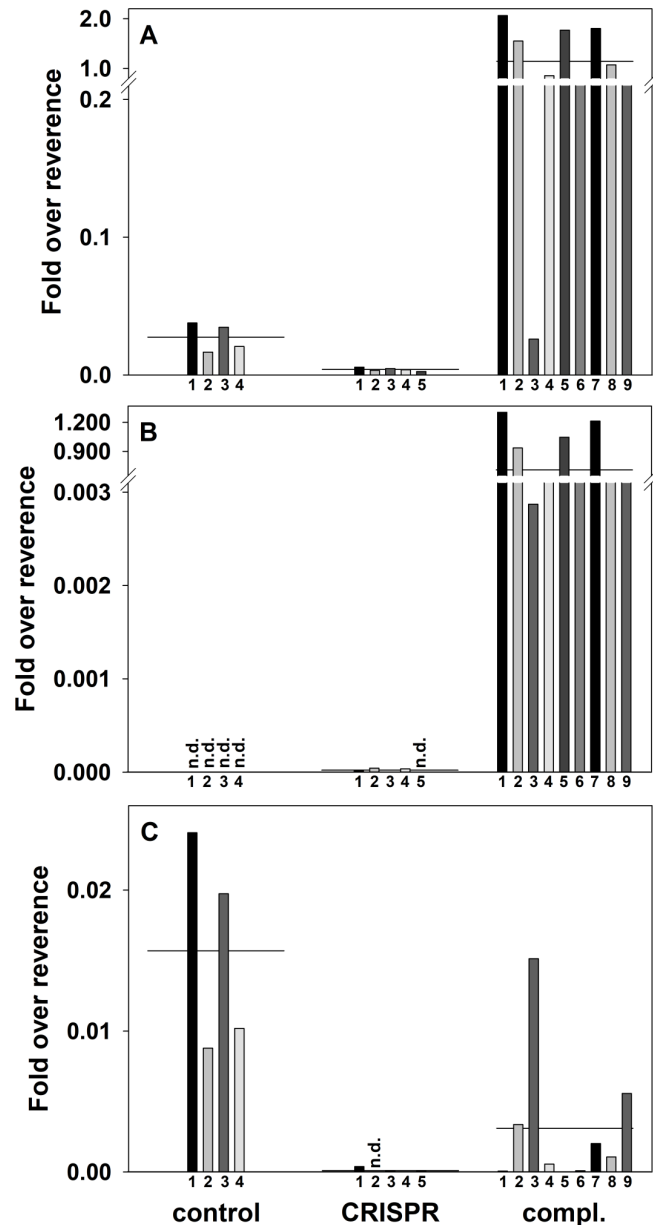


Fig 4. Expression of *BcFLA1* as affected by CRISPR/Cas9-induced gene editing and complementation, respectively. Total *BcFLA1* expression (A), expression of *BcFLA1a_{mut}* (B) and expression of the wildtype *BcFLA1* gene (C). Each bar represents the expression in one single transgenic root measured by three technical replicates with the horizontal line representing the corresponding mean of all biological replicates. Numbers indicate the respective transgenic roots. Fold over reverence = $2^{-\Delta\Delta Ct}$; n.d. = not detected.

<https://doi.org/10.1371/journal.pone.0185429.g004>

GFP should be under the control of promoters highly active in hairy roots. Hairy roots are most likely chimeras depending on how early the mutation took place. In the case of an early mutation, the expected number of variants in a single transgenic root is smaller. The fact that we found only a few indel events in one individual root (Fig 2) suggests that the proteins

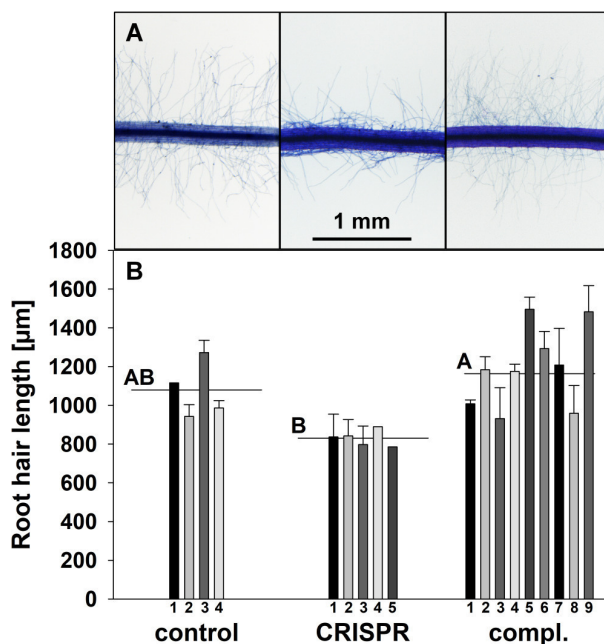


Fig 5. Root hair length of *B. carinata* 'Bale' as affected by downregulation and complementation of *BcFLA1*, respectively. Root hairs stained by Toluidine Blue (A) and root hair length in µm (B). Each bar represents the mean of the root hair length of root tips from the transgenic 1st and 2nd order lateral root of a single independent event with the error bars indicating the standard error. Numbers indicate the respective transgenic roots. Horizontal lines represent the mean of all transgenic roots. Letters denote significant differences at $P < 0.05$ (Tukey test).

<https://doi.org/10.1371/journal.pone.0185429.g005>

needed to induce the mutations were active at an early stage of the transformation so that the mutations, induced in a few founder cells, are carried through the whole root cell population. In conjecture, it was shown previously that the sequence of the protospacer, as well as the location of the target site within the gene, is crucial for the efficiency of the CRISPR construct [14,34]. We designed gRNAs to target a sequence near the 5' end of *BcFLA1* (S1C Fig) to ensure that the functionally important protein structures, possibly encoded at the beginning of the gene, are affected by the induced deletions. This is consistent with a prior study, in which the 3' target was less efficient in the gene knockout compared to the 5' end [14]. A GC content of at least 50% should guarantee a strong binding of the gRNA to the target sequence leading to a higher editing efficiency as observed in a previous study [21]. The latter also determined that pairing of more than 6 bp between the target sequence and the gRNA should be avoided in order to prevent the building of stem-loop structures so that the protospacer is free to bind the target sequence. In a recent publication, the effect of every base at each position of the protospacer was determined for the activity of the gRNA [34]. Interestingly, the higher activity of gRNA1 compared to gRNA2 in our study (only two deletion events were detected for the latter, see Fig 3 and S2 Fig) might be explained by the guanine at position 20 of gRNA1 directly before the PAM sequence as well as the cytosine as variable base of the PAM sequence (S1C Fig), which both are described to result in higher efficiency. In contrast, gRNA2 does not contain these bases at the respective positions and exhibits a thymine as the variable base in the PAM sequence, which was shown to be disfavored [34].

The relaxed specificity of a gRNA might be compensated by using a complementation construct in parallel

The usage of the CRISPR technology for functional characterization of a gene might be limited by off-target effects affecting additional genes, thereby impeding the association of a mutation with a phenotype. This is especially of concern in a non-model plant without a fully sequenced genome or in cases where the phenotypic consequences of a mutation are evaluated directly without entering the germline, rendering a genetic separation of off-target mutations impossible. In this context, it is noticeable that gRNA1 additionally resulted in gene indel events in another allele of *BcFLA1*, called *BcFLA1b* (S2 Fig). This allele also has a thymine as a variable base in the PAM (S1C Fig), so that gRNA1 possibly had a higher activity for *BcFLA1a* than for *BcFLA1b*. Nevertheless, in *BcFLA1b* the first gRNA was also active even though there was a mismatch at position 15 (6 bp away from the 3' end) and therefore in the part close to the PAM sequence, which was considered as crucial for the specificity [8,10,11]. However, according to Semenova *et al.* [9], exactly this position is surrounded by, but does not belong to, the so-called 'seed sequence', which reflects the base positions in which a perfect base pairing is essential. Moreover, this is consistent with the results of [35], who found that single mismatches, even at the 3' end of the gRNA, may not lead to a lower efficiency of the gRNA, depending on the target site. We did not systematically screen for off-target effects. However, with the hairy root technique, independent transformation events for each transgenic root were obtained so that the homogeneity of the phenotypic changes indicates that the potential side effects are rather low unless the off-target effects are as frequent as the target effects. Additionally, the impact of such effects on the interpretation of the overall results might be severely reduced by using a complementation approach in parallel. We ectopically expressed *BcFLA1a_{mut}* that was resistant to the induction of indels by the Cas9/gRNA combination used for targeting the wildtype *BcFLA1* expressed in parallel (S1C Fig). Both *BcFLA1* genes code for the same amino acid sequence, therefore, the mutant version of *BcFLA1* could complement the loss-of-function caused by indels in the wildtype *BcFLA1*. The success of this approach was monitored by qPCR which was able to distinguish transcripts originating from two different versions of the gene (Fig 4). The expression of *BcFLA1a_{mut}* was high in transgenic roots carrying the complementation construct and therefore seemed unaffected by the Cas9/gRNA designed to target wildtype *BcFLA1* (Fig 4B). However, the reduction of the wildtype *BcFLA1* expression in the complementation samples was not as clear as in the CRISPR samples (Fig 4C). This may be due to a reduced efficiency of the CRISPR/Cas9 mechanism in the complementation vector. It is conceivable that the strong overexpression of *BcFLA1a_{mut}* leads to a reduced expression of the Cas9 protein because the expression of both is under the control of the same ubiquitin promoter from parsley. Another reason may be that the wildtype primer also binds to *BcFLA1a_{mut}* with a very low rate, which was not detectable in the agarose gel (S3 Fig), but measurable by the more sensitive qPCR. Even if this rate is very low, it may have an influence because of the extremely high expression of the ectopically expressed *BcFLA1a_{mut}*. Another possibility is a feedback regulation of the overexpressed *BcFLA1a_{mut}* on the wildtype *BcFLA1* expression, suggesting that the expression of *BcFLA1a_{mut}* under control of the native promoter might be a significant improvement of the system.

The size of deletions caused by CRISPR in hairy roots is highly variable

In this study, we found deletions ranging from 1 to 112 bp, some of which were easily detectable in a fragment analysis by agarose gel electrophoresis. Indeed, it was possible to identify deletions and sometimes even insertions for many of the samples (Fig 3, S2 Fig) with this approach. Even though there are examples of larger deletions [36,37], CRISPR/Cas9 studies

applied in systems other than hairy roots, usually led to deletions of only a few bases as long as only one gRNA was used [6,7]. Perhaps, the hairy root transformation leads to larger deletions than other transformation methods, for example, because the DSB repair mechanisms in hairy roots may be less efficient than in other plant tissues or the activity of nucleases degrading the DNA strands before the repair is higher. However, in another paper working with CRISPR/Cas9 in hairy roots, mostly the usual 1 or 2 bp indels and only one 5 bp and one 7 bp deletion were observed at two different sites targeted by one gRNA each [17]. Furthermore, Michno *et al.* [16] detected, with one exception of 32 bp, only small (up to 8 bp) CRISPR/Cas9-induced deletions in hairy roots in overall three different target sites and two different plant species. Hence, it is also possible that the target site is decisive for the type and size of the observed gene editing events, as expected by Jacobs *et al.* [14]. The latter also applied CRISPR/Cas9 in a hairy root system and observed on average smaller deletions than in our study, but depending on the target sites, large indels were also detected. Furthermore, the authors observed similar mutations for the same target sites in somatic embryos, independent of the transformation method. Wang *et al.* [18] also applied CRISPR/Cas9 in hairy roots and observed 1–2 bp indels induced by a single gRNA, and larger deletions using two gRNAs. Interestingly, deletions larger than the sequence between the two gRNAs were detected. Summing up, the type and size of indels differs strongly depending on the target site and sometimes also between hairy roots of the same target and even within a single hairy root. The latter was also observed by Iafaldano *et al.* [15], who detected for the same target in one hairy root culture exclusively 1 bp indels and in three others varying indels between 1 and up to 22 bp.

Sensitive detection of indels with fragment analysis *via* PAGE

For the detection of small indels by fragment analysis the resolution of agarose gels is too low, therefore we tried to identify a method that is sensitive, semi-quantitative, cost-effective and able to estimate the size of the deletions. Jacobs *et al.* [14] used next generation sequencing to detect and quantify all mutations that were induced by CRISPR/Cas9 in hairy roots. This is a highly effective, though a costly approach. Additionally, cleaved amplified polymorphic sequence (CAPS) assays were used to detect the mutations [15–18]. However, this method requires a restriction site in the gRNA target sequence which is destroyed by a deletion in the mutant. This limits the number of suitable target sequences so that the design of specific and efficient gRNAs is more difficult. Another method, the high-resolution melting curve analysis, is effective and sensitive in detecting mutations [38], but cannot determine the exact number and size of indels. We decided to combine an amplicon size analysis strategy with the high resolution of PAGE. Indeed, the size of the deletions was determined with one base pair resolution and deletions were revealed in all samples (Fig 2). In all cases, there was no signal indicative of the wildtype allele without deletions detectable. The results from the fragment analysis by PAGE were in agreement with the Sanger sequencing obtained previously from fragments isolated from agarose gel electrophoresis (Fig 3). Additionally, the reduction of *BcFLA1* transcript measured by qPCR is an independent line of evidence that suggests a successful induction of site-directed indels (Fig 4). However, this method cannot be used to quantify the effect. We believe that fragment analysis by PAGE is uniquely suited for the detection of indels, especially when the chance of occurrence of a wildtype allele in the sample should be minimized. However, a direct evaluation of the fragment analysis by PAGE by next-generation sequencing might help to further establish the sensitivity and the accuracy of the method. Furthermore, we would like to emphasize that the amount of DNA loaded is crucial for sensitivity and accuracy of the fragment analysis by PAGE. In fact, overloading of the gel ensures that there are indeed no additional amplicons at a low level. However, this impedes a good

estimation of the deletion size. Furthermore, the resolution of the PAGE is substantially increased by use of amplicons smaller than 250 bp.

Effect of indels in *BcFLA1* on root hair length

Because of the proposed role of *BcFLA1* in the root hair elongation, we expected an influence of its loss-of-function on the root hair length. Indeed, the root hairs of the transgenic CRISPR roots were by tendency shorter compared to that of the wildtype (Fig 5). In order to exclude off-target effects, we additionally complemented the gene function of *BcFLA1* by simultaneously overexpressing a mutant *BcFLA1a_{mut}* gene, which was immune against the CRISPR/Cas9 mechanism. The consequence was not only the recovery of the wildtype root hair length but even the formation of slightly longer root hairs (Fig 5). This fits with the expression of *BcFLA1* in the complementation samples, which was on average 40-times higher than in the wildtype (Fig 4A). In consideration of the proposed role of *BcFLA1* in the root hair formation, the effect of the complementation of *BcFLA1* and the fact that off-target effects in plants are less frequent [6,7,14], the effect on the root hair length can be traced back to a successful disruption of *BcFLA1* gene function by CRISPR/Cas9. We believe the direct phenotyping of roots harboring deletions originating from CRISPR/Cas9, in combination with the hairy root technique, is a versatile tool, especially when the root is a focus of the research. We envision a great impact on studies e.g. in plant nutrition, plant pathogen, and plant symbiont interaction as well as root developmental biology.

Conclusion

The objective of this paper was the application of the CRISPR/Cas9 system by performing a stable transformation of *Brassica carinata* using the hairy root technique. Gene editing could be verified indirectly by qPCR as well as directly by Sanger sequencing and fragment analysis by PAGE. Additionally, we could demonstrate a phenotypic effect of the gene editing on the root hair length. Fragment analysis by PAGE turned out to be an especially successful method to uncover all gene editing events.

Supporting information

S1 Fig. Expression cassette and *BcFLA1* gRNAs. Expression cassette of CRISPR+35S::GFP+-fla1-guides (A), the complementation vector (B) and gRNAs for CRISPR/Cas9 targeting *BcFLA1* (C). For the control, the pB-CRISPR+35S::GFP vector without gRNAs was introduced into the plants. The gRNA sequences are displayed in the reverse and complement form for a better understanding.

(TIF)

S2 Fig. CRISPR/Cas9-induced deletions and insertions in *BcFLA1*. Grey background indicates the region of the gRNAs; violet letters indicate differing bases in *BcFLA1b*, from which the first two were used to discriminate between both alleles; inserted bp (red numbers) and deleted bp (on the right). Data from the independent experiment replication.

Compl = complementation. Numbers on the left indicate the respective transgenic roots.

(TIF)

S3 Fig. *BcFLA1* primers are specific for the mutant and the wildtype version of the gene, respectively. Primers for the validation of the gene editing with large products (A) and small products (B). Primers used for expression analysis (C). C = control sample, P = plasmid used for the complementation, N = no template control.

(TIF)

S4 Fig. Pretest of the qPCR primers confirming the primer specificity and revealing a successful downregulation and complementation. Expression of the endogenous control *AtUBC9* (A), *BcFLA1a_{mut}* (B), the wildtype *BcFLA1* (C) and both versions of *BcFLA1* (D). Data from independently replicated experiment.
(TIF)

S5 Fig. Expression of *BcFLA1* as affected by CRISPR/Cas9-induced gene editing and complementation, respectively. Total *BcFLA1* expression (A), expression of *BcFLA1a_{mut}* (B) and expression of the wildtype *BcFLA1* gene (C). Each bar represents the expression in one single transgenic root measured by three technical replicates with the horizontal line representing the corresponding mean of all biological replicates. Data from independently replicated experiment. Numbers indicate the respective transgenic roots. Fold over reverence = $2^{-\Delta\Delta Ct}$; n.d. = not detected.
(TIF)

S1 Text. Preparation of the constructs.

(DOCX)

S2 Text. Expression analysis.

(DOCX)

S1 Table. Primers used for construct preparation.

(DOCX)

S2 Table. qPCR primers. Wildtype and mutant-specific primers were designed on the two gRNA regions harboring three differing bases at the 3' end (underlined). The unspecific primers are located at the 3' end of *BcFLA1*.

(DOCX)

S3 Table. Primer pairs for the verification of gene editing. For M13 SSR-PCR forward primers were tagged with M13 tail GTAAAACGACGGCCAGT. Numbers in brackets indicate amplicon size including the M13 tail.

(DOCX)

Acknowledgments

The authors are very grateful to Ruth Lintermann (Freie Universität Berlin) for excellent technical assistance with the cloning of the vector and Neelke Goldenstein for her help with the transformation procedure. For helpful advice regarding the PAGE the authors would like to thank Ina Menz and Marcus Linde. We would like to thank Mounashree and Zachary Mullin-Bernstein for proofreading the manuscript.

Author Contributions

Conceptualization: Thomas W. Kirchner, Manfred K. Schenk, Marco Herde.

Data curation: Thomas W. Kirchner, Marco Herde.

Formal analysis: Thomas W. Kirchner.

Funding acquisition: Manfred K. Schenk.

Investigation: Thomas W. Kirchner, Markus Niehaus.

Methodology: Thomas W. Kirchner, Markus Niehaus, Thomas Debener, Manfred K. Schenk, Marco Herde.

Project administration: Manfred K. Schenk, Marco Herde.

Resources: Manfred K. Schenk.

Supervision: Manfred K. Schenk, Marco Herde.

Visualization: Thomas W. Kirchner.

Writing – original draft: Thomas W. Kirchner.

Writing – review & editing: Thomas W. Kirchner, Thomas Debener, Manfred K. Schenk, Marco Herde.

References

1. Shan G. RNA interference as a gene knockdown technique. *Int J Biochem Cell Biol.* 2010; 42: 1243–1251. <https://doi.org/10.1016/j.biocel.2009.04.023> PMID: 19442757
2. Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V. Editing plant genomes with CRISPR/Cas9. *Curr Opin Biotechnol.* 2015; 32: 76–84. <https://doi.org/10.1016/j.copbio.2014.11.007> PMID: 25437637
3. Zhang D, Li Z, Li J-F. Targeted gene manipulation in plants using the CRISPR/Cas technology. *J Genet Genomics.* 2016; 43: 251–262. <https://doi.org/10.1016/j.jgg.2016.03.001> PMID: 27165865
4. Barrangou R, Birmingham A, Wiemann S, Beijersbergen RL, Hornung V, van Smith AB. Advances in CRISPR-Cas9 genome engineering. Lessons learned from RNA interference. *Nucleic Acids Res.* 2015; 43: 3407–3419. <https://doi.org/10.1093/nar/gkv226> PMID: 25800748
5. Knoll A, Fauser F, Puchta H. DNA recombination in somatic plant cells. Mechanisms and evolutionary consequences. *Chromosome Res.* 2014; 22: 191–201. <https://doi.org/10.1007/s10577-014-9415-y> PMID: 24788060
6. Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang D-L, et al. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. *Proc Natl Acad Sci U S A.* 2014; 111: 4632–4637. <https://doi.org/10.1073/pnas.1400822111> PMID: 24550464
7. Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, et al. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol J.* 2014; 12: 797–807. <https://doi.org/10.1111/pbi.12200> PMID: 24854982
8. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013; 339: 819–823. <https://doi.org/10.1126/science.1231143> PMID: 23287718
9. Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, Wanner B, et al. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc Natl Acad Sci U S A.* 2011; 108: 10098–10103. <https://doi.org/10.1073/pnas.1104144108> PMID: 21646539
10. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* 2012; 337: 816–821. <https://doi.org/10.1126/science.1225829> PMID: 22745249
11. Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol.* 2013; 31: 233–239. <https://doi.org/10.1038/nbt.2508> PMID: 23360965
12. Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol Adv.* 2015; 33: 41–52. <https://doi.org/10.1016/j.biotechadv.2014.12.006> PMID: 25536441
13. Ding Y, Li H, Chen L-L, Xie K. Recent advances in genome editing using CRISPR/Cas9. *Front Plant Sci.* 2016; 7: 703. <https://doi.org/10.3389/fpls.2016.00703> PMID: 27252719
14. Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnol.* 2015; 15: 16. <https://doi.org/10.1186/s12896-015-0131-2> PMID: 25879861
15. Iaffaldano B, Zhang Y, Cornish K. CRISPR/Cas9 genome editing of rubber producing dandelion *Taraxacum kok-saghyz* using *Agrobacterium rhizogenes* without selection. *Ind Crops Prod.* 2016; 89: 356–362. <https://doi.org/10.1016/j.indcrop.2016.05.029>

16. Michno J-M, Wang X, Liu J, Curtin SJ, Kono TJ, Stupar RM. CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using a new web-tool and a modified Cas9 enzyme. *GM Crops Food*. 2015; 6: 243–252. <https://doi.org/10.1080/21645698.2015.1106063> PMID: 26479970
17. Ron M, Kajala K, Pauluzzi G, Wang D, Reynoso MA, Zumstein K, et al. Hairy root transformation using *Agrobacterium rhizogenes* as a tool for exploring cell type-specific gene expression and function using tomato as a model. *Plant Physiol*. 2014; 166: 455–469. <https://doi.org/10.1104/pp.114.239392> PMID: 24868032
18. Wang L, Wang L, Tan Q, Fan Q, Zhu H, Hong Z, et al. Efficient inactivation of symbiotic nitrogen fixation related genes in *Lotus japonicus* using CRISPR-Cas9. *Front Plant Sci*. 2016; 7: 1333. <https://doi.org/10.3389/fpls.2016.01333> PMID: 27630657
19. Veena V, Taylor CG. *Agrobacterium rhizogenes*. Recent developments and promising applications. *In Vitro Cell Dev Biol Plant*. 2007; 43: 383–403. <https://doi.org/10.1007/s11627-007-9096-8>
20. Kahl G, Molina C, Rotter B, Jüngling R, Frank A, Krezdorn N, et al. Reduced representation sequencing of plant stress transcriptomes. *J Plant Biochem Biotechnol*. 2012; 21: 119–127. <https://doi.org/10.1007/s13562-012-0129-y>
21. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, et al. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. *Mol Plant*. 2015; 8: 1274–1284. <https://doi.org/10.1016/j.molp.2015.04.007> PMID: 25917172
22. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 2003; 31: 3406–3415. <https://doi.org/10.1093/nar/gkg595> PMID: 12824337
23. Xie K, Minkenberg B, Yang Y. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc Natl Acad Sci U S A*. 2015; 112: 3570–3575. <https://doi.org/10.1073/pnas.1420294112> PMID: 25733849
24. Vieweg MF, Frühling M, Quandt H-J, Heim U, Bäumlein H, Pühler A, et al. The promoter of the *Vicia faba* L. leghemoglobin gene Vflb29 is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants. *Mol Plant Microbe Interact*. 2004; 17: 62–69. <https://doi.org/10.1094/MPMI.2004.17.1.62> PMID: 14714869
25. Vandesompele J, Preter K de, Pattyn F, Poppe B, van Roy N, Paepe A de, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002; 3: 1–12. <https://doi.org/10.1186/gb-2002-3-7-research0034>
26. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. *Nucleic Acids Res*. 2012; 40: e115. <https://doi.org/10.1093/nar/gks596> PMID: 22730293
27. Schuelke M. An economic method for the fluorescent labeling of PCR fragments. *Nat Biotechnol*. 2000; 18: 233–234. <https://doi.org/10.1038/72708> PMID: 10657137
28. Seifert GJ, Roberts K. The biology of arabinogalactan proteins. *Annu Rev Plant Biol*. 2007; 58: 137–161. <https://doi.org/10.1146/annurev.arplant.58.032806.103801> PMID: 17201686
29. Dadami E, Moser M, Zwiebel M, Krczal G, Wassenegger M, Dalakouras A. An endogene-resembling transgene delays the onset of silencing and limits siRNA accumulation. *FEBS Lett*. 2013; 587: 706–710. <https://doi.org/10.1016/j.febslet.2013.01.045> PMID: 23380068
30. Harper BK, Mabon SA, Leffel SM, Halfhill MD, Richards HA, Moyer KA, et al. Green fluorescent protein as a marker for expression of a second gene in transgenic plants. *Nat Biotechnol*. 1999; 17: 1125–1129. <https://doi.org/10.1038/15114> PMID: 10545923
31. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Res*. 2014; 42: 10903–10914. <https://doi.org/10.1093/nar/gku806> PMID: 25200087
32. Zischewski J, Fischer R, Bortesi L. Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. *Biotechnol Adv*. 2017; 35: 95–104. <https://doi.org/10.1016/j.biotechadv.2016.12.003> PMID: 28011075
33. West AG, Gaszner M, Felsenfeld G. Insulators. Many functions, many mechanisms. *Gene Dev*. 2002; 16: 271–288. <https://doi.org/10.1101/gad.954702> PMID: 11825869
34. Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, et al. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol*. 2014; 32: 1262–1267. <https://doi.org/10.1038/nbt.3026> PMID: 25184501
35. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat Biotechnol*. 2013; 31: 822–826. <https://doi.org/10.1038/nbt.2623> PMID: 23792628

36. Lawrenson T, Shorinola O, Stacey N, Li C, Ostergaard L, Patron N, et al. Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol.* 2015; 16: 258. <https://doi.org/10.1186/s13059-015-0826-7> PMID: [26616834](https://pubmed.ncbi.nlm.nih.gov/26616834/)
37. Fauser F, Schiml S, Puchta H. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J.* 2014; 79: 348–359. <https://doi.org/10.1111/tpj.12554> PMID: [24836556](https://pubmed.ncbi.nlm.nih.gov/24836556/)
38. Wang K, Mei DY, Liu QN, Qiao XH, Ruan WM, Huang T, et al. Research of methods to detect genomic mutations induced by CRISPR/Cas systems. *J Biotechnol.* 2015; 214: 128–132. <https://doi.org/10.1016/j.jbiotec.2015.09.029> PMID: [26419205](https://pubmed.ncbi.nlm.nih.gov/26419205/)

General Discussion

Plants react to Pi deficiency with a series of different mechanisms leading to a higher Pi utilization and uptake efficiency (Raghothama, 2000). A complex signaling network of transcription factors, enzymes, phytohormones, small RNAs and other small molecules, underlies these low Pi responses. Components of this network can be regulated transcriptionally and post-transcriptionally and can be involved in both local and systemic signaling pathways (Plaxton and Tran, 2011). Even an interaction with signaling pathways regulating the homeostasis of other nutrients was described (Medici *et al.*, 2015). In recent years, many elements of the Pi starvation responses (PSR) have been identified, but the network is still far from being complete.

The aim of this study was to identify regulatory components involved in the development of longer root hairs under Pi deficiency, which plays an important role in enhancing the Pi uptake in *Brassicaceae*. For it, we compared genome-wide expression profiles of two *B. carinata* cultivars differing in their root hair length under Pi deficiency. Since a low expression level of regulatory genes and a moderate alteration of their transcript levels is enough to cause strong effects in a regulatory network for example due to feedback loops, we used MACE for the preparation of the expression profiles, which allows a high resolution and accurate quantification (Kahl *et al.*, 2012). Hundreds of differentially expressed genes were observed and indeed, we were able to confirm the regulation of nearly all selected candidate genes by qPCR. A split-root experiment not only revealed the involvement of both local and systemic signaling pathways in the Pi deficiency-induced root hair development, but also gave hints in which of both signaling pathways the candidate genes are involved. Furthermore, cultivation of the plants under N- and K deficiency led to the identification of P-specific genes, most probably acting in an independent pathway, which merges with the general root hair elongation pathway at a so far unknown position. Targeting of these genes by CRISPR/Cas9 led to an overall strong reduction of the transcript level of the respective genes in the transgenic hairy roots. Shorter root hairs in the *bcfla1* roots under Pi starvation compared to the wildtype indicated a role of *BcFLA1* in a P-specific root hair elongation pathway, which was supported by restoration of the wildtype root hair length when a synthetic, CRISPR-resistant *BcFLA1* gene was simultaneously overexpressed. Furthermore, the fragment analysis by PAGE allowed

us to determine the exact number of base pair indels in order to make a precise statement on the occurrence of a knockout.

The following sections will discuss certain aspects of this work as an addition to the discussion in Chapter I regarding the functioning of BcFLA1 and its position in the Pi deficiency signaling network, the possible role of the other candidate genes which were not selected for the knockout, as well as the potential and limits of the applied methods, arisen problems and future studies.

1. Functioning and position of BcFLA1 in the Pi deficiency signaling network

According to the results of this study, BcFLA1 may be involved in a pathway, which translocates the low Pi signal to the root hair elongation pathway (Fig. 1). This P-specific pathway may merge with a pathway translocating environmental cues to a putative root hair-specific transcription factor (RHF) which senses both developmental and environmental signals and controls downstream root hair genes, thus leading to a balanced root hair elongation (Hwang *et al.*, 2016). However, we were not able to assign an exact place for BcFLA1 in the Pi starvation signaling network. It has to be evaluated, if BcFLA1 acts up- or downstream of auxin. According to Salazar-Henao and Schmidt (2016) Pi acts rather upstream of auxin, which would indicate the same for BcFLA1. However, *BcLRL3L* was also P-specific reacting in this study, but its relative in Arabidopsis seems to act downstream of auxin since it was auxin-responsive (Salazar-Henao and Schmidt, 2016). Maybe there are several points in the environmental pathway which are influenced by Pi deficiency. To locate BcFLA1 more precisely in this network, it should be evaluated in future studies, if BcFLA1 also responds to auxin.

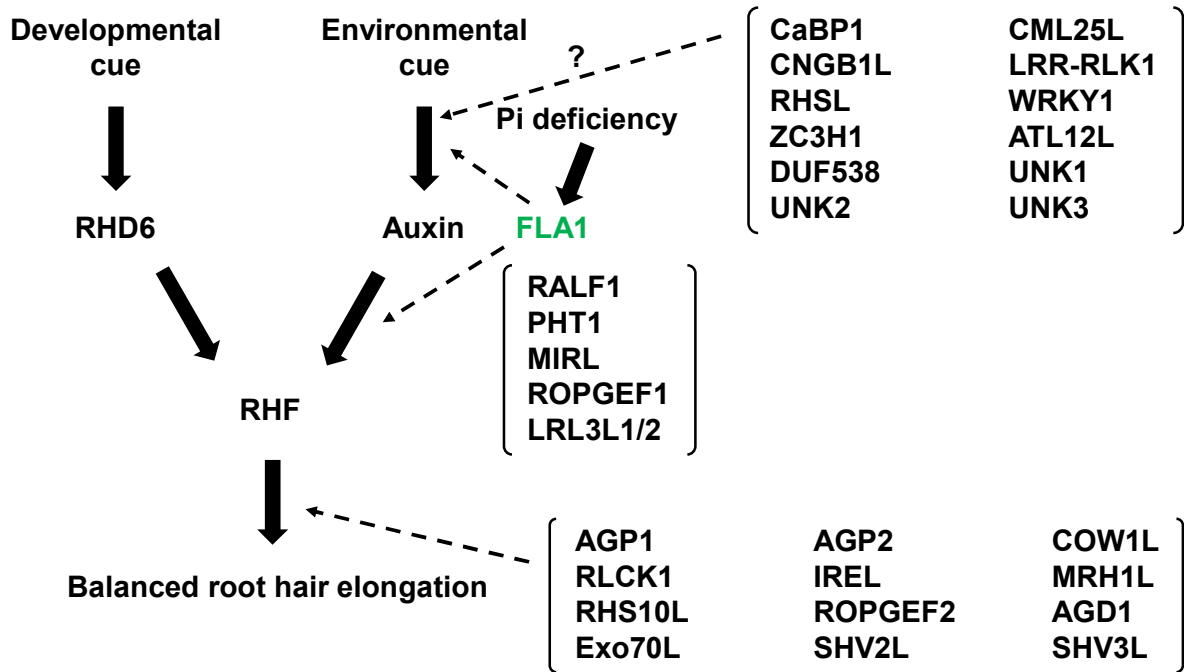


Fig. 1: Scheme showing the potential location of BcFLA1 and the other candidate gene products in the root hair signaling network. BcFLA1 may transfer the Pi starvation signal to the universal environmental pathway which merges with the developmental pathway in the form of a putative root hair-specific transcription factor (RHF). The latter processes both developmental and environmental signals to enable a balanced root hair elongation. Dashed arrows indicate possible target sites.

Another open question is the functional mechanism of the putative signal perception and transduction by BcFLA1. A topology prediction of BcFLA1 revealed that the C-terminal end of BcFLA1 is embedded in the plasma membrane via the GPI-anchor and that the rest of the protein is outside the cell (Fig. S1). Since the protein consists of two AG-rich regions surrounding the fasciclin domain, it could be speculated, that the first AG-rich region of BcFLA1 passes through the cell wall, the fasciclin domain is located on the surface of the cell wall with contact to the extracellular matrix, and the second AG-rich region is again located in the cell wall. Low Pi signal perception could therefore be mediated by the interaction of the fasciclin domain with signal molecules in the extracellular matrix. Then the signal may be transmitted to a putative cell wall protein kinase, whose extracellular domain may be connected to the AG regions of BcFLA1 within the cell wall, like it was proposed in a model regarding another FLA protein in Arabidopsis (Basu *et al.*, 2016). This may in turn lead to autophosphorylation of the cytosolic kinase domain of the putative protein kinase, leading to downstream signaling events for the Pi deficiency-induced root hair elongation.

2. Role of the other candidate genes in the root hair elongation pathway

As discussed in Chapter I, even if *BcFLA1* was the only gene whose downregulation led to a reduced root hair length under Pi starvation, a role of the other downregulated candidate genes in the Pi deficiency-induced root hair elongation cannot be ruled out (Fig. 1). The same applies to the rest of the candidate genes which were not further investigated by knockdown. Even though they reacted to both Pi and N deficiency, which indicated rather a general role during root hair elongation, it is also possible that they act in an environmental pathway independent of the general root hair elongation pathway in which both Pi and N starvation signals are processed. Or at least some of them could be regulatory components shared by both P- and N-specific pathways which, apart from that, may act separately. The convergence of P and N signaling has already been described in terms of the transcription factor HYPERSENSITIVE TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1 (HRS1), which was shown to be regulated by both NO_3^- and Pi (Medici *et al.*, 2015). In addition, the upregulation of these candidate genes was overall higher during Pi than during N deficiency although the root hair length was higher under N deficiency, which rather speaks against a downstream role in the general root hair elongation pathway. Due to the complex network of regulatory mechanisms it is worthwhile to further investigate the most-promising of the remaining candidate genes. Therefore, in the following paragraphs the individual genes of the candidate gene groups (Chapter I Table S7) are discussed regarding their involvement in the root hair growth and results are concluded in Fig. 1.

Arabinogalactan proteins

As mentioned in Chapter I section 4.1, arabinogalactan proteins can be involved in cell wall remodeling and synthesis, but also in signaling processes (Seifert and Roberts, 2007). Considering that *BcAGP1* and *BcAGP2* responded to both Pi and N deficiency and that cell wall remodeling plays an important role during cell growth, these AGPs may indeed have rather a general role during root hair elongation. This assumption is supported by (i) the positive effect of the relative of *BcAGP2* in Arabidopsis (*AtAGP24*) on the cell wall rigidity (Mabuchi *et al.*, 2016), which indicates a role in cell wall remodeling. Furthermore, (ii) the expression of the relative of *BcAGP1* in Arabidopsis root hairs (*AtAGP3*) was dependent on RSL4 (Salazar-Henao and Schmidt, 2016), indicating that *AtAGP3* acts downstream of RSL4, so that both developmental and environmental signals should be processed to *AtAGP3* (Datta *et al.*, 2015).

Additionally, (iii) both AGPs consist of a N-terminal signal peptide, a region with AG motifs and a C-terminal GPI-anchor (Table S1), but they lack a special domain, which is predestinated for signaling as in BcFLA1.

Calcium-related proteins

The calcium-related genes *BcCaBP1* and *CALMODULIN-LIKE 25-LIKE (BcCML25L)* both encode a calcium-binding EF-hand family protein, which may regulate root hair elongation via Ca^{2+} signaling. The counterpart of *BcCaBP1* in Arabidopsis was not related to root hair growth or Pi starvation until now, so that it would be interesting to evaluate, if it is involved in general root hair growth mechanisms or if it acts in the environmental pathway. Interestingly, the relative of *BcCML25L* in Arabidopsis (*AtCML25*) was downregulated in response to Pi starvation (Lin *et al.*, 2011), therefore exhibiting an opposite expression pattern compared to *BcCML25L*. Since *atcml25* knockout mutants exhibited longer root hairs exclusively under Pi deficiency (Lin *et al.*, 2011), a P-specific role could be assumed. However, recently, *AtCML25* was assigned a role in pollen tube elongation by regulating K^+ influx via Ca^{2+} activity (Wang *et al.*, 2015), which could indicate rather a general role in cell elongation. *CYCLIC NUCLEOTIDE-GATED CATION CHANNEL BETA-1-LIKE (BcCNGB1L)* seems to encode for a cyclic nucleotide-gated cation channel (CNGC). These channels are permeable for K^+ and Ca^{2+} and are regulated by binding of cyclic nucleotides (Leng *et al.*, 1999) or calmodulin (Köhler and Neuhaus, 2000). Therefore, they are thought to be involved in various signal transduction pathways (Leng *et al.*, 1999; Köhler and Neuhaus, 2000). Due to the unspecific response of *BcCNGB1L* to both Pi and N deficiency, *BcCNGB1L* could play a general role during root hair growth either by developmental signaling or by establishing the Ca^{2+} gradient or by setting the K^+ concentration for the turgor. However, until now there is no study describing an involvement of these channels in root hair development. But, in a study investigating CNGC genes in rice, the involvement of CNGCs in *inter alia* hormonal and abiotic stress responses was predicted (Nawaz *et al.*, 2014), so that a role of *BcCNGB1L* in the environmental pathway of the root hair elongation would also be conceivable. As the last gene of the group of calcium-related genes *CAN OF WORMS 1 (BcCOW1)* encoding a phosphatidylinositol transfer protein (PITP) was selected from the MACE gene list. Since, *AtCOW1* was already suggested to have an essential role in establishing the calcium gradient during root hair elongation (Böhme *et al.*, 2004), and

additionally a regulating role during the Pi deficiency-induced root hair growth could also be assumed, because PITPs can regulate phosphoinositides, which are known to be involved in various signaling pathways (Böhme *et al.*, 2004; Balla, 2013). However, the upregulation of *BcCOW1* by both Pi and N deficiency rather pleads for the general role during root hair elongation and this assumption is supported by a role of AtCOW1 downstream of RSL4 as mentioned for AtAGP3 above (Salazar-Henao and Schmidt, 2016).

Protein kinases

From the protein kinases, the counterparts of *LEUCINE-RICH REPEAT RECEPTOR-LIKE SERINE/THREONINE PROTEIN KINASE 1* (*BcLRR-RLK1*) and *ROOT HAIR-SPECIFIC 10-LIKE* (*BcRHS10L*) in Arabidopsis exhibited a high edge count in the co-expression analysis of Lan *et al.* (2013) like the relative of *BcMRIL*, thus indicating a key role during root hair growth. This assumption is supported by the fact, that the relative of *BcLRR-RLK1* in Arabidopsis was neither responsive to auxin, nor dependent on a functional RSL4 protein, indicating that it acts upstream of auxin and RSL4 (Salazar-Henao and Schmidt, 2016). *BcRHS10L* encodes for a proline-rich extensin-like receptor kinase, which possesses both an extracellular domain with AG motifs and an intracellular kinase domain (Table S1). Recently, the relative of *BcRHS10L* in Arabidopsis (*AtRHS10*) was shown to negatively regulate the root hair growth duration and therefore the final root hair length (Hwang *et al.*, 2016; Cho, 2016). Interestingly, the AG repeats in the extracellular region of AtRHS10 were necessary for this regulation. The authors postulated, that changes in the cell wall integrity caused by the turgor pressure in the tip-growing root hair cell, are sensed by the AGP motifs leading to a signal transduction to the intracellular kinase domain, which is then autophosphorylated and therefore able to activate downstream targets. Therefore, the predicted way of the signal transduction into the cell is similar to that predicted for BcFLA1 with the exception, that the kinase domain is located in the same protein containing the AG motifs. However, since this role of AtRHS10 was suggested to be a general mechanism during root hair elongation (Hwang *et al.*, 2016; Cho, 2016), and taken into account that *BcRHS10L* was upregulated under both P- and N-deficient conditions, it may act rather downstream of the putative RHF as it was expected by Hwang *et al.* (2016), meaning that Pi and N deficiency only intensified its general function leading to the enhanced root hair elongation. The same applies to

MORPHOGENESIS OF ROOT HAIR 1 (BcMRH1L) and *INCOMPLETE ROOT HAIR ELONGATION-LIKE (BcIREL)*, whose relatives in Arabidopsis were shown to be necessary for a proper root hair elongation (Oyama *et al.*, 2002; Jones *et al.*, 2006). The sixth protein kinase, BcRLCK1, may also have a general role in cell growth, because its counterpart in Arabidopsis connected the root hair-related sub cluster with the pollen tube-related sub cluster in the co-expression analysis of Lan *et al.* (2013).

GTPase-related

Besides *BcROPGEF1*, *BcROPGEF2*, *ADENOSINE DIPHOSPHATE (ADP) RIBOSYLATION FACTOR (ARF)-GTPASE ACTIVATING PROTEIN (GAP) DOMAIN-CONTAINING PROTEIN 1 (BcAGD1)* and *EXOCYST COMPLEX COMPONENT EXO70-LIKE (BcEXO70L)* were grouped to GTPase-related proteins. The relative of *BcROPGEF2* in Arabidopsis, *AtROPGEF10*, marginally affected root hair length and had strong effects on the root hair initiation, but its action was classified to the developmental root hair pathway (Huang *et al.*, 2013b). *BcAGD1* encodes for a gene belonging to another group of GTPase-activating proteins, whose members are part of different signaling pathways and may also coordinate actin and membrane remodeling (Nie *et al.*, 2003). Furthermore, *BcAGD1* possesses a C2 domain, which is known to be involved in membrane trafficking and signaling (Leonard, 2013). One member of the AGD family was already shown to be implicated in the control of the root hair polarity (Yoo *et al.*, 2012). *BcEXO70L* belongs to the EXO70 family, whose members can interact with Rho GTPases (Wu *et al.*, 2010) and function in polarized cell growth by regulating the secretion of specific vesicles (He *et al.*, 2007). The exocyst can direct secretory vesicles from the Golgi apparatus to specific locations on the plasma membrane (He and Guo, 2009). Most recently, a member of the EXO70 family was shown to regulate the tip growth in pollen tubes of Arabidopsis (Synek *et al.*, 2017). Based on this information and taken into account the unspecific response to Pi and N-deficiency, *BcROPGEF2*, *BcAGD1* and *BcEXO70L* may have rather a general role during root hair growth downstream of the putative RHF.

Root hair affecting

ROOT HAIR-SPECIFIC-LIKE (BcRHSL), *BcSHV2L* and *BcSHV3L* were grouped as root hair affecting genes. The relative of *BcRHSL* in Arabidopsis was already shown to be expressed in root hair cells in a large transcriptomic analysis, in which the

expression of *AtRHS* was induced by both RHD6 and auxin as well as ethylene (Bruex *et al.*, 2012), indicating rather a role downstream of RHF. However, an exact function in root hair growth was not attributed to *AtRHS* until now. *AtSHV2* and *AtSHV3* are named after the root hair phenotype of their knockout mutants, which possess shorter root hairs than the corresponding wildtypes. Both genes encode for GPI-anchored proteins and were suggested to be necessary for the transition to the tip growth in root hairs (Parker *et al.*, 2000; Jones *et al.*, 2006). *BcSHV2L* possesses a COBRA domain, which was proposed to play a key role in oriented cell expansion (Schindelman *et al.*, 2001; Roudier *et al.*, 2002). *BcSHV3L* encodes a glycerophosphoryl diesterphosphodiesterase (GPDP)-like protein (Borner *et al.*, 2003). Both *AtSHV2* and *AtSHV3* were suggested to have signaling / regulating roles during cell growth by affecting the cellulose synthesis (Jones *et al.*, 2006; Yeats *et al.*, 2016), which indicates a general function during root hair growth, supported by the unspecific response of *BcSHV2L* and *BcSHV3L* in this study. However, another GPDP protein was suggested to release Pi from phospholipids under Pi deficiency (Cheng *et al.*, 2011). If *BcSHV3L* would have this additional role, this could explain the by tendency stronger upregulation of *BcSHV3L* under Pi- compared to N-deficient conditions.

Transcription factors

Besides the two genes encoding for bHLH transcription factors, a gene encoding for a WRKY transcription factor, *WRKY TRANSCRIPTION FACTOR 1* (*BcWRKY1*), was selected from the MACE gene list. Interestingly, its relative in Arabidopsis (*AtWRKY42*) was not related to root hair growth until now. Instead, it is thought to be involved amongst others in modulation of the Pi homeostasis by regulating the expression of the Pi transporter PHT1;1 (Su *et al.*, 2015). Therefore, a connection between its role in regulating the Pi homeostasis and its putative role in root hair growth indicated in this study could be hypothesized.

Zinc finger proteins

Similar to *BcRHSL*, the relative of *ZINC FINGER CCCH DOMAIN-CONTAINING PROTEIN 1* (*BcZC3H1*) in Arabidopsis (*AtZC3H18*) was already related to root hair growth (Bruex *et al.*, 2012) without knowing its exact function. The family of ZC3H proteins has an important role in RNA processing and many members responded to biotic and abiotic stresses with an altered expression (Wang *et al.*, 2008).

ARABIDOPSIS TÓXICOS EN LEVADURA (ATL) 12-LIKE (BcATL12L) encodes for a putative RING-H2 finger protein, which may function as a E3 ubiquitin ligase (Serrano and Guzmán, 2004; Serrano *et al.*, 2006), therefore having the potential to alter the occurrence, activity and/or location of proteins. Due to their potential involvement in post-transcriptional and post-translational modification / regulation, respectively, *BcZC3H1* and *BcATL12L* could be candidates for further investigations.

To conclude, a further investigation of *BcCaBP1*, *BcCML25L*, *BcCNGB1L*, *BcLRR-RLK1*, *BcRHSL*, *BcWRKY1*, *BcZC3H1* and *BcATL12L* is most promising, since the current state of knowledge suggests a role in the environmental root hair elongation pathway (Fig. 1). The same applies to the unknown proteins as they contain unknown domains, which may have regulatory functions and/or may belong to new groups of regulators.

3. Potential and limits of the applied methods

MACE turned out to be an appropriate method to obtain information about the regulation of a large number of genes in response to Pi deficiency, which could be largely confirmed by qPCR. However, since we did not apply a usual RNAseq method using the same RNA samples, it is not possible to draw a suitable comparison regarding the resolution of the MACE. A disadvantage of the MACE is that it produces only the 3' ends of the genes, so that the gene sequences had to be extended by 5'-RACE. This was cumbersome, especially because often several attempts were needed for a successful amplification of the lacking sequence. For some genes, no or only a sequence belonging to another isoform was amplified. Gene isoforms were a general problem, since we were working with an unsequenced non-model organism without knowledge of the isoforms existing for a gene. Furthermore, it was often impossible to assign a gene from *B. carinata*, which was annotated to a designated *B. rapa / napus* gene, to a specific *A. thaliana* gene, since in several cases numerous *B. rapa / napus* genes were assigned to only one *A. thaliana* gene. This impedes a secure statement about the gene function based on the well-characterized Arabidopsis genes. However, this is a general disadvantage of working with non-model organisms. But, the approach of comparing *B. carinata* cv. Bale and cv. Bacho under different Pi regimes to obtain root hair-related genes, which play a role at least *inter alia* during Pi starvation, was successful, since no gene responded to the non-root hair affecting K

deficiency and many known root hair-related genes from *Arabidopsis* were among the candidate genes.

As discussed in Chapter II, the combination of the hairy root technique with CRISPR/Cas9 is a suitable method to rapidly investigate potential root effects of a gene knockout, and the fragment analysis by PAGE represented an appropriate method for the indel detection. However, the transformation efficiency of the *B. carinata* plants was very low, so that a large number of plants had to be inoculated with the agrobacteria. Therefore, the transformation method needs to be optimized for *B. carinata* when conducting future experiments, especially because a large number of transgenic roots is needed for the root hair length determination due to the strong variability in the root hair length, which was observed in some cases.

This study was based on a transcriptomic analysis. However, post-transcriptional and post-translational regulation plays an important role in the low Pi signaling network. For example, transcripts of the E2 ubiquitin conjugase UBC24 are destructed after binding of miR399, which in turn impedes the degradation of downstream targets of UBC24 (Plaxton and Tran, 2011). Furthermore, a central transcription factor of the Pi homeostasis, PHR1 is active only after sumoylation (Briat *et al.*, 2015). Moreover, due to the potential involvement of a row of protein kinases, post-translational modifications seem to play a key role also in the root hair response to Pi starvation. Additionally, Thieme *et al.* (2015) observed a large number of mobile mRNAs, that are transported to distant tissues, where they may be translated into proteins or work directly as regulating RNAs, thus being part of systemic signaling in response to stresses. For example, the transcript of *AtIPS2*, which is involved in the regulation of the Pi homeostasis, was shown to be bidirectional mobile. Thus, gene transcript level in a cell will not necessarily reflect the gene action in a cell. (Thieme *et al.*, 2015). Therefore, to completely understand the network of regulatory mechanisms leading to the Pi deficiency-induced root hair elongation, it is indispensable to also have a look at the protein level.

4. Outlook

The results from this study indicated a role of BcFLA1 in a P-specific signaling pathway leading to an increased root hair length under Pi deficiency. For further characterization, it will be useful to locate the BcFLA1 protein in the root tissue via immunostaining by designing a specific antibody for BcFLA1, or by fusing the *BcFLA1* gene with for example a Strep-tag[®], thus allowing the detection with a fluorescent or enzymatic marker. Furthermore, since phytohormones play key roles in stress responses, it could be evaluated, if *BcFLA1* responds to phytohormone supply with an altered expression level, thus indicating its site of action in relation to the respective phytohormones. Hints about signaling cascades will also be obtained by investigating the effect of the down- or upregulated *BcFLA1* gene on the expression of the other candidate genes in the transgenic hairy roots. However, as mentioned in Chapter I section 4.3, the root hair number has to be taken into account, since it may be altered due to the modified expression. To locate BcFLA1 more precisely in the regulatory network for Pi deficiency-induced root hair elongation, the study of protein-protein interactions is necessary. This could also be done by Strep-tag[®] fusion, because the latter allows the purification of protein complexes. Protein-protein interactions *in vivo* can be investigated by the two-hybrid system.

Five genes did not exhibit a root hair response after knockdown of their gene function in the transgenic hairy root. However, as mentioned before, the root hair response may not have been visible due to a replaced gene function by redundantly acting isoforms. If gene isoforms are known like in the case of *BcLRL3L*, the tRNA-gRNA system applied in this study could be used to target several isoforms of one gene with numerous gRNAs, so that these isoforms will be knocked out simultaneously, thus reducing the possibility of redundantly acting isoforms. Additionally, the five genes could be further investigated by overexpression.

Some of the candidate genes were not investigated by knockout in this study, but have the potential to be involved in the environmental root hair pathway (Fig. 1), so that a functional analysis of these genes by knockdown and overexpression is promising.

Since post-transcriptional and post-translational regulation as well as mRNA mobility seem to play an important role in the Pi deficiency-induced root hair growth, differentially abundant proteins in root tips of cv. Bale and cv. Bacho under Pi deficiency compared to sufficient Pi conditions may be investigated by a two-

dimensional gel electrophoresis and the analysis of the differentially abundant spots by mass spectrometry.

Furthermore, because of the important role of miRNAs in the P signaling network, the separately isolated small RNAs from the pool samples of the MACE experiment could be used to generate genome-wide miRNA expression profiles as it was done for the mRNAs, to identify differentially expressed miRNAs putatively involved in the Pi deficiency-induced root hair growth.

References

- An L, Zhou Z, Sun L et al.** 2012. A zinc finger protein gene *ZFP5* integrates phytohormone signaling to control root hair development in Arabidopsis. *The Plant Journal for Cell and Molecular Biology* **72**, 474–490.
- Ayadi A, David P, Arrighi J-F et al.** 2015. Reducing the genetic redundancy of Arabidopsis PHOSPHATE TRANSPORTER1 transporters to study phosphate uptake and signaling. *Plant Physiology* **167**, 1511–1526.
- Balla T.** 2013. Phosphoinositides: Tiny lipids with giant impact on cell regulation. *Physiological Reviews* **93**, 1019–1137.
- Baluska F, Salaj J, Mathur J et al.** 2000. Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Developmental Biology* **227**, 618–632.
- Bari R, Pant BD, Stitt M, Scheible W-R.** 2006. PHO2, MicroRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology* **141**, 988–999.
- Barrangou R, Birmingham A, Wiemann S, Beijersbergen RL, Hornung V, van Smith AB.** 2015. Advances in CRISPR-Cas9 genome engineering: Lessons learned from RNA interference. *Nucleic Acids Research* **43**, 3407–3419.
- Basu D, Tian L, Debrosse T et al.** 2016. Glycosylation of a fasciclin-like arabinogalactan-Protein (SOS5) mediates root growth and seed mucilage adherence via a cell wall receptor-like kinase (FEI1/FEI2) pathway in Arabidopsis. *PLoS ONE* **11**, e0145092.
- Bates TR, Lynch JP.** 1996. Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. *Plant, Cell & Environment* **19**, 529–538.

References

- Belhaj K, Chaparro-Garcia A, Kamoun S, Patron NJ, Nekrasov V.** 2015. Editing plant genomes with CRISPR/Cas9. *Current Opinion in Biotechnology* **32**, 76–84.
- Bibikova TN, Blancaflor EB, Gilroy S.** 1999. Microtubules regulate tip growth and orientation in root hairs of *Arabidopsis thaliana*. *The Plant Journal for Cell and Molecular Biology* **17**, 657–665.
- Bibikova TN, Jacob T, Dahse I, Gilroy S.** 1998. Localized changes in apoplastic and cytoplasmic pH are associated with root hair development in *Arabidopsis thaliana*. *Development* **125**, 2925–2934.
- Bibikova TN, Zhigilei A, Gilroy S.** 1997. Root hair growth in *Arabidopsis thaliana* is directed by calcium and an endogenous polarity. *Planta* **203**, 495–505.
- Bialeski RL.** 1973. Phosphate pools, phosphate transport, and phosphate availability. *Annual Review of Plant Physiology* **24**, 225–252.
- Böhme K, Li Y, Charlot F et al.** 2004. The Arabidopsis *COW1* gene encodes a phosphatidylinositol transfer protein essential for root hair tip growth. *The Plant Journal for Cell and Molecular Biology* **40**, 686–698.
- Boisson-Dernier A, Franck CM, Lituiev DS, Grossniklaus U.** 2015. Receptor-like cytoplasmic kinase MARIS functions downstream of CrRLK1L-dependent signaling during tip growth. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 12211–12216.
- Borner GHH, Lilley KS, Stevens TJ, Dupree P.** 2003. Identification of glycosylphosphatidylinositol-anchored proteins in Arabidopsis. A proteomic and genomic analysis. *Plant Physiology* **132**, 568–577.
- Bortesi L, Fischer R.** 2015. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnology Advances* **33**, 41–52.

References

- Bouché N, Bouchez D.** 2001. Arabidopsis gene knockout: Phenotypes wanted. *Current Opinion in Plant Biology* **4**, 111–117.
- Bremer M.** 2010. Molecular mechanisms of root hair growth induced by Pi deficiency in *Brassica carinata*. Dissertation, Gottfried Wilhelm Leibniz Universität Hannover, Hanover.
- Briat J-F, Rouached H, Tissot N, Gaymard F, Dubos C.** 2015. Integration of P, S, Fe, and Zn nutrition signals in *Arabidopsis thaliana*: Potential involvement of PHOSPHATE STARVATION RESPONSE 1 (PHR1). *Frontiers in Plant Science* **6**, 290.
- Bruex A, Kainkaryam RM, Wieckowski Y et al.** 2012. A gene regulatory network for root epidermis cell differentiation in Arabidopsis. *PLoS Genetics* **8**, e1002446.
- Bustos R, Castrillo G, Linhares F et al.** 2010. A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. *PLoS Genetics* **6**, e1001102.
- Campbell L, Turner SR.** 2017. A comprehensive analysis of RALF proteins in green plants suggests there are two distinct functional groups. *Frontiers in Plant Science* **8**, 37.
- Carol RJ, Dolan L.** 2002. Building a hair: Tip growth in *Arabidopsis thaliana* root hairs. *Philosophical Transactions of the Royal Society B: Biological Sciences* **357**, 815–821.
- Carol RJ, Takeda S, Linstead P et al.** 2005. A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. *Nature* **438**, 1013–1016.
- Chandrika NNP, Sundaravelpandian K, Yu S-M, Schmidt W.** 2013. ALFIN-LIKE 6 is involved in root hair elongation during phosphate deficiency in Arabidopsis. *New Phytologist* **198**, 709–720.

References

- Cheng Y, Zhou W, El Sheery NI et al.** 2011. Characterization of the Arabidopsis glycerophosphodiester phosphodiesterase (GDPD) family reveals a role of the plastid-localized AtGDPD1 in maintaining cellular phosphate homeostasis under phosphate starvation. *The Plant Journal for Cell and Molecular Biology* **66**, 781–795.
- Chiou T-J, Lin S-I.** 2011. Signaling network in sensing phosphate availability in plants. *Annual Review of Plant Biology* **62**, 185–206.
- Cho H-T.** 2016. Arabinogalactan protein motif-containing receptor-like kinases are likely to play the negative feedback factor to maintain proper root hair length. *Plant Signaling & Behavior* **11**, e1226454.
- Cho H-T, Cosgrove DJ.** 2002. Regulation of root hair initiation and expansin gene expression in Arabidopsis. *The Plant Cell* **14**, 3237–3253.
- Cong L, Ran FA, Cox D et al.** 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823.
- Cordell D, Drangert J-O, White S.** 2009. The story of phosphorus: Global food security and food for thought. *Global Environmental Change* **19**, 292–305.
- Cosgrove DJ.** 1997. Relaxation in a high-stress environment: The molecular bases of extensible cell walls and cell enlargement. *The Plant Cell* **9**, 1031–1041.
- Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R.** 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology* **139**, 5–17.
- Dadami E, Moser M, Zwiebel M, Krczal G, Wassenegger M, Dalakouras A.** 2013. An endogene-resembling transgene delays the onset of silencing and limits siRNA accumulation. *FEBS Letters* **587**, 706–710.

References

- Daras G, Rigas S, Tsitsekian D, Iacovides TA, Hatzopoulos P.** 2015. Potassium transporter TRH1 subunits assemble regulating root-hair elongation autonomously from the cell fate determination pathway. *Plant Science* **231**, 131–137.
- Datta S, Kim CM, Pernas M et al.** 2011. Root hairs: Development, growth and evolution at the plant-soil interface. *Plant and Soil* **346**, 1–14.
- Datta S, Prescott H, Dolan L.** 2015. Intensity of a pulse of RSL4 transcription factor synthesis determines Arabidopsis root hair cell size. *Nature Plants* **1**, 1–6.
- Desbrosses G, Josefsson C, Rigas S, Hatzopoulos P, Dolan L.** 2003. AKT1 and TRH1 are required during root hair elongation in Arabidopsis. *Journal of Experimental Botany* **54**, 781–788.
- Ding Y, Li H, Chen L-L, Xie K.** 2016. Recent advances in genome editing using CRISPR/Cas9. *Frontiers in Plant Science* **7**, 703.
- Dolan L, Duckett CM, Grierson C et al.** 1994. Clonal relationships and cell patterning in the root epidermis of Arabidopsis. *Development* **120**, 2465–2474.
- Du C, Li X, Chen J et al.** 2016. Receptor kinase complex transmits RALF peptide signal to inhibit root growth in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E8326-E8334.
- Duan Q, Kita D, Li C, Cheung AY, Wu H-M.** 2010. FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 17821–17826.
- Duff SM, Plaxton WC, Lefebvre DD.** 1991. Phosphate-starvation response in plant cells: De novo synthesis and degradation of acid phosphatases. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 9538–9542.

References

- Eticha D, Schenk MK.** 2001. Phosphorus efficiency of cabbage varieties. In: Horst WJ, Schenk MK, Bürkert A *et al.*, eds. *Plant nutrition – Food security and sustainability of agro-ecosystems through basic and applied research*, 542–543.
- Etienne-Manneville S, Hall A.** 2002. Rho GTPases in cell biology. *Nature* **420**, 629–635.
- Faik A, Abouzouhair J, Sarhan F.** 2006. Putative fasciclin-like arabinogalactan-proteins (FLA) in wheat (*Triticum aestivum*) and rice (*Oryza sativa*): Identification and bioinformatic analyses. *Molecular Genetics and Genomics MGG* **276**, 478–494.
- Fausser F, Roth N, Pacher M *et al.*** 2012. In planta gene targeting. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 7535–7540.
- Feng Z, Mao Y, Xu N *et al.*** 2014. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 4632–4637.
- Feng Z, Zhang B, Ding W *et al.*** 2013. Efficient genome editing in plants using a CRISPR/Cas system. *Cell Research* **23**, 1229–1232.
- Foreman J, Demidchik V, Bothwell JHF *et al.*** 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422**, 442–446.
- Franco-Zorrilla JM, Valli A, Todesco M *et al.*** 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. *Nature Genetics* **39**, 1033–1037.
- Galway ME, Heckman JW, Schiefelbein JW.** 1997. Growth and ultrastructure of *Arabidopsis* root hairs: The *rhd3* mutation alters vacuole enlargement and tip growth. *Planta* **201**, 209–218.

References

- Galway ME, Masucci JD, Lloyd AM, Walbot V, Davis RW, Schiefelbein JW.** 1994. The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. *Developmental Biology* **166**, 740–754.
- Gericke S, Kurmies B.** 1952. Die kolorimetrische Phosphorsäurebestimmung mit Ammonium-Vanadat-Molybdat und ihre Anwendung in der Pflanzenanalyse. *Zeitschrift für Pflanzenernährung und Bodenkunde* **59**, 235–247.
- Gilroy S, Jones DL.** 2000. Through form to function: Root hair development and nutrient uptake. *Trends in Plant Science* **5**, 56–60.
- Grunwald U, Guo W, Fischer K et al.** 2009. Overlapping expression patterns and differential transcript levels of phosphate transporter genes in arbuscular mycorrhizal, Pi-fertilised and phytohormone-treated *Medicago truncatula* roots. *Planta* **229**, 1023–1034.
- Haruta M, Sabat G, Stecker K, Minkoff BB, Sussman MR.** 2014. A peptide hormone and its receptor protein kinase regulate plant cell expansion. *Science* **343**, 408–411.
- He B, Guo W.** 2009. The exocyst complex in polarized exocytosis. *Current Opinion in Cell Biology* **21**, 537–542.
- He B, Xi F, Zhang J, TerBush D, Zhang X, Guo W.** 2007. Exo70p mediates the secretion of specific exocytic vesicles at early stages of the cell cycle for polarized cell growth. *The Journal of Cell Biology* **176**, 771–777.
- Huang G-Q, Gong S-Y, Xu W-L et al.** 2013a. A fasciclin-like arabinogalactan protein, GhFLA1, is involved in fiber initiation and elongation of cotton. *Plant Physiology* **161**, 1278–1290.
- Huang G-Q, Li E, Ge F-R et al.** 2013b. Arabidopsis RopGEF4 and RopGEF10 are important for FERONIA-mediated developmental but not environmental regulation of root hair growth. *New Phytologist* **200**, 1089–1101.

References

- Huang L, Shi X, Wang W, Ryu KH, Schiefelbein J.** 2017. Diversification of root hair development genes in vascular plants. *Plant Physiology*, pp.00374.2017.
- Hwang Y, Lee H, Lee Y-S, Cho H-T.** 2016. Cell wall-associated ROOT HAIR SPECIFIC 10, a proline-rich receptor-like kinase, is a negative modulator of Arabidopsis root hair growth. *Journal of Experimental Botany* **67**, 2007–2022.
- Ishida T, Kurata T, Okada K, Wada T.** 2008. A genetic regulatory network in the development of trichomes and root hairs. *Annual Review of Plant Biology* **59**, 365–386.
- Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA.** 2015. Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology* **15**, 16.
- Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA.** 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nature Biotechnology* **31**, 233–239.
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E.** 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821.
- Johnson KL, Jones BJ, Bacic A, Schultz CJ.** 2003. The fasciclin-like arabinogalactan proteins of Arabidopsis. A multigene family of putative cell adhesion molecules. *Plant Physiology* **133**, 1911–1925.
- Jones MA, Raymond MJ, Smirnov N.** 2006. Analysis of the root-hair morphogenesis transcriptome reveals the molecular identity of six genes with roles in root-hair development in Arabidopsis. *The Plant Journal for Cell and Molecular Biology* **45**, 83–100.

References

- Jones MA, Shen J-J, Fu Y, Li H, Yang Z, Grierson CS.** 2002. The Arabidopsis Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. *The Plant Cell* **14**, 763–776.
- Jun L, Xiaoming W.** 2012. Genome-wide identification, classification and expression analysis of genes encoding putative fasciclin-like arabinogalactan proteins in Chinese cabbage (*Brassica rapa* L.). *Molecular Biology Reports* **39**, 10541–10555.
- Jungk A.** 2001. Root hairs and the acquisition of plant nutrients from soil. *Journal of Plant Nutrition and Soil Science* **164**, 121–129.
- Kahl G, Molina C, Rotter B et al.** 2012. Reduced representation sequencing of plant stress transcriptomes. *Journal of Plant Biochemistry and Biotechnology* **21**, 119–127.
- Kersey PJ, Allen JE, Armean I et al.** 2016. Ensembl Genomes 2016: More genomes, more complexity. *Nucleic Acids Research* **44**, D574-D580.
- Ketelaar T, Faivre-Moskalenko C, Esseling JJ et al.** 2002. Positioning of nuclei in Arabidopsis root hairs: An actin-regulated process of tip growth. *The Plant Cell* **14**, 2941–2955.
- Knoll A, Fauser F, Puchta H.** 2014. DNA recombination in somatic plant cells: Mechanisms and evolutionary consequences. *Chromosome Research* **22**, 191–201.
- Kodera H, Hatamoto M, Abe K, Kindaichi T, Ozaki N, Ohashi A.** 2013. Phosphate recovery as concentrated solution from treated wastewater by a PAO-enriched biofilm reactor. *Water Research* **47**, 2025–2032.
- Köhler C, Neuhaus G.** 2000. Characterisation of calmodulin binding to cyclic nucleotide-gated ion channels from *Arabidopsis thaliana*. *FEBS Letters* **4710**, 133–136.

References

- Krouk G, Lacombe B, Bielach A et al.** 2010. Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. *Developmental Cell* **18**, 927–937.
- Kumar S, Verma S, Trivedi PK.** 2017. Involvement of small RNAs in phosphorus and sulfur sensing, signaling and stress: Current update. *Frontiers in Plant Science* **8**, 285.
- Kwasniewski M, Chwialkowska K, Kwasniewska J, Kusak J, Siwinski K, Szarejko I.** 2013. Accumulation of peroxidase-related reactive oxygen species in trichoblasts correlates with root hair initiation in barley. *Journal of Plant Physiology* **170**, 185–195.
- Kwasniewski M, Szarejko I.** 2006. Molecular cloning and characterization of beta-expansin gene related to root hair formation in barley. *Plant Physiology* **141**, 1149–1158.
- Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ.** 2006. Root structure and functioning for efficient acquisition of phosphorus: Matching morphological and physiological traits. *Annals of Botany* **98**, 693–713.
- Lan P, Li W, Schmidt W.** 2013. Genome-wide co-expression analysis predicts protein kinases as important regulators of phosphate deficiency-induced root hair remodeling in Arabidopsis. *BMC Genomics* **14**, 210.
- Leng Q, Mercier RW, Yao W, Berkowitz GA.** 1999. Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. *Plant Physiology* **121**, 753–761.
- Leonard TA.** 2013. C2 domain proteins. In: Kretsinger RH, Uversky VN, Permyakov EA, eds. *Encyclopedia of metalloproteins*. New York: Springer New York, 309–318.

References

- Li P, Zhang S, Li F et al.** 2017. A phylogenetic analysis of chloroplast genomes elucidates the relationships of the six economically important *Brassica* species comprising the Triangle of U. *Frontiers in Plant Science* **8**, 111.
- Lin S-I, Chiang S-F, Lin W-Y et al.** 2008. Regulatory network of microRNA399 and PHO2 by systemic signaling. *Plant Physiology* **147**, 732–746.
- Lin W-D, Liao Y-Y, Yang TJW, Pan C-Y, Buckhout TJ, Schmidt W.** 2011. Coexpression-based clustering of Arabidopsis root genes predicts functional modules in early phosphate deficiency signaling. *Plant Physiology* **155**, 1383–1402.
- Ma Y, Yan C, Li H et al.** 2017. Bioinformatics prediction and evolution analysis of arabinogalactan proteins in the plant kingdom. *Frontiers in Plant Science* **8**, 66.
- Mabuchi A, Soga K, Wakabayashi K, Hoson T.** 2016. Phenotypic screening of Arabidopsis T-DNA insertion lines for cell wall mechanical properties revealed ANTHOCYANINLESS2, a cell wall-related gene. *Journal of Plant Physiology* **191**, 29–35.
- Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu J-K.** 2013. Application of the CRISPR-Cas system for efficient genome engineering in plants. *Molecular Plant* **6**, 2008–2011.
- Marchler-Bauer A, Bo Y, Han L et al.** 2017. CDD/SPARCLE: Functional classification of proteins via subfamily domain architectures. *Nucleic Acids Research* **45**, D200–D203.
- Marschner P.** 2012. *Marschner's mineral nutrition of higher plants*, 3rd edn. Amsterdam, Boston: Elsevier/Academic Press.
- Marzec M, Szarejko I, Melzer M.** 2015. Arabinogalactan proteins are involved in root hair development in barley. *Journal of Experimental Botany* **66**, 1245–1257.

References

- Masucci JD, Schiefelbein JW.** 1994. The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process. *Plant Physiology* **106**, 1335–1346.
- Masucci JD, Schiefelbein JW.** 1996. Hormones act downstream of TTG and GL2 to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. *The Plant Cell* **8**, 1505–1517.
- McQueen-Mason SJ, Cosgrove DJ.** 1995. Expansin mode of action on cell walls: Analysis of wall hydrolysis, stress relaxation, and binding. *Plant Physiology* **107**, 87–100.
- Medici A, Marshall-Colon A, Ronzier E et al.** 2015. AtNIGT1/HRS1 integrates nitrate and phosphate signals at the *Arabidopsis* root tip. *Nature Communications* **6**, 6274.
- Miura K, Rus A, Sharkhuu A et al.** 2005. The *Arabidopsis* SUMO E3 ligase SIZ1 controls phosphate deficiency responses. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 7760–7765.
- Młodzińska E, Zboińska M.** 2016. Phosphate uptake and allocation - a closer look at *Arabidopsis thaliana* L. and *Oryza sativa* L. *Frontiers in Plant Science* **7**, 1198.
- Morato do Canto A, Ceciliato PH, Ribeiro B et al.** 2014. Biological activity of nine recombinant AtRALF peptides: Implications for their perception and function in *Arabidopsis*. *Plant Physiology and Biochemistry* **75**, 45–54.
- Mudge SR, Rae AL, Diatloff E, Smith FW.** 2002. Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*. *The Plant Journal for Cell and Molecular Biology* **31**, 341–353.
- Murphy E, De Smet I.** 2014. Understanding the RALF family: A tale of many species. *Trends in Plant Science* **19**, 664–671.

- Myrach T, Zhu A, Witte C-P.** 2017. The assembly of the plant urease activation complex and the essential role of the urease accessory protein G (UreG) in delivery of nickel to urease. *The Journal of Biological Chemistry* **292**, 14556–14565.
- Nawaz Z, Kakar KU, Saand MA, Shu Q-Y.** 2014. Cyclic nucleotide-gated ion channel gene family in rice, identification, characterization and experimental analysis of expression response to plant hormones, biotic and abiotic stresses. *BMC Genomics* **15**, 853.
- Nie Z, Hirsch DS, Randazzo PA.** 2003. Arf and its many interactors. *Current Opinion in Cell Biology* **15**, 396–404.
- Oppermann E.** 2004. Funktionelle Diversifizierung in Auxin-gesteuerten Wurzelfunktionen bei einer Tandem-Duplikation in der Genfamilie der Phospholipase A von *Arabidopsis thaliana*. Dissertation, Gottfried Wilhelm Leibniz Universität Hannover, Hanover.
- Oyama T, Shimura Y, Okada K.** 2002. The *IRE* gene encodes a protein kinase homologue and modulates root hair growth in *Arabidopsis*. *The Plant Journal for Cell and Molecular Biology* **30**, 289–299.
- Parker JS, Cavell AC, Dolan L, Roberts K, Grierson CS.** 2000. Genetic interactions during root hair morphogenesis in *Arabidopsis*. *The Plant Cell* **12**, 1961–1974.
- Petersen TN, Brunak S, Heijne G von, Nielsen H.** 2011. SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nature Methods* **8**, 785–786.
- Pierleoni A, Martelli PL, Casadio R.** 2008. PredGPI: A GPI-anchor predictor. *BMC Bioinformatics* **9**, 392.
- Pitts RJ, Cernac A, Estelle M.** 1998. Auxin and ethylene promote root hair elongation in *Arabidopsis*. *The Plant Journal for Cell and Molecular Biology* **16**, 553–560.

References

- Plaxton WC, Tran HT.** 2011. Metabolic adaptations of phosphate-starved plants. *Plant Physiology* **156**, 1006–1015.
- Qi X, Sun J, Zheng H.** 2016. A GTPase-dependent fine ER is required for localized secretion in polarized growth of root hairs. *Plant Physiology* **171**, 1996–2007.
- Raghothama KG.** 2000. Phosphate transport and signaling. *Current Opinion in Plant Biology* **3**, 182–187.
- Rengel Z, Marschner P.** 2005. Nutrient availability and management in the rhizosphere: Exploiting genotypic differences. *New Phytologist* **168**, 305–312.
- Rigas S, Debrosses G, Haralampidis K et al.** 2001. *TRH1* encodes a potassium transporter required for tip growth in Arabidopsis root hairs. *The Plant Cell* **13**, 139–151.
- Ringli C, Baumberger N, Diet A, Frey B, Keller B.** 2002. ACTIN2 is essential for bulge site selection and tip growth during root hair development of Arabidopsis. *Plant Physiology* **129**, 1464–1472.
- Roudier F, Schindelman G, DeSalle R, Benfey PN.** 2002. The COBRA family of putative GPI-anchored proteins in Arabidopsis. A new fellowship in expansion. *Plant Physiology* **130**, 538–548.
- Sakano K, Yazaki Y, Mimura T.** 1992. Cytoplasmic acidification induced by inorganic phosphate uptake in suspension cultured *Catharanthus roseus* cells: Measurement with fluorescent pH indicator and ³¹P-nuclear magnetic resonance. *Plant Physiology* **99**, 672–680.
- Salazar-Henao JE, Schmidt W.** 2016. An inventory of nutrient-responsive genes in Arabidopsis root hairs. *Frontiers in Plant Science* **7**, 237.
- Salazar-Henao JE, Velez-Bermudez IC, Schmidt W.** 2016. The regulation and plasticity of root hair patterning and morphogenesis. *Development* **143**, 1848–1858.

- Schaefer BC.** 1995. Revolutions in rapid amplification of cDNA ends: New strategies for polymerase chain reaction cloning of full-length cDNA ends. *Analytical Biochemistry* **227**, 255–273.
- Scheffzek K, Ahmadian MR.** 2005. GTPase activating proteins: Structural and functional insights 18 years after discovery. *Cellular and Molecular Life Sciences CMLS* **62**, 3014–3038.
- Scheible W-R, Rojas-Triana M.** 2015. Sensing, signalling, and control of phosphate starvation in plants: Molecular players and applications. *Annual Plant Reviews* **48**, 25–64.
- Schindelman G, Morikami A, Jung J et al.** 2001. COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in Arabidopsis. *Genes & Development* **15**, 1115–1127.
- Seifert GJ, Roberts K.** 2007. The biology of arabinogalactan proteins. *Annual Review of Plant Biology* **58**, 137–161.
- Semenova E, Jore MM, Datsenko KA et al.** 2011. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 10098–10103.
- Serrano M, Guzmán P.** 2004. Isolation and gene expression analysis of *Arabidopsis thaliana* mutants with constitutive expression of *ATL2*, an early elicitor-response RING-H2 zinc-finger gene. *Genetics* **167**, 919–929.
- Serrano M, Parra S, Alcaraz LD, Guzmán P.** 2006. The ATL gene family from *Arabidopsis thaliana* and *Oryza sativa* comprises a large number of putative ubiquitin ligases of the RING-H2 type. *Journal of Molecular Evolution* **62**, 434–445.

References

- Shan G.** 2010. RNA interference as a gene knockdown technique. *The International Journal of Biochemistry & Cell Biology* **42**, 1243–1251.
- Shin H, Shin H-S, Chen R, Harrison MJ.** 2006. Loss of *At4* function impacts phosphate distribution between the roots and the shoots during phosphate starvation. *The Plant Journal for Cell and Molecular Biology* **45**, 712–726.
- Song L, Yu H, Dong J, Che X, Jiao Y, Liu D.** 2016. The molecular mechanism of ethylene-mediated root hair development induced by phosphate starvation. *PLoS Genetics* **12**, e1006194.
- Steibel JP, Poletto R, Coussens PM, Rosa GJ.** 2009. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. *Genomics* **94**, 146–152.
- Stetter MG, Schmid K, Ludewig U.** 2015. Uncovering genes and ploidy involved in the high diversity in root hair density, length and response to local scarce phosphate in *Arabidopsis thaliana*. *PLoS ONE* **10**, e0120604.
- Su T, Xu Q, Zhang F-C et al.** 2015. WRKY42 modulates phosphate homeostasis through regulating phosphate translocation and acquisition in *Arabidopsis*. *Plant Physiology* **167**, 1579–1591.
- Synek L, Vukašinović N, Kulich I et al.** 2017. EXO70C2 is a key regulatory factor for optimal tip growth of pollen. *Plant Physiology* **174**, 223–240.
- Tan L, Showalter AM, Egelund J, Hernandez-Sanchez A, Doblin MS, Bacic A.** 2012. Arabinogalactan-proteins and the research challenges for these enigmatic plant cell surface proteoglycans. *Frontiers in Plant Science* **3**, 140.
- Thibaud M-C, Arrighi J-F, Bayle V et al.** 2010. Dissection of local and systemic transcriptional responses to phosphate starvation in *Arabidopsis*. *The Plant Journal for Cell and Molecular Biology* **64**, 775–789.

References

- Thieme CJ, Rojas-Triana M, Stecyk E et al.** 2015. Endogenous Arabidopsis messenger RNAs transported to distant tissues. *Nature Plants* **1**, 15025.
- Ticconi CA, Lucero RD, Sakhonwasee S et al.** 2009. ER-resident proteins PDR2 and LPR1 mediate the developmental response of root meristems to phosphate availability. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 14174–14179.
- Untergasser A, Cutcutache I, Koressaar T et al.** 2012. Primer3--new capabilities and interfaces. *Nucleic Acids Research* **40**, e115.
- Vance CP, Uhde-Stone C, Allan DL.** 2003. Phosphorus acquisition and use: Critical adaptations by plants for securing a nonrenewable resource. *New Phytologist* **157**, 423–447.
- Vaughn JN, Ellingson SR, Mignone F, Arnim Av.** 2012. Known and novel post-transcriptional regulatory sequences are conserved across plant families. *RNA* **18**, 368–384.
- Vijayakumar P, Datta S, Dolan L.** 2016. ROOT HAIR DEFECTIVE SIX-LIKE4 (RSL4) promotes root hair elongation by transcriptionally regulating the expression of genes required for cell growth. *The New Phytologist* **212**, 944–953.
- Viklund H, Bernsel A, Skwark M, Elofsson A.** 2008. SPOCTOPUS: A combined predictor of signal peptides and membrane protein topology. *Bioinformatics* **24**, 2928–2929.
- Wada Y, Kusano H, Tsuge T, Aoyama T.** 2015. Phosphatidylinositol phosphate 5-kinase genes respond to phosphate deficiency for root hair elongation in *Arabidopsis thaliana*. *The Plant Journal for Cell and Molecular Biology* **81**, 426–437.
- Wang D, Guo Y, Wu C, Yang G, Li Y, Zheng C.** 2008. Genome-wide analysis of CCCH zinc finger family in Arabidopsis and rice. *BMC Genomics* **9**, 44.

References

- Wang S-S, Diao W-Z, Yang X et al.** 2015. *Arabidopsis thaliana* CML25 mediates the Ca²⁺ regulation of K⁺ transmembrane trafficking during pollen germination and tube elongation. *Plant, Cell & Environment* **38**, 2372–2386.
- Won S-K, Lee Y-J, Lee H-Y, Heo Y-K, Cho M, Cho H-T.** 2009. Cis-element- and transcriptome-based screening of root hair-specific genes and their functional characterization in *Arabidopsis*. *Plant Physiology* **150**, 1459–1473.
- Wu H, Turner C, Gardner J, Temple B, Brennwald P.** 2010. The Exo70 subunit of the exocyst is an effector for both Cdc42 and Rho3 function in polarized exocytosis. *Molecular Biology of the Cell* **21**, 430–442.
- Wu J, Kurten EL, Monshausen G, Hummel GM, Gilroy S, Baldwin IT.** 2007. NaRALF, a peptide signal essential for the regulation of root hair tip apoplastic pH in *Nicotiana attenuata*, is required for root hair development and plant growth in native soils. *The Plant Journal for Cell and Molecular Biology* **52**, 877–890.
- Wymer CL, Bibikova TN, Gilroy S.** 1997. Cytoplasmic free calcium distributions during the development of root hairs of *Arabidopsis thaliana*. *The Plant Journal for Cell and Molecular Biology* **12**, 427–439.
- Xie K, Minkenberg B, Yang Y.** 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 3570–3575.
- Yeats TH, Sorek H, Wemmer DE, Somerville CR.** 2016. Cellulose deficiency is enhanced on hyper accumulation of sucrose by a H⁺-coupled sucrose symporter. *Plant Physiology* **171**, 110–124.
- Yi K, Menand B, Bell E, Dolan L.** 2010. A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. *Nature Genetics* **42**, 264–267.

References

- Yoo C-M, Quan L, Cannon AE, Wen J, Blancaflor EB.** 2012. AGD1, a class 1 ARF-GAP, acts in common signaling pathways with phosphoinositide metabolism and the actin cytoskeleton in controlling Arabidopsis root hair polarity. *The Plant Journal for Cell and Molecular Biology* **69**, 1064–1076.
- Zang L, Zheng T, Chu Y et al.** 2015. Genome-wide analysis of the fasciclin-like arabinogalactan protein gene family reveals differential expression patterns, localization, and salt stress response in Populus. *Frontiers in Plant Science* **6**, 1140.
- Zhang D, Li Z, Li J-F.** 2016. Targeted gene manipulation in plants using the CRISPR/Cas technology. *Journal of Genetics and Genomics = Yi chuan xue bao* **43**, 251–262.
- Zhang H, Zhang J, Wei P et al.** 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnology Journal* **12**, 797–807.
- Zhang K, Raboanatahiry N, Zhu B, Li M.** 2017. Progress in genome editing technology and its application in plants. *Frontiers in Plant Science* **8**, 177.
- Zhou H, Liu B, Weeks DP, Spalding MH, Yang B.** 2014. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Research* **42**, 10903–10914.

Supplementary Material

1. Chapter 1



Fig. S1: Exemplary 2-cm root tip of *B. carinata* cv. Bale grown under Pi-deficient conditions.

Table S1: Number of genes in the different annotations of the MACE tags according to the filter steps.

filter \ annotation	upregulation		downregulation	
	<i>B. rapa</i> (GenXPro)	<i>B. napus</i> (CLC)	<i>B. rapa</i> (GenXPro)	<i>B. napus</i> (CLC)
database entries with dedicated tags	46,685	62,555	46,685	62,555
at least 2-fold regulated in Bale	3,796	3,550	3,419	4,062
at least 3 normalized tags in Bale	1,511	1,470	884	1,170
regulation in Bacho between 0.7 and 1.5-fold	463	411	348	516
Bale -P at least 1.5-fold higher / lower than Bacho -P	313	270	199	258

Supplementary Material

Table S2: Partial cDNA sequences of candidate genes for Pi deficiency-induced root hair growth obtained by MACE. In case of genes occurring in both annotations after filtering the dataset, only the sequence obtained from the *B. rapa* annotation is shown. Primers used for qPCR (underlined).

	Bale consensus sequence (5' → 3')	Bacho consensus sequence (5' → 3')
BcEF-1-a1	AACTTCACTTCCCAGGTCATCATCATGAACC ACCCCGGTCAGATTGGTAAACGGTTACGCTCC AGTTCCTGACTGCCACACCTCCCACATTGCC GTCAAGTTCTCTGAGATCCTCACCAAGATTG ACAGGCGTTCTGGTAAAGAGATCGAGAAGGA GCCCAAGTTCTTGAAGAATGGTGACGCTGGT <u>ATGGTGAAGATGACTCCAACCAAGCCCATGG</u> TTGTTGAGACCTTCTCTGAGTACCCACCCCT TGGACGTTTCGCTGTGAGGGACATGAGGCAG <u>ACTGTTGCAGTCGGTGTTCATCAAGAGCGTTG</u> ACAAGAAGGACCCAACCGGAGCCAAGGTGAC CAAGGCTGCCGTCAAGAAGGGTGCCAAGTG	GTCAAGTTCTCTGAGATCCTCACCAAGATTG ACAGGCGTTCTGGTAAAGAGATCGAGAAGGA GCCCAAGTTCTTGAAGAATGGTGACGCTGGT <u>ATGGTGAAGATGACTCCAACCAAGCCCATGG</u> TTGTTGAGACCTTCTCTGAGTACCCACCCCT TGGACGTTTCGCCGTGAGGGACATGAGGCAG <u>ACTGTTGCAGTCGGTGTTCATCAAGAGCGTTG</u> ACAAGAAGGACCCAACCGGAGCCAAGGTGAC CAAGGCTGCCGTCAAGAAGGGTGCCAAGTG
BcAGP1	GAGTTTTCTCAGTTTCTCTATCTCTACAGA CAACGAAAGAAGTTTGAACAGAGATCGATG GCAGCTCTTAAGACAATGCAAGCTYTGTATCT TYCTTGGTCTATTGGCCACGTCTGTATGGC TCARGCTCCGGCTCCAGCACCCATCATGGTT <u>CTCCCACCGGTAGAGTCTCCCTCTCCTCCTC</u> CTGCTATTACACCAACCGCTGAGCCACCTTC TCCGGTACCGGTTGCTTACCACCGGTTATG ATTCCCAGGCAACTCCAGCTCCGGCGACTC CTCCCACCGTCTCACCACCGACTAAGTCTCC CAAACCTTCCCCTGTGCTTCTCCCCGAAA CCAGAAGCTATGGCTCCAGGCCCATCAGGCC CAACACCATCACCAGCTCCGGCTCCTGACGG ACCAATCGCTGATTCAGCATTGACTAACAAA GCTTTCCTTGTGAGCACTGTCAATGCAGGAG CCTGTACGCTGTGCTTTTGGCTTAAGAGTG CCCTGAGTATATTTACCCTTTTCTATTTATC ATTTGGTTGTTCTTGTGATAATTTGTTTGAA GATTTTTCTCTGTTGCTCTAATACAGTTTG TGTTTCTTCTCTTTGTTGGTGTTAATGTTT TCTTTTTCTTCTTTTATAACACTAATTGAC CATGGRATTAACCTGAGGAGGAGATTCTGAT TTTAATTCTGAAAAATCTTAACTATGAATTA TGCGTTTTGATT	GAGTTTTCTCAGTTTCTCTATCTCTACA GACAACGAAAGAAGTTTGAACAGAGATCGA TGGCAGCTCTTAAGACAATGCAAGCTTTGAT CTTCTTGGTCTATTGGCCACGTCTGTATG GCTCAAGCTCCGGCTCCAGCACCCATCATGG <u>TTCTCCCACCGGTAGAGTCTCCCTCTCCTCC</u> TCCTGCTATTACACCAACCGCTGAGCCACCT TCTCCGGTACCGGTTGCTTACCACCGGTTA TGATTCCCAGGCAACTCCAGCTCCGGCGAC TCCTCCCACCGTCTCACCACCGACTAAGTCT CCAAAACCTTCCCCTGTGCTTCTCCCCGAA AACCAGAAGCTATGGCTCCAGGCCCATCAGG CCCAACACCATCACCAGCTCCGGCTCCTGAC GGACCAATCGCTGATTCAGCATTGACTAACAA AAGCTTTCCTTGTGAGCACTGTCAATGCAGG AGCCTTGTACGCTGTGCTTTTGGCTTAAGAG TGCCCTGAGTATATTTACCCTTTTCTATTTA TCATTTGGTTGTTCTTGTGATAATTTGTTTG AAGATTTTTCTCTGTTGCTCTAATACAGTT TGTGTTTCTTCTCTTTGTTGGTGTTAATGT TTTCTTTTTCTTCTTTTATAACACTAATT GACCATGGGATTAACCTCAGGATGAGTTTCT GATTTTAATTCTGAAAAATCTTAACTATGAA TTATGCGTTTTGAT
BcAGP2	GCAACAAATATTTTCTCATTCACTCAAAGTT CAAACCAAAGCAAACACAACCTTTTTTACG AAGAAGATGATGATGAAGAAGATGTTTGTT AGATCGCAGTGTGTTTGTCTATTGGCTACGAT CGCAGCCGTCTCCGGCCACGAAGGTCACGTT CACAGCCCGGCTCCGGCACCGGGACCGGCAA CTAACTCAGCTGTGCTTCCAACCACCAACAT GTTACCCGATTGGCTTTTGTGCTGCGTAGCG CTTGTCTTGGTCTCAACCACTGAGTTCATC ATCACTACTGCATTCAGCGTTTCTTGTGTTGG TTCAGTTTCTATGAGATTGTTCTTGTGACTT TCAATATTCTTTTTGTTTTATCAAAGTTTCA TTTTGTTGTTTATCTTAT	CAAACAGCAACAAATATTTTCTCATTCACTC AAAGTTCAAACCAAAGCAAACACAACCTCTT TTTACGAAGAAGATGATGATGAAGAAGATGT TTGTTTACGATCGCAGTGTGTTTGTCTATTGGC TACGATCGCAGCCGTCTCCGGCCACGAAGGT CACGTTTACAGCCCGGCTCCGGCACCGGGAC CGGCAACTAACTCAGCTGTGCTTCCAACCAC <u>CAACATGTTTACCAGGATTGGCTTTTGTGCTG</u> <u>GTAGCGCTTGTCTTGGTCTCAACCACTGAG</u> TTCATCATCACTACTGCATTCAGCGTTTCTT GTTTGGTTTCAAGTTTCTATGAGATTGTTCTCT TGACTTTCAATATTCTTTTTGTTTTATCAAA GTTTCATTTTGTGTTTATCTTAT

Supplementary Material

BcFLA1a	<p>GGAACCCTAAACAACTAAGCCACGAAGACC AAGTCAAACCTCATCCTTTACCATGTTAGTCC CAAGTTCTACACATTGGATGATCTTCTCTCC GTRAGTAACCCGGTWAGGACTCAGGCTTCAG GACGAGACGGCGCGGTTTATGGACTTAATTT CACCAGTCAAGGGAACCAAGTCAACGTATCA ACCGGTGTCGTTGAAACACGTGTCAGTAATG MATTAAGAAAAGAACGTCTCTTGCGRTTTTA CGTGGTGGACATGGTMTTGTGGCCGAAGAG ATGTTTGGAGAAAACAAGGTCTCGCCAGCGG CACCTGGTCCRAAATCAAATTCTCCTGATGT CTCCGATGACCAAGAGAAAGCAGGGGCTCCA <u>TCGGATAAGTCTGGCTCCGGTGAGATGAAGG</u> CTGGATTAGGGTTKGCTCTTGACTTGTTTT CTTATGTTTTGAAACTCGTCGTTGATTTGGG TTTTCTATATGTTTTTTTTCTTAATTTGTTTT TATATATTTTATTTTATTGTGGATTGTTATA AAAGAAATTTTGTGGATATGATGCATGGAT TGAAAACCTGAAATAAGAGG</p>	<p>AAACAAACTAAGCTACGACGACCAAGTCAAA CTCATTTCTTTACCATGTTAGTCCCAAGTTCT ACACATTGGATGATCTTCKCTCCGTAAGTAA CCCGGTAAGGACTCAAGCTTCAGGACGAGAC RGC GCGGTATATGGACTTAATTTACCAGTC AAGGGAACCAAGTCAACGTATCAACCGGTTT <u>CGTTGAAACACGTGTCAGTAATGCATTAAGA</u> AAAGAACGTCTCTTGC GGTTTACGTGGTGG ACATGGTATTGTTGCCGGAGGAGATGTTTGG AGAAAACAAGGTCTCACCAGCGGCACCTGGT CCAAAATCAAATTCTCCTGATGTCTCCGATG ACCAAGAGAAAGCAGGGGCTCCATCGGATAA <u>GTCTGGCTCCGGTGAGATGAAGGCTGGATTA</u> GGGTGGCTCTTGACTTGTTTTCTTATGTT TGAAACTCGTCGTTGATTTGGGTTTCTATA TGTTTTTTTTCTTAATTTGTTTTTATATATT TTATTTTATTGTGGATTGTTATAAAAGAAAT TTTGTGGATATGATGCATGGATTGAAAACCT GAAATAAGAG</p>
BcCaBP1	<p>GAAGTTCTGATTATTGTGATCGTTGGCCTCA TAATAGGCGTACCGGCCTATGTTAGATCAAC TTATCCGTTTTGGATATGTGTCTTGGCATT GCTCTCTACTTCTTTTCACTTGTCCTCGTCT <u>ATCTTCATTTTAATTCTCGAGACAAGAANGG</u> AACGTTACCTCTAACATATAGTCACTGAAT GTTTCCAAGTTTTTTCTCTCTCT</p>	<p>TCTGATTATTGTGATCGTTGGCCTCATAATA GGCGTACCGGCCTATGTTAGATCAACTTATC CGTTTTGGATATGTGTCTTGSCATTTGCTCT CTACTTCTTTTTCACTTGTCCTCGTCTATCTT <u>CATTTTAATTCTCGAGACAAGATCGGAACGC</u> TCACCTCTAACATATAGTCACTGAATGTTTC CAAGTTTTTTCTCTCTCTGAAGATTTTGTGC TTTTCTTCCCTTTAGATTACAGTAAGTATT TTTAAAGTTCCGTCTGCGTTGTATGTT</p>
BcCML25L	<p>CGGTCGATMTCGGCGGARGAGCTGCATGAG GTTYTGAGGAGTCTTGGAGATGAGTGTTCGA <u>TCGCGGAGTGTAGGAAGATGATTGGTGGTGT</u> GGATAAGGATGGAGATGGGACGATTGATTTT GAGGAGTTTAAGATTATGATGACGTTGGGAT <u>CAAGGCGTGATAAYGTTATGGGTGGWGGTGG</u> TCAGAGGTAGAGTTTAAAYGGCCGAGTGATGT TTGTGTTTCGTCTTTGTCTTCCTYATTTTCTC GTTTTGTTYGTCCATTGTTATTATTCCATCA TTGTTTCAWTTTTTTGKTCTTGTCTTTGG GAGAATCACAT</p>	<p>GGGAACGGRTCTATATCGGCGGAGGAGCTGC ATGAGGTTCTGAGGAGTCTKGGAGATGAGTG <u>TTCGATCGCGGAGTGTAGGAAGATGATTGGT</u> GGCGTGGATAAGGATGGAGATGGGACGATTG ATTTTGAGGAGTTTAAAGATTATGATGACGTT <u>GGGATCAAGGCGTGATAATGTTATGGGWGGT</u> GGTNNNCAGAGGTAGAGTTTAAATGGCCGAGT GATGTTTGTGTTTCGTCTTCTTCCCTCCTC GTTTTCTCGTTTTGTTTCATCCATTGTTATTA TTCCGTTATTGTTTCAATTTTTTTTTCTTGT TCTCT</p>
BcCNGB1L	<p>GGAAGTTGTGAAAGGTAAGAAGGAATCTCCA TCAGCTTACAACGATGTAATAGCAAGTAAGA TGCAAGAGAACCCAAGGAAGAACAAGTTTT GGCTCTTGCTGGGGCTTTTCAAACCGTTATT GACTATGAAACTGCCGCATCTAAGTGATACA TGTCCTCAAAGAGACA</p>	<p>GGAGAAACAGGAGAAGGAAAGAAGAGGGAAG TTGTGAAAGGTAAGAAGGAATCTCCATCAGC TTACAACGATGTAATAGCAAGTAAGATGCAA GAGAACCCAAGGAAGAACAAGTTTTGGCTC TTGCTGGGGCTTTTCAAACCGTTATTGACTA TGAAACTGCCGCATCTAAGTGATACATGTCC CAAAGAGACA</p>

Supplementary Material

BcRALFL1	<p>ACAGAATCAAGTTTCAGAGAGAGAGAGAAAC AAATTCACGAAAGACGACTAAAGAAATCTCA AAAAGCCTCTGATTCAGCYAGAAGGTTTGGT CAAAGAARAAGATGAACTCTCGCGGATCTA CGCCGTCATCGCGCTCCTSGCGCTCGTAATC TCWGCCGTCAACGCAACCGGAGGCTTCGGAG ATTCGCTTGACTTCGTACGGAGCGGATCTTC TTCGCTCTTCTCYGGATGCGAAGGTTTCGATC GCTGAGTGTATAGCCGAGGAAGAGGAGATGG <u>AGTTCGATTAGATATCAGCCGCGCATTTTT</u> <u>AGCGCAGAAGAAGTACGTTAGCTACGGTGCG</u> <u>ATGAGGAAGAACAGTGTGCCTTGCTCGCGAC</u> GTGGAGCTTCGTATTACAACCTGCCAGCGTGG CGCTCAGGCGAATCCTTACCGCCGTGGATGC AGCACCATTACCAGGTGCAGGCGTTGAAGAG GAAGAAGATGATGAGTCCAATTCCTGTTTTG CCCTTGCGTGTTCGCTTTATTTTCTTCWTT TTTTAATTTATTTCCGGGATATATGGGTTTG ATTTACACATTGATGAAAACCTATA</p>	<p>AATCAAGTTTCAGAGAGAGAGAGAAACAAAT TCACGAAAGACGACTAAAGAAATCTCAAAAA GCCTCTGATTCAGCCAGAAGGTTTGGTCAAA GAAAAAGATGAACTCTCGCGGATCTACGCC GTCATCGCGCTCCTGGCGCTCGTAATCTCTG CCGTCAACGCAACCGGAGGCTTCGGAGACTC GCTCGATTTTCGTACGGAGCGGATCTTCGTCG CTCTTCTCTGGATGCGAAGGCTCGATCGCTG AGTGTATAGCCGAGGAAGAGGAGATGGAGTT <u>CGATTAGATATCAGCCGCGCATTTTTAGCG</u> <u>CAGAAGAAGTACGTTAGCTACGGTGCGATGA</u> <u>GGAAGAACAGTGTGCCTTGCTCGCGCGTGG</u> AGCTTCGTATTACAACCTGCCAGCGTGGCGCT CAGGCGAATCCTTACMCCGTGGATGCAGCA CCATTACCAGGTGCAGGCGTTGAAGAGGAAG AAGATGATGAGTCCAATTCCTGTTTTGCCCT TGCGTGTTCGCTTTATTTTCTTCTTTTTTTT AATTTATTTCCGGGATATATGGGTTTGATTT ACACATTGATGAAAACCTATA</p>
BcCOW1L	<p>AACTCAGTTTGTATGAGGAATCTATGCCTA AGCAGAACCAAGCTCAATTTGCAGCTCCCGT CTCGAGCTCAGAGTACATGTTGATGGTGA CGTATGGCTGAGCTGGAAGAAAAGTGCATGT TCCTTGACTTGAAACCGGCTAATGTTGACAC TGAAAAAGAGGAGAAACTTCAGGCGGCGCTT <u>AACCGTGTTCAAGTACTTGAGCAAGAGTTGA</u> CTGAGACCAAGAAGGCTTTGGAAGAAACCTT <u>TGTGGGCCAGAAGGAGATTTTTGG</u></p>	<p>GAATTGTTGCAATGGTTAGGCTATCAAAGA CGTTCCACGYAAACTCACAGAGGCTGCTTTA TATGGAACTCWGTTTGTATGAGGAWTCTA TGCCTAAGCAGAATCAAGCTCAATTTGCAGC TCCCGTCTCGAGCTCAGAGTACATGTTGATG GTGAAACGTATGGCTGAGCTGGAAGAAAAGT GCATGTTCCCTTGACTTGAAACCGGCTAATGT TGACACTGAAACAGAGGAGAAACTTCAGGCG GCGCTTAACCGTGTTCAAGTACTTGAGCAAG AGTTGAGTGAGACCAAGAAGGCTTTGGAAGA AACCCTTGTTGGGCCAGAAGGAGATTTTTGG</p>
BcPHT1	<p>CCTTTTTCTTTGCTAACTTTGGTCCAAACGCAAC CACCTTCATCGTACCGGCTGAAATCTTCCAGCC AGGCTAAGGTCCACATGCCACGGAATATCAGCCG CAACAGGCAAGGCCGGAGCCATCGTGGGAGCTTT TGGTTTCTTATACGCAGCTCAACCACAAGACAAG ACCAAGACRGATGCAGGATATCCACCGGGCATCG GAGTCAAGAACTCTCTGATCATGCTTGGTGTCA TAACCTTTGTCGGTATGCTCTTACCTTCCTTGTC CCTGARCCSAARGGAAAGTCCCTTGAAGAGCTMT CCGGTGAGACTGAGGTTGAGAAATGACCACGCCG TAAGATCTTCAGTTGTATTTATTATTTGTTTGTG</p>	<p>TACGACCACTGGATCAAACCAGACAACCGTATCG GCTTCGTTGTTATGTACTCTCTCACCTTTTTCTT TGCAAATTTTGGTCCAAACGCGACCACCTTCATT GTACCGGCTGAAATCTTCCGGCCAGACTAAGGT CCACATGCCATGGAATATCAGCTGCAACAGGTAA GGCTGGAGCCATCGTGGGAGCTTTTGGTTTTCTA TACGCGGCTCARCCACARGACAAGACCAAGACAG ATGCAGGATATCCACCGGGCATCGGAGTSAAGAA CTCTCTGATCATGCTTGGTGTCAATTAACCTTTGTT GGTATGCTCTTACCTTCCTTGTCCCTGAGCCGA AAGGAAAGTCCCTTGAAGAGCTMTCCGGTGARAC TGAGGTTGAGAA</p>

Supplementary Material

BcPHF1	<p>CCTGTGGTCAATGTTTATGACATTAGTACAT GGAAGAAGCTTGGGTTCAAGAAGCTGTCAAG GAAGAGTGCATCGACAATGGCMGTCAGCTTG GATGGCAAATATATTGCKTTGGGTGGCAAAG ATGGAGATATATCTGTYGCTGARGTGAAAAC AATGGARATCTACCACTACAGTAAGAGGCTG CATCTGGTGCAGACCATTGCTTCACTTGAGT TCTGCCCTAGTGAAAGGGTAATGCTGACAAC ATCGAGTGAATGGGGTGAGATGGTGACCAAA CTCACTGTACCAAAGGAGTGGAAGAGTGGC AGATATATGCRCTGTTGTTTTGCTTGTTCAT GGCGTCAGTGGTACTTGACATACGTRTTCCTC GAGAACTCAGATTCGTTTTTGGAAAGTTGCCAA TGGGGAAAGATCAAAGAAGACCAAAGATWAG <u>TCTTTTTGGAGATTCATCCACTACGCCTGCT</u> GAGGATCATAATAAGTGGAACTTGGACCTAT AGATTGAAAGAGATTTATGTAACCTCACACG <u>AGACGTTTCCTGTCTTTTTTTTTTTTTTTTT</u> C</p>	<p>ACCTTTTCTGTTCTGTGCTGCTCAGAGAGGT GATGTTCCCTGTGGTGAATGTTTACGACATTA GTACATGGAAGAAGCTTGGGTTCAAGAAGCT GTCAAGGAAGAGTGCATCGACAATGGCAGTS AGCTTGGATGGCAAATATATTGCTTTGGGTG GCAAAGATGGAGATATATCTGTGCTGAAGT TAAAACAATGGAGATCTACCACTACAGCAA AGGCTGCATCTTGGTCAAACCATTGCTTCAC TTGAGTTCTGCCCTAGTGAAAGGGTAATGCT GACAACATCGAGTGAATGGGGTGAGATGGTG ACCAAACCTCACTGTACCAAAGAGTGGAAG AGTGGCAGATATATGCGCTGTTGTTTTGCTT GTTTCATGGCGTCAGTGGTACTTGACATACGTG TTCTTCGAGAACTCAGATTCGTTTTTGGAAAGT TACCAATGGGGAAAGATCAGAGAAGACCAA GATTAGTCTTTTTGGAGATTCATCCACTACG CCTGCTGAGGATCATAATAAGTGGAACTTGG ACCTATAGATTGAAAGAGATTTATGTAACCTC <u>CACACGAGACGTTTCCTGTCTTTTTTTTTTTT</u> TTTTTTCGAAACTTCACTGCATGTTTTA</p>
BcRLCK1	<p>TTATGATGTTTGCAGGATGTGATGAAGGATC CATGTATTGCGGCTGACGGATACACTTATGA CCGGAAGCCATAGAGAAGTGGATGGAGGAC CACCGTAGCTCGCCGGTGACTGATTCCCCCT TAGAAAACATGACCCTTTTTGCCCAAYCACAC TCTTCATGCAGCCATTGTGCGAGTGGTGCCGT AGAAATCAGTAAGGCAAACAAAAGCGAGCGC ACCGTGAGGAATGATGTTAWTGGAGATTATG ATATTATTATGGAGAATGATTGAAGCAATYA TATGAAATTGTTGTGATTATATATCC</p>	<p>AGGATGTGATGAAGGATCCATGTATTGCGGC <u>TGACGGATACACTTATGACCGGAAAGCCATA</u> GAGAAGTGGATGGAGGACCACCGTAGCTCGC CGGTGACTGATTCCCCCTTAGAAAACATGAC CCTTTTGCCCAACCACACTCTTCATGCAGCC <u>ATTGTGCGAGTGGTGCCGTAGAAATCAGTAAG</u> GCAAACAAAAGCGAGCGCACCGTGAGGAATG ATGTTAATGGAGATTATGATATTATTATGGA GAATGATTGAAGCAATTATATGAAATTGTTG TGATTATATATCC</p>
BcMRIL	<p>GCCTTGTGTGTTTCAGTAYGAGGCGGATTTCC GGCCTAACATGACCATCGTTGTCAAGGCACT TCAGCCTCTCCTTAACTCYAAACCKGCCGGT CCTGAGTCTTCTTCCCTGAATTACCAGTTAAC CAATTCTCTTGGTTTTGTTTTGTTGTTGTT CTGGCTCTCTYCCCTCTCTTTCTTCTTGTGTTG TAAGATGTTGTCATTATATTTTTCTGTCAAC AAGATGTT</p>	<p>AACAGTTGGCTGCCGTGGCGGCCTTGTGTGT TCAGTATGAGGCGGATTTCCGGCCTAACATG <u>ACCATTGTTGTGTCAMGGCACTYCAGCCTCTCC</u> TTAACTCCAAACCGGCCGGTCTGAGTCTTC TTCTGAAATTACCAGTTAACCAATTCTCTTG GTTTTGTTTTTGTGTTGTTCTGCTCTCTT CCTCTCTTTCTTCTTGTGTAAGATGTTGT CATTATATTTTTCTGTCAACAAGATGTTGTC</p>
BcLRR-RLK1	<p>GAACTGAGTGGAATGCTGACGTGTTTGATAA GGAGATGAGAGCAGGGAAAGAACAGGAAGGG CAGATGCTGAAGCTGCTCAAGATTGGGTTGA GRTGYTGTGACTGGGACGTGGAGAGGAGAAT GGAGCTTACAGAGGCTGTTGATCGGATAGAR GAAGTTGATCACAGAGAGGCAGGTGGAAGTC <u>AAGAGAGTTTTTCGATCTTCGTACGTGACTGC</u> TAGTGATGGTGAAAATCGTTTTTCAAGAGCC ATGACTGGRGAGTTCTCCCTCGTGTAATAGC TTCACTGTCTCATTGATGTTTGTGTTGTTT TGTTTGTAGGTAAAAGACTGATCACCATTTT TATTTGCATTCTATTATTTTTCTTAGTTATGG TGGATGAGTAAGCTTTT</p>	<p>CGAGAAGTGAAGTGAACGCTGACGTGTTTGA TAAGGAGATGAGAGCAGGGAAAGAACAGGAA GGTCAGATGCTGAAGCTGCTCAAGATTGGGT TGAGGTGTTGTGACTGGGACGTGGAGAGGAG <u>AATGGAGCTTACAGAGGCTGTTGATCGGATA</u> GAAGAAGTTGATCACAGAGAGGCAGGTGGAA GTCAAGAGAGTTTTTCGATCTTCGTACGTGAC TGCTAGTGATGGTGAAAATCGTTTTTCAAGG GCCATGACTGGAGAGTTCTCCCTCGTGTAAT AGCTTCACTGTCTCATTGATGTTTGTGTTGTT TTMTGTTTGTAGGTAAAAGACTGATCACCAT TTTTATTTGCATTCTATT</p>

Supplementary Material

BcIREL	<u>GCTTCGATCAACTATGATCTTGTCCAAAAGA</u> <u>ACGCTAAGGAATCAACAGAAGCTGCAAACCC</u> <u>ATCAGCCCCTTGACCAAATCCGAAAAGAAAA</u> <u>ACTAGCTCTGTGCCCGAAGTATGATGAAAAA</u> <u>AATGTGTATTGAGCATCTACTTCAAGTGCTG</u> <u>ATTGGTTT</u>	<u>AGAACCTCTCACAACTTGCTTCGATCAACTA</u> <u>YGATCTTGTCCAAAAGAACGCTAAGGAATCW</u> <u>ACAGAGGCTTCAAACCCATCAGCCCCTTGAC</u> <u>CAAAACCGAAAAGAAAACTAGCTCTGGGCC</u> <u>CCAAGTGTGATGATGAATATGTGTATYGAGC</u> <u>ATCTACTTCAAGTGCTGATTGGTTTCTGGT</u>
BcMRH1L	<u>CTGGAAAATGACGTTTTAGGAGAGATGATAG</u> <u>ACCCATCGTTAAAGACATTCAAAGAAGAAGA</u> <u>GCTTGAAGTGATATGTGATGTGATACGAGAA</u> <u>TGTCTGAAACCAGACCAGAGACATCGACCTT</u> <u>CAATGAAAGATGTAGCGAACAGCTAAAACA</u> <u>AGTTATTAACATAACTCCTGAAAAAGCTACT</u> <u>CCAAGATCATCTCCTCTTTGGTGGGCAGAAC</u> <u>TCGAGATCTTGTCTCTGAAGCAACTTGACC</u> <u>CTCAATGAAATGCTCTTTCTCTTATACGTTG</u> <u>TACCAAAAAATTTCTTTTTTTCTTTCTTCTG</u> <u>GTTTCTTGTATCAGTTTTCTTATACGAGCTT</u> <u>TTGGTTCTTGATTCTTCACATTGAGTTTTGTT</u> <u>TTGGATTAGTGTGTTGCTTCTTGATGGATTTG</u> <u>ATATAAACGAGTTTCGAT</u>	<u>GGCATCAAACATCTGGAAAATGACGGTTTA</u> <u>GGAGAGATGATAGACCCATCGTTAAAGACAT</u> <u>TCAAAGAAGAAGAGCTTGAAGTGATATGTGA</u> <u>TGTGATACGAGAATGTCTGAAACCAGACCAG</u> <u>AGACATCGACCTTCAATGAAAGATGTAGCGG</u> <u>AACAGCTAAAACAAGTTATTAACATAACTCC</u> <u>TGAAAAAGCTACTCCAAGATCATCTCCTCTT</u> <u>TGGTGGGCAGAACTCGAGATCTTGTCTCTG</u> <u>AAGCAACTTGACCCTCAATGAAATGCTCTTT</u> <u>CTCTTATACGTTGTACCAAAAAATTTCTTTT</u> <u>TTTTCTTCTGGTTTTCTTGTATCAGTTTTCTTA</u> <u>TACGAGCTTTTTGGTTCTTGATTCTTCACATT</u> <u>GAGTTTGTGTTTTGGATTAGTGTGTTGCTTCTTG</u> <u>ATGGATTTGATATAAACGAGTTTCGAT</u>
BcRHS10L	<u>AGGTTGTGAGAGCACTAGACAGTGAAGGGGA</u> <u>CATGGGAGATATCAGCAACGGATCMAAAGTG</u> <u>GGACAWAACARWARTGCTTATGACTCTKGTC</u> <u>AGTATAACWCAGACGACAGGAAAATGGCKTT</u> <u>TGGTTTTGAYGAYAGTTCAGATTCAGGGATG</u> <u>CACAGTGGAGACTACTCTGTCCRAAGCTCCA</u> <u>GGAAAGGATCCAATGGAGCCTCTACTGAGTT</u> <u>CACAAGGARYGAATCTGAGAACMGAACCTTC</u> <u>AATAACCGGCGGTTCTGATTCAAATACAAGT</u> <u>GAAGTTATTGTACCTCATGTATGAGCTCCCT</u> <u>GTACAGCATTAAATGCTCTTTGTATTTTTTTTT</u> <u>TGTGTGAGATAAATTTTTCTTGCATAGGGGC</u> <u>TACCTTTAGTTTTGCTT</u>	<u>GAGAGCATTGGACAGTGAAGGGGACATGGGA</u> <u>GATATCAGCAACGGATCCAAAGTGGGACATA</u> <u>ACAAAAATGCTTATGACTCTTGTGAGTATAA</u> <u>CTCAGACGACAGGAAAATGGCTTTTGGTTTT</u> <u>GACGATAGTTCAGATTCAGGGATGCACAGTG</u> <u>GAGACTACTCTGTCCGAAGCTCCAGGAAAGG</u> <u>ATCCAATGGAGAATCTACTGAGTTCACAAGG</u> <u>AGTGAATCTGAGAACAGAACTTCAATAACC</u> <u>GGCGGTTCTGATACAAATACAAGTGATGTTA</u> <u>TCATACCTCATGTTTGAGCTCCCTGTACAGC</u> <u>ATTTATGCTCTTTGTATTTTTTTTTTTTTGGGA</u> <u>GATGGCATTCTTGCATAGCT</u>
BcPIP5K1	<u>GTAATGGACCATTAATTAGACTTGGGGAAAG</u> <u>TACACCCGCAAAAGCAGAGCAGGTGAGCAGA</u> <u>TTTGARGAAGAAGTGTGGGAAGATGACAGCA</u> <u>TTGACAACCTCRAATCCAAAGAATACAAGGAA</u> <u>GGAGGTTGTTGACGTGATCCTGTACTTCGGT</u> <u>ATTATTGATATTCTTCAAGACTATGATATCA</u> <u>CCAAAAAGTTAGAGCACGCCTACAAGTCACT</u> <u>CCATGCAGATCCCACCTTCTATATCAGCTGTT</u> <u>GATCCCAAGCTTTATTCCAAAAGGTTTAGAG</u> <u>ACTTTWTAACAAGATATTTGTTGAAGACAA</u> <u>ATGAGAAAATATAATATGCAACAGCTGCACA</u> <u>AGTTTAAAGACCAAGAATCTAAAGATCCACC</u> <u>AAG</u>	<u>GTAATGGACCATTAATTAGACTTGGGGAAAG</u> <u>TACACCCGCAAAAGCAGAGCAGGTGAGCAGA</u> <u>TTTGAGGAAGAAGYGTGGGAAGATGACAGCA</u> <u>TTGACAACCTCAAATCCAAAGAATACAAGGAA</u> <u>GGAGGTTGTTGACGTGATCCTGTACTTCGGT</u> <u>ATTATTGATATTCTTCAAGACTATGATATCA</u> <u>CCAAAAAGTTAGAGCACGCCTACAAGTCACT</u> <u>CCATGCAGATCCCACCTTCTATATCAGCTGTT</u> <u>GATCCCAAGCTTTATTCCAAAAGGTTTAGAG</u> <u>ACTTTATAACAAGATATTTGTTGAAGACAA</u> <u>ATGAGAAAATATAATATGCAACAGCTGCACA</u> <u>AGTTTAAAGACCAAGAATCTAAAGATCCACC</u> <u>AAGAGTTATATTG</u>
BcROPGEF1	<u>GCTTCTTGATATCTTGAAGATTTTCATCGGAG</u> <u>CATGAAGCGCTTGAGCTWGCTGACAAAGTYG</u> <u>AGGCTTCACTGGTAACATGGAGGCGTAAGAC</u> <u>YGGAGGGTTGGCTCACTCTAAATCCTCATGG</u> <u>GACATGATGAAAGATATGGGCGGCACGCTG</u> <u>GGAAATGACAAGAACCACATTCTCGCRGCTCG</u> <u>AGCTAGGAGCTTACTGTTTTGTCTGAAACAG</u> <u>AGATTCCCTGAACTCTCTCAGACCTCACTTG</u> <u>ACATCTGCAAGATTCAGTATAACCGAG</u>	<u>TTCTTGATATCTTGAAGATTTTCATCGGAGCA</u> <u>TGAAGCGCTTGAGCTAGCTGACAAAGTTGAG</u> <u>GCTTCACTGGTAACATGGAGGCGTAAGACCG</u> <u>GAGGGTTGGCTCACTCTAAATCCTCATGGGA</u> <u>CATGATGAAAGATATGGGCGGCACGCTGGG</u> <u>AATGACAAGAACCACATTCTCGCAGCTCGAG</u> <u>CTAGGAGCTTACTGTTCTGTCTGAAACAGAG</u> <u>ATTCCCTGAACTCTCTCAGACCTCACTTGAC</u> <u>ATCTGCAAGATTCAGTATAACCGAGTGAGTG</u> <u>GTCCCTCATAG</u>

Supplementary Material

BcROPGEF2	<p>GACTTCATAGGGTGGAGATTGTCATCAGAWA CAAAGAAGCATAAGTTCGATGAGTGATATAGA GTTCTTTCYATAGAACKGAGCAGGAGAAGCCG ATGATGAARTCTCCAAGAGCTCTACCGAAGA AACTTTCATATCTATCAAAGTTAGAGAACAT GAGAAGCMCGAGTGAGAGACACTRATAATGA TAACATGTAAGTGTAGACGAGAGCAATATGT ATATAGAAGAAGAAAMMAACAAAGAGAGGG AGCGGGGGGGGAGAGAGTAGGGGAGGTTAA AGTGTGTGTACATATAATGAAGGCACT</p>	<p>GACTTCATAGGGTGGAGATTGTCATCAGATA CAAAGAAGCATAAGTTCGATGAGTGATATAGA GTTCTTCCATAGAAGTGGAGCAGGAGAAGCCG ATGATGAAATCTCCAAGAGCTCTACCCAAGA AACTTTCATATCTATCAAAGTTAGAGAACAT GAGAAGCACGAGTGAGAGACACTGATAGTGA AAACAGGTACGTGTAGACGAGAGCAATATGT ATATAGAAGAAGAAAAAACAACAAAGAGGG AGCGGGGGGGGGGAGAGTAGGGGAGGCTAA AGTGTGTGTAAATATAATGAAGGCACTG</p>
BcAGD1	<p>TAGACATAGAACCCTGGTGGTGMAGCAAT AGAGTACGAGACATCRASCATAAGAGAAACG AGGCGGCTGGGGAGTTGGCTGGCGTGCAAAG AGGATACATCGGTGAGWGATGGAATAGTCTC ACATGAAGAAGYGAAAGTGAACAAGACATT TCACTAAGGCTTCAAAAAGTGGAGAGAGGTG TTCTTGAGATCCAGATTGAATGTCTTCCACT AACTCAATGATAAACACTTTTCGTTTTCTCCC TCGTTTTCTTATTGTTGTTTATATTGATAGT GAAGAGATGCTACTTTTTTCTTGAGATACCT AAAACCCTATGTACAAAACGCATAATT</p>	<p>GCTAGTGTACGACAAGGACAAGTTCACGACG GATGATTTTCATGGGAGAGGCAGAGATAGACA TAGAACCATTGGTGAGTGCAGCAATAGAGTA CGAGACATCAACCATAAGAGAAACGAGGCGG CTGGGGAGTTGGCTGGCGTGCAAAGAGGATA CATCGGTGAGTGATGGAATAGTCTCACATGA AGAAGTGAAGTGAACAAGACATTTCTACTA AGGCTTCAAAAAGTGGAGAGAGGTGTTCTTG AGATCCAGATTGAATGTCTTCCACTAECTCA ATGATAAACACTTTTCGTTTTCTCCCTCGTTT TCTTATTGTTGTTTATATTGATAGTGAAGAG ATGCTACTTTTTTCTTGAGATACCTRAAACC CTATGTACAAAACGCATAATTTGAT</p>
BcEXO70L	<p>AGGAACTATCACAAAAGCTACCAGAGAGAGA CGTGGGGGAAGCTGCTGGGGTTCTTRGGRCA CGAGGGGCTWATGCACAACGGGAAGATAGTS AAGCCTAATCTYAAGNNNNNNNNNNNNNNNN TTAATGCAACGTTTGATGAGATACATAAGAC GCAGACCACGTGGGTGGTAAATGATGAGCAG CTTCAGAGTGAGCTGAGGGTTTCTATTACTG CAGTTATGATACCTGCCTACAGGGCTTTTCAT GGCTAGGTTTGGACAGTACTTGGATCCTGGT CGCCAGACTGAGAAGTATGTCAAGTACCAGC CTGAGGATATTGAGGATCTCATTGACGAGCT CTTCGARGGMAACACTTCATCCTCTTCAACT GCCACGGCCAAGCGAAGAACATAGATYCTTT CTTCTTTTCTTGACGCAACATTTTCTTAAC TTTCCCAAACCTCATGTCTGCTTCTACTGT GTTTGTCTGAATGGCACAACCTGGATGTGTA AATGTAGTTATAATCAGTTCATCATTATG AATAAA</p>	<p>AGAGAGAGACGTGGGGGAAGCTGCTKGGGTT CTTRGGGCAYGAGGGGCKTATGCAYAAYGGG AAGATAGTKAAGCCTAATCTYAAGGAGAGGT TTAAAAGCTTTAATGCAACGTTTGATGAGAT ACATAAGACGCAGACCACGTGGGTGGTTAAT GATGAGCAGCTTCAGAGTGAGCTGAGGGTTT CTATTACTGCAGTTATGATACCTGCCTACAG GGCTTTCATGGCTAGGTTTGGACAGTACTTG GATCCTGGTCGACAGACGGAGAAGTATGTYA AGTACCAGCCTGAGGATATTGAGGATCTCAT TGAYGAGCTTTTTGAAGGAAACACTTCCTCC TATTCAACGGCCACGGCCAAGAGAAGAACAT AGGTCCTTTCTTCTTTTCTTGCAGTAACACT TTTCTTAAATGTTTCAAACCTTTCATGTCTTC TTCTACTGTTGTTTTGTCTGAATGCCACAA CTTGATGTGTAATGTAGTTATTAATCAGT TCCATCATTATGAATAAA</p>

Supplementary Material

BcRHSL	<p>GGGCTTCAACAACAACACTGCTCTGGCCCGAGA AAACAACCCGGTTGTACTTCGATCTACCCGTG GAGATGATCAAATGTGGCCGTGGAAGCGGCG <u>GAGAGGTCCAAGAACCAACGAGTTTGGGTTT</u> CTTCTTCAGCAAGGAAACAGAAAAGTTTRCCW TTGAGAAGTGTWCWAAAGACTTCTCGGTGCGG AAAGACAACAAAGGGGTTGTTGCGGTGAGAC ATCACCGCATCGCCGTGTCAGGTTTTTCWACC ACGACCTCGGTTTCATGCCCGACTTCACCGC GGTCGTGTATCACCCACGCTTGCTTAAAGC TAGAGATGACTTCAACACGTTCTTAGCAGCA CAGAACGCGTGAAGAACTTAAGAAAACATAG CACTATAATAAGACTTACTATAATATATGTT CGTATCTACAAGTAACCGGCATGTATATAAC AATGATGAAGGATTACGATATTGATCCTCCA CATG</p>	<p>TCGTTTGAGAAGTTTGAATGTGGCTCGTGGG CTTCAACAACAACACTGCTCTGGCCCGAGAAA CAACCGGTTGTACTTCGATCTACCCGTGGAG ATGATCAAATGTGGCCGTGGAAGCGGCGGAG <u>AGGTCCAAGAACCAACGAGTTTGGGTTTCTT</u> CTTCGACAAGGAAAAGAGAAAAGTTTACCTTTG AGAAGTGTCTAAAGACTTCTCGGTGCGAAA GSCAACAAMGGGGTTGTTGCGGTGAGACATC ACCGCATCGCCGTGTCAGGTTTTCAACCACG ACCTCGGTTTTCATGCCCGACTTCACCGCGGT CGTGTATCACCCACGCTTGCTTAAAGCTAG AGATGACTTCAACACGTTCTTAGCAGCACAG AACGCGTGAAGAACTTAAGAAAACATAGCAC TATAATAAGACTTACTATAATATATGTTTCTG ATCTACAAGTAACCGGCATGTATATAACAAT GATGAAGGATTACGATATTGATCCTCCACAT GTA</p>
BcSHV2L	<p>GGCTGGACCGCGAGGATCACCATTTTTCAACT GGGGAGAAATCGATTTCCCTGACTGGTTTTCT AGCGGTTTCAGATGAAGAAACCAGCCATTCGT GGATTCGAGAAAGCGTATTCGTTCAACGCGA GYCTCTTGAGTATAGAAGGAGGTGTTAACA CACCATTTTCATGGAAGGTCTTCCCGGTTTG GAGTATCTTGTGGCKGAACGTGATGAGTTAG ATCCRAAGAAGAATCTTAGGGTTCCTGGTAA GCAACAGTCTGTGATACAGTTCAGYAAGAAA CTTACACCGGGGATTAATGTGCCGGAACGTG ACGGATTTCCGGCGAAAGTTATATTCAACGG TGAGGAATGTTTGCTTCCCGATGTGCTTCCA CTTCCAAGTGGTGAAGAAGAAAATGGGTTTG ATACTATGGTTTTGTTGTGTATGGTGATTTT TGTTGTGGCGTTGGTAATTTAATTTGTGATCT TTCTAGAATTTATATAATGTGATTTTCTTGA TTGATTAGTTTGTGTTTGTAAAAATAAATTGT TCAGTTGACA</p>	<p>TTTTCAACTGGGGAGAAATCGATTTCCCTGA CTGGTTTCTAGCGGTTTCAGATGAAGAAACCA GCCATTCGTGGATTCGAGAAAAGCGTATTCGT <u>TCAACGCGAGCCTCTTGAGTATAGAAGGAGG</u> TGTTAACAACACCATTTTCATGGAAGGTCTT CCCGTTTTGGAGTATCTTGTGGCTGAACGTG ATGAGTTAGATCCAAAGAAGAATCTTAGGGT TCCTGGTAAGCAACAGTCTGTGATACAGTTC AGTAAGAACTTACACCGGGGATTAATGTGC <u>CGGAACGTGACGGATTTCCGGCGAAAGTTAT</u> ATTCAACGGTGAGGAATGTTTGCTTCCCGAT GTGCTTCCACTTCCAAGTGGTGAAGAAGAA ATGGGTTTGATACTATGGTTTTGTTGTGTAT GGTGATTTTTGTTGTGGCGTTGGTAATTTAA TTGTGATCTTTCTAGAATTTATATAATGTGA TTTTCTTGATTGATTAGTTTTGTTTTGTAAAA TAAAATTGTTTCAGTT</p>
BcSHV3L	<p>GGAATAGATGCTTGGGAAGCAAAGAAACTCC ACCTTACATGGCTCCTGTTCAACCAGGTGGA CTCTTACAASTTGTGAGTCATGCGTCYTTAC CTCCAGCCGMAGCTCCRAACCCGGTGTTCAC <u>AGATGCTGATGTTACTGAGCCRCCACTGCCT</u> CCRGTTACAGCAAAAAGCCCCAACCTCAACCC CCCCTGGAACACCATCAAAACCATCAACCAA TGCACCAGCACCTAGTGGACAAACCCGCCTC ACTCTATCTCTTCTCCTTTCTGTTTTTSCAA TGGCTATTGCCTCTCTTCTACTTCTGTGATC AWACCCTTGATCACACATCTTTGTGTGTGAC AATGTCAGATCTTAACAGCCGGAACCATAAT GTTTTCTCATGTTTATATTCTAGTTCTGTCC TA</p>	<p>GAATAGATGCTTGGGAAGCAAAGAAACTCCA CCTTACATGGCTCCTGTTCAACCAGGTGGAC TCTTACAAGTTGTGAATCATGCGTCTTTACC TCCAGCCGAAGCTCCGAACCCGGTGTTCACA <u>GATGCTGATGTTACTGAGCCGCCACTACCTC</u> CGGTTACAGCAAAAAGCCCCAACCTCAACCCC CCCTGGAACACCATCAGAACCATCAACCAAT GCACCAGCACCTAGTGGACAAACCCGCCTCA CTCTATCTCTTCTCCTTTCTGTTTTTCCAAT GGCTATTGCCTCTCTTCTACTTCTGTGATCA AACCTTGATCACACATCTTTGTGTGTGACA ATGTCAGATCTTAACAGCCGGAACCATAATG TTTTCTCATGTTTATATTCTAGTTCTGTCC A</p>

Supplementary Material

BcLRL3L1	<p>GGGAATGGAAACGCAACAGGATCATCCAACG ARAGCCTAAGGACAACGGAACAGAGGGTGGC GAAACTGATGGAAGAAGACATGGGATCAGCA ATGCAGTATCTTCAAGGGAAGGGGCTTTGTC TAATGCCCATCTCTCTCGCAACTGCTATGTC ATCTTCATCTGCTCACTCTCGTGGAGCTCTC TTCAACCCGACCTCAAATGTCTGTAGCAACAG AGGAKGCAAATGTAGTAGTAGCAGCTGCACC <u>TGAAGCCTCCTCTCCTATGGATGATGTCTCCGC</u> <u>CTCCAACAAGGCCTAAACCATAGGATCTCTC</u> ATTGTTTCTTCATTTTTTTTTTCGTTTACAGT CAAAACMTTAATATTCATTATCTATCATCTA TTTTCACTGTTAAGGATTAATAATAAAAAA TACTTA</p>	<p>ATAGCACCAAACATCATCATGGTTTTTGGTA ATGCAGGTAGGAGGACGGCTCAACGCACTCT CAGTACCGTCCAATGGCATAGTAAACGGGAA TGGAAACGCACCAGGATCATCCAACGAAAGC CTAAGGACAACGGAACAGAGGGTGGCGAAAC TGATGGAAGAAGACATGGGATCAGCAATGCA GTATCTTCAAGGGAAGGGGCTTTGTCTAATG <u>CCCATCTCTCTCGCAACTGCTATGTCATCTT</u> <u>CATCTGCTCACTCTCGTGGAGCTCTCTTCAA</u> <u>CCCGACCTCAAATGTCTGTAGCAACAGAGGAG</u> <u>GCAAATGTAGTAGCAGCAGCTGCACCTGAAG</u> <u>CCTCCTCTCCTATGGATGATGTCTCCGCCTC</u> <u>CAACAAGGCCTAAACCATAGGATCTCTCATT</u> <u>GTTTCTTCATTTTTTTTTTCGTTTACAGTCAA</u> <u>AACATTAATWTTTCATTATCTATCATCTATTT</u> <u>TCACTGTTAAGGATTAATAATAAAAAA</u></p>
BcLRL3L2	<p>GCAATGGCTTAAACGGGAAAGGAAACGCAAC AGGATCWTCCAACGAGAGCTTAAGGTCAACA GAAAATAGGGTGGCAAARCTGATGGAAGAAG ACATGGGATCTGCRATGCARTACCTTCAAGG GAAAGGGCTTTGCTTAATGCCCATCTCTCTC <u>GCAACTGCAATGTCATCTACATCTAATCACT</u> <u>CTCGTGGAGCTCTCTTTAACCCCAAATCCAA</u> TGTGTTAGCAGCAGAGGAGAAAAATGTAGCA GCAGTAGCTGCACCTGAAGACTCCTCTCCTA <u>TGGATGATGTATCTGCCTCCAAGGGCTA</u></p>	<p>GGAGCACGGCTCAACGCCCTCTCTGGACCGT GCAATGGCTTAAACGGGAAAGGAAACGCAAC AGGATCTTCCAACGAGAGCTTAAGGTCAACA GAAAATAGGGTGGCAAAGCTGATGGAAGAAG ACATGGGATCTGCAATGCAGTACCTTCAAGG GAAAGGGCTTTGCTTAATGCCCATCTCTCTC <u>GCAACTGCAATGTCATCTACATCTAATCACT</u> <u>CTCGTGGAGCTCTCTTTAACCCCAAATCCAA</u> TGTGTTAGCAGCAGAGGAGAAAAATGTAGCA GCAGTAGCTGCACCTGAAGACTCCTCTCCTA <u>TGGATGATGTATCTGCCTCCAAGGGCTAA</u></p>
BcWRKY1	<p>CAACAGTCCAAGTTCTCCGGTTTGCAGATAC CATCTCAGTCGMGAACGCTGGCGAGAGTGT CAGCGCCGTTATGGCCGCCAATCCGAACCTT GCAGCGGCTCTRGCCGCAGCCATCACGTCTA <u>TTATCAACGGTTCGAAAAAAAAAAAAAAAAATGG</u> GAGCAGTAACAGCAGTAATAATAACGTTTCG ACGAGCAGCGGTGACAGGCAATRACATTTTT AAAAATAAATGTTTTAGGTTTATTTATCGAC CGATTTTGAATTGTTTTGTCTTTTTTTTTTAG AACATTTGGATTCTAGTTATGGCCTTTTAG CTTCTTC</p>	<p>ATCTCAGTCGCTGAACGCTGGCGAGAGCGTC AATGCTGCTATGCCCGCCAATCCGAACCTTG CAGCGGCTCTGGCCGCAGCCATCACGTCTAT <u>TATCAACGGTTCGAGTAATCACMAGAATGGG</u> AGCAGTAACAGTAGTAATAATAATGTTACGA CGAGCAGCGGTGACAGGCAATAACATTTAAA AAAAAAAAATGTTTTAGGTTTGTTTATCGGCC GATTTTGAATTGTTTTGTCTTTTTTTTTTAGA <u>ACATTTGGATTCTAGTTATGGCCTTTTAGC</u> TTCTTCTTTTTTTT</p>
BcZC3H1	<p>GAGAAATCCATTGTGAACCTWTGGACAACCAA GTCTTGAAYCTACCAGAGAGTCCGTTTTCTT CCCTTTCTGGGAAAGAGATTTTCGACAGTTAC <u>KTAGAGAKGAAGAGATTAATACCTAACCAAA</u> AGAGARAARAGAAGATAGGAGCATAAAGGTT AGAGKAAATTTACAAAGATCWAATAATCAT C</p>	<p>GAATCCATTGTGAACCTATGGACAACCAAGT CTTGAATCTACCAGAGAGTCCGTTTTCTTCC CTTTCTGGGAAGGAGATTTTCGACAGTTACGT <u>AGAGATGAAGAGATTAATACCTAACCAAAAG</u> AGAGAAGMGAAGATAGGAGCATAAAGGTTAG AGGAAATTTACAAAGATCAAATAATCATC</p>

Supplementary Material

BcATL12L	<p>CTAGAGAAAACGCGGTTTRGTGGAAGCAACAG <u>CGGTTTGGACGCTTCTACAGCTTCCACATCG</u> AGAAGTGATGTGATTGCTGCGATGGAGGAAA GAAGACGAAGGATGTGGTTGCCGATTGCTAG AAAAACAGCACAGTGGTTYGTTAACAGAGAG AAAARGAATGAACTAAAAACGACAGGACAAG ATCTTAACGTTTTAGTTTTCTTTTAAAGGTGCG TTGACAATTTGAAGTTATTTTTTACAGAGATA ATTAAGCGTGCTGAATAAGATTAGACTAAAT GCTTGGTGTTTATGACTTTTGTGTACATACA TTGTGGATTGATTATGAAGAAAGTTTTGGCC AATGGTGATATACATTTATTAATTGGATTT</p>	<p>GTGACTTGCGTGACAAGGAAGAGATTGATAT GAAGAAAATGGTTGATAACAAAGATTCTACT AGAAGATCGGTTTCTGAGATCACGGCTGTTC CTAGACTCAGCGTTGTTACTACAGCGGCTAG AGAAAACGCGGTTGGTGGAAAGCAACAGCGGT <u>TTGGACGCTTCTACAGCTTCCACATCGAGAA</u> <u>GTGATGTGATTGCTGCGATGGAGGAAAGAAG</u> <u>ACGAAGGATGTGGTTGCCGATTGCTAGAAAA</u> ACAGCACAGTGGTTTGTAAACAGAGAGAAAA AGAATGAACTAAAAACGACAGGACAAGATCT TAACGTTTAGTTTTCTTTTAAAGTTCGTTGA CAGTTTGAAGTTATTTTTTACAGAGATAATTA AGCGTGCTGAATAAGATTAGACTAAATGCTT GGTGTATTATGACTTTGTTGTACATACATTGT GGATTGATTATGAAGAACGTTTTGGCCAATG GTGATATACATTTATTAATTGGATTT</p>
BcDUF538	<p>CCTGTGGGCATCTTCCCACGGGATGCTACAA ACTACGAGTTCGATGAACAGACMAAGAACT <u>GACGGTGCTGATCCCATCAGTCTGCGAAGTT</u> GGCTACAAGGACTCATCCGTCTCAAATTCA CCACAACAGTAACGGGATATCTGGAGAAAGG GAAGCTAAGTGAGGTGGAAGGGATGAAGACA AAAGTGATGATATGGGTCAAAGTCACAAGCA TCTCTGCGGACTCGTCAAAGGTGTATTTTAC TGCGGGGATGAAGAAGAGCAGAAACCGAGAT GCTTATGAGGTTCTAAGAGATGGCGTTTCGAG TTGATAAATTCTA</p>	<p>AGGAACAATGCAGAAGCCGTTGCCGAGTTG CTGAAAGAATATGATTTGCCTGTGGGTATCT TCCCAGGCGATGCTACAACTACGAGTTCGA TGAGCAGACAAAGAACTGACGGTGCTGATC <u>CCATCAGTCTGCGAAGTTGGCTACAAGGACT</u> CATCCGTCTCAAATTCACCACAACAGTAAC GGGATATCTGGAGAAAGGGAAGCTAAGTGAG GTGGAAGGGATGAAGACAAAAGTGATGATAT GGGTCAAAGTCACAAGCATCTCTGCGGACTC <u>GTCAAAGGTGTATTTTACTGCGGGGATGAAG</u> AAGAGCAGAAACCGAGATGCTTATGAGGTTT TTAGAGATGGCGTTCGAGTTGATAAATTCTA</p>
BcDUF620	<p>GTTGACTCGGTTAACATAGCACACGGTGGTC GAACGTTGACCACGCTTTATCGTTATGGAGG AGCTGTTAACCACCGGAGACGAATAGAGGAG AARTGGACGATTGAGGAAGTGGATTTTAATA TTTGTGGTTTTGTGTTTGGAGAGCTTCTTGCC <u>ACCGTCGGATATGAACAATGATCATTAGCTA</u> CGGTGAGATCGTTACCCTCTT</p>	<p>GTTGACTCGGTTAACATAGCACACGGTGGTC GAACGTTGACCACGCTTTATCGTTATGGAGG AGCTGTTAACCACCGGAGACGAATAGAGGAG AARTGGACGATTGAGGAAGTGGATTTTAATA TTTGTGGTTTTGTGTTTGGAGAGCTTCTTGCC <u>ACCGTCGGATATGAACAATGATCATTAGCKA</u> CGGTGAGATYGTACCCTCTTCTCTGATCT ATCTTTCATTGTTAGAATATTGGTTTGGTTT TGAAAGTTAGGTTTCTTAAAAAAATGG</p>
BcUNK1	<p>GCAAAGCTAGTGTTCTTTTTCAAAGAGTTAAT TAAATCCCTTTTCATATTATATAAAAATGGG AATCAAACCTCAGCCGTGATCATTCAACTTCA GCCATCCACGACAACAATCACCACCAAGAGT TTGAAGACCAGAAGAACAAGACGAAGATGGG TGAGGATACGAGTCGAAATCAATCAAACAAC <u>TTCAAGATCCCACCTCATTATCCAAAGTATA</u> CAAAGAATGATTATGAGAAAAATGCCAGAGTG <u>GCAATTGGATCAGCTTTTGGAGAGAGTATGGC</u> TTGCCGATCATCGGAGACTCCAACGAGAAGA GGAAGTTTGTGATCGGAGCTTTTCTTTGGTC ACTGGAGAAAGAGCACTAGATGTTGTGATCC GTGTAAGGCCCACTGTCCTTATTATATTGTC ATATATACCCAAAAGAAATTTGTTAT</p>	<p>TACGAGTCGAAATCAATCAAACAACCTTCAAG <u>ATCCCACCTCATTATCCAAAGTATACAAAGA</u> ATGATTATGAGAAAAATGCCAGAGTGGCAATT GGATCAGCTTTTGGAGAGATATGGCTTGCAG ATCATCGGAGACTCCAACGAGAAGAGGAAGT TTGTCATCGGGCTTTTCTCTGGTCGCCGGA GAAGGATCACCAGATGTTATGATC</p>

Supplementary Material

BcUNK2	<p>ATCTTTATCYGAAWCCGGGWCATCGGTRTAT AAGAAACAATACACGAGTGACGATGAGCTSG ATGACTTAGAYACATCCATTAACCTCTATAAT CTCTGCTCCTGGAACAACCAGTTCATCAGAG TGGATGCCTAAAGGATAYRGACGCAGAAAGA CTGTAAGATATCARCTCCTTAGAGAAATATG GAAGGAAGATGGATTACAATGACTCTTCTCC TTTTTTTGTCTTGGCTATATATATCCTTATGA TGAAAACAATTCTTAAGTTTCTTGGTTGACA TGTATGTATATCAAGCTGCTACTTTGTAAAC TCTATTCTTGATATCCTAAATGATTAGCCTA AAGAAGCTAACCTTTTCAATC</p>	<p>ATCTTTCTCAGAAGTCGGGTCATCGGTATAT AAGAAACAATACACGAGTGACGATGAGCTGG ATGACTTAGATACATCCATAAACTCTATCAT CTCTGCTCCTGGAACAACCAGTTCATCAGAG TGGATGCCTAAAGGATACAGACGCAGAAAGA CTGTAAGATATCAACTCCTTAGAGAAATATG GAAGGAAGATGGATTACAATGACTCTTCTCA TTTTTTTGTCTTGGCTATATATATCCTTATGAT GAAAACAATTCTTAAGTTTCTTGGTTGACAT GTATGTATATCGAGCTGCTACTTTGTAAACT CTATTCTTGATATCCTAAATGATTAGCCTAA AGAAGCTAACCTTTTCAATC</p>
BcUNK3	<p>CTCACCGATCACTTGGTGCTCAATATTATCA GAGATCGCCGCCTCCACCCCAAGAAAAGA TGAATCTGAAACCACTGGTCGTGAAGATGAC TCGTAAGTTGGTGTCTGTTACTTGGGTTCT GATGTTTGTTTTATGTTTTCAAGATATGTCT AATGTTTTAAGGCATTATCTGTGTACTIONTATT CTAAATAAAAAAAAAACGGTGGTACTTCTCAT CAAGATTATGTTTTACATGGTATATGTTTT CCAAAGCAATTTATGTGTGTGTTTTCTCTGT TTCTTTCTTGTACCACCTTCAAATTGGTGA CAATAAAGATAAATTGTTTTCAAATAACAAATG CAGTATTTGTTTTTTCATATTTTTTGTTTTT GTWACTAATAATACACCAGAAAGAGTTG</p>	<p>TATTCTAAATAAAAAAAAAACGGTGGCACTTC TCATCAAGATTATGTTTTACATGGTATATG TTTTCCAAAGCAATTTATGTTTGTGCTTTCT CTGTTTCTTT----- ATTGGTGACAATAAAGATAAATTGTTTCAA</p> <p>Number of tags was too low to get a complete consensus sequence of the primer region ('-' = region without sequence information).</p>
BcPP1	<p>GAGGAAGCAGAACGGATAAAACAATCGGATG GACGGTTGTTTTGCCTAGATGATGAACCGGG AGTGTACCGGGTGGGCATGCCAAATGGCAGA TCGCTTGGTTTAGCTGTCTCAAGAGCGTTTTG GAGATTACTGCCTTAAAGACTTTGGTTTTAGT CTCTGAACCGGAAGTGACATACAGMAAGATA ACCAGCAAGGACCAGTTTCTCATCTTGGCAA CCGATGGGGTAATATATATTTCAATATAATC AGTTTTGAATCAAAAGCAAGATAAAAACATA TAAATTTATAATCATGTATGTGTGTGTGTGT AGATGTGGGATGTGATGACAAAT</p>	<p>TATTACTACCCGGTTAAACAGAGGAAGCAGA ACGGATAAAACAATCGGATGGACGGTTGTTT TGCCCTAGATGATGAACCGGGAGTGTACCGGG TGGGCATGCCAAATGGCAGATCGCTTGGTTTT AGCTGTCTCAAGAGCGTTTGGAGATTACTGC CTTAAAGACTTTGGTTTTAGTCTCTGAACCGG AAGTGACATACAGAAAGATAACCAGCAAGGA CCAGTTTCTCATCTTGGCAACCGATGGGGTA ATATATATTTCAATATAATCAGTTTTGAATC AAAAGCAAGATAAAAACAYATAAATTTATAA TCATGTRTGTGTGTGTATATAGATGT</p>

Table S3: qPCR primers. Primers for the candidate genes for Pi deficiency-induced root hair growth and *BcEF-1-a1* were designed on the partial cDNA sequences obtained by MACE. *AtUBC9* primers derived from the study of Czechowski *et al.* (2005).

Gene	Sequence forward / reverse (5' → 3')	Amplicon size
<i>AtUBC9</i>	TCACAATTTCCAAGGTGCTGC TCATCTGGGTTTGGATCCGT	61
<i>BcEF-1-a1</i>	GGTGACGCTGGTATGGTGAA TGATGACACCGACTGCAACA	127
<i>AtIPS2</i>	TGAGGATTGTGTGTGAATGGA GAACCGAAGCTTGCCAAAGG	118
<i>BcAGP1</i>	CCAGCACCCATCATGGTTCT TAACCGGTGGTGAAGCAACC	109
<i>BcAGP2</i>	TCGCAGTGTGTTTGTCTATTGGC TGTTGGTGGTTGGAACGACA	126

Supplementary Material

<i>BcFLA1</i>	TCGTTGAAACACGTGTCAGT ACTTATCCGATGGAGCCCCCT	189
<i>BcCaBP1</i>	GGCGTACCGGCCTATGTTAG TGAAGATAGACGAGGACAAGTGA	95
<i>BcCML25L</i>	GATGAGTGTTTCGATCGCGGA ATCACGCCTTGATCCCAACG	117
<i>BcCNGB1L</i>	AGGTAAGAAGGAATCTCCATCAGC CACTTAGATGCGGCAGTTTCA	138
<i>BcRALFL1</i>	AGGAAGAGGAGATGGAGTTCGA TGTTCTTCCTCATCGCACCG	90
<i>BcCOW1L</i>	GCGCTTAACCGTGTTCAAGT CCAAAATCTCCTTCTGGCCCA	91
<i>BcPHT1</i>	CCATCGTGGGAGCTTTTGGT TGACACCAAGCATGATCAGAGA	117
<i>BcPHF1</i>	GTCTTTTTGGAGATTCATCCACTAC ACGTCTCGTGTGGAGTTACATAA	100
<i>BcRLCK1</i>	GCGGCTGACGGATACACTTA ACTCGACAATGGCTGCATGA	139
<i>BcMRIL</i>	TTTCCGGCCTAACATGACCA AGAGAGCCAGAACAACAACAA	139
<i>BcLRR-RLK1</i>	GGAGAATGGAGCTTCACGAGG ACGAAGATCGAAAACCTCTCTTGAC	90
<i>BcIREL</i>	TGATCTTGTCAAAAGAACGCT ACCAATCAGCACTTGAAGTAGA	147
<i>BcMRH1L</i>	CCAGACCAGAGACATCGACC GTTCTGCCACCAAAGAGGA	115
<i>BcRHS10L</i>	AGTGAAGGGGACATGGGAGA GGACAGAGTAGTCTCCACTGT	166
<i>BcPIP5K1</i>	TGGACCATTAATTAGACTTGGGGA CAACAACCTCCTTCTTGATTTCTT	131
<i>BcROPGEF1</i>	TGGGAATGACAAGAACCACAT GAGGTCTGAGAGAGTTCAGGG	90
<i>BcROPGEF2</i>	ACTTCATAGGGTGGAGATTGTCA TCACTCGTGCTTCTCATGTTCT	175
<i>BcAGD1</i>	TAAGAGAAACGAGGCGGCTG CCTCTCTCCACTTTTTGAAGCC	133
<i>BcEXO70L</i>	AGAGAGAGACGTGGGGGAAG CCCTCAGCTCACTCTGAAGC	183
<i>BcRHSL</i>	CAACAACAACCTGCTCTGGCC TTGGTTCTTGGACCTCTCCG	105
<i>BcSHV2L</i>	AAGCGTATTTCGTTCAACGCG GGCACATTAATCCCCGGTGT	199
<i>BcSHV3L</i>	CCGGTGTTACAGATGCTGA GCTGGTGCATTGGTTGATGG	113
<i>BcLRL3L1/2</i>	CCCATCTCTCTCGCAACTGC TCCATAGGAGAGGAGGCTTCA	140
<i>BcWRKY1</i>	GCCATCACGTCTATTATCAACGG GCTAAAAGGCCATAACTAGAATCCA	200
<i>BcZC3H1</i>	ATCCATTGTGAACCTATGGACA TCTCTACGTAACGTGCGAAATCTC	96
<i>BcATL12L</i>	GAAGCAACAGCGGTTTGGAC GGCAACCACATCCTTCGTCT	94
<i>BcDUF538</i>	AAACTGACGGTGCTGATCCC TACACCTTTGACGAGTCCGC	185

Supplementary Material

<i>BcDUF620</i>	CATAGCACACGGTGGTCGAA GTTTCATATCCGACGGTGGCA	157
<i>BcUNK1</i>	CAATCAAACAACCTTCAAGATCCCAC TCAAAGCTGATCCAATTGCCA	94
<i>BcUNK2</i>	TCTCTGCTCCTGGAACAACC GTCATTGTAATCCATCTTCCTTCCA	117
<i>BcUNK3</i>	AAAACGGTGGTACTTCTCATCA CACCAATTTTGAAGGTGGTACAAGA	112
<i>BcPP1</i>	TGGACGGTTGTTTTGCCTAGA CCAAACGCTCTTGAGACAGC	96

Table S4: Primers used for 5' RLM-RACE.

Oligo name	Primer sequence (5' → 3')
BcFLA1_RACE_outer	CAATCCATGCATCATATCCAAC
BcFLA1_RACE_inner	ACCCAAATCAAACGACGAGT
BcCML25L_RACE_outer	TGTGATTCTCCCAAAGAACAAG
BcCNGB1L_RACE_outer	ACATGTATCACTTAGATGCGGCAGT
BcCNGB1L_RACE_inner	TCTTCCTTGGGTCTCTTGCA
BcRALFL1_RACE_outer	CGCAAGGGCAAACAGGAAT
BcRALFL1_RACE_inner	CTCTTCAACGCCTGCACCT
BcCOW1L_RACE_outer	TCCAAAGCCTTCTTGGTCTCA
BcCOW1L_RACE_inner	ACTTGAACACGGTTAAGCG
BcPHT1_RACE_outer	TGGTCATTTCTCAACCTCAGTCT
BcPHT1_RACE_inner	GGACAAGGAAGGTGAAGAGCA
BcMIRL_RACE_outer	ACAACAAGAAGGAAAGAGAGG
BcMIRL_RACE_inner	AGAGAGCCAGAACAACAACA
BcLRR-RLK1_RACE_outer	GCTTACTCATCCACCATAACTAAGA
BcLRR-RLK1_RACE_inner	GGTGATCAGTCTTTTACCTACAAAC
BcIREL_RACE_outer	TCATACTTCGGGCACAGAGC
BcIREL_RACE_inner	GGATTTGGTCAAGGGGCTGA
BcRHS10L_RACE_outer	AATGCTGTACAGGGAGCTCA
BcRHS10L_RACE_inner	TGAATCAGAACCGCCGTTAT
BcROPGEF1_RACE_outer	CTCGGTTATACTGAATCTTGCAGA
BcROPGEF1_RACE_inner	AGGTCTGAGAGAGTTCAGGGA
BcROPGEF2_RACE_outer	GTGCCTTCATTATATGTACACACAC
BcROPGEF2_RACE_inner	TTAACCTCCCCTACTCTCTCC
BcAGD1_RACE_outer	TCTGGATCTCAAGAACACCTCTC
BcAGD1_RACE_inner	CCTCTCTCCACTTTTTGAAGCC

Supplementary Material

BcRHSL_RACE_outer	ACATGCCGGTTACTTGTAGA
BcRHSL_RACE_inner	GTTCTTCACGCGTTCTGTGC
BcSHV2L_RACE_outer	AATTACCAACGCCACAACAA
BcSHV2L_RACE_inner	ACCCATTTCTTCTTCCACCACT
BcSHV3L_RACE_outer	ACATTATGGTTCCGGCTGTT
BcSHV3L_RACE_inner	ACAAAGATGTGTGATCAAGGGT
BcLRL3L1_RACE_outer	TGAGAGATCCTATGGTTTAGGC
BcLRL3L1_RACE_inner	TATGGTTTAGGCCTTGTTGGA
BcLRL3L2_RACE_outer	TTAGCCCTTGGAGGCAGATA
BcLRL3L2_RACE_inner	ACATCATCCATAGGAGAGGAGTCT
BcWRKY1_RACE_outer	GCTAAAAGGCCATAACTAGAAATCCA
BcWRKY1_RACE_inner	TGTTATTGCCTGTCACCGCT
BcZC3H1_RACE_outer	CTCTAACCTTTATGCTCCTATCTTC
BcZC3H1_RACE_inner	CCAGAAAGGGAAGAAAACGGA
BcATL12L_RACE_outer	ATCACCATTGGCCAAAACCTT
BcATL12L_RACE_inner	GTCTAATCTTATTCAGCACGCT
BcUNK3_RACE_outer	CACCAATTTTGAAGGTGGTACA
BcUNK3_RACE_inner	TCTTGATGAGAAGTACCACCGT

Table S5: Primers used for amplification of the Bale gDNA sequences.

Gene	Sequence forward / reverse (5' → 3')	Amplicon size
<i>BcFLA1</i>	TTCAAACGCAATGGCAACCA ACCCAAATCAAACGACGAGTTTC	752
<i>BcRALFL</i>	AGTTTCAGACAGAGAGAGAGAGA CGCAAGGGCAAACAGGAAT	500
<i>BcMIRL</i>	ATGTTCTGTTGCGGAGGT TCAGGAAGAAGACTCAGG	1972
<i>BcROPGEF1</i>	ACTCGATCATTACACAGAGTCTCG CAGAACACAATCATAACCAACACAC	2470
<i>BcLRL3L1</i>	AGAGATGGAGAAGGGGAATGG CCTATGGTTTAGGCCTTGTTGG	1221
<i>BcLRL3L2</i>	AGAGATGGGAAATGAGAATGGAGA GCCCTTGGAGGCAGATACAT	1371

Supplementary Material

Table S6: Primers used for construct preparation.

Oligo name	Primer sequence forward / reverse (5' → 3')
BcFLA1_gR1	TAGGTCTCCAGAGGAAGACAGGTTTTAGAGCTAGAA ATGGTCTCACTCTCCACGGAGTGCACCAGCCGGGAA
BcFLA1_gR2	TAGGTCTCCTCGCCGTGATGTGTTTTAGAGCTAGAA ATGGTCTCAGCGATCCTTGAATGCACCAGCCGGGAA
BcRALFL1_gR1	TAGGTCTCCTCATCGCGCTCCGTTTTAGAGCTAGAA ATGGTCTCAATGACGGCGTAGTGCACCAGCCGGGAA
BcRALFL1_gR2	TAGGTCTCCAACGCAACCGGTTTTAGAGCTAGAA ATGGTCTCAGTTGACGGCAGATGCACCAGCCGGGAA
BcPHT1_gR1	TAGGTCTCCGCTTGGTCACCAGTTTTAGAGCTAGAA ATGGTCTCAAAGCTTCTTGGCTGCACCAGCCGGGAA
BcPHT1_gR2	TAGGTCTCCGGAAGTGAACCGTTTTAGAGCTAGAA ATGGTCTCATCCCCCTCATGTTGCACCAGCCGGGAA
BcMIRL_gR1	TAGGTCTCCTGAAGGCTCTTAGTTTTAGAGCTAGAA ATGGTCTCATTCACCGATCAGTGCACCAGCCGGGAA
BcMIRL_gR2	TAGGTCTCCATTTACCTCACGTTTTAGAGCTAGAA ATGGTCTCAAAATCGGAGTCATGCACCAGCCGGGAA
BcROPGEF1_gR1	TAGGTCTCCTCTTCACCGTACGTTTTAGAGCTAGAA ATGGTCTCAAAGAGACGGAGCTGCACCAGCCGGGAA
BcROPGEF1_gR2	TAGGTCTCCGGTGAGATAAACGTTTTAGAGCTAGAA ATGGTCTCACACCGGTTTCGACTGCACCAGCCGGGAA
BcLRL3L1_gR1	TAGGTCTCCGAGATGAGCCCCGTTTTAGAGCTAGAA ATGGTCTCATCTCTTCCCCCTTGCACCAGCCGGGAA
BcLRL3L1_gR2	TAGGTCTCCGTGAGAGCGTGGGTTTTAGAGCTAGAA ATGGTCTCATCACCGTCCCCGTGCACCAGCCGGGAA
BcLRL3L2_gR1	TAGGTCTCCAAGGAGGGAGAGGTTTTAGAGCTAGAA ATGGTCTCACCTTGCTGGGACTGCACCAGCCGGGAA
BcLRL3L2_gR2	TAGGTCTCCAAGAGGCGTCGAGTTTTAGAGCTAGAA ATGGTCTCATCTTCCCCGACTGCACCAGCCGGGAA

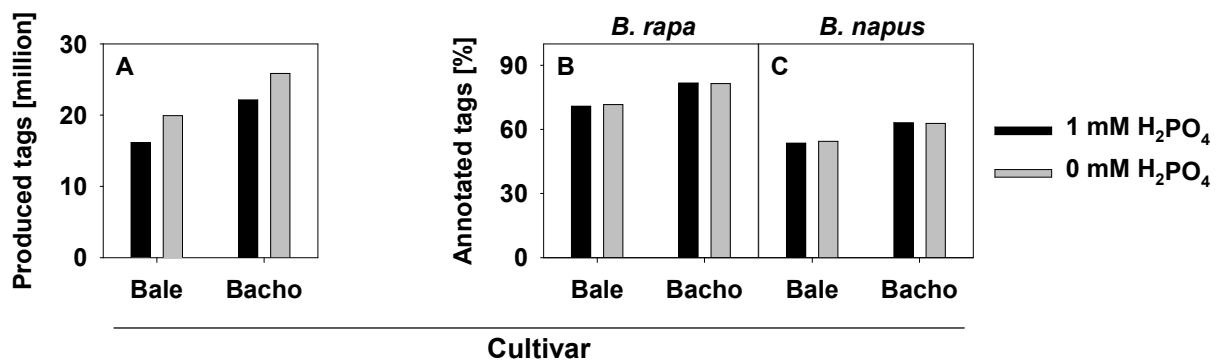


Fig. S2: Produced tags during MACE in million (A) and relative number of tags annotated to *Brassica rapa* (B) and *Brassica napus* (C).

Supplementary Material

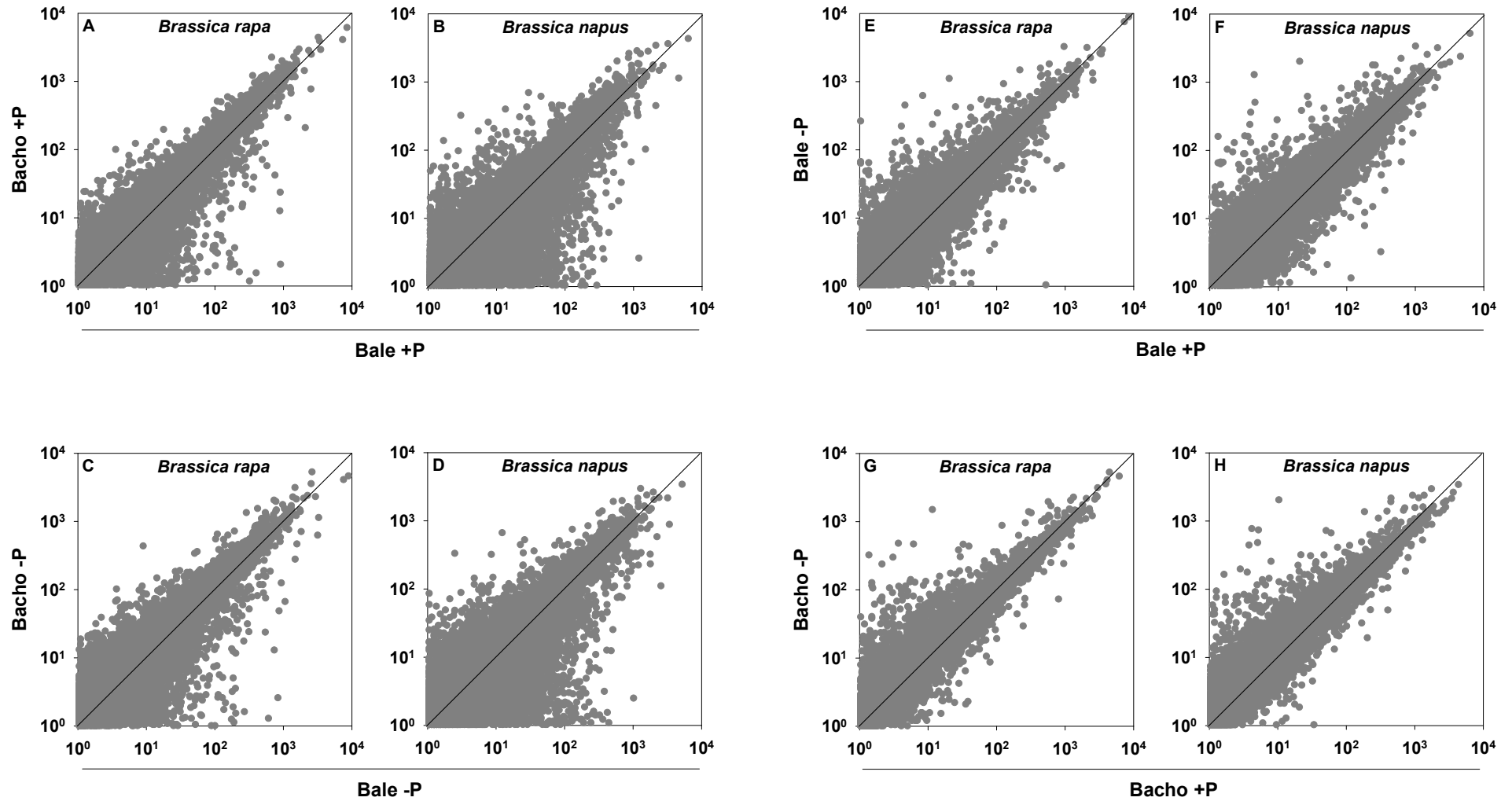


Fig. S3: Number of normalized tags annotated to the different genes depending on cultivar, treatment and annotation. Comparison within +P (A+B), -P (C+D), Bale (E+F) and Bacho (G+H).

Supplementary Material

Table S7: Selected candidate genes for the Pi deficiency-induced root hair growth in *Brassica carinata*.

Gene name abbr.	Annotated gene id	<i>A. thaliana</i> gene id	Description	Tags (normalized to 1 m.)				Fold change	
				Bale		Bacho		Bale -P / +P	Bacho -P / +P
				+P	-P	+P	-P		
Endogenous control									
<i>BcEF-1-a1</i>	Bra031605	AT5G60390	Elongation factor 1 alpha 1	58.0	55.0	55.2	55.2	0.9	1.0
Arabinogalactan proteins									
<i>BcAGP1</i>	TC167185	AT4G40090	Arabinogalactan protein 1	144.5	857.6	190.9	180.8	5.9	0.9
<i>BcAGP2</i>	Bra025551	AT5G40730	Arabinogalactan protein 2	80.4	316.7	105.9	110.8	3.9	1.0
<i>BcFLA1</i>	Bra033722	AT5G44130	Fasciclin-like arabinogalactan protein 1	53.4	116.1	26.9	22.3	2.2	0.8
Calcium-related proteins									
<i>BcCaBP1</i>	Bra032925	AT1G29020	Calcium-binding EF-hand family protein 1	1.2	3.8	0.8	0.9	3.1	1.2
<i>BcCML25L</i>	TC189239	AT1G24620	Calmodulin-like 25-like	2.3	8.0	1.8	2.4	3.5	1.4
<i>BcCNGB1L</i>	Bra009801 ES266808	AT5G24880	Cyclic nucleotide-gated cation channel beta-1-like	11.1 4.1	41.1 20.0	8.8 4.7	12.9 6.6	3.7 4.9	1.5 1.4
<i>BcRALFL1</i>	Bra001149 TC167929	AT3G05490	Rapid alkalization factor-like 1	116.9 109.4	269.8 230.7	154.4 56.0	160.4 60.6	2.3 2.1	1.0 1.1
<i>BcCOW1L</i>	ES907398	AT4G34580	Can of worms 1-like	4.7	34.7	10.0	12.3	7.4	1.2
Phosphate transport									
<i>BcPHT1</i>	Bra027492	AT5G43360	Inorganic phosphate transporter 1	3.2	49.8	5.1	5.5	15.8	1.1
<i>BcPHF1</i>	TC192478	AT3G52190	Phosphate transporter traffic facilitator 1	35.5	71.0	20.8	30.6	2.0	1.5
Protein kinases									
<i>BcRLCK1</i>	Bra022543	AT5G51270	Receptor-like cytoplasmic kinase 1	3.3	9.7	3.2	3.3	2.9	1.0
<i>BcMRIL</i>	Bra004665	AT2G41970	Receptor-like cytoplasmic kinase MARIS-like	4.9	13.0	4.4	4.1	2.7	0.9
<i>BcLRR-RLK1</i>	Bra011255	AT4G31250	Leucine-rich repeat receptor-like serine/threonine protein kinase 1	3.9	16.0	3.7	3.9	4.1	1.1
<i>BcIREL</i>	Bra010074	AT5G62310	Incomplete root hair elongation-like (serine/threonine protein kinase)	1.3	9.4	2.0	2.7	7.3	1.3
<i>BcMRH1L</i>	TC212815	AT4G18640	Morphogenesis of root hair 1-like (serine/threonine protein kinase)	22.6	46.1	23.9	28.6	2.0	1.2
<i>BcRHS10L</i>	Bra007915	AT1G70460	Root hair specific 10-like (proline-rich extensin-like receptor kinase)	6.3	15.9	6.8	5.3	2.5	0.8
<i>BcPIP5K1</i>	Bra007756	AT2G26420	Phosphatidylinositol phosphate 5-kinase 1	4.8	24.0	2.6	6.0	4.9	2.3

Supplementary Material

GTPase-related									
<i>BcROPGEF1</i>	Bra004945	AT2G45890	Rop guanine nucleotide exchange factor 1	10.1	24.8	9.4	11.1	2.4	1.2
<i>BcROPGEF2</i>	Bra020048	AT5G19560	Rop guanine nucleotide exchange factor 2	1.7	16.3	1.6	1.8	9.3	1.1
<i>BcAGD1</i>	Bra001277	AT3G07940	Adenosine diphosphate (ADP) ribosylation factor (ARF)-GTPase activating protein (GAP) domain-containing protein 1	3.0	10.5	3.8	4.1	3.4	1.1
<i>BcEXO70L</i>	TC204552	AT5G13990	Exocyst complex component EXO70-like	6.7	23.1	11.3	11.7	3.4	1.0
Root hair affecting									
<i>BcRHS1</i>	Bra005382	AT2G34910	Root hair specific-like	4.5	24.0	9.6	10.4	5.4	1.1
<i>BcSHV2L</i>	Bra036147	AT5G49270	Shaven 2-like	13.6	48.4	12.9	16.2	3.6	1.3
<i>BcSHV3L</i>	Bra019092	AT4G26690	Shaven 3-like (glycerophosphodiester phosphodiesterase-like, GDPDL)	8.9	31.0	15.3	17.8	3.5	1.2
Transcription factors									
<i>BcLRL3L1</i>	Bra006788	AT5G58010	<i>Lotus japonicus</i> roothairless1-like 3 (LRL3)-like 1	8.5	24.2	7.5	8.5	2.9	1.1
<i>BcLRL3L2</i>	Bra020398	AT5G58010	<i>Lotus japonicus</i> roothairless1-like 3 (LRL3)-like 2	7.2	21.0	8.8	10.8	2.9	1.2
<i>BcWRKY1</i>	Bra029491	AT4G04450	WRKY transcription factor 1	1.0	4.7	1.6	1.1	4.5	0.7
Zinc finger proteins									
<i>BcZC3H1</i>	Bra013181	AT2G05160	Zinc finger CCCH domain-containing protein 1	2.4	6.7	2.1	2.6	2.8	1.3
<i>BcATL12L</i>	TC178182	AT2G20030	Arabidopsis Tóxicos en Levadura (ATL) 12-like (RING-H2 finger protein)	6.2	19.9	7.6	7.7	3.2	1.0
Unknown									
<i>BcDUF538</i>	TC174694	AT5G01610	Protein containing domain of unknown function (DUF) 538	2.6	10.8	2.0	2.6	4.1	1.3
<i>BcDUF620</i>	Bra008179	AT1G75160	Protein containing domain of unknown function (DUF) 620	0.2	5.5	0.4	0.4	28.3	1.2
<i>BcUNK1</i>	Bra028503 ES907952	AT5G41761	Unknown protein 1	0.4	13.1	0.1	0.2	33.9	1.1
				0.8	28.8	0.1	0.2	34.2	1.3
<i>BcUNK2</i>	EE450922	AT5G43230	Unknown protein 2	3.4	22.6	3.1	4.3	6.7	1.4
<i>BcUNK3</i>	Bra009749	AT5G24313	Unknown protein 3	2.6	19.6	0.0	0.0	7.6	0.9
Protein phosphatases									
<i>BcPP1</i>	Bra022179	AT3G16800	Protein phosphatase 1	33.6	15.8	32.5	32.6	0.5	1.0

Supplementary Material

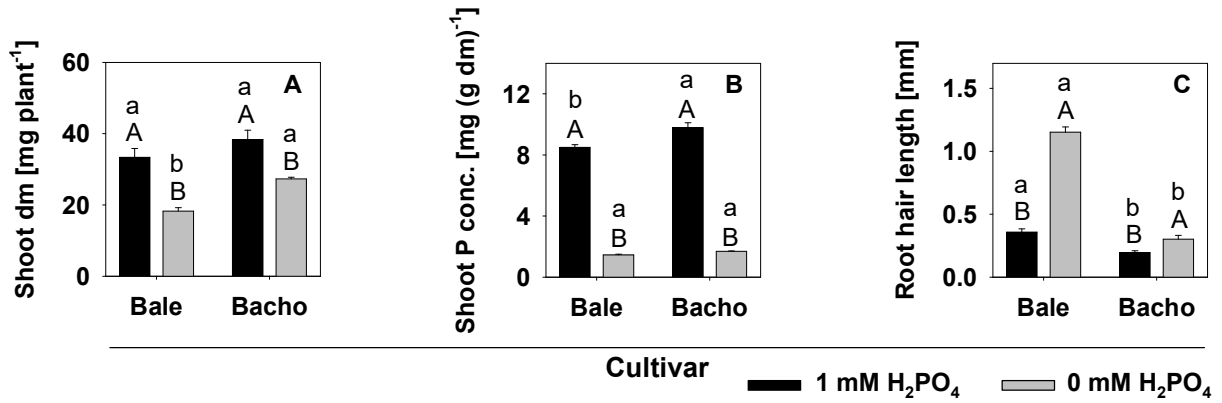


Fig. S4: Shoot dry matter (A), shoot P concentration (B) and root hair length (C) of *B. carinata* cv. Bale and cv. Bacho as affected by P supply in the 2nd experiment. Small letters denote significant differences at $P < 0.05$ between cultivars at the same P-level; capital letters between P-treatments of the respective cultivar (Tukey test). Columns represent means and bars SE; $n=6$ (A), 3 (B) and 12 (C).

Supplementary Material

Table S8: Relative expression of candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by P supply determined by qPCR in samples obtained from the MACE (1st) experiment. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Stars denote significant differences compared to +P within the cultivar (significance codes: 0 '**' 0.001 '**' 0.01 '*' 0.05).**

Gene name (abbr.)	Relative expression			
	Bale		Bacho	
	+P	-P	+P	-P
Arabinogalactan proteins				
<i>BcAGP1</i>	1.00	***4.83	1.57	0.97
<i>BcAGP2</i>	1.00	***3.06	1.14	1.34
<i>BcFLA1</i>	1.00	1.22	0.91	0.66
Calcium-related				
<i>BcCaBP1</i>	1.00	0.75	1.25	0.94
<i>BcCML25L</i>	1.00	1.06	0.71	***0.47
<i>BcCNGB1L</i>	1.00	***1.85	0.73	0.75
<i>BcRALFL1</i>	1.00	***1.73	1.24	0.99
<i>BcCOW1L</i>	1.00	***2.39	1.85	1.58
Phosphate transport				
<i>BcPHT1</i>	1.00	***7.55	1.33	***0.30
<i>BcPHF1</i>	1.00	***2.18	1.54	2.17
Protein kinases				
<i>BcRLCK1</i>	1.00	**1.35	0.73	*0.57
<i>BcMRIL</i>	1.00	1.25	1.90	*1.41
<i>BcLRR-RLK1</i>	1.00	1.32	0.75	0.62
<i>BcIREL</i>	1.00	1.45	0.70	0.65
<i>BcMRH1L</i>	1.00	***1.43	1.09	1.03
<i>BcRHS10L</i>	1.00	*1.39	1.55	***0.94
<i>BcPIP5K1</i>	1.00	***2.34	0.53	***0.73
GTPase-related				
<i>BcROPGEF1</i>	1.00	***1.80	1.00	0.94
<i>BcROPGEF2</i>	1.00	***4.83	1.57	0.97
<i>BcAGD1</i>	1.00	1.13	0.84	0.40
<i>BcEXO70L</i>	1.00	0.84	0.81	0.57
Root hair affecting				
<i>BcRHSL</i>	1.00	***2.53	2.18	1.62
<i>BcSHV2L</i>	1.00	***2.24	1.45	1.32
<i>BcSHV3L</i>	1.00	***1.84	0.81	***0.67
Transcription factors				
<i>BcLRL3L1/2</i>	1.00	***2.06	1.73	1.56
<i>BcWRKY1</i>	1.00	1.00	0.56	**0.31
Zinc finger proteins				
<i>BcZC3H1</i>	1.00	***1.73	1.06	0.91
<i>BcATL12L</i>	1.00	1.45	1.95	1.42
Unknown				
<i>BcDUF538</i>	1.00	***3.21	2.59	3.24
<i>BcDUF620</i>	1.00	1.22	0.41	0.31
<i>BcUNK1</i>	1.00	***7.00	0.04	***0.02
<i>BcUNK2</i>	1.00	***1.81	1.22	1.18
<i>BcUNK3</i>	1.00	***4.28	0.00	0.00
Protein phosphatases				
<i>BcPPP1</i>	1.00	***0.39	0.44	***0.28

Supplementary Material

Table S9: Relative expression of candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by P supply determined by qPCR in samples obtained from the independently conducted 2nd experiment. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Stars denote significant differences compared to +P within the cultivar (significance codes: 0 ** 0.001 *** 0.01 ** 0.05).**

Gene name (abbr.)	Relative expression			
	Bale		Bacho	
	+P	-P	+P	-P
Arabinogalactan proteins				
<i>BcAGP1</i>	1.00	***4.05	1.13	0.82
<i>BcAGP2</i>	1.00	***6.06	1.29	***2.79
<i>BcFLA1</i>	1.00	***6.25	4.34	4.41
Calcium-related				
<i>BcCaBP1</i>	1.00	***4.14	6.20	7.68
<i>BcCML25L</i>	1.00	***2.26	1.22	1.38
<i>BcCNGB1L</i>	1.00	*1.98	1.02	1.06
<i>BcRALFL1</i>	1.00	***2.36	1.80	1.64
<i>BcCOW1L</i>	1.00	***2.22	1.57	1.57
Phosphate transport				
<i>BcPHT1</i>	1.00	***12.00	1.67	***0.58
<i>BcPHF1</i>	1.00	***2.33	1.99	*3.30
Protein kinases				
<i>BcRLCK1</i>	1.00	1.55	0.81	0.85
<i>BcMRIL</i>	1.00	***2.59	3.46	4.46
<i>BcLRR-RLK1</i>	1.00	***1.81	0.88	0.87
<i>BcIREL</i>	1.00	***2.98	1.66	1.68
<i>BcMRH1L</i>	1.00	1.26	1.36	1.17
<i>BcRHS10L</i>	1.00	1.73	1.61	1.30
GTPase-related				
<i>BcROPGEF1</i>	1.00	***2.65	1.47	1.55
<i>BcROPGEF2</i>	1.00	***5.31	1.38	1.74
<i>BcAGD1</i>	1.00	**2.30	1.21	0.97
<i>BcEXO70L</i>	1.00	*2.26	4.49	3.09
Root hair affecting				
<i>BcRHSL</i>	1.00	***6.29	4.83	5.57
<i>BcSHV2L</i>	1.00	***4.02	2.05	3.02
<i>BcSHV3L</i>	1.00	1.69	0.93	0.82
Transcription factors				
<i>BcLRL3L1/2</i>	1.00	***2.75	1.26	1.81
<i>BcWRKY1</i>	1.00	***6.08	1.70	1.92
Zinc finger proteins				
<i>BcZC3H1</i>	1.00	**2.05	1.06	1.31
<i>BcATL12L</i>	1.00	***3.04	2.45	3.23
Unknown				
<i>BcDUF538</i>	1.00	***5.62	2.82	***6.16
<i>BcDUF620</i>	1.00	***6.55	1.08	***2.03
<i>BcUNK1</i>	1.00	***37.13	0.01	***0.02
<i>BcUNK2</i>	1.00	***2.64	2.02	2.40
<i>BcUNK3</i>	1.00	***10.19	0.00	0.00
Protein phosphatases				
<i>BcPPP1</i>	1.00	***0.19	0.41	***0.19

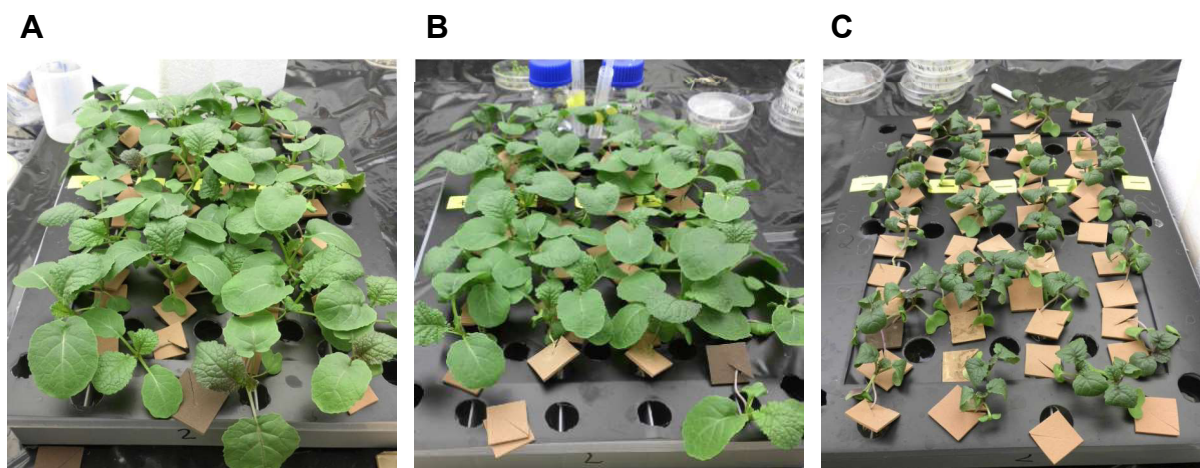


Fig. S5: *B. carinata* cv. Bale plants after an eight-day-cultivation in a split-root system containing 1 mM Pi on both sides (A), 1 mM Pi on one side and 0 mM Pi on the other side (B) and 0 mM Pi on both sides (C).

Supplementary Material

Table S10: Relative expression of candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale as affected by P supply in a split-root system determined by qPCR. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Different letters denote significant differences between the treatments.

Gene name (abbr.)	Relative expression			
	++	+	-	--
Arabinogalactan proteins				
<i>BcAGP1</i>	b1.00	b1.43	b2.12	^a 12.05
<i>BcAGP2</i>	b1.00	^b 0.94	b1.10	^a 5.10
<i>BcFLA1</i>	b1.00	^b 0.97	^{ab} 2.12	^a 5.31
Calcium-related				
<i>BcCaBP1</i>	b1.00	b1.12	^a 5.62	^a 6.63
<i>BcCML25L</i>	b1.00	b1.52	^a 5.07	^a 7.64
<i>BcCNGB1L</i>	^c 1.00	^c 1.24	b2.29	^a 4.12
<i>BcRALFL1</i>	b1.00	b1.00	b1.12	^a 2.42
<i>BcCOW1L</i>	^c 1.00	^{bc} 1.30	b1.93	^a 5.30
Phosphate transport				
<i>BcPHT1</i>	^c 1.00	b2.24	b4.02	^a 30.91
Protein kinases				
<i>BcRLCK1</i>	b1.00	b1.24	^a 3.12	^a 5.08
<i>BcMRIL</i>	b1.00	b1.40	^a 3.96	^a 6.29
<i>BcLRR-RLK1</i>	b1.00	b1.18	^a 3.24	^a 5.78
<i>BcIREL</i>	b1.00	b1.25	^a 4.39	^a 7.66
<i>BcMRH1L</i>	^c 1.00	^{bc} 1.16	b1.64	^a 3.02
<i>BcRHS10L</i>	^c 1.00	^c 1.10	b1.97	^a 3.58
<i>BcPIP5K1</i>	b1.00	b1.19	^a 3.13	^a 6.20
GTPase-related				
<i>BcROPGEF1</i>	^c 1.00	^c 1.32	b2.91	^a 5.70
<i>BcROPGEF2</i>	^c 1.00	^c 1.48	b3.82	^a 9.62
<i>BcAGD1</i>	b1.00	b1.34	^a 5.07	^a 7.47
<i>BcEXO70L</i>	b1.00	b1.18	^a 6.82	^a 7.48
Root hair affecting				
<i>BcRHSL</i>	^c 1.00	^c 1.30	b2.85	^a 7.23
<i>BcSHV2L</i>	^c 1.00	^{bc} 1.43	b2.30	^a 6.42
<i>BcSHV3L</i>	b1.00	b1.07	b1.16	^a 2.83
Transcription factors				
<i>BcLRL3L1/2</i>	^c 1.00	^{bc} 1.21	b2.22	^a 5.05
<i>BcWRKY1</i>	b1.00	b1.14	^a 3.00	^a 4.40
Zinc finger proteins				
<i>BcZC3H1</i>	b1.00	^b 0.98	^a 2.43	^a 4.18
<i>BcATL12L</i>	b1.00	b1.32	^a 4.49	^a 6.87
Unknown				
<i>BcDUF538</i>	^c 1.00	^{bc} 1.10	b2.60	^a 6.61
<i>BcUNK1</i>	^c 1.00	^c 1.46	b5.54	^a 25.83
<i>BcUNK2</i>	b1.00	b1.51	^a 4.15	^a 8.67
<i>BcUNK3</i>	^c 1.00	^{bc} 2.01	b4.74	^a 24.79

Supplementary Material

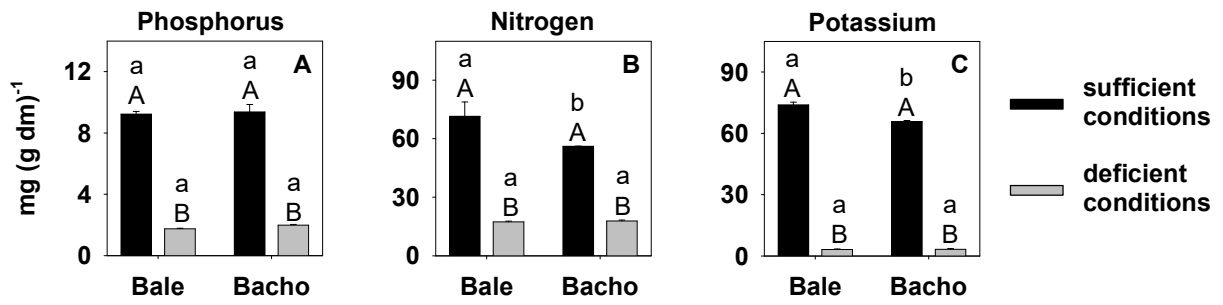


Fig. S6: P (A), N (B) and K (C) concentration in the shoot of *B. carinata* cv. Bale and cv. Bacho as affected by P supply. Small letters denote significant differences at $P < 0.05$ between cultivars at the same nutrient-level; capital letters between the treatments of the respective cultivar (Tukey test). Columns represent means and bars SE; $n=3$.

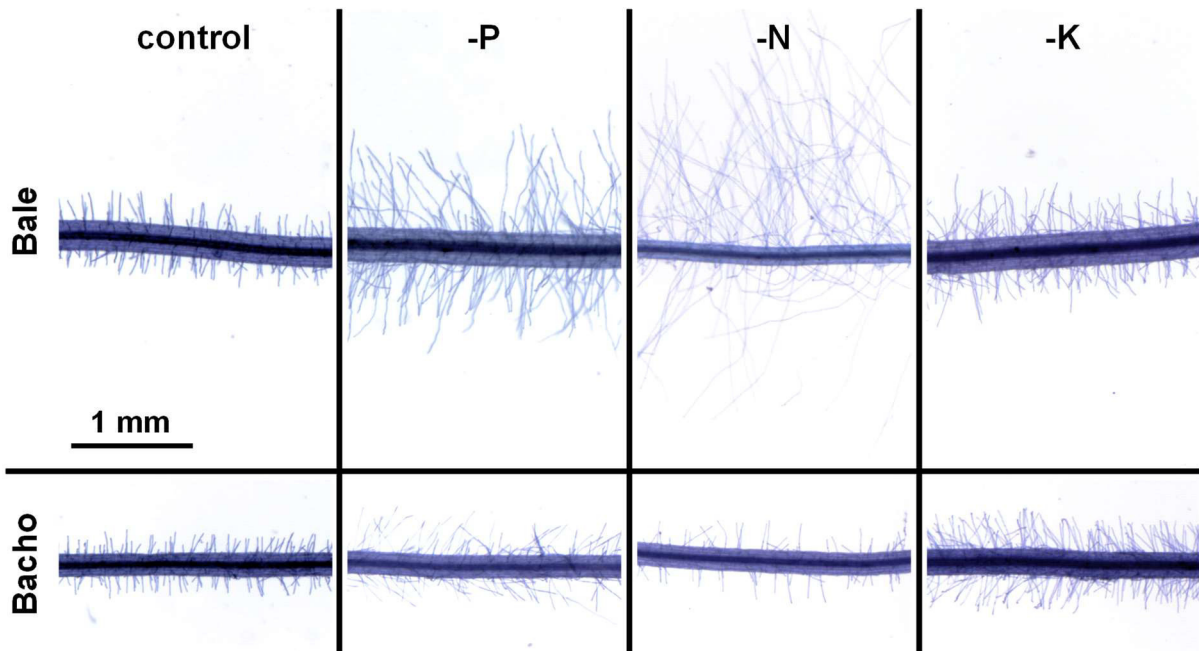


Fig. S7: Representative root hairs of *B. carinata* cv. Bale and cv. Bacho as affected by different nutrient deficiencies.

Supplementary Material

Table S11: Relative expression of candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR. *AtUBC9* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Stars denote significant differences compared to the control (C) within the cultivar (significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05).**

Gene name (abbr.)	Relative expression							
	Bale				Bacho			
	C	-P	-N	-K	C	-P	-N	-K
Arabinogalactan proteins								
<i>BcAGP1</i>	1.00	***8.21	***4.13	1.04	2.02	1.35	1.73	1.41
<i>BcAGP2</i>	1.00	**6.48	**2.85	0.99	2.87	3.92	1.77	**1.14
<i>BcFLA1</i>	1.00	***5.07	1.40	0.88	4.51	2.83	3.10	2.82
Calcium-related								
<i>BcCaBP1</i>	1.00	***6.47	***5.00	1.35	6.90	9.25	8.26	7.16
<i>BcCML25L</i>	1.00	2.33	2.77	0.65	1.02	0.67	0.87	0.72
<i>BcCNGB1L</i>	1.00	***4.57	***2.94	1.50	1.98	1.96	2.72	1.68
<i>BcRALFL1</i>	1.00	***2.50	*1.48	0.87	1.87	1.91	1.49	***1.01
<i>BcCOW1L</i>	1.00	***4.96	***2.95	1.14	3.25	2.90	2.65	2.55
Phosphate transport								
<i>BcPHT1</i>	1.00	***25.86	**2.84	1.44	4.71	2.74	***1.66	3.05
Protein kinases								
<i>BcRLCK1</i>	1.00	***4.40	**3.64	0.80	1.45	1.03	1.35	1.03
<i>BcMRIL</i>	1.00	*1.90	0.99	0.60	2.54	2.80	2.16	1.78
<i>BcLRR-RLK1</i>	1.00	***3.69	*1.96	0.73	1.19	1.49	1.17	1.14
<i>BcIREL</i>	1.00	***6.03	***3.30	1.39	2.76	2.90	3.00	2.89
<i>BcMRH1L</i>	1.00	**2.67	*2.15	1.90	2.41	2.50	3.21	3.11
<i>BcRHS10L</i>	1.00	*3.40	2.48	1.19	2.13	1.41	2.64	2.50
<i>BcPIP5K1</i>	1.00	***7.23	***4.42	1.88	1.57	2.35	2.21	1.57
GTPase-related								
<i>BcROPGEF1</i>	1.00	***2.56	1.42	0.78	1.28	1.52	1.23	1.08
<i>BcROPGEF2</i>	1.00	***9.34	***4.58	1.32	2.56	1.76	1.57	2.98
<i>BcAGD1</i>	1.00	***4.46	**2.52	1.66	2.24	2.60	2.63	2.26
<i>BcEXO70L</i>	1.00	*4.36	**5.87	2.49	4.00	3.62	6.62	4.38
Root hair affecting								
<i>BcRHSL</i>	1.00	*4.16	2.00	0.95	3.55	3.73	2.88	4.51
<i>BcSHV2L</i>	1.00	***7.09	**4.62	1.71	4.28	4.75	3.43	2.84
<i>BcSHV3L</i>	1.00	***6.07	*2.98	2.17	2.40	2.48	1.68	2.49
Transcription factors								
<i>BcLRL3L1/2</i>	1.00	***2.82	1.23	0.86	1.62	1.69	0.98	1.19
<i>BcWRKY1</i>	1.00	*4.72	***11.59	3.80	1.77	1.58	3.19	2.18
Zinc finger proteins								
<i>BcZC3H1</i>	1.00	1.38	1.45	0.50	0.64	0.50	0.68	0.67
<i>BcATL12L</i>	1.00	*2.05	1.90	1.28	2.94	3.17	2.76	2.76
Unknown								
<i>BcDUF538</i>	1.00	***3.87	*2.68	0.74	2.17	2.38	1.61	1.51
<i>BcUNK1</i>	1.00	***40.69	*13.27	1.05	0.04	0.04	0.06	0.08
<i>BcUNK2</i>	1.00	**4.40	2.69	0.49	2.25	2.21	2.15	1.49
<i>BcUNK3</i>	1.00	**16.32	3.91	0.96	0.00	0.00	0.00	0.00

Supplementary Material

Table S12: Relative expression of candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR. *BcEF-1-a1* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Stars denote significant differences compared to the control (C) within the cultivar (significance codes: 0 '**' 0.001 '***' 0.01 '**' 0.05).**

Gene name (abbr.)	Relative expression							
	Bale				Bacho			
	C	-P	-N	-K	C	-P	-N	-K
Arabinogalactan proteins								
<i>BcAGP1</i>	1.00	***5.32	2.29	0.91	1.18	1.30	1.55	1.72
<i>BcAGP2</i>	1.00	***4.32	1.37	0.75	1.96	3.34	1.56	1.17
<i>BcFLA1</i>	1.00	*3.20	0.87	0.71	3.13	2.56	2.80	3.21
Calcium-related								
<i>BcCaBP1</i>	1.00	4.08	1.25	1.10	4.80	8.36	7.48	7.50
<i>BcCML25L</i>	1.00	1.67	1.24	0.84	0.91	1.24	1.20	1.42
<i>BcCNGB1L</i>	1.00	***2.96	1.63	1.32	1.16	1.89	2.43	2.05
<i>BcRALFL1</i>	1.00	1.62	0.82	0.77	1.09	1.84	1.33	1.23
<i>BcCOW1L</i>	1.00	**3.21	1.63	1.01	1.90	2.79	2.37	3.11
Phosphate transport								
<i>BcPHT1</i>	1.00	***16.76	1.57	1.26	2.76	2.64	1.48	3.73
Protein kinases								
<i>BcRLCK1</i>	1.00	*3.16	1.63	1.03	1.30	1.90	1.85	2.01
<i>BcMRIL</i>	1.00	1.38	0.59	0.57	2.01	3.00	2.24	2.21
<i>BcLRR-RLK1</i>	1.00	***2.67	1.16	0.70	0.95	1.59	1.22	1.42
<i>BcIREL</i>	1.00	***4.03	1.81	1.05	1.89	2.47	2.65	2.82
<i>BcMRH1L</i>	1.00	1.71	1.19	1.63	1.64	2.26	2.83	3.18
<i>BcRHS10L</i>	1.00	***2.46	1.46	1.14	1.69	1.51	2.74	3.11
<i>BcPIP5K1</i>	1.00	***4.35	*2.69	1.54	0.99	1.97	1.78	1.69
GTPase-related								
<i>BcROPGEF1</i>	1.00	1.86	0.84	0.74	1.02	1.62	1.29	1.34
<i>BcROPGEF2</i>	1.00	**6.70	2.06	1.70	2.29	3.22	2.16	5.84
<i>BcAGD1</i>	1.00	**2.97	1.35	1.24	1.53	2.21	2.33	2.56
<i>BcEXO70L</i>	1.00	2.91	3.14	1.87	2.73	3.09	5.85	4.95
Root hair affecting								
<i>BcRHSL</i>	1.00	2.68	1.11	0.82	2.41	3.37	2.54	4.61
<i>BcSHV2L</i>	1.00	***4.56	**2.56	1.48	2.91	4.30	3.02	2.90
<i>BcSHV3L</i>	1.00	***4.31	1.77	*2.03	1.88	2.65	1.77	3.24
Transcription factors								
<i>BcLRL3L1/2</i>	1.00	*2.04	0.73	0.82	1.28	1.80	1.02	1.48
<i>BcWRKY1</i>	0.57	0.93	0.94	1.32	1.59	2.90	4.38	4.28
Zinc finger proteins								
<i>BcZC3H1</i>	1.00	0.99	0.65	0.64	0.57	0.93	0.94	1.32
<i>BcATL12L</i>	1.00	1.31	1.05	1.11	1.99	2.86	2.43	2.81
Unknown								
<i>BcDUF538</i>	1.00	2.84	1.28	1.03	2.00	4.65	2.33	3.27
<i>BcUNK1</i>	1.00	**29.88	6.35	1.46	0.04	0.08	0.08	0.17
<i>BcUNK2</i>	1.00	*3.23	1.29	0.80	2.08	4.32	3.11	3.24
<i>BcUNK3</i>	1.00	11.98	1.87	1.34	0.00	0.00	0.00	0.00

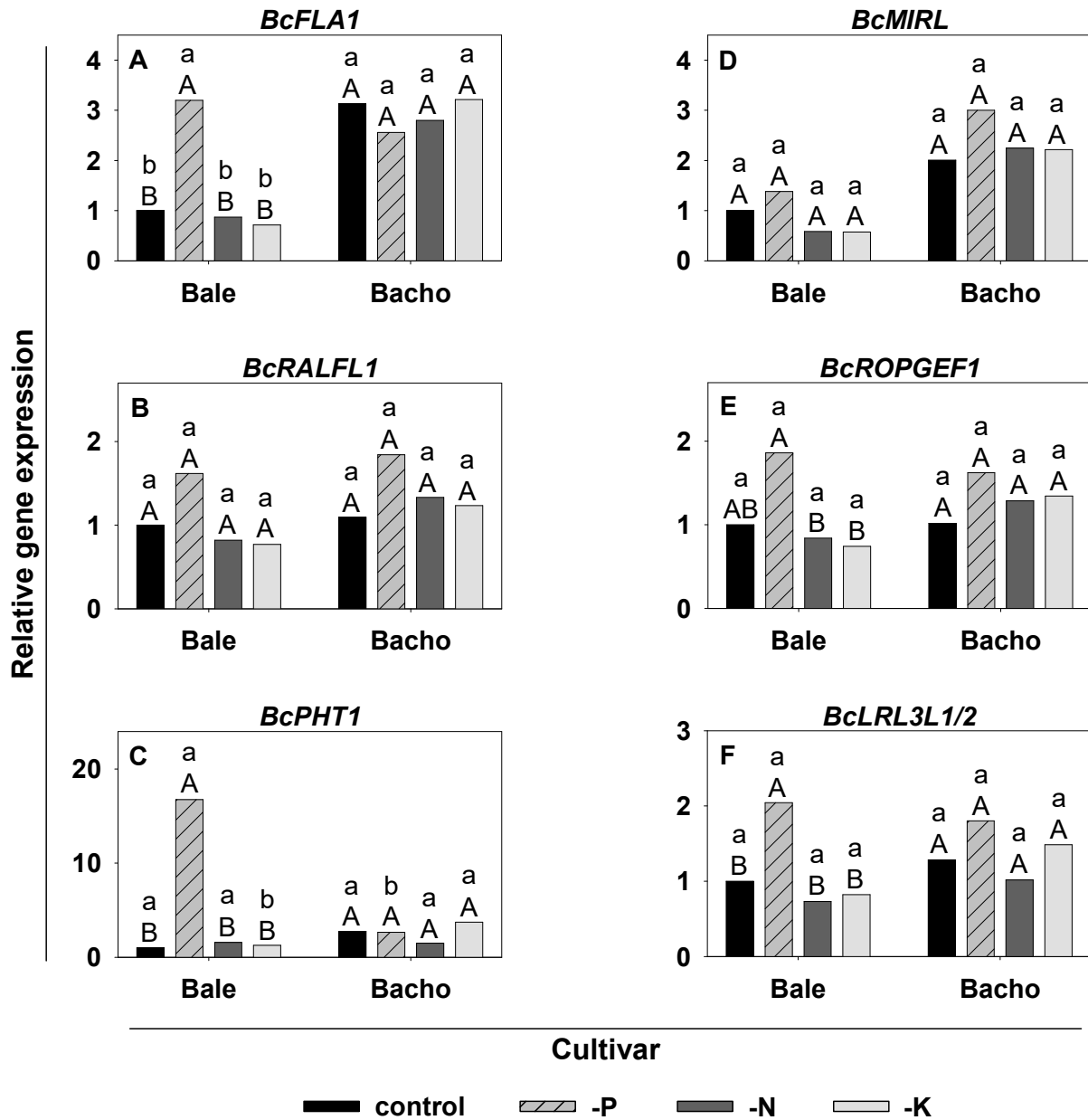


Fig. S8: Relative expression of selected candidate genes for increased root hair length under Pi deficiency in *B. carinata* cv. Bale and cv. Bacho as affected by nutrient deficiency determined by qPCR. *BcEF-1-a1* was used as endogenous control. Significance was calculated according to Steibel *et al.* (2009). Significant differences at $P < 0.05$ are denoted by small letters between cultivars for the same nutrient treatment and capital letters between nutrient treatments for the same cultivar. Columns represent means; $n=3$.

Supplementary Material

Table S13: Bale gene sequences obtained by 5' RLM-RACE (candidate genes, which were not further investigated by knockout). RACE inner primer (bold); additionally used sequencing primers (underlined). In case of *BcCML25L* only the RACE outer primer was used.

Bale consensus sequence (5' → 3')	
<i>BcCML25L</i>	<p>AAAGAGATTTATTCCTGAATTAGTTGCGAGAATCCGAAATTAGCTAGCGATCCAGAGATTCATT CGCTCCCCTCAAGAATCACCACAAAATACAAATACATCTCTCNTTCTCACACACAAAACGAGATT CTGAACCATGTTCCACAAGAAAAACCAATCCAATGGTTCGGGCGGCGGCTCGGTATCCCGAACC GGAGCAGACTCTCCGTACCTACAGAAAGCACGCTCGGGTAAAACCGAGATCCGAGAACTCGAAG CGTTTTTCAAAAAATTTCGACGTGAACGGAGACGGCAAGATCTCGTCCAAAGAGCTCGGCGCGAT CATGGCGAGTCTAGGCAACGAGGTTCCGGAGGAGGTCTTGGAGAAGGCGATCACGGAGATAGAT CGTAAAGGAGACGGTTACATAAACTTCGAGGAGTTCGTCGAGCTGAACACGAAAGGGATGGATC AGAACGACGTGCTCGAGAATCTCAAGGACGCGTTCTCCGTTTACGATATCGACGGGAACGGAGC GATATCGGCGGAGGAGCTGCATGAGGTTTTGAGGAGTTTGGGGGATGAGTGTTCGATTGGGGAG TGCAGGAAGATGATCGGNTGGTGTGGATAAGGATGGTGATGGGACGATTGATTTTCGAGGAGTTT AAGATTATGATGACGATGGGATCGAGACGTGATAACGTCATGGGTGGTGGTGGTGGTCAGAGGT AGAGTGTTCACTTTTACGGCGGGGTGATGATCGTCTTCCCTCTTCGTGTTCTCGTTTTGTTTGT CCATTGTTTTATTATTCATCATTGTTTCATTTTTTTGTTTCTTGTTCTTTGGGAGAATCACA</p>
<i>BcCNGB1L</i>	<p>AAAAATCGAATCTCATTTTATACAAACCCTAGTTCCTTTCCAAGGAGATTCGTTTCCTTGACAC ACACGTCTTCTTTCTTTTCCCTCCCAAAAAAGCTATTTATTTGTTCTTCTTCCCTTTGATCGATA TTTCTTGAGGTCATGGCAACGACATCAAACNCGCAAAAAGCAAAGGTGNNCAACAATAACCTAA CCAGAACAAAATCACTCGGTAAAAAACCAAAGCAACCATCCTCGGTATCATCAGAAGATGGATC TGATCAGAAACCTGAGAAGCCTTTACCAAATATCTGAAACCAACCATCAGTTCAAGACCTGAC CCAGTCAAGTTCTTGAAGAAGAATGATCAGGATAACCAGAAGCTTCTCCGTAGAAGATCTTTTG ATCGACCACCATCTGCTTTGACTTCTGCTTCTGCTCCTTCTCCTCGTATCCAGAAAACCCTCAA CGTTTCTCCTTCTCTGCCACGGGACAAACCCGCGGTTCCAGAGAGAAGCCTGCCACAGCTCAA CGGTCTTCTTCTTTCCATGGAAGCAGAAGTGTTCCAAGAGGAAGCACTTCTCTGAAATCGCCTC ATGTTGCTCCAAAGAAGAGCGGGTTGAGCAGTAGTAGCGCTTCTTTGAAGAGTAAGAAGGAAGG TTCTGAAAATGTTGCTGGAAAGAAAGCACCTGAGAAAGAGATTGTGTTGGATACTGCTTCTTTG TCATCTGCTCAGGAAGATGACGAGGAGATTCTCAAGGTTGAGAGTGATGTACAAGTTGCTGGTG ATATAGAAGAACCCTAAAGGTGAAAACGAAAATAAAGAGGTTGCATGTAGAGGTTGTGCATGGGGA TACTTCTGGTGAAGAGAATGAGAGTGGTGGATCAACATTTGTTGTTGCCAAAGAAAATGAAAA GNAGAAGAAGAAAGATTGATCANTGANCNAACTCAGAAGAGAATATAGAGGAAAAGAAAGAAC AAGAGAAAATAGAGGAAAAGAAAGAACAAGAGAATAACCAAGAGAACAAGGTTGAACTCGCTGA AGAGGTTACACCAAAGGTTGATGAATATGAGACTTCGGAGAAGGCCGATGCTGACATGGATACA AAAGAGGTTGAGAAGGTCGAAGAAGTTGAAAGTGTGGTTCGAGACGAAGGAAGAAAAAGAAG TGAAAGAAGAAAAGGAAGTGGAGAGAGAGGAGAAGGATAAAGTTAAAGAAGAAGAATC AGGAGAAGGAAAAGAAGAAGGAGTAGTGAAAGGTAAGAAGGAATCTCCATCAGCCTACAACGAT GTAATAGCAAGTAAGATTGCAAGAGAACCCAAGGAAGA</p>

Supplementary Material

BcCOW1L

AAAGTCTACATGTTATGAAGTAGTTTCAGATGAGCCTCTCCATTATCTTCACCCCCCTTACCC
TTCCACAAACACTCTTCTCCAATAAGGTAATCAATTGCCGTGTGCAACAAAGTGCTTTAAAAGT
CATCTATCCATGGCTGAGACCAAACCTGAAATAGAGATGTCAGAGGAGGAGAGGAAGATAGTAA
AGATGTGTTCACTGAAGAAGAAAGCCATAAGCGCATCAAACAGATTCAAGAACTCATTTCAAGAA
AAAGGGTCGAAGAAGCACCAGCAGAGTCATGTCCGCACCGATTGAGGACGACATCGATGCAGAA
GATCTTCAAGCTCTTGATGCTTCCCGTCAAGCTCTTGTCCCTTGATGAGCTCTTGCCCTCCAACC
TCGATGATCTCCATACGATGCTTCGATTTCTGAGGGCAAGAAAGTTTGACATTGAGAAAGCTAA
GCAAATGTGGAGTGATATGATTAATTGGAGGAAAGATTTCCGGTGCTGACACAATCATCGAGGAT
TTTGAGTTNGAAGNGATCGATGATNTCATGAAGCATTACCNTCAAGGCTNTCATGGAGTAGNCA
AGGAAGGCAGNCCAGNNTNCATTTGNNNGATTAGGTCAAATCGATGCTANCAAGCTGCTTCAAGT
AACACAATGGACCGGTACGNAAATNTCATGTGAGNGAGTTCGAGAAGACTTTCAAGATCAAG
TTCCTTGCTTGTCTGTGCTGCGCTGCCAAAAAGCACATTGACCAGAGCNCAACTATTCTTGACGTCC
AAGGCGTGGGACTTAAGAACTTCAGCAAAATCTGCGAGAGAGCTTCTTCAAAGGCTTNTCAAGAT
TGACAATGAAAATTATCCTGAGACGTTGAACCGGATGTTTATTATCAATGCGGGTTTCAGGGTTT
CGGCTCTTGTGGAGCACTGTTAAGTCGTTTCTTGTATCCCAAGACTACTGCAAAGATTTCATGTTT
TTGGTAACAAATATCATAGCAAATTGCTTGAAGTCATCGATGCTAGTGACTTGCCAGAGTTCTT
TTGGAGGTGCTTGCACTTGTGAAGATAAAGGGAGGTTGTATGCGCTCAGACAAAGGTCCATGGA
ATGATCCTGAGGTTCTCAAAATGGCTATAAACCGTGAATCCAAGTGCTCACCAATTTCAGAAGA
TGAACACAAGCATGTTGACCAAGGAAGAACAACCTTCAGTTCCGAAAGTCTTGAAAGAAACAAT
AAGAAAAGGGATGAAGACAATGTGCATGAGAAGCAAGTTGCAACCATTGACAAGTCCGTGATGG
AGTGGCCTACAAAACTCAGAAAATCGAAAATTTTCTGTCTCTAAAGGGTTGGAGTGCTACGT
GAGGAAGGGGGCACCGAAGAAAGGGGATGGGTTACTTGTGGGAGGGGTCATGGCCTTTGTGATG
GGAATTGTTGCAATGGTTAGGCTATCAAAAGACGTTCCACGTAAACTCACAGAGGCTGCTTTAT
ATGGAACTCTGTTTGTATGAGGATTCTATGCCTAAGCAGAATCAAGCTCAATTTGCAGCTCC
CGTCTCGAGCTCAGAGTACATGTTGATGGTGAACGTATGGCTGAGCTGGAAGAAAAGTGCATG
TTCCTTGACTTGAAACCGGCTAATGTTGACACTGAAACAGAGGAGAACTTCAGGCGGCGCTTA
ACCGTGTTCAGTACTTGAGCAAGAGTTGACT**GAGACCAAGAAGGCTTTGGA**

Supplementary Material

BcLRR-RLK1	<p>AATTTTTGTTTTGTTTTCTTCAAGATGATTCTTCATGACAACAAACCTGCGGTGGTATACTCTC TTTTAGTATTATTACTCGCCGTTAGCTTCTTCGTTCCCATCTCCAGCGACGGTGACGCTGATGC TCTTTTGAAGTTCAAGTCATCTCTCGTGAACGCCACCGTCCTCACCGGATGGGGGGACTCCGGT GATCCTCCATGCACCCGGGAAAAAAGGGAGCAACTCGAAATGGAAAGGAGTCATGTGTTCTGCCG GCGTCGTTTACGCTCTCCGTCTAGAGAACATGAGCCTTGCGGGGACGCTTGACGTGCAAGCGCT AGGCTCCATGCGCGGTCTCAAGAGCGTTAGCTTCATGCGCAACGGTTTAGAAGGTCCGATACCG CGTGGACTCGACGGGCTTGGATCTCTTGTGCATCTTTACTTGGCGCATAACCGGTTTTCCGGCG AGATTGACGGTGGTTTTGTTGATGGAATGAAGGATTTGGTGAAGGTCATCTTGAAGGAAACCG GTTTTCCGGTGAGATTCCCTAAGTCGTTGGGGAAGTTGCCGAAGCTGACTGAGCTGAATCTTGAG GATAATTTGTTCCACCGGGAAGATACCTCCATTTAATCAGAAGAATCTTGTACCCTAACGTCG CTAATAATCGTTTAGAGGGTCGATTCCGGTAACTCTCGGCCTCATGAACATCACCTTCTTCTT AGGTAACAAGGGGCTGTGTGGACCGCCTTTGCTTCCCTGTAGACACCCCTCGTCCACCGTTGGTT GCAGTGTTCCTCCTTGCCCTCACCGTCTCGCCGTATAGTCCTCGTAACCGTCTTCTGTTCG TTTGCATCCTCAGCCGTCGTCAACGCAAAGGTCCCACCACGACCGCGGCCACAGCCCCAGCCT CGGCCTCGGCACAGTCTACGGACCAAGCGAGCAACAGCAACAAAACAGCGAGAARAGCTCGCAG GACTCCAAGGTCTACAGGAAGCTAGCCAACGAAGCTKKGCAGCGANNNNNNNNNNNNNNNNNN CCAGTGTATCCGAGAGAGCCCTGCCTCGAGAGGAAGATCAGCGGAAGCTGCATTTTCGTTCCGA ATGATCAGGAGAAGTTCACGCTTCCAGGATATGCTCCGTGCGTCCGCGGAGGTTCTCGGCAGCG GGGGTTTTGGATCGTCGTACAAGCGGCTCTTACGGGAAGCCGCGCGGTGGTTGTGAAGCGGTT TAGGTTTTATGAATAATATCAGGAGAGAGGAGTTTTACGATCATATGAAGAAGATCGGACGGTTA TCGCACCCTAATCTACTTCCATTGATAGCATTCTACTACAGAAAAGATGAGAAGCTTCTCGTCA CCAATTACATTTCCAATGGCAGCCTCGCCAATCTCCTTCATGCAAACCGAACACCCGGTCAAGT GGTTTTGGATTGGCCAATCCGGTTAAAGATTGCAAGAGGAGTCACAAGAGGCTTAGCTTATCTC TACAGAACATTTCCCTGATCTGAATCTCCCTCACGGCCATCTCAAATCATCCAACGTGTTGCTCG ACCACGACTTCGAGCCGCTTCTAACAGACTACGCTCTCGTGCCAGTGGTCAACAAGGAGCAATC TCACCAGTTCATGGTGGCGTACAAGTCAACCAGAGTTCACTCAGCAAGACCGAACATCAAGAAAG TCCGACGTCTGGAGCCTAGGAATCTTGATCCTCGAGATACTAACGGGGAAGTTTCCAGCTAACT ACCTCCGGCAAGGGAAAGGAGCTGATGACGAGCTAGCTACTTGGGTGAGTCAGTAGCGAGAAC TGAGTGAATGCTGACGTGTTTGATAAGGAGATGAGAGCAGGGAAAGAACAGGAAGTTCAGATG CTGAAGCTGCTCAAGATTGGGTTGAGGTGTTGTGACTGGGACGTGGAGAGGAGAATGGAGCTTC ACGAGGCTGTTGATCGGATAGAAGAAGTTGATCACAGAGAGGCAGGTGGAAGTCAAGAGAGTTT TCGATCTTCGTACGTGACTGCTAGTGATGGTAAAATCGTTTTTTCAAGAGCCATGACTGGAGAG TTCTCCCTCGTGTAATAGCTTCACTGTCTCATTGATGTTTGTGTTTCTGTTTGTGGGTAAA AGACTGATCACC</p>
BcIREL	<p>GAAATCAATCAGGATGGTCACATCAAGTTGACAGACTTTGGGCTTTCCAAGGTTGGTCTTATCA ACAGCACAGATGACTTATCAGGTGATCCGTCATTGGGGAATAGTGAATTTTTAGCAGAAGATGG AAACCCTCAACATTCACAAGACAAAGATAGCCGTA AAAA ACATGCAGTTGTGCGAACACCTGAT TATCTTGCACCTGAAATACTTCTTGGAAATGGGTGATGGTAAAACCGCTGATTGGTGGTCAGTAG GTGTTATTCTCTTTGAGGTTCTCGTGGGTATTCCTCCTTTCAATGCAGAAACCCACAGCAAAT CTTTGAAAATATAATCAACAGAGATATACCATGGCCTAATGTGCCAGATGAGATATCTTATGAA GCATATGATCTGATCAACAAGCTGCTAACCGAAAACCTGTCCAAAGACTAGGTGCTACGGGAG CTGGAGAGGTGAAGCAGCATCATTTTTTCAAAGATATTA ACTGGGACACGCTTGCCAGGCAAAA GGCTATGTTTGTACCATCAGCTGAACCACAAGACACTAGCTATTTTCATGAGCCGGTATATATGG AACCCGGAAGACGAAAATGTTTCATGGAGGCAGCGATTTTGTGATGACCTTACAGACACATGCAGCA GCAGCTCCTTACCACACAGGAGGAAGATGGTGTGATGAGTGTGGTAGCATAGCAGAATTAGCAA TGGACCCAATCTTGCTGTGAAGTATTCCTTCAGCAATTTTTCTTTCAAGAACCTTTCACAACTG GCTTCGATCAACTACGATCTTGTCCAAAAGAACGCTAAGGAATCAACAGAAGCTGCAAACCCAT CAGCCCCTTGACCAAATCC</p>

Supplementary Material

BcRHS10L

AAACTCTCTCTCCAGCTTATTTCCAGGCCCTAGCTTTCATAAACTCTGACTTATTGTAATTATT
ATTATTATTTTTCTGTAAATGGGATCCATTTTCAGATTTTTAATTATTAGTTGTTTCCAGTTG
TAGAGTGGAAATATAATTTATTTTCATCTGATTATTCAACTGTATCTGGTATAGTTCTTCTGTA
TCGATTGATAGTTTCATTCATTAGAGACTTCTGAGTGGTGTCTGTTCTGGGGAAGAATCAGAATCA
GAATCAGAACAAGAAAATGTCTGACTCGCCGACTTCTTCCCCACCCGCACCCTCCGCCGACTCC
AGTCCGCCGCCAGATAACTCCACCGGTGGTCTGTCTCCTCCACCTACTGATTCCCTCACCACCTC
CTTCCCCACCGACTGACTCAGCACCGCCGCCGAGTATCCCATCTCCACCGCCAGCGACTCCTCC
TCCGGAATCCAATCCCCCGCTCCACCGCTGATTCCCCACCCCTCCTCCCCGGATGCTCCA
CCTCCATCTGATCCTCTACCACCGTTGACTCCGGTCTCCACCACCGGAGCCACAACTCTC
CTCCTCCCCCTGAGGAATTTGAATCTCCACCGCCTCCGCCGAATGAAGACAACACCTCTCCTCC
TTCACCTCCGGAACAATCACCTCCTCCTCCTCAGGCGCTTCCCCAAAGGGAGGATCCAAA
AAACCGAAAAATCACAGCACCAACCAATTCTCCTCCGGCTCCTCCAAATGCGCCTTCTCACG
CACTACCACCCAAGTCCACCGCTGCAGGAGGACCTCTCAAATCCCCATCTCGCGGCGTCCCCAG
CTTCCCCGCACCTCCTCCAACCAGCAATGACGGTGGCTATCAAGGCAAGACTATGGCCGGAATG
GCAGTCGCCGGATTTCGAATCATCGCCTTAATCGCCGTCGTGTTCTTAGTCAGAAGAAAGAAAA
AGAGAAACGTAGATGTATATACTGACTCCAGTACTTGCCTCCTTCTAATTCTCCATCAAGTC
AGATGGATTTTTTATACGGACAGAATACGACAAAAGGAGGGTACTCTGGTCTGGTGGCTACAAT
ACACAACAACAACAATCCAATACAGGAAACAGCTTTGGGAGCCAAAGAGGTTATACGCAGTCAG
GGAGTACACCTGATTTCAGCTGTGATGGGAAGTGGTCAAACGCATTTTCGCCTACGAAGAGCTAAT
GGAGATAACGCAAGGATTTCGTAAGCAAAACATACTCGGAGAAGGTGGGTTCGGGTGTGTCTAC
AAAGGTAATACTACACGACGGGAAACTAGTTGCTGTGAAGCAGCTTAAGGTTGGGAGTGGACAAG
GTGACCGCGAGTTTAAAGCAGAGGTTGAGATCATTAGCCGTGTTACCATCGTCATTTGGTTTC
TCTGGTTGGTTATTGTATCTCGGATGTCCAGAGGTTGCTTATCTATGAGTATGTTCCAAACCAA
ACCTTGGAGCATCATTTGCATGGGAAGGGAAGGCCAGTCTTAGACTGGGCTAGGAGAGTCCGAA
TCGCTATAGGTTCTGCCAAAGGTTTGGCGTATTTGCATGAAGACTGCCACCCAAAAATCATTCA
CAGGGACATAAAGTCGGCAAACATACTGCTGGACGATGAGTTTGAAGCTCAGGTTGCTGACTTT
GGACTTGCAAAACTCAGTGATTCAACACAAACCCATGTATCAACTCGCGTTATGGGAACCTTTG
GGTACTTGGCACCAGAATATGCACAAAGCGGGAAACTAACAGATAGATCAGATGTTTTCTCCTT
TGGGGTTGTTCTCTTAGAACTTATAACTGGACGCAAACCAGTTGATCAGTACATGCCTTTGGGA
GAAGAGAGTTTGGTTCGAATGGGCTCGTCCACTGCTTCATAAAGCCATTGAGACCGGTGATTTCA
GCGATCTGGTTGATAGACGGCTCGAAAAACATTACGTGGAGAATGAAGTTTTTCAGAATGATTGA
AACAGCTGCTGCATGTGTTAGACATTCCGGTCCAAAACGTCCACGCATGGCTCAGGTTGTGAGA
GCATTGGACAGTGAAGGGGACATGGGAGATATCAGCAACGGATCCAAAGTGGGACATAACAATG
CTTATGACTCTTGTGAGTATAACTCAGACGACAGGAAAAATGGCTTTTGGTTTTGACGATAGTTC
AGATTCAGGGATGCACAGTGGAGACTACTCTGTCCGAAGCTCCAGGAAAGGATCCAATGGAGAA
TCTACTGAGTTCACAAGGAGTGAATCTGAGAACAGAACTTCA**ATAACCGGCGGTTCTGATTCA**

Supplementary Material

BcROPGEF2	<p>AAAATTC AAGTGCACCAACTCAAACAAATTTAAAAAAGCCGAAAAAATACATGTAAAACCCAA AGTATTTTTTTTTTTTTTGCATCTTTTGTATTATGTTAAAGAACAGAGACAAAAGAGAGCGAATGA CTAGTTCTTGATCTGCACTTCTCTTTTGGCTTTCTTATTCTCCATTTTGTGGATTACATTTTCTT CTTTTTTGTGTTTACATATGGTCTGATAAAAAAACACAAAAATCTAGAAATAAAAAGAGTGATT GGGAGAGATAGGCTGAGAGGAGTATTTGGGGATGGTGCAGACGTTTGGACGCAAACCTAAGTTG GCCAAGATCTTTCAGCTTTAGAAAGATGTTAGATGGTCTGAACTCAGGAAACTCATCCTTCTCT TCTCGCGGGGAAGGCAGGCAAACACCTGACCACGAACTTGCCGTACATGCTACACCATCGGGCC AGGGGACTCGTAGAGGCAACCAAATAGGATATCAGACATGGAAGTGATGAAGGAAAGATTTCGC AAGACTGCTGCTTGGAGAGGACATGTCAGGAGGTAGTGAAGGCGTGACATCTGCGTTAGCTTTG TCAAATGCCATCACCAACCTCGCTGATTCGATGTTTGGTGAGCAGATGAAATTGCAGCCTATGT ATCCGGAGACAAAAGATATTTGGAGGAAAGAATTGGACTGGTTATTGTCTGTGGTTGATCACAT TGTTTCAGTTTGTTCCTTCAAACAAATGGCCAAAAATGGGACATTCCTGAGATCATGGTGACA AAACAAAGAGATGATCTGCTGATGAACATTCCAGCCTTACGCAAGCTTGGACTCGGTTCTTCTCG AGACTTTGGACAACCTTCAAGGATCAGAAAAGACTTTTGGTATGTCCCAAGAGATGTTGAGGATAC AGAGAGCAATGGAGAGTGGAGAAGGGATGAGAAGCTGGTGGTTACCAGTTGTTAAGGTTCCATCG GATGGTTTTGTCTGATGAATCACGTAAGCTGTTGTATAGTCAGAAAGATTCTGTGTACAGGTCC TTAAAGCCGCCGAGCCATCAATGCTGTAGTATTGTCAGAAATGCACATTCCTGACAACCTACAT TGACTCTCTTCAAAGAATGGGAAGACGAGCCTGGGAGATTTTCTTTACAAGAACATAACGGAT GAGTACTTTGATCCTGACTACTTCATCTCTTTCTTGGACTTGTCAACGGAATACAAAGTTCTTG ATCTTAAGAACAGGATTGAGGCTTCCATGGTGATATGGAAGAGGAAGATGAACCAGAAAGAGAA AGACGGGAAATCACAGTGGGGATCATCTGTTAGCTTAGAGAAGAGGGAGCTTTTTCGAGTCCGA GCTGAGACCATCTTGTTATGCTCAAACAGCAGTTCCCTAGGATCCACAATCCTCACTTGAGA TCTCCAAGATCAAACCAACAAGGACGTGGGACAGGCGATTCTGGAGAGCTATTCAAGAGTATT AGAAAATCTGGCTTCGAAGATATTGTCAAGAATAGATGATGTTCTTGAAGCTGATTTGTTAGTT CAAAGAAAAGTGATGGCAGAGGCAGAGAGAAGATCAGAAAGCGAGGCTGAATATGAATACGAAG AAAGCGAAAAGGTGGCATCAGCAGAAACACCAAACCTCGAGGAAACTATCAGACTTCATAGGGTG GAGATTGTCAATCAGAAACAAAGAAGCATAGTTCGATGAGTGATATAGAGTTCTTCTACAGAACG GAGCAGGAGAAGCCGATGATGAAGTCTCCAAGAGCTCTACCGAAGAAACTTTTCATATCTATCAA AGCTAGAGAACATGAGAAGCCCGAGTGAGAGACACTAATAATGATAACATGTAAGTGATAGACGA GAGCATATGTATATAGAAGAAGAAAAACAAGAGAGGGAGCCGAGAGAGTAGGGGAGGTTAA</p>
BcAGD1	<p>GATTTGCATTTTTCATATTCGACCCTTGTTTACGGGGATGTCGTGAAAAATCTTTCAGACAAAA ACCTGTTTGAATGTCTCTCTCGTGTTCATTGTCTCGGCTCTAGTTCGAAAATGTCTGTCTGGT CAAGAAAATGTCTATCCTGTGCAAGTTCCCTGGGTGCGGTGCTTGCTTATATGACCTTTTATGTT CTGACACACCAAGATGGACTCTTCAAATGGATTTGCAGACATCTTCTTCTTCTGATCCTCGAGA TCGGTTGGAGAAGTTGATGAAGCAACCTGGTAATAAATATTGTGCTGACTGTGGATCTCCAAA CCGAAATGGGTATCTTTAAACCTCGGTGTATTTCATCTGCATTAAGTGTTCTGGTGTACACAGAA GCCTTGGTGTTCACATATCAAAGGTTTTATCAGTTAAGCTAGATGAGTGGACAGATGATCAAGT TGATATGCTCGTAGGGTGCAGTGGAAACACTGCAGTGAACCAAAGATTGCAAGCTTGCACATC GACCAGTTAAAGAAACCCAAACCTGATTCATAATGAGGAACGCAATGATTTTCATCAGGAAGA AATATGAGCTGCACCGGTTTATGGATCCAAAAGACAGTGCCTTGTTCACATCAGCAGCCTAG CAGAGTAAATAATCCACCAGCGTCTTTATGTTCTTCTAGCCACCGTTCTGCGAAAAACCGTATT GGTCATGCTCTCAGGAATAGCTGGGGAAGAAGAGAATCTGATCAAAAAGGCACAAAGAAGAGCA ATTCTCTGGCAGGTATGGTTGAGTTTGTGGATTGATTAAGGTTAATGTGGTTAAAGGAACAAA CCTTGCAGGTTGAGATGTGATGACCAGTGATCCTTATGTCATCCTTTCTCTTGGCCAACAATCG GTAAAAACAAGGTTGATCAAGAACAACCTGAATCCTGTGTGGAATGAGACGTTGATGCTTTTCGA TACCTGAGCAGGTGCCTCCTCTCAAAGTGCTAGTGTACGACAAGGACAAGTTACGACGGATGA TTTCATGGGAGAGGCAGAGATAGACATAGAACCATTGGTGAGTGCAGCAATAGAGTACGAGACA TCAACCATAAGAGAAACGAGGCGGCTGGGGAGTTGGCTGGCGTGCAAAGAGGATACATCGGTGA GTGATGGAATAGTCTCACATGAAGAAGTGAAAGTGAACAAGACATTTCTACTAAGGCTTCAAAA AGTGGAGAGAGG</p>

Supplementary Material

BcRHSL	<p>TAAGCATCTCACCTCTTACTTTTTCCTTCACCTTTGTTAGTATATTACCTTAGATTACTAAGTTT ATTATGTCTCCTCCATCGTTGATTGTGTTAGAAGATGGTCTAACCAACAAATGGAGCAATGACG AGTGGCAGCGTCTCGGCGTTTCTCCGGAGAAAACCCCTAACCTACTTTCAATTTCTTAGTGAA GAATCCGCTGACAGTTTACCAGTTCTCTCCCGCCATCTCTTAAAGATGAAAGCTTCAGGATG GTTTTACCTTTGGCCATGTCTCCGCCAAGAGACAACGCCGTGCCGCTTCCTGTTCTTCCCGAGC CGATGATGAAGCCACGGAAGAAGCTTGGCCACCAAGAATCCATGTTTTCTTTTGGAAAAGCTAG GTGTCCCATGAAAACTTCATCCACGAAGAAGAAGAGTTCAAGTGCAACGCCTTCTGTCTC TCCCTCCGTGGATTTGGCAAGCAAAAACATGTGAGGTCAACGAAAAGCAACGACGATTCGTCTG TAAAAAGAAGAAGATGACCAAAGCGTCATCGTTTTTCACTCCACAGTTTCTTAAATGCCTC GTTTGAGAAGTTTGAATGTGGCTCGTGGGCTTCAACAACAACCTGCTCTGGCCCCGAGAAAACAAC CGTTTGTACTTCGATCTACCCGTGGAGATGATCAAATGTGGCCGTGGAAGCGGCGGAGAGGTCC AAGAACCAACGAGTTTGGGTTTCTTCTTCGACAAGGAAAAGAGAAAGTTACCTTTGAGAAGTGT TCTAAAGACTTCTCGGTCCGAAAGGCAACAAGGGGTTGTTCCGGCTGAGACATCACCGCATCGC CGTGTACAGTTTTTCAACCACGACCTCGGTTTCATGCCGACTTCACCGCGGTGCTGTATCACCC CACGCTTGCTTAAAGCTAGAGATGACTTCAACACGTTCTTAGCAGGCACAGAACCGGTGAAGAAC</p>
BcSHV2L	<p>AAAAAGCTTACCATTGGAGAAAGCACAATGGGGTTTCTCCTTCCGATCTTATTAGGAGTTTTCC TCTTAATCACAGCTACGCCACCATCCTTGTCTCAGCTCCCTCCGGAGATAGACCCTCCGGCTCC GGCGCCGATTTACCCCTCAGAACTTTGTAACGGAATATTCCTCTCTACACATTCATTTCTCGGC CGTCAGATCCCTCCAAACGACACAACCGATCAGCCTTATCGTTTTCGAGTCAGTACTAACGGTTC TCAACAACGGCCGTGAAGAGCTCAAAGAGTGGAGAGTGTTCGTTGGTTTCCAACATCACGAGAT CCTTACTTCCGCCACCGATGCGATCATCGTCAACGGGACAGATCTTCTGCTCCGGTAGGAAAC GGCACCATCTTCGCTGGCTATCCTGTTTCTGACCTCAAACGGCCATTCAAACGGCTGGAGACT TAAAACAGATGACGGCTACGATTGAGCTCGTAGGGACTCAATTCATGGTGACTCCTCCGGCCAT TCCACTGCCATCCAATATATCGCTTGTAAACGATGGTTGGAGCTGTCTGAGCCTACCGCTACT CCTCTAAGTAAAAGACAGATAACGACATGCTGCATAAGAGACCCAACATTTGAAGTCAACNCNA CAACCATCACGGACAAGTTCCCTCCACGACAACCCGGAGACTTGACCATAATGTACGACGNGAT CCGAGCCTACGACCAGAATACTAGCCGAAGTAACCATGGAGAACCNCAATCCACTAGGCCGG CTCGACCATTGGGAACTAAGTTTTCGACTGGATGCGAGAAGAGTTCATACAAAAAATGCAAGGAG CGTATCCAACGGTCGTGGATGCCACAAAATGCATCTTCGGTCCACAGTCCCAAATCTACACAGG ACTAGATTTTGGCGACGTTCTCACATGTGAGAGACGGCCCATCATTGNGGACTTACCTCCACC AAAGCAGAGGACCCGGTTCTAGGTAAAATCCCTTCTTGTGTANAAATGGTACGATCTTACCAC GAACCATGGACCCTTCAAATCAGCATCAGTCTTCACCATGCAAGTCGCCAAAATGCCTCCTGA TTTTAACCGGTCCGNGCTNTCCCCGCCTCAAACCTGGAGGATCAAAGGAACGCTCAACCCGGAT TACTCATGCGGGCCACCTGTTCCGGTCAGCCCAACTTTGTATCCGGATCCGAGCGGTATGCCCA CCAACAAAACGTCTTTTGGCAGCTGGCAAATAGTATGCAACATCACACACGCCAAAACGGAAAC ACAAAATGCTGCGTTTCTACTCAGCTTTCTTTAATGACTCAATCATCCCTTGTAACACATGC GCTTGCGGTTGCGTTTCCGAAACGCGACGCACATGCAGCGCCGAGACTCCCTCCTTACTCATCC CTTCAGATGAAGAAACCAGCCATTCGTGGATTTCGAGAAAGCGTATTCGTTCAACGCGAGCCTCT TGAGTATAGAAGGAGGTGTTAACAACACCATTTTCATGGAAGGTCTTCCCGGTTTGGAGTATCT TGTGGCTGAACGTGATGAGTTAGATCCAAAGAAGAATCTTAGGGTTCTGGTAAGCAACAGTCT GTGATACAGTTCAGCAAGAACTTACACCGGGTATTAATGTGCCGGAACGTGATGGGTTTCCGG CGAAAGTTATATTC AACGGT GAGGAATGTTTGCTTCCCGATGTGCTTCCACTTCCAAGTGGTGG AAGAAGAAATGGGT</p>

Supplementary Material

BcSHV3L

AAAGAACTAAGAAGACCCCTCTCTCTCTCTAACAATCTCCTTCGGTCTCCGAGAAAAAAAAAAG
AATAAGAAGAAGCCGAAAAAGTCAACGGCGGGAGGAAGAACGACAGGTTATTACCAAATTCGG
CGAGGTAATCCGACAATGCGAGGGTTACTTCTACTCTGTGGTGTGTTTTGATTTCAGCTGCTTG
CTGTTCAAACCGATGCTCAAGGATCTAAAAGTAAATGGCAGACGCTCACCGGTTTCTCTCCTCG
TGTGATTGCTCGTGGAGGGTTCTCTGGATTGTTCCAGATTTCGAGCATCGATGCCTACAACCTC
GCAATGCTGACAAGTGTAGAAGACGTTGTTCTATGGTGTGATCTTCAACTAACCAAAGACGGAG
CTGGGATCTGCTTCCCTGGTTTAAACAATGAGCAATGCTTCTAATATTGAAGCTGCTTACCCAAA
TCGTACAAACACTTACCTTGTTAATGGAGTCTCTACACAGGGCTGGTTCACCATCGATTTCTCC
TTGAAAGATCTCAACAAAGTTAACCTGATCAGAGGTATATTATCTCGGTCCGAGAGATTCNATG
GAAACGGATACTCGATCTTGACTGTTCAANNATGTAANACAGAGCTGAAACCACAGGCCTTTG
GTTAAACGTTGAGCAGAGGCCTTCTACGCGCAGCATAACCTCAGCATGACCACCTTTCTAACC
ACAACCTCAAACCAATTATCATCGACTTCATCTCTTCCCCTGAAGTCAACTTCTTCAAGAAAA
TCGCTGGACGTTTCGGGCGTGAAGGACCGATCTTCGTGTTCCGTTTTCTCGAGAAAGAAACATT
CGAGCCGACGACAAACAGAACATACGGTTCTATCTTGAGTAACTTGACTTTTCGTCAAACGTTT
GCTTCAGGGATTCTTGTTCCCAAGTCTTACGTGTTGCCACTAGATGACAAGCAGTACTTGCTTC
CGTCTACTTCTTTGGTTCAAGATGCTCACAAAGCAGGACTTGAAGTGTGTTGTGTCAGGTTTTGC
TAATGATGTTGATATTGCACATGACTACAGTTATGATCCTGTCTCTGAGTATCTATCTTTTGTG
GATAATGGTAACTTCTCTGTGATGGTATGCTCTCTGATTTTCCATAAGTGCATCTGCCTCCG
TCGAATGCTTCTCCACATAGGCAGAAACGCTACAAAACAAGTGGATTTTCTTGTTATATCCAA
AAATGGTGCAGTGGAGACTACCCAGGATGTACAGACTTGGCATATGCCAAGGCAATCAAAGAT
GGTGCTGATGTTATAGACTGTTCTGTCCAAATGTCCAGTGACGGTACACCCTTTTGTCCAGTT
CAATCGATCTTGGGAACACCACAATGGTGCACCAACTCCTTTGAGAAACCGTTCAACAAATGT
TCCTGAACTTAGCTCACTTGGTGGTATATACACTTTTAGTCTCACCTGGCCTGAGATCCAGACC
TTGACTCCTGCTATATCAAACCCATACAGAACATAACAATATGTTTAGGAATCCAAACGAGAGAA
ACGCTGGGAAGCTTGTTTCCCTTACTGAGTTCCTAAACTTGGCAAAGAACTCCACCTCTCTTTC
CGGTGTTTTGATCAGTGTGGAGAATGCAGCATAACCTGAGAGAGAACCAAGGGATAGACGTGGTG
AAAGCGGTTCTTGACACACTCACACAAACCGGCTACACCAACATCACAAACAAAAAGGTTATGA
TTCAATCAACAAACAGCTCGGTTCTACTCGACTTCAAGAAGCAGAGCCGGTACGAGACTGTGTA
CAAAGTCGAAGAAACCATCCGTGACATTCTAGACTCTGCTATAGAAGACATAAAGGCATTCGCT
AGTGCTGTTGTCATCGTAAAATCATCGGTCTTTCTGACTCAGAGGGTTTTGTACAGGGGCAA
CCAATGTTGTGGAGAGGCTGCAAAGTCTCAGCTTCCGGTATATGTTGAGCTGTTCCAGAATGA
GTTTGTGTCTCAACCTTATGACTTCTTCTCTGACGCAAATGTTGAGATAAACTCTTATGTTACT
GGAGCTGGTGTAAATGGAACCGTCACTGAGTTCCTTACACAGCAGCTAGATACAGAAAGAATA
GATGCTTGGGAAGCAAAGAAACTCCACCTTACATGGCTCCTGTTCAACCAGGTGGACTCTTACA
AGTTGTGAATCATGCGTCTTTACCTCCAGCCGAAGCTCCGAACCCGGTGTTCACAGATGCTGAT
GTTACTGAGCCGCCACTACCTCCGGTTACAGCAAAGCCCCAACCTCAACCCCTGGAACCCAT
CAGAACCATCAACCAATGCACCAGCACCTAGTGGACAAACCCGCCTCACTCTATCTCTTCTCCT
TTCTGTTTTTCCAATGGCTATTGCCTCTCTTCTACTTCTGTGATCAA**ACCCTTGATCACACATC**
TTTGT

Supplementary Material

BcWRKY1	<p>AAAAATCACACACACGCACACACTAAGATAAATACTTACCTTTTTCTTATCCGGCAGCTTACACA TCCTCTTTACTTACTAGGGTTTCAGATAACAAATCAAGCAAGATCATCGTCCGTACTTACTGAT CATACACAAAAAAATCAAAGTTGTCTATCAAGAAGTTAGAATTATCTATATATATATCGAGAG AGATGTTTTCGTTTTCCAGTGAGTCTTGGAGGTCAAGGTGACAATTTGAAGCAGACACAACCATC GGATGAGCAGCATCATCGGCCAATAATGGATGAGGTTGACTTTTTTCAGGTCCGAGAGAAGAGAT GATCAGAATGTCATCACCGAGGAACTCATAGGCTTCATGTCAAAGGGAAAATTCACGTGTTG ACGATGATGATGATCGTTCCATGGGTATCAATACTGGACTTAATCTTCTCACTGCGAATACGGG AAGCGACGAGTCATTGGTGGATGATGGATTGTCTGTTGATATGGAGGAGAAAACGTACAAAGATT GAGAATACACAACCTTCGAGAAGAGCTAAAGAAGTGAATGAAGAGAATCAAAGACTAAAGGAAA TGCTAAGTCAAACAACCAAMAACCTTCAAYTCCTTGAGATGCAACTTGTTGSTGTGATGAGGCA AGAAGAAGATCATCATCACCTGGCTACGATCGAGAGCAAAGACAAGGACACTAACCGACATGAA GCTCCTGAGATGGTTCCAAGACAGTTCATTGATTTGGGACTACCATCTGCTGAAGTATCATCCG ATGAGAGGATAACGTCAAGATCTCCCCAATCCCTTCTAGAGGACTCTAGCTCACGTCAGCGCGC AAAAGAGTACTTGAGATAGAAGAAAGCCCAGAGAACGAATCGAACGGCTGGGGAAAACCTAGC AAAGTTTCGAAACACAATGCCTCTTCCAGCAATGGCAATGGAAATGCTATCGATCAATCGGCCG CTGAAGCCACCATGCGTAAAGCCCCTGTTTCGGTCCGTGCAAGGTCCGAAGCTCCCATGTTAAG CGATGGATGTCAATGGARAAAATACGGACAGAAAATGGCAAAGGGAAACCCGTGCCCTCGAGCT TATTACCGCTGCACGATGGCCGTTGGATGTCCCGTTTCGTAASCAAGTGAACGTTGCSCARACG ACAGATCTATTTCTATAACAACCTACGAAGGAAACCATAACCATCCATTACCTCCGGCAGCTAT GTACATGGCTTCAACCACAACCGCAGCCGCAAGCATGCTCCTCTCAGGCTCCACAATGTGCAAC CAAGACGGCTTAATGAACCCAACAACCTCTTCGCTCGAACCATGTTGACTTGTTCCTCAAGCA TGGCCACGATCTCAGCCTCGGCCCATTTCCAACCATTACACTAGACCTCACAGAATCAGCGTC GGACGTTAACGGTCCGACGAATAACAACCCACTGATGCAATTCGCTCAACGGTCTGGTTTCGCG GAGTTGAACCAATCGGGCTTGCCTCAGATGATGGGCCAGGCTTTGTATTATAACCAACAACAGT CCAAGTTCTCCGGTTTGCATATACCATCTCAGTCGCTGAACGCTGGCGAGAGCGTCAATGCTGC TATGCCCGCCAATCCGAACCTTGCAGCGGCTCTGGCCGAGCCATCACGTCTATTATCAACGGT TCGAGTAATCACCAGAATGGGAGCAGTAACAGTAGTAATAATAATGTTACGACGAGCAGCNGGA CAGGCAATAACA</p>
BcZC3H1	<p>AAAGTCCATGAGAAACGATGAAAGTATATGAGTCTATGAACATGGAGGAAATTCACGAACAGAT CCATTAAGAGGACTTTTTGAGATAAGACGTTATTATTAGAGATGAATTTACAGAATCAATGA ACGTTGTGCACAACAGAATCCAACAACCTCGAACCTGAAAACGCTTCGAAAATCATTGGTTATCT CTTGTGATGCAAGAACATGGCGACCGGGACATGATCCGTCTCGCCTTCTGCCCTGATTCTGTG ATGCGTTCCATGATCAACTTCGTTAAATGCGAAGTACGCTAAAGATCCTCGTTACCACAGCCCTC CTTCTGATCACCTCCCTATTCGTAATAGTTTCGGAACCTTCACCGGTTCAATCCAGCCTCC CTCGGCATCGTTTTCTCCTCCTTCGAGAACCGGTGTTTGGGAGAATTCAACTGAGGTTGATTCA CTCCAGTTCTTGAACCTTTGACGATTCAATGACGAGCCCTGAGTTCTCTAGTGGCTTCTTCTCTC GGGATCACCAATGTCTGCCTTTGAAGACGAGCAGGAGATCACCGAGTTTACCCGAGTTCCCGGT GAAAATATGTCACTACTTCAGCAAAGGGCACTGCAAGCAGGCAACAATTGCCGCTACTTCCAC GGGACAGATCATACCGGAGAGAGAGGTTTCTCTCAGATGTTTAATCCAAACAACGTAAGTGATG AAGAGCACGTTGTTTTCTCCTGGATCACTAGAGAAGCTTGAAGGAGAGATCATCGAACTGCTGAA ATCAAGAAGAGGCGCTCCAATTTCCATAGCTTCGTTGCCAATGATGTACTTCGAAAAATACGGT AGGACCCTTCAAGCTGAAGGATATCTCACAGAGTCACAAAGACATGGCAAAGCTGGCTATAGCC TCACCAAGCTTCTTGCTCGTTGAAGAACCATCCGTCTCATCGACAGGCTCATGGGCAGCA CTCGGTTATATTAGCAGAAGATGTACCAAAGTTTGTGGAATACATGGAAGAGAGAAAACGAACAC GGAGCGATCCTTGCTGGTTCCAACAGATTTACCTGACATTCCCAGCAGAGAGTAGTTTACCGG AGCATGATGTCTCAAACACTTCTCCAAGTTTGGACTCGTGAAGATGTGAGGATTCTTGTGCA ACAGAAGAGAATGTTTGGATTGTAACATTTGTTTACACCGAAACCGTCAAACACATTCTTGCT AAAGGCAATCCTCATTTCATCTGTGGGGCTCGTGTCTCGTCAAGCCTTACCGGGAAAAATCAC GCTCTAGTCGATATCTTGACAATAACAAGCCTCTTACGGGATGCGGTATGGCTCCCAATACAT CGACAGAGACATGGAGATGAACACATTGCCAGCGCGTGAAGTGAAGGCTCAAGACTAATGAGA AAGCAGTTTCTTGAGGAACATGAGCAATCGGTTTCAAAGTCTTGCCTACTAATTACTCCTATC TCGGCTTCTCCGATGACTTTAAACTAACTGCAGATGGTATTTATATAGGTAACACTAATTGAAT ATATAAAATAATACACAATCAATTGCTTGATGTTTATAAATTTGTAGCGGAGCTAGAGGAACAAG CAGGACGGTTGAGCTATCTGCTGGAGTATTTGAACACCGAAGACAACGTCCTAAACATATCCAC TAACTACAAAGACACTGATCGAATCCATTGTGAACCTATGGACAACCAAGTCTTGAATCTACCA GAGAGTCCGTTTTCTTCCCTTTCTGG</p>

Supplementary Material

BcATL12L	<p>AANNGTTCNCNACGATGAATCCNCCANANGCAATCNCNCGCTCTTCTTCTCCATCTTCYTCTT CTTAGATCNYGTCTCCGCKCANTCTCCTCCTCCTCCYCCCTCCTCYTTYCTGTNCGCNYCNWC GGCCTCTTTCANSCNWSYTYANCCNYCNCKSSASYGYCYCCNTCKTCTTNSCNCTCACCT TCGTTCTCCTCATCTACGCCAAATGCTTCCACAACGATCTCAGATCGGAGACCTACGAAGACGG AGAGATACGACGACTAGATCGTCTATGGCAAGGACTCTTCAGCCAATCTTCTCGATTATCAGGT CTCGACAAAGCAGCAATCGAGTCTCTCCCTTTTTTCAGATTCTCTGCTTTGAAAGGATCGAAAC AAGGGCTTGAATGTTCTGTTTGTGTTGCTAAATTTGAAGATGTTGAGATTCTTAGGCTTCTGCC TAAATGCAAACACGCTTTTCATATAGAGTGTATTGATGAGTGGCTTGAGCAGCACGCTACGTGC CCTTTGTGTAGAAAACAGAGTCTCCATTGAGGACGAGCTCTCTGTTTTTGGTGGTGGTAGTAACA ATAGTATGAGAATCATGAGTCAGTTGTCTGAGTCTAGAGAGGAAGAATCGAGCATGGAGGTTTA CGTCGAACGCGAAGAAGGAATCAGAGACGGGTCTTCGAGATTTCAGCAGCTTCCGGAAAGATACTC AACTTTGGTAAAAACGATAACTCGTTGTGCTTGTGGAACAAGGTAACGAGAAATGTCTGCACA AGTTCAATCACAGGATTGTTGTTTTAGATGCTGTGTTTAAAGAACCGTTGGAGTAATGTGACTCC AGCTGATTTGACGTTTTTGGACATCGAACATGTTGAGTTCGACGTCAAGCGACAGATTCTCGTCG GTGGATCGAGTGCAGAGAGGTGACTTGCCTGACAAGGAAGAGATTGATATGAAGAGAATGGTTG ATAACAAAGATTCTACTAGAAGATCGGTTTCTGAGATCACGGCTGTTCCCTAGACTCAGCGTTGT TACAACAGCGGCTAGAGAAAACGCGGTTGGTGGGAAGCAACAGCGGTTTGGACGCTTCTACAGCC TCCACATCGAGAAGTGATGTGATTGCTGCGATGGAGGAAAGAAGACGAAGGATGTGGTTGCCGA TTGCTAGAAAAACAGCACAGTGGTTTTGTTAACAGAGAGAAAAAGAACGAACATAAAACGACAGG ACAAGATCTTAACGTTTAGTTTTCTTTTAAAGGTCGTTGACAATTTGAAGTTATTTTTACAGAG ATAATTAAGCGTGCTGAATAAGATTAGAC</p>
BcUNK3	<p>AAAATCCATCCATCACCAGAAAACAAAAACAAAACAAGAAAATGGCCTCTTGGAACCATCTCTT GAAATGTTGTTGTATTCTTCTAGCTATACTCATGGTCGTGCAGGCTCAAACCAACCGGAAACTT CTTGACAACAACAACAACAACACACCTGCTGCATCATCTATGCATGCTCGTCAAGATTATCTTC CTTCTTCTTCTCACCGATCACTTGGTGTCAATATTATCAGAGATCGCCGCCTCCACCCCCAAG AAAAAGATGAATCTGAAACCACTGGTTCGTGAAGATGACTCGTAAGTTGGTGTGATGTTACTTGG GTTCTGATGTTTGTGTTTTATGTTTTCAAGATATGTCTAATGTTTTAAGGCATTATCTGTGACTT ATTCTAAATAAAAAAAAACGGTGGTACTTCTCATCAAGA</p>

Table S14: Bale gDNA sequences of candidate genes for Pi deficiency-induced root hair growth. Primers used for amplification (bold) and gRNA binding sites with PAM (underlined). Since about 450 bp at the 3' end of *BcROPGEF1* were not obtained by MACE, a reverse primer designed on the annotated *B. rapa* gDNA sequence was used to amplify and sequence the *BcROPGEF1* gDNA.

	Bale sequence (5' → 3')
BcFLA1a	<p>TTCAAACGCAATGGCAACCATTTCATCTAACTCTAGCTCCTCTCCTCATCCTTGCCGCTGTCTTC <u>CTCTCCACGGAGATAACTGCTCAGCCGCTGCTCCAGCTCCTGGTCCTGCCGCTCCGACCAACA</u> <u>TCACGGCGATCCTTGAAAAGGCGGTCAATTCGCTACTTTTATCCATCTTCTAAACACAACACG</u> AGTCGAAACCAAATCAACATCCAAGTCAACAGCTCATCAGAAGGCATGACCGTGTGTTGCACCA ACAGACAACGCTTTCCAAAACCTTAAACCAGGAACCCTAAACAACTAAGCTACGACGACCAAG TCAAATCATTCTTTACCATGTTAGTCCCAAGTCTACACATTGGATGATCTTCTCTCCGTAAG TAACCCGTAAGGACTCAAGCTTCAGGACGAGACAGCGCGGTATATGGACTTAATTTACCAGT CAAGGGAACCAAGTCAACGTATCAACCGGTTTCGTTGAAACACGTGTCAGTAATGCATTAAGAA AAGAACGTCCTCTTGCGGTTTACGTGGTGGACATGGTATTGTTGCCGAGGAGATGTTTGGAGA AAACAAGGTCTCACCAGCGGCACCTGGTCCAAAATCAAATTCCTGATGTCTCCGATGACCAA GAGAAAGCAGGGGCTCCATCGGATAAGTCTGGCTCCGGTGAGATGAAGGCTGGATTAGGGTTGG CTCTTGGACTTGTGTTTTCTTATGTTTGAAACTCGTCGTTTGAATTTGGGT</p>

Supplementary Material

BcFLA1b	<p>TTCAAACGCAATGGCAACCAGTCACTCTAACTCTAGCTCCTCTCCTCATCCTCGCCACTGTCCCTC CTCTCCACGGAGGTAAC TGCTCAGCCCGCCGCTCCGGCTCCTGGCCCTGCCGGTCCCGTCAACA TCACTGCCATCCTAGAAAAAGGAGGTCAATTGCTACTCTTATCCGTCTTTTAAACACAACACA AGTCGGAAACCAAATCAACATCCAAGTCAACAGTTCATCAGAAGGCATGACCGTGTTCACCA ACAGATAACGCTTTCCAAAACCTTAAACCCGGAACCCTAAACAAACTAAGCCACGAAGACCAAG TCAAACATCATCCTTTACCATGTTAGTCCCAAGTCTACACATTGGATGATCTTCTCTCCGTGAG TAACCCGGTTAGGACTCAGGCTTCAGGACGAGACGGCGCGGTTTATGGACTTAATTTACCAGT CAAGGGAACCAAGTCAACGTATCAACCGGTGTGCTTGAAACACGTGTGAGTAATGAATTAAGAA AAGAACGTCTCTTTGCGATTTACGTGGTGGACATGGTCTTGTGCGGGAAGAGATGTTTGGAGA AAACAAGGTCTCGCCAGCGGCACCTGGTCCGAAATCAAATTTCTCTGATGTATCCGATGACCAA GAGAAAGCAGGGGCTCCGTCCGATAAGTCTGGCTCCAGTGAGATGAAGGCTGGATTAGGGTTG CTCTTGAGTTGTTTTCTTATGTTGAAACTCGTCGTTTGATTGGGT</p>
BcRALFL1	<p>AGTTTCAGACAGAGAGAGAGAGAACAAATTCACGAAAGACGACTAAAGAAATCTCAAAAAGCC TCTGATTCAGCCAGAAGGTTTTGGTCAAAGAAAAAGATGAACTCTCGCGCGATCTACGCCGTCA TCGCGCTCCTGGCGCTCGTAATCTCTGCCGTCAACGCAACCGGAGGCTTCGGAGACTCGCTCGA TTTCGTACGGAGCGGATCTTCGTGCTCTTCTCTGGATGCGAAGGCTCGATCGCTGAGTGTATA GCCGAGGAAGAGGAGATGGAGTTCGATTCAGAGATCAGCCGGCGCATTCTAGCGCAGAAGAAGT ACGTTAGCTACGGTGCGATGAGGAAGAACAGTGTGCCTTGCTCGCGGCGTGGAGCTTCGTATTA CAACTGCCAGCGTGGCGCTCAGGCGAATCCTTACAGCCGTGGATGCAGCACCATTACCAGGTGC AGGCGTTGAAGAGGAAGAAGATGATGAGTCCAATTCTGTTTTGCCCTTGCC</p>
BcMIRL	<p>ATGTTCTGTTGCGGAGGTGCGGATGAGGAACCCGCGGTCCGCCAGCAAACCAGTATTCATCAG CGCCTCCTAACAAAGGCCGAAATAACAATTTTGGCGGTAACAATCCCTAACTTCTCCTCGCGCC TAACCCCTTATCCCTTGCTTTGTTGTGATTAAGCGTTTGCCTTTGCGATGCGTTTAGGCGGTGGG AACAGAGGAGAGCCGAGGAATACAAACGCACCTAGATCAGGAGGTCCTGCAAAGGTTTTACCTA TAGAGATCCCTTCTATTCCATTGGATGAGCTGAACAAAATATCAGGTAACCTCGGAAACAAGGC ACTGATCGGTGAAGGCTCTTATGGACGTGTCTTCCAAGCTAAGTATAATGGAGGCGATGTTGCT ATTAAGAAGCTTGATGCTAGCTCTTCTGAAGAACCTGACTCCGATTTACCTCACAGGTACACA CATAAAAATATTACAACCCTTCAGCGTAATTAATTTAACCAATGATTAATCTAATAATTGTTTT AAGTAATTTCTCTATTAAANTGGCAAGTTATCGGTGGTGTACGGCTTAAAAATGAACATTTTG TGAACTGATGGGGTACTGCTTGGAAAGCAAACCTACCGCATTCTTGTCTACGAGTTCGCAACTAA AGGTTCCCTTACACGATGTGTTACATGGTACGTGAATTTAACTCTCACTACGCATGTTTTGTTAT GGACTATATGTGATATTGGATTGGTCTTTAGGGAGAAAGGGCGTGCAAGGAGCTGAGCCAGGAC CGGTGCTGAACCTGGAACCAAAGGGTCAAGATCGCATATGGAGCAGCCAAAGGGCTTGAGTTCCCT TCATGAGAAGGTCCAGCCACCAATAGTCCACAGGGACGTAAGGTCGAGCAATGTCTTGTGTTTT GATGACTTTGTGGCCAAAATGGCTGATTTCAACTTGACCAACGCATCTTCAGATACTGCCGGA GGCTTCATTCTACTCGTGTATTGGGAACGTTTGGCTATCACGCTCCCAGTACTGTACCATAAA CCAATTAATTAGTTCATCTCTTTTTATCATCTCATATTGTTCTAGTAAAGATTATGATTTGTT CTTGATAATTTACAAGGTAATCTTGTGTTAGGTATGCTATGACGGGACAGATAACGCAGAAGA GTGATGTGTATAGTTTTGGTGTGTGCTATTGGAGCTCTTAACAGGAAGAAAACCTGTAGATCA TACCATGCCTAAAGGCCAACAAAGTCTTGTACTTGGGTACGTGCACATATTAATTAATATAAA TATGTATGTTCGAAATGATTAATGCCCTTATTATGCTATTATAGAAGTTATAAATCCTTTTACT TTTGTCTGTTTTTGCAGGCAACTCCGAGACTAAGTGAAGACAAAGTCAAACAATGCATAGATC CTAAGCTTAACAATGACTTCCCTCCCAAAGCAGTAGCGAAGGTCCGTTATTTTTGGTATTTTGG CTCTTACTAACCGTTAAATAGTTATTAACGCACCTAGATGTTAATTTGAAAGATGCATAGAAAC CTATTTCACTATTTGCATGTAGATAGTTTCAAAATGATGTTTCCAGGCTTCTTTAGTAGCTAAATC TTTTGTATCGAGTTTCTTGAACCTTCAACTTAAATTTAGATGGAAGTACGTAAATTTATCTGTTT TTATTCATGATGTAAGTTTTTTGAAATGGGGATGGTATATTTAGATAAGATTTTTTTTTTGT TTTACTCATACTGAATCATAGCTACCTAAAAGTCTAAGCTAATGAGTAATGAGGAAGGATCTTG TGGTTTTATTAGTTTTGTAATGCTGACTTTTTTTTTCTTTGTGGGTGGTCAACAGTTGGCTGCCG TGGCGGCTTGTGTGTTTCAAGTATGAGGCGGATTTCCGGCCTAACATGACCATTGTTGTCAAGGC ACTTCAGCCTCTCCTTAACTCAAACCGGCCGGTCCTGAGTCTTCTTCCTGA</p>

Supplementary Material

BcROPGEF1

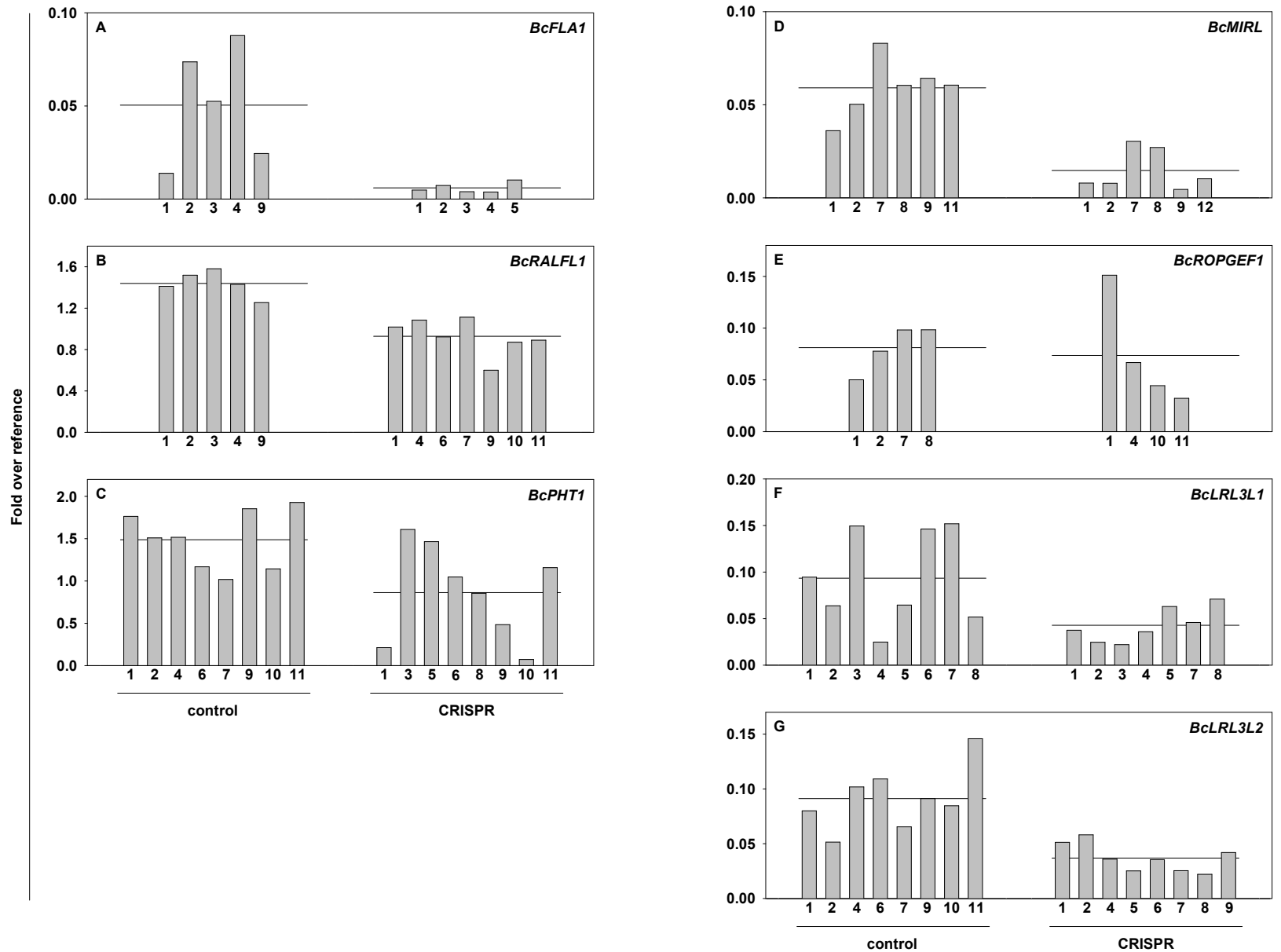
ACTCGATCATTACACAGAGTCTCGATGGAGAGCTCACCGAGTTCGGACCAAACGAGGTATCTG
CCTCTGAAACTCCGACAAGCTCCGTCTCTTACCGTACCGGAGAACGTA CT CAGATATCTCCGG
TTTATCTCACCGGTTTCGACGTT CAGAGCTTCTATAACCGGCCGTCAAACACAAACGCCGTTGTC
CATGAAGAAGATCTCTCCGAAGACGCCGTGGAACCAAAGATAACGTCGACGGCGATGGAGAAG
ATCATGACCGGGACAGTGACATTGACTCTGCAGAAGACGCAGGTGAACTCTTTAGAAAAC TTTT
ATCACTCTGTTTCATTTTCGATTGATCCAGGTTTTTGT TAGCGCAGAGCTGGAGATGATGAGGGA
AAGATTCGCGAAGCTGTTGCTCGGTGAAGATATGTCGGGAAGTGGGAAAGGAGTTTGC ACTGCT
GTTACTGTCTCTAACTCCATAACTAATCTCTATGGTTCGTTTCGTCTTCTTAAATTTTCATTGTTA
TTTTTTTTTGTCTCCTTTGCTCTGTTTTCGAAAACAGAGTATTTGATAATGGGTATTGTTTTGC
AGCGACTGTGTTTGGACAGAGTTTGAGATTGCAACCATTGAGTACAGAGAAGAAGGATCTATGG
AAACGTGAGATGAATTGTTTTATGTCTATATGTGATTACATTGTTGAAGTTGTTCCAAGATCTC
TTGGTAATAATGTTGAGGTAAAAACA ACTACCTTACTTGTGTCTCACGCAATCTAAAAATAAAA
AACCTTNAAGTTTGATCTTTTTTTTTTGTTCAAAATTTTCANATAACGGAAACAAA ACTAAGAT
CCGACATTTCTCATGAACCTCCCTGCTTTGAGAAA ACTCGATAACATGCTCATGNNNNNNNNNN
NNATATATTGGATGGT
TTTACGGAGAATGAGTTCGGTACATGGAGAGAGGAAGCTCATCGATGAACTCTAACAATGGTG
GTAGAGATTCTGGATCGTTTTCGGAAAGTTGTGGTTCAAAGGAAAGACGAGAAGTGGTGGCTTCC
TGTGCCATGTGTTCTGCTGAAGGTTTATCAGAAAATGAACGAAAGAATTTGCGTCACAAACGT
GACTGTGCTAATCAGATACATAAGGCTGCTTTGGCCATTAACGACTCCACTCTAAATGACATGG
ATATTCCTGACTCTTACCTCACC ACTCTCCCAAAGNNNNNTTAAAAATCCNNATTTATGTAACC
CAATTTGGTTTTAAATGGTTCAAATCAATTTAATTTGGTTACGTCGATCTAAATGATCTAAA
CCAATTAACGAAGTAATATAAAAGTGAATTATGACTAGTTTAGTTATATCGTCTTACCATCA
ATTTATCTATAAAAAA ACTATGATACANTTTAATTTTGTTTATTAATATAAGTTCCACACGTTTA
TTTGATTGTTTCAATATATCAAAAATTTAATATTT CATCAAAA TAGNTTTTTTAATCAAACACA
TAANTTTTCCTATACAAAACGCTACTAACGANTTTTGAACGTTGTTATTAATCCTATATGATA
ACATTATGTACATGTGAAAATATTGTAATCAATAAGGTATTTTTTGTCTTTTTTTGAATAGAGTG
GGAAAGCAAGTGTAGGAGACGTGATATAACAAGCAACTGTGCACAGCTGAGAAGTTTTATCCAGA
CCAGCTTCTTGATATCTTGAAGATTT CATCGGAGCATGAAGCGCTTGAGCTAGCTGACAAAGTT
GAGGCTTCACTGGTAACATGGAGGCGTAAGACCGGAGGGTTGGCTCACTCTAAATCCTCATGGG
ACATGATGAAAGATATGGGCGGCGACGCTGGGAATGACAAGAACCACATTCTCGCAGCTCGAGC
TAGGAGCTTACTGTTCTGTCTGAAACAGAGATTCCCTGAACTCTCTCAGACCTCACTTGACATC
TGCAAGATTCAGTATAACCGAGTGAGTGGTCCTCATAGAACCTTTTTACATTTTGTTTTAAGAT
TAATATTTATTTGATTATCAAATATCTATGTAGGCATGGACGTTTGGTTTTTCATTCTTTTGGT
TCTTGTGATATATGAATCGTTCGGTTATTTATAGATTTCCGGTTCAGTTATTTCCGTTTTTCAGTT
CGGTTCCGATAGCAATGTTAGGAACCGGCTAATATCTTATAAAAATTCAGTTCCAATTTTGTGT
GTGTTTTTTTTGGTTTGAATAGGATGTGGGCAAGGCTG TACTGGAGAGCTATTCGAGAGTTT TAG
AAGGTTTGGCTTACAATGTTGTGTCTGGATTGATGATGTTCTTTACGTGGACAGAACAGTGAG
GAACAGAGATGATTAAGGTTTCAACTAAGTTTTCTTCTTCTGAGTGTAATGAGAATACAAA
ACAAAATGTTTTTT**GTGTGTTGGTTATGATTGTGTTCTG**

Supplementary Material

BcLRL3L1	<p>AGAGATGGAGAAGGGGAATGGAGAAGGAAAGGGGGAGTTCATAAACCAAACAGTAACGGTTTC TTCCATGACTCCATGTCAATGCTTTCCTCTCTTCCCTCCCTGGGGCTCATCTCTTCCCCCTT CTCCTCCGCCGCAATTCCTCCTCCACGCTCTCACCGTCCCCGACAAGTTCCATCAACCTCAGGC ACCAAAC TATTACCTTACATTACAAAACACCAAATGTATTAATGTTTATGATTCCTCGAATGA CATCTTTTGTGTTGTTGTTTGTCAATGTCAGGAGTCTGGTGGTCCAACAACGGGTAGCCAAGAT GGGTTGCAGCATGCACAAGGGACAGTGTGACCACCACGAGCCCACCTGTGGCCCGCCAAAAGC CGAGGGTGCAGCCAGGAGAGGCCAAGCCACCGATCCTCACAGCATTGCTGAGCGGGTATCTCC ATTGTTCACTTAACTCCCATGTCTCTGTTTGTGTTACTCATTGTATTGTTTTTGTGGCAGT TGAGAAGGGAACGCATTGCAGAGAGGATGAAGTCTCTCCAAGAAGTGGTTCCCAACACCAACAA GGTACTCAATATTATCATGATTTTACAAGTACTGTCTGAATCTGGACAATAACCGAATTGGTT GATGATGTAATGCAGACGGACAAGGCATCAATGCTGGACGAGATCATAGAGTATGTTAGATTCC TTCAGCTTCAAGTCAAAGTACTAAGCATGAGCAGATTGGGAGCTGCAGGGGGAATTCGTCCACG CCTCAATGGTCTCACCTCTGATGTGAGTTTCTAAGTCCCTTAATGCCTTTTTTAAAGTGACATAG CACCAAACATCATCATGGTTTTTGGTAATGCAGGTAGGAGGACGGCTCAACGCCTCTCAGTAC CGTCCAATGGCATAAGTAAACGGGAATGGAACGCACCAGGATCATCCAACGAAAGCCTAAGGAC AACGGAACAGAGGGTGGCGAAACTGATGGAAGAAGACATGGGATCAGCAATGCAGTATCTTCAA GGGAAGGGGCTTTGTCTAATGCCCATCTCTCTCGCAACTGCTATGTCATCTTCATCTGCTCACT CTCGTGGAGCTCTCTTCAACCCGACCTCAAATGTCGTAGCAACAGAGGAGGCAAATGTAGTAGC AGCAGCTGCACCTGAAGCCTCCTCTCCTATGGATGATGTCTCCGCCTCCAACAAGGCCTAAACC ATAGG</p>
BcLRL3L2	<p>AGAGATGGGAAATGAGAATGGAGAAAGAAAGGGAGAGTT CATAAACCAAACAACAATGACTTC TTCCTTGATTCAATGTCAATGCTCTCCTCTCTCCCTCCTTGCTGGGACTCTTCTCTTCCCCCTC CTCATCCTCCTCCTCCGCACGCTCTGACCGTTCGACGCTCTTTCCCCGACCAGTTCCATCAACC TCAGGCACCAAAC TATCACATTACATCATGATAAAGAATGTTTTTGATTCCCTGCATCACATCT TCTTGTGTTATTGTTTGTCAATGCAGGAGTCAAGTGGTCCAACAATAGGCAGCCAAGATGGGTT GCAGGGACAAGGGACAGTCTCGACCACGAGTGCACCTGTGGCTCGTCAAAGCCAAGGGTGCAG GCTAGGAGAGGTCAAGCAACCGATCCTCACAGCATCGCTGAGCGGGTATATATCTTCATTGTTC ACTTCTTCCATCTCCTCTATTATTATGTGTTTCTGATTGTATTGTTTTGACTAGTTGAGAAGAG AACGCATTGCAGAGCGTATGAAGTCTCTTCAAGAAGTGGTTCCCAACACCAACAAGGTA CTCAA TACTTTCCTGATTTATATAACTCTTGTCTGGATCTGCGTTAACCTAATTGGTTGATGCAGACGG ACAAGGCATCGATGCTGGACGAGATCATCGAGTATGTTAGATTCCCTTCAGCTGCAAGTCAAGGT ACTAAGCATGAGCAGATTGGGAGGTGCAGGTGCAGTTGGTCCACGCCTCAACGGTCTCACTTCC GAGGTCAGTTTCTTATTCTCTTATTACAGGGTTAGATCTGAGATTTAGGAAACTACAGTATAAAC ATTTTTACCTTAATTTTAAATTTTTTAAAAAAATGAGGTCATACGCCCTATACATAATACCTC TTTAAATGTTATGAAACAAAATAGCAAGTCTTAAATATGGAGATAAGCAAATGTTTTATTTC GGATGTTGCCAGAACCGCCTGTCTTTATGGCTTTTTATATTAACAACCATCATGGTTTTTGGG TAATGTAGGTAGGAGCACGGCTCAACGCCCTCTCTGGACCGTCAATGGCTTAAACGGGAAAGG AAACGCAACAGGATCTTCCAACGAGAGCTTAAAGTCAACAGAAAATAGGGTGGCAAAGCTGATG GAAGAAGACATGGGATCTGCAATGCAGTACCTTCAAGGGAAAGGGCTTTGCTTAATGCCCATCT CTCTCGCAACTGCAATGTCATCTACATCTAATCACTCTCGTGGAGCTCTCTTAAACCCAAAATC CAATGTGTTAGCAGCAGAGGAGAAAATGTAGCAGCAGTAGCTGCACCTGAAGACTCCTCTCCT ATGGATGATGTATCTGCCTCCAAGGGC</p>

Supplementary Material

Fig. S9: Expression of the 7 candidate genes for Pi deficiency-induced root hair elongation (A-G) as affected by CRISPR/Cas9-induced gene editing determined by qPCR. Each column represents the expression in root tips of one single transgenic root measured by three technical replicates with the horizontal line representing the corresponding mean of all biological replicates (all transgenic roots). Fold over reference = $2^{-\Delta\Delta Ct}$.



Supplementary Material

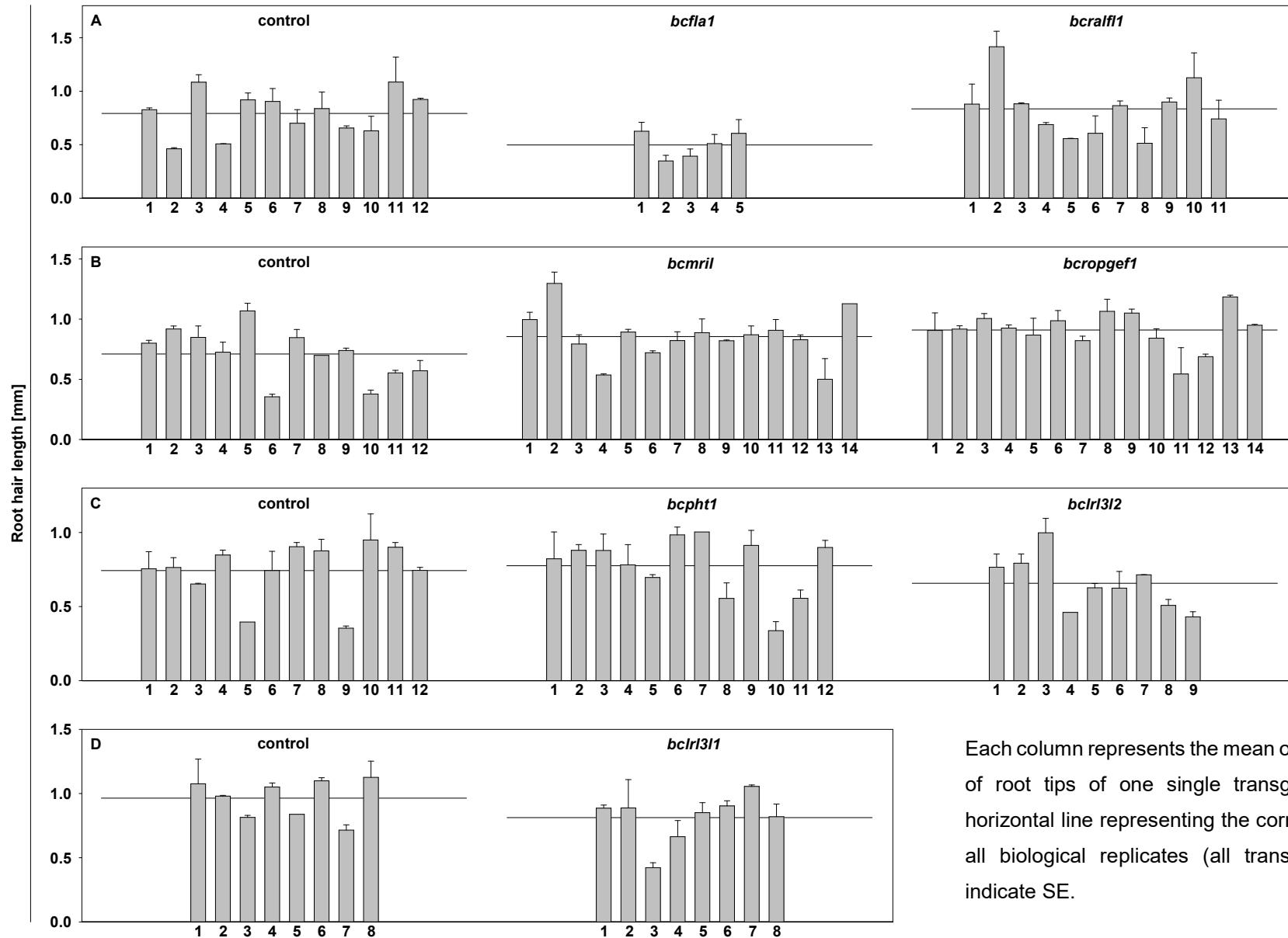


Fig. S10: Root hair length of the transgenic 1st and 2nd order lateral hairy roots after targeting the respective candidate genes for Pi deficiency-induced root hair elongation (A-D) by CRISPR/Cas9.

Each column represents the mean of the root hair length of root tips of one single transgenic root with the horizontal line representing the corresponding mean of all biological replicates (all transgenic roots). Bars indicate SE.

Supplementary Material

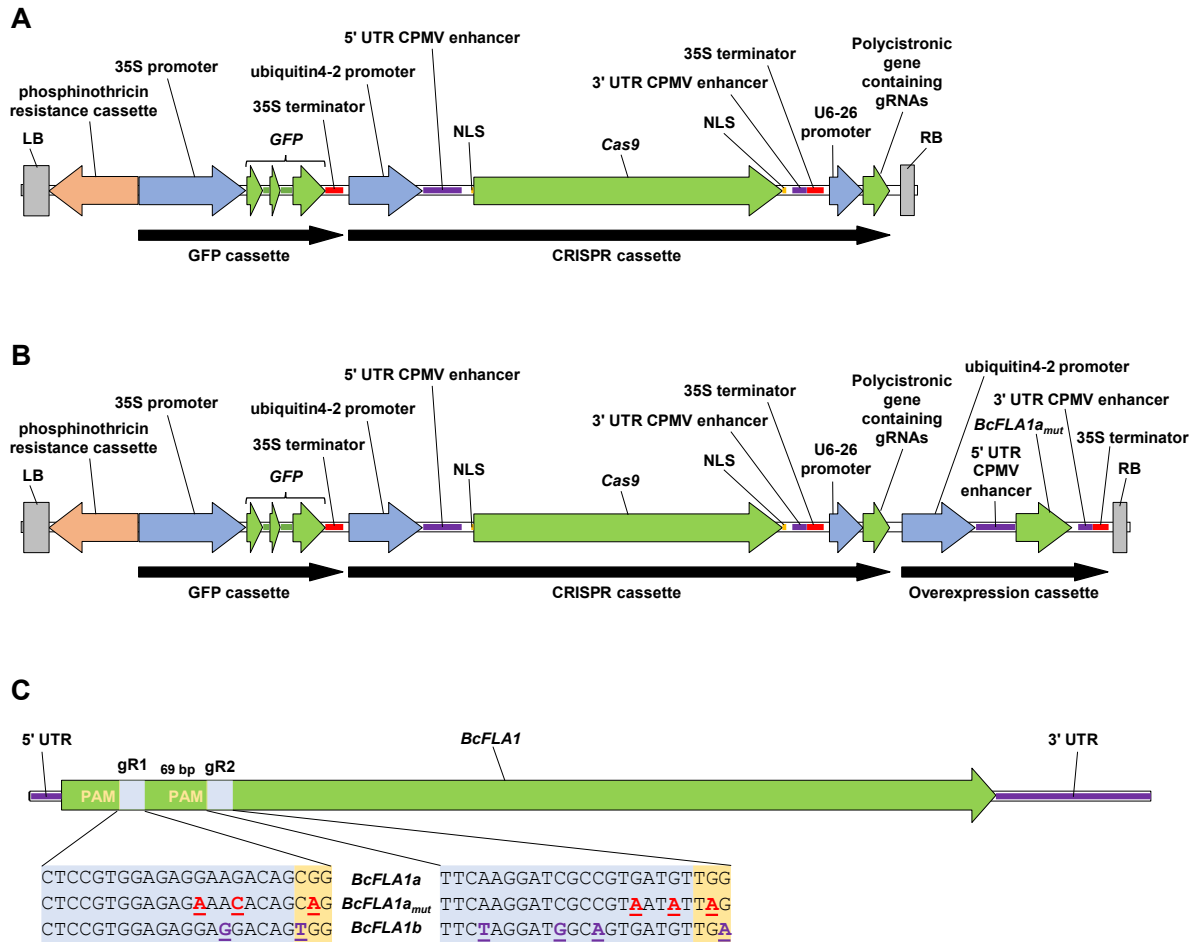
Table S15: Amino acid sequences encoded by the two *BcFLA1* alleles. Colors indicate identified motifs. N-terminal signal peptide (green) was identified with SignalP 4.1 (Petersen *et al.*, 2011), putative AG glycomodules (red) were marked according to Ma *et al.* (2017), fasciclin domain (yellow) was marked as in Jun and Xiaoming (2012) and GPI-anchor (purple) was identified with PredGPI (Pierleoni *et al.*, 2008).

BcFLA1a	<p>MATIH^{LT}LA^{PLL}LILAAVFLSTEITA^QPAAPAPGPAAP^TNI^{TA}ILEKGGQFATFIHLLN^TTRVGN</p> <p>QINI^QVN^{SS}SE^{GM}TVFAP^{TD}NAFQNLKPG^{TL}NKLSYDDQV^{KL}LILYHVS^{PK}FY^{TL}DD^{LL}SV^{SN}PV</p> <p>RTQASGR^{DS}AVYGLN^{FT}SQGNQ^{VN}VSTG^{FV}ET^{RV}SNALR^{KER}PLAVYV^{VD}MV^{LL}PEEMFGENKV</p> <p>SPAAP^GPKSN^{SP}DVSD^{DD}QEKAG^{AP}SDK^{SG}SGEMKAGLGLALGLV^{FL}CL^{KL}VV</p>
BcFLA1b	<p>MATSH^{LT}LA^{PLL}LILATVLLSTE^{VTA}QPAAPAPGPA^GVP^{NI}TAILEKGGQFATLIRLLN^TTQVGN</p> <p>QINI^QVN^{SS}SE^{GM}TVFAP^{TD}NAFQNLKPG^{TL}NKLSHEDQV^{KL}LILYHVS^{PK}FY^{TL}DD^{LL}SV^{SN}PV</p> <p>RTQASGR^{DG}AVYGLN^{FT}SQGNQ^{VN}VSTG^{VV}ET^{RV}SNELR^{KER}PLAIYV^{VD}MV^{LL}PEEMFGENKV</p> <p>SPAAP^GPKSN^{SP}DVSD^{DD}QEKAG^{AP}SDK^{SG}SEM^{KAG}LG^{FAL}GV^{VFL}CL^{KL}VV</p>

2. Chapter 2

S1 Table: Primers used for construct preparation.

Oligo name	Primer sequence forward / reverse (5' → 3')
gRNA1-F	TAGGTCTCCAGAGGAAGACAGGTTTTAGAGCTAGAA
gRNA1-R	ATGGTCTCACTCTCCACGGAGTGCACCAGCCGGGAA
gRNA2-F	TAGGTCTCCTCGCCGTGATGTGTTTTAGAGCTAGAA
gRNA2-R	ATGGTCTCAGCGATCCTTGAATGCACCAGCCGGGAA
L5AD5-F	CGGGTCTCAGGCAGAAGACTAGATTGAACAAAGCACCAGTGG
L5AD5-R	TAGGTCTCCAAACGAAGACAAAAACAAAAAAAAGCACCGACTCG
S5AD5-F	CGGGTCTCAGGCAGAAGACTAGATT
S5AD5-R	TAGGTCTCCAAACGAAGACAAAAAC
Ubi-sense	TATGGCGCGCCAAAAATTACGGATATGAATATAGGC
Ubi-anti	ATACTCGAGGCTGCACATACATAACATATCA
Cas9-sense	TTATGAATTCATGGACTATAAGGACCAC
Cas9-anti	CTATCCCGGGTTACTTTTTCTTTTTTGC
guide-RNA-sense	TATCGGGTACCGAGCTCGGATCCA
guide-RNA-anti	TATCTACGCGTCTCGAGCGGCCGCCAGTG
MCS1	GGCCGGACGTCCCTTAGGTACCCTACACGCGTCTAGACCGGTGTTT
MCS2	AAACACCGGTCTAGACGCGTGTAGGGTACCTAAGGGACGTCC
GFP-1	TTTCCCGCCTTCGGTTTGGGCCTGCAGGTTAGGGCACGATGTCCTG
GFP-2	GAAAAAGATTAGTTTTTCATTGTTAATTAAGTTAGACAG
GFP-3	AATGAAAATAATCTTTTTCTCTTTCTCATCTTTTC
GFP-4	TAAATATGTCGTCATCTTCTTTTTTTTTTTGG
GFP-5	AGAAGATGACGACATATTTATATATTTAGAAAAATTTAACTTTTAAAC
GFP-6	GTTGCACGCCGCCATCTTCGATGTTGTG
GFP-7	CGAAGATGGCGGCGTGCAACTCGCTGAT
GFP-8	TTCATATCCGTAATTTTTGGCCGCTCTAGAAGTGTTGG
35S-Sbfl-sense	AACCTGCAGGGAATCCAATCCACAAAAATCTGAGCTTA
35S-Pacl-anti	AATTAATTAATCGATAATTGTAAATGTAA



S1 Fig: Expression cassettes and *BcFLA1* gRNAs. Expression cassette of CRISPR+35S::GFP+fla1-gRNAs (A), the complementation vector (B) and gRNAs for CRISPR/Cas9 targeting *BcFLA1* (C). For the control, the pB-CRISPR+35S::GFP vector without gRNAs was introduced into the plants. The gRNA sequences are displayed in the reverse and complement form for a better understanding.

S1 Text: Preparation of the constructs.

Primer Ubi-sense and Ubi-anti (S1 Table) were used to amplify the ubiquitin4-2 promoter from parsley using plasmid pPZP221-I-SceI_co as a template (kind gift of the Puchta lab in Karlsruhe, see Fauser *et al.* (2012)) with attached *Ascl* and *XhoI* restriction sites and cloned into the *Ascl*/*XhoI* restricted V69 vector (containing modified 5' and 3' UTRs of RNA-2 from the Cowpea Mosaic Virus as translational enhancers, see Myrach *et al.* (2017) for a detailed description of V69) replacing the 35S promoter with the ubiquitin4-2 promoter resulting in V69-Ubi. The primers Cas9-sense and Cas9-anti were used to amplify the Cas9 gene from 35S-Cas9-SK (kindly provided by Jian-Kang Zhu, Shanghai Center for Plant Stress Biology, China, see Feng *et al.* (2013) and Mao *et al.* (2013)) with attached *EcoRI*/*XmaI* sites and the Cas9 gene was cloned into *EcoRI* and *XmaI* linearized V69-Ubi resulting in V69-Ubi:Cas9. With

hybridized oligos MCS1 and MCS2 a multiple cloning site with MluI, KpnI, AgeI and AatII sites was added to the PmeI and NotI linearized V69-Ubi:Cas9 resulting in V69-Ubi-Cas9-MCS. The U6-26 promoter together with the guide scaffold (separated by a BbsI cloning site allowing the cloning of the target sequence) was amplified from the vector AtU6-26-SK (also kindly provided by Jian-Kang Zhu) with primer guide-RNA-sense and guide-RNA-anti adding flanking KpnI and MluI sites and the KpnI/MluI restricted PCR product was ligated in the KpnI/MluI linearized V69-Ubi:Cas9-MCS resulting in V69-Ubi:Cas9-MCS-U6. For amplification of a fluorescent marker with introns for reduced silencing we used pG104-GFPendo (kindly provided by Michael Wassenegger, see Dadami *et al.* (2013) for details) as a template. The gene cassette including *rbcS* promoter and terminator contained BpI sites, that were removed by amplification of four overlapping PCR-products (GFP-1+GFP-2, GFP-3+GFP-4, GFP-5+GFP-6, GFP-7+GFP8), that were assembled together with the AscI linearized V69-Ubi:Cas9-MCS-U6 vector using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs, Ipswich, USA) creating the vector V69-Ubi-Cas9-MCS-U6-*rbcS*:GFP (V110). The backbone of the binary vector was switched by cloning the complete cassette into pBGWFS (BpI sites were removed previously) using PmeI and Eco47III, resulting in pB-Ubi-Cas9-MCS-U6-*rbcS*:GFP (V111). For expression of the fluorescent marker in roots we amplified a 35S promoter devoid of BpI sites from pICH51266 (a gift from Sylvestre Marillonnet and Nicola Patron (Addgene plasmid # 50267)) with attached SbfI and PacI sites (using primer 35S-SbfI-sense and 35S-PacI-anti) and ligated it with the PacI/SbfI restricted vector resulting in pB-CRISPR+35S::GFP (V112).

For the complementation, *BcFLA1a_{mut}* was produced synthetically by IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA) and contained a ClaI restriction site at the beginning and a PstI restriction site at the end for the insertion into the MCS of V69 vector resulting in H279. Then the overexpression cassette from H279 was cut by AscI and PmeI and, after blunting the AscI digested end, inserted into the CRISPR+35S::GFP+*fla1*-guides (H278), which was digested by PmeI, resulting in H280.

S2 Text: Expression analysis.

cDNA synthesis was performed using the 'PrimeScript™ 1st strand cDNA Synthesis Kit' (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. 1 µg of the isolated RNA was applied in a 20 µL reaction. The cDNA was diluted 1:1 with H₂O, quantified (NanoPhotometer® P-Class P 300, Implen, Munich, Germany) and stored at -85°C.

Apart from the endogenous control gene *UBIQUITIN CONJUGATING ENZYME 9* (*AtUBC9*; AT4G27960) (Czechowski *et al.*, 2005), qPCR primers were designed using Primer3Plus (Untergasser *et al.*, 2012) (S2 Table) in consideration of the following quality standards: amplicon size between 90 and 200 bp, primer size between 20 and 25 bp, primer T_m between 58 and 62°C and a GC content between 40 and 60 %. The specific primers were designed on the two gRNA regions with three differing bases at the 3' end of the primers, whereas the binding sites for the unspecific primers are located at the 3' end of *BcFLA1*. Confirmation of the primer specificity and the pretest were carried out by PCR using the 'DCS DNA HotStart Polymerase' (DNA Cloning Service e.K., Hamburg, Germany) according to the manufacturer's instructions and a primer concentration of 0.25 µM. The amplicons were separated on a 4 % (w/v) non-denaturing agarose gel containing 0.004 % (v/v) Midori Green Advance (Nippon Genetics Europe, Dueren, Germany) for 35-40 min at 7.6 V cm⁻¹ in TAE buffer.

Besides *AtUBC9* (Czechowski *et al.*, 2005), qPCR was performed using a second endogenous control, *ELONGATION FACTOR 1 ALPHA 1* (*BcEF-1-a1*), which exhibited a uniform expression in the MACE dataset. The partial cDNA sequence of *BcEF-1-alpha1* obtained by MACE analysis, was submitted to the NCBI database under accession number MF061227.

For primer efficiency tests, qPCR was performed using seven different cDNA amounts per reaction (200, 40, 8, 1.6, 0.32, 0.064, 0.0128 ng cDNA), whereas consistently 100 ng cDNA per reaction were used for expression analysis. Only primer pairs with an efficiency between 90 and 110 % were used.

Three technical replications were performed per transgenic root. The components per 10 µL reaction were as follows: 5 µL 2x 'SYBR® Premix Ex Taq™ (Tli RNaseH Plus)' (Takara Bio Europe SAS, Saint-Germain-en-Laye, France), 0.25 µM forward and reverse Primer, 0.5 µL H₂O and 4 µL of the diluted cDNA.

Supplementary Material

The quantitative reverse transcriptase polymerase chain reaction (qPCR) runs were performed in the CFX96 C1000 Thermal cycler (Bio-Rad, Hercules, CA, USA) with an initial step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C or 65°C for 30 s as well as a closing step of 95°C for 10 s and a final melting curve procedure with a stepwise increment of 0.5°C ranging from 65°C to 95°C.

S2 Table: qPCR primers. Wildtype and mutant-specific primers were designed on the two gRNA regions harboring three differing bases at the 3' end (underlined). The unspecific primers are located at the 3' end of *BcFLA1*. *AtUBC9* primers derived from the study of Czechowski et al. (2005).

Description	Sequence forward / reverse (5' → 3')	T _a [°C]
<i>AtUBC9</i>	TCACAATTTCCAAGGTGCTGC TCATCTGGGTTTGGATCCGT	65
<i>BcEF-1-alpha</i>	GGTGACGCTGGTATGGTGAA TGATGACACCGACTGCAACA	60
<i>BcFLA1</i> (unspec. qPCR)	TCGTTGAAACACGTGTCAGT ACTTATCCGATGGAGCCCCT	65
<i>BcFLA1a_{mut}</i> (qPCR)	CTCATCCTTGCTGCTGTGTTT CAAGGATCGCCGTAATATTAGTCCG	65
<i>BcFLA1</i> (wildtype-spec. qPCR)	TCATCCTTGCCGCTGTCTTC CAAGGATCGCCGTGATGTTG	65

S3 Table: Primer pairs for the verification of gene editing. For M13 SSR-PCR forward primers were tagged with M13 tail 'GTAAAACGACGGCCAGT'. Numbers in brackets indicate amplicon size including the M13 tail.

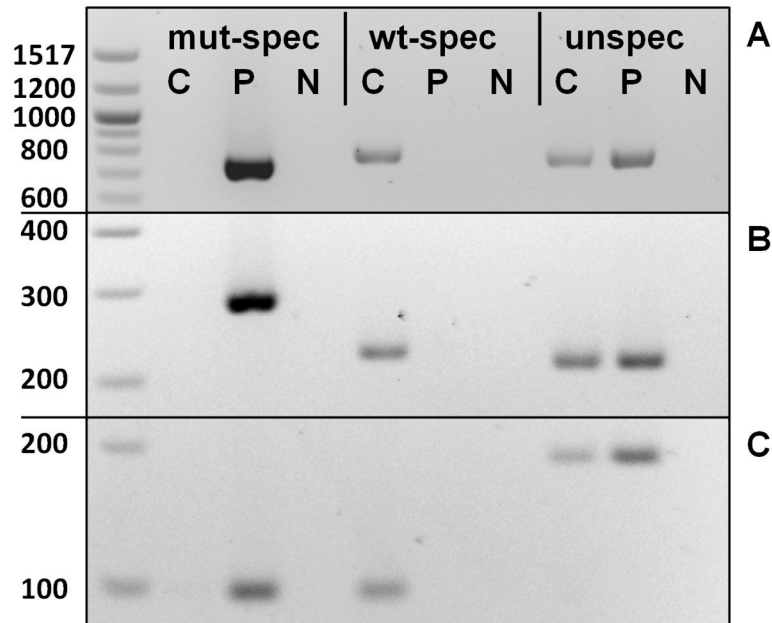
Description	Sequence forward / reverse (5' → 3')	T _a [°C]	Amplicon size [bp]
<i>BcFLA1</i> (unspec. large)	TTCAAACGCAATGGCAACCA ACCCAAATCAAACGACGAGTTTC	60	752 (769)
<i>BcFLA1a_{mut}</i> (large)	TGCGGCGCCATTAAATAACG TTGAGTCCTTACCGGGTACTT	60	713 (730)
<i>BcFLA1</i> (wildtype-spec. large)	AACTCAAACTTCAAACGCAATGGC GAAACCCAAATCAAACGACGAGT	63	665 (682)
<i>BcFLA1</i> (unspec. small)	TTCAAACGCAATGGCAACCA TTCCGACTCGTGTTGTGTTT	60	200 (217)
<i>BcFLA1a_{mut}</i> (small)	CCCCTGGTTTTTCGAACTTGG TTCCGACTCGTGTTGTGTTT	60	237 (254)
<i>BcFLA1</i> (wildtype-spec. small)	AACTCAAACTTCAAACGCAATGGC TTCCGACTCGTGTTGTGTTT	63	210 (227)

Supplementary Material

```

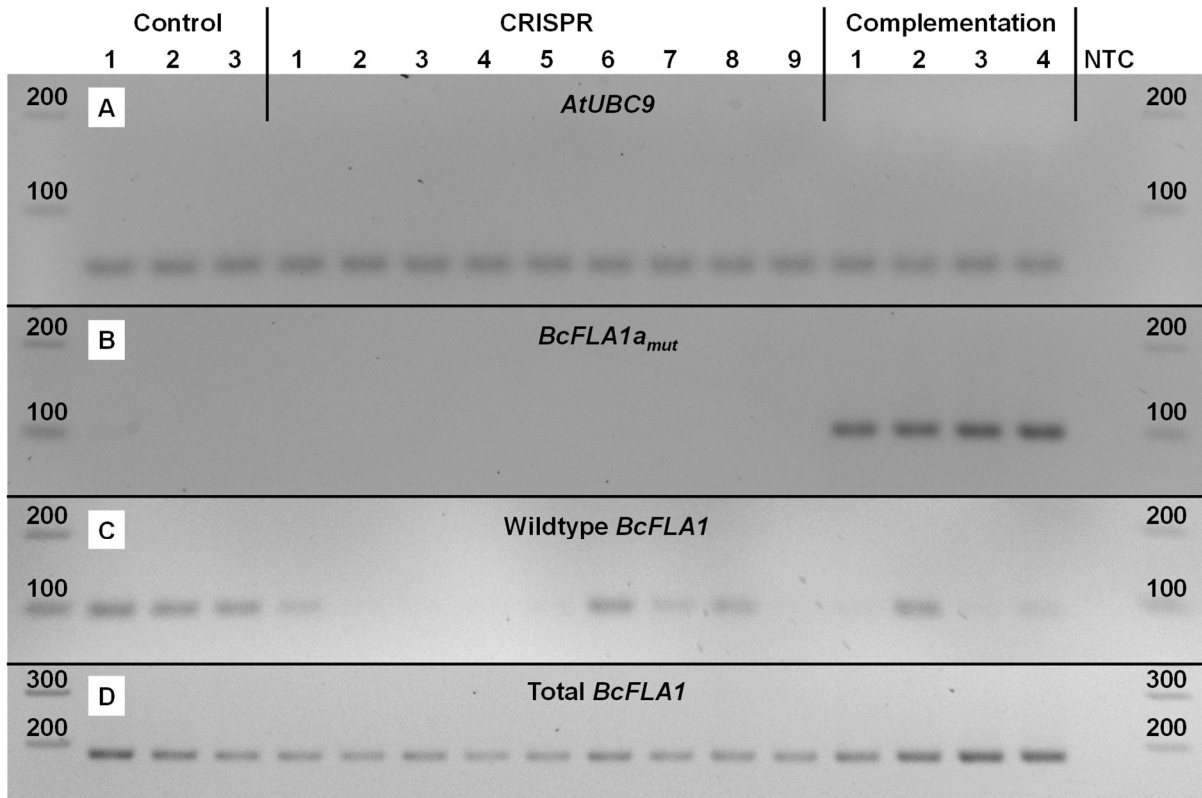
          PAM      gRNA1      30 bp      PAM      gRNA2      22 bp
BcFLA1a  TCTAACTCTAGCTCCTCTCCTCATCCTTGCCGCTGTCTTCCCTCCACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC
BcFLA1b  TCTAACTCTAGCTCCTCTCCTCATCCTGCCA CTGCTCCTCTCCACGGAGGTAAGTCTC/.. /GTCCCGTCAACATCCTGCAATCCTTGAAAA/.. /ATCC
CRISPR 1L TGTAACGTAGTTCCTGTC-----/14/-----GTCCTCCTCTCCACGGAGGTAAGTCTC/.. /GTCCCGTCAACATCCTGCAATCCTTGAAAA/.. /ATCC - 15
CRISPR 1S  TCTAA-----CCACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC - 39
CRISPR 2  TCTAACTCTAGCTCCTCTCC-----ACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC - 26
CRISPR 3  TCTAACTCTAGCTCCTCTCCTCATCCTGCCA CTG-----ACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /-CC -112
CRISPR 4  TCTAACTCTAGCTCCTCTCC-----ACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC - 26
CRISPR 5L  TCTAACTCTAGCTCCTCTCCTCATCCTGCCA CTG-----/68/-----ATAACTGCTC/.. /GTCCCGTCAACATCCTGCAATCCTTGAAAA/.. /ATCC - 17
CRISPR 5S  TCTM-----CTCGGAGWTATCTTCCCTCCACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC - 21
CRISPR 7L  TCTAATTCTAGCTCCTCTCCTCATCCTCTCCWNTG--TCCCTCCACGGAGNATAACTGCTC/.. /GTCCNNTCAACATCACKGCATCCTWGAAAA/.. /ATCC - 3
CRISPR 7S  TCTAACTCTAGCTCCTCTCCTCAT-----/6/-----AACTGCTC/.. /CTC-----ACGGCGATCCTTGAAAA/.. /ATCC - 41
CRISPR 9  TCTAACTCTA-----TC/.. /GTCCCGTCAACATCCTGCAATCCTTGAAAA/.. /ATCC - 50
Compl 3   TCTAACTCTAGCTCCTCTCCTCATCCTTGCCGCT--NCCTCTCCACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC - 4
Compl 4   TCTAACTCTAGCTCCTCTCCTCATCCTTGCCGCTG--TCCTCTCCACGGAGATAACTGCTC/.. /CTCCGACCAACATCACGGCGATCCTTGAAAA/.. /ATCC - 3
    
```

S2 Fig: CRISPR/Cas9-induced deletions and insertions in *BcFLA1*. Grey background indicates the region of the gRNAs, blue background the PAM; violet letters indicate differing bases in *BcFLA1b*, from which the first two were used to discriminate between both alleles; inserted bp (red numbers) and deleted bp (on the right). Data from the independent experiment replication. Compl = complementation; ././ = break. Numbers on the left indicate the respective transgenic roots.

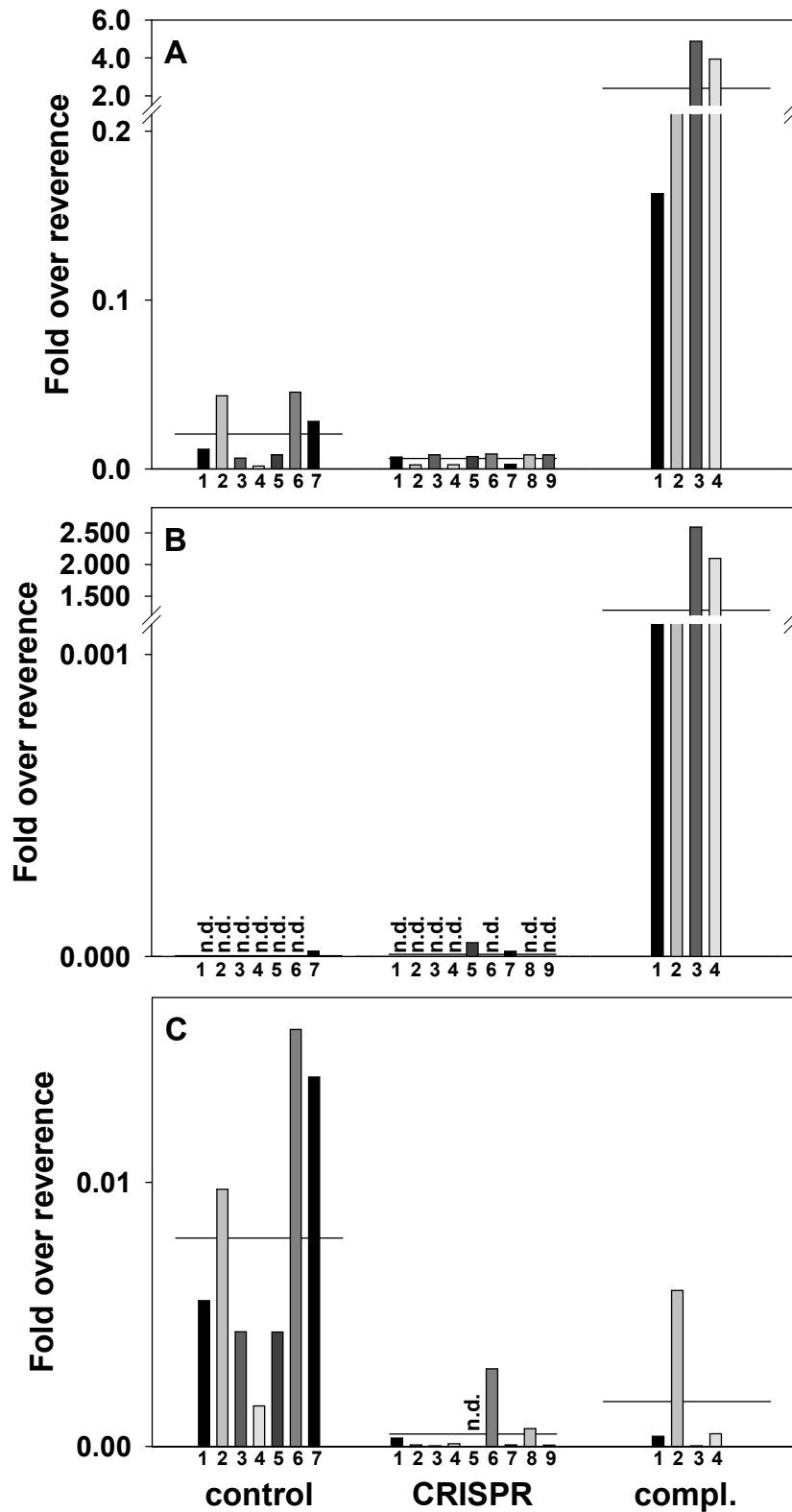


S3 Fig: *BcFLA1* primers are specific for the mutant and the wildtype version of the gene, respectively. Primers for the validation of the gene editing with large products (A) and small products (B). Primers used for expression analysis (C). C = control sample, P = plasmid used for the complementation, N = no template control.

Supplementary Material



S4 Fig: Pretest of the qPCR primers confirming the primer specificity and revealing a successful downregulation and complementation. Expression of the endogenous control *AtUBC9* (Czechowski *et al.*, 2005) (A), *BcFLA1a_{mut}* (B), the wildtype *BcFLA1* (C) and both versions of *BcFLA1* (D). Data from independently replicated experiment.



S5 Fig: Expression of *BcFLA1* as affected by CRISPR/Cas9-induced gene editing and complementation, respectively. Total *BcFLA1* expression (A), expression of *BcFLA1a_{mut}* (B) and expression of the wildtype *BcFLA1* gene (C). Each bar represents the expression in one single transgenic root measured by three technical replicates with the horizontal line representing the corresponding mean of all biological replicates (transgenic roots). Data from independently replicated experiment. Numbers indicate the respective transgenic roots. Fold over reverence = $2^{-\Delta\Delta Ct}$; n.d. = not detected.

3. General Discussion

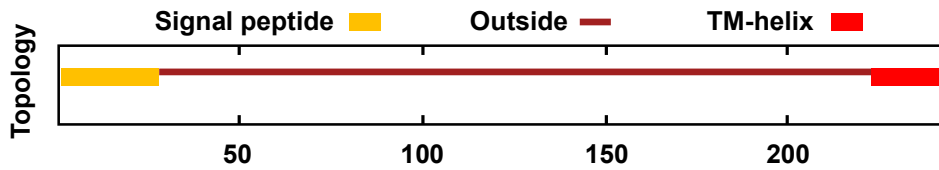


Fig. S1: Topology prediction of BcFLA1 according to SPOCTOPUS (Viklund *et al.*, 2008).

Table S1: Amino acid sequence of BcAGP1, BcAGP2 and BcRHS10L. Colors indicate identified motifs. N-terminal signal peptide (green) was identified with SignalP 4.1 (Petersen *et al.*, 2011), putative AG glycomodules (red) were marked according to Ma *et al.* (2017), GPI-anchor (purple) was identified with PredGPI (Pierleoni *et al.*, 2008), transmembrane domain (grey) was predicted by TMHMM Server v. 2.0, and kinase domain (turquoise) was predicted by NCBI Conserved Domain Search (Marchler-Bauer *et al.*, 2017).

BcAGP1	<p>MAALKTMQALIFLGLLATSCMAQAPAPAPIMVLPPVESPSPPPAITPTAEPSPVPVASSPVMIP PEPTPAPATPPTVSPPTKSEKTSPPVASEPKPEAMAFGPSGPTSPAPAPDGPIADSALTNKAFL VSTVIAGALYAVVLA</p>
BcAGP2	<p>MMMKKMFVQIAVFCLLATIAAVSGHEGHVHSPAPAPGPA TN SAVVPTTNMFTGLAFAAVALVLG LNH</p>
BcRHS10L	<p>MSD SPTS SPPAPS ADS SP PPDNSTGGS AP PPTDSS PPS PPTDS AP PPS IPSPFPATFP PPSNP PPPPPDSP P P P P P D A P P S D L P P V D S G S P P P E P T N S P P P E E F E S P P P P N E D N T S P P S P P E Q S P P P P Q A P S E K G G S K K P K K S P A P T N S P P A P P N A P S H A L P P K S T A A G G P L K S P S R G V P S F P P P P P T S N D G G Y Q G K T M A G M A V A G F A I I A L I A V V F L V R R K K R N V D V Y T D S Q Y L P P S N F S I K S D G F L Y G Q N T T K G G Y S G P G G Y N T Q Q Q S N T G N S F G S Q R G Y T Q S G S T P D S A V M G S G Q T H F A Y E E L M E I T Q G F A K Q N I L G E G G F G C V Y K G K L H D G K L V A V K Q L K V G S G Q D R E F K A E V E I I S R V H R H L V S L V G Y C I S D V Q R L L I Y E Y P N Q T L E H H L H G K R P V L D W A R R V R I A I G S A K G L A Y L H E D C H P K I I H R D I K S A N I L L D D E F E A Q V A D F G L A K L S D S T Q T H V S T R V M G T F G Y L A P E Y A Q S G K L T D R S D V F S F G V V L L E L I T G R K P V D Q Y M P L G E E S L V E W A R P L L H K A I E T G D F S D L V D R R L E K H Y V E N E V F R M I E T A A A C V R H S G P K R P R M A Q V V R A L D S E G D M G D I S N G S K V G H N N A Y D S C Q Y N S D D R K M A F G F D D S S D S G M H S G D Y S V R S S R K G S N G E S T E F T R S E S E N R N F N N R R</p>

Danksagung

Zuallererst möchte ich mich sehr bei **Prof. Dr. Manfred Schenk** für die hingebungsvolle Betreuung bedanken, welche seit seinem Ruhestand eher noch intensiver wurde. Die während der Arbeit aufgetretenen Probleme und Erkenntnisse machten viele Diskussionen unabdinglich, aber auch über die Grenzen der Arbeit hinaus waren es immer sehr angenehme Gespräche.

Prof. Dr. Thomas Debener und **Prof. Dr. Helge Küster** möchte ich danken, dass sie als Mitglieder meines Betreuungsteams innerhalb des Graduiertenkollegs bei auftretenden Problemen für Diskussionen zur Verfügung standen und in Form der Fortschrittsberichte wichtigen Input für das weitere Vorgehen lieferten. Außerdem möchte ich mich bei beiden für die Übernahme des Korreferats bedanken.

Prof. Dr. Traud Winkelmann möchte ich danken, dass sie sich so kurzfristig bereit erklärt hat, den Vorsitz der Promotionskommission zu übernehmen.

Bedanken möchte ich mich außerdem bei **Prof. Dr. Claus-Peter Witte**, dessen Übernahme der Institutsleitung eine reibungslose Fortführung meiner Arbeit ermöglichte.

Vielen Dank auch an **Dr. Marco Herde**, durch dessen Knowhow eine schnelle Etablierung des CRISPR/Cas-Systems möglich war und auch sonstige Probleme und Fragen immer schnell geklärt werden konnten.

Bei **Martin Hinrichs** möchte ich mich neben der angenehmen Arbeitsatmosphäre im Büro für die Einführung in die *hairy root*-Transformation sowie sonstige kleinere und größere Hilfen bedanken.

Danken möchte ich außerdem den drei Studenten, welche ich während meiner Arbeit betreuen durfte. **Kim Rössig** für die Durchführung des Split-Root-Versuches, **Timo Lauterbach** für die Durchführung des Versuches zur Nährstoffspezifität und **Markus Niehaus** für die Durchführung der CRISPR-Versuche. Letzterem möchte ich ganz besonders auch für seinen Einsatz in der Zeit nach seiner Masterarbeit danken, in welcher er half, die letzten noch fehlenden Analysen für die Einreichung des Manuskripts zu Kapitel II fertigzustellen.

Aus der AG Debener möchte ich **Ina Menz** und **Dr. Marcus Linde** für ihre Hilfe rund um die PAGE sowie **Enzo Klein** für die Einführung in die CLC-Software danken.

Aus der AG Küster danke ich **Rico Hartmann** und **Marian Uhe** u.a. für ihren Input bezüglich der *hairy root*-Technik.

Bei **Neelke Goldenstein** bedanke ich mich für ihre Hilfe vor allem bei der Pflanzentransformation.

Außerdem geht mein Dank an **Dr. Nieves Medina Escobar** für alles Organisatorische rund ums Labor, **Herbert Geyer** für sämtliche organisatorischen Dinge in der zentralen Versuchsanlage, **Ingrid Dusy** und **Iris Wienkemeier** für alle administrativen Angelegenheiten, **André Specht** für informatische Angelegenheiten sowie durchgeführte ICP-Analysen und **Hildegard Thölke**, welche u.a. dafür gesorgt hat, dass im Labor immer ausreichend Materialien vorhanden waren, oder ggf. schnell geliefert wurden. Auch bei den ehemaligen technischen Mitarbeitern des Instituts, **Anne Herwig**, **Hartmut Wieland** und **Birgit Stedeler** möchte ich mich für jegliche Hilfestellungen bedanken.

Generell geht ein großes Dankeschön an alle aktuellen und ehemaligen **Mitarbeiter des IPE** für die angenehme Arbeitsatmosphäre und Hilfen aller Art.

Abschließend möchte ich mich noch ganz besonders bei **meiner Familie** für jegliche Unterstützung bedanken. Auch, wenn es in letzter Zeit nicht mehr so häufig möglich war, euch zu besuchen, haben mir die Aufenthalte in der Heimat immer wieder die nötige Kraft für das weitere Voranschreiten mit meiner Arbeit gegeben.

Lebenslauf

Persönliche Daten

Name: Thomas Wolfgang Kirchner
Geburtsdatum: 15.05.1986
Geburtsort: Duderstadt
Familienstand: ledig
Staatsangehörigkeit: deutsch

Ausbildung

10/2011 – Heute **Leibniz Universität Hannover (LUH), Institut für Pflanzenernährung (IPE),
Wissenschaftlicher Mitarbeiter** (Hannover, DE)
August 2014 **Beijing Forestry University, Summer School** (Peking, China)
2009 – 2011 **LUH, IPE, Studentische Hilfskraft** (Hannover, DE)
2009 – 2011 **LUH, M. Sc. in Pflanzenbiotechnologie** (Hannover, DE)
2006 – 2009 **LUH, B. Sc. in Pflanzenbiotechnologie** (Hannover, DE)
2005 – 2006 **Harz-Weser-Werkstätten, Zivildienst** (Duderstadt, DE)
1998 – 2005 **Eichsfeld-Gymnasium Duderstadt, Abitur** (Duderstadt, DE)
1996 – 1998 **Astrid-Lindgren-Schule, Orientierungsstufe** (Duderstadt, DE)
1992 – 1996 **Janusz-Korczak-Grundschule** (Duderstadt, DE)

Publikationsliste

Zeitschriftenartikel

Klug B, Kirchner TW, Horst WJ. 2015. Differences in Aluminium Accumulation and Resistance between Cultivars of the Genus *Fagopyrum*. *Agronomy* **5**, 418–434.

Posterbeiträge

Kirchner TW, Rössig KL, Bremer M, Schenk MK. 2014. Pi deficiency-induced root hair growth in *Brassica carinata* – local or systemic signaling? Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung, Halle-Wittenberg, Germany.

Kirchner TW, Rössig KL, Schenk MK. 2015. Molecular mechanisms of phosphate starvation-induced root hair growth in *Brassica carinata*. Rhizosphere 4, Maastricht, the Netherlands.

Kirchner TW, Niehaus M, Herde M, Schenk MK. 2016. Down-regulation of candidate genes by CRISPR/Cas9 to reveal their role in Pi deficiency-induced root hair elongation in *Brassica carinata*. Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung. Stuttgart-Hohenheim, Germany.

Kirchner TW, Niehaus M, Herde M, Schenk MK. 2017. A fasciclin-like arabinogalactan protein is involved in the Pi deficiency-induced root hair elongation in *Brassica carinata*. International Plant Nutrition Colloquium. Copenhagen, Denmark.

Vorträge

Kirchner TW, Klug B, Erban A, Kopka J, Horst WJ. 2012. Physiological and molecular analysis of the spatial characteristics of aluminum uptake and translocation in roots of buckwheat (*Fagopyrum esculentum*). Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung, Bonn, Germany.