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**Random and CRISPR-Cas mutagenesis to create oilseed rape with
reduced seed phytic acid content**

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M.Sc. Niharika Sashidhar
aus Kurnool, Andhra Pradesh, India
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Dekan: Prof. Dr. Dr. Christian Henning

1. Berichterstatter: Prof. Dr. Christian Jung
2. Berichterstatter: Prof. Dr. Frank Kempken

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List of Abbreviations

2D	Two dimensional
3D	Three dimensional
%	Percent
°C	Degree Celsius
μl	Microliter
μm	Micrometer
X ²	Chi square
A	Adenine
ABC	ATP binding cassette
Amp	Ampicillin
ANOVA	Analysis of variance
<i>A. thaliana</i> , <i>At</i>	<i>Arabidopsis thaliana</i>
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
<i>Bar</i>	Bialaphos resistance
BLAST	Basic local alignment search tool
<i>B. napus</i> , <i>Bn</i>	<i>Brassica napus</i>
<i>B. oleracea</i> , <i>Bo</i>	<i>Brassica oleracea</i>
bp	Base pairs
BRAD	<i>Brassica</i> database
<i>B. rapa</i> , <i>Br</i>	<i>Brassica rapa</i>
C	Cytosine
Cas	CRISPR-associated
cDNA	Complementary DNA
CDS	Coding sequence
cm	Centimeter
CRISPR	Clustered regularly interspaced short palindromic repeats
CTAB	Cetyltrimethyl ammonium bromide
cv	Cultivar
C _t	Cycle threshold
d	Day
DAP	Days after pollination
DAG	Diacyl glycerol
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DSB	Double-stranded break
DW	Dry weight
EC	Exome capture
<i>E. coli</i>	<i>Escherichia coli</i>
EMS	Ethyl methanesulfonate
IMP	<i>Myo</i> -Inositol monophosphatase
InDel	Insertions and deletions
Ins	Inositol
InsP1	Inositol monophosphate
InsP2	Inositol bisphosphate
InsP3	Inositol trisphosphate
InsP4	Inositol tetrakisphosphate

List of Abbreviations

InsP5	Inositol pentakisphosphate
InsP6	Inositol hexakisphosphate Phytic acid
InsP7	Inositol pyrophosphate
InsP8	Inositol pyrophosphate
IPK1	Inositol pentakis-phosphate 2-kinase
IPK2	Inositol multi phosphate kinase
ITPK	Inositol tetrakis-phosphate kinase
g	Relative centrifugal force
gm	Gram
h	Hour
kb	Kilobase (s)
min	Minutes
MIPS	<i>myo</i> -inositol phosphate synthase
MIK	<i>myo</i> -inositol kinase
ml	Milliliter
mM	Millimolar
MRP	Multi drug resistance protein
NGS	Next generation sequencing
ng/ μ l	Nano gram per micro liter
nmol	Nano molar
PA	Phytic acid
PCR	Polymerase Chain Reaction
P _i	Inorganic phosphorus
Ptd	Phosphatidyl
PGK2	2-phosphoglyceric acid kinase
PHT	Phosphate transporters
PSV	Protein storage vacuoles
RNA	Ribonucleic acid
RPM	Rotations Per Minute
RT-qPCR	Quantitative real-time PCR
TILLING	Targeted Induced Local Lesions IN Genomes
TbyS	TILLING by Sequencing
<i>Taq</i>	<i>Thermus aquaticus</i>
RNase	Ribonuclease
SNP	Single Nucleotide Polymorphism
x	Fold coverage
V	Volt
W	Watt
WGS	Whole genome sequencing
w/v	Weight / Volume

1. General introduction

1.1. Biology and genomics of *Brassica napus* L.

Brassica napus L. (oilseed rape or rapeseed) cultivation has started in the 16th century in Europe and later in the 20th century, it became one of the most important oil crops in the world (Robson et al. 2002). Varieties from *B. napus* are not only grown for the production of cooking oil and biodiesel, but also as vegetables, for condiments and animal feed. Rapeseed is cultivated in three different growth types such as winter, semi-winter and spring types, depending on the growing region with an average lifecycle of 3-12 months. The difference between the growth types is underlined by the vernalization requirement and subsequent onset of seed formation. The seeds are majorly formed after self-pollination with occasional outcrossing (Vollmann and Istvan 2010).

B. napus is an allotetraploid which formed by spontaneous interspecific hybridization of *B. oleracea* and *B. rapa* between ~6700 years (Sun et al. 2017) and ~7500 years (Chalhoub et al. 2014) ago, based on two synonymous substitution estimations and a Bayesian Markov chain Monte Carlo simulation, respectively (Lu et al. 2019). A recent genome wide association study with 588 *B. napus* accessions including transcriptome analyses from 11 different tissues revealed that the *B. napus* A genome originated from European turnip, whereas the C genome originates from a common ancestor of kohlrabi, cauliflower, broccoli, and Chinese kale (Lu et al. 2019). The genome size of *B. napus* is ~1.13 Gbp with 38 chromosomes ($2n = 4x$). During evolution, several genome duplications occurred and in the genus *Brassica*, an additional triplication event caused a complex genetic framework, where each gene is encoded by several paralogous genes. As a result, the ancestral function of a gene is separated to different gene copies, which is referred to as sub-functionalization (Schiessl et al. 2019). Alternatively, gene copies might gain a new function during evolution referred to as neo-functionalization (Xia et al. 2016).

The cultivation of so called double zero or '00' varieties that are low in erucic acid and glucosinolates which are derived from the mother plants cv. Liho and cv. Bronowski, respectively have narrowed the germplasm. In order to circumvent this situation, new crosses between *B. oleracea* and *B. rapa* were performed to develop the so called re-synthetic rapeseeds (Girke et al. 2012). Crosses between *B. napus* and *B. rapa* were also performed to obtain early maturing cultivars in Canada, Japan and China. In addition, crosses were performed between *B. napus* and *B. oleracea* to increase the existing gene pools with no consequences on fertility of the hybrids (Rahman et al. 2015). Furthermore, use of resynthesized rapeseed can be beneficial in hybrid production as it is encouraged due to the underlined heterotic effects (Rahman 2013).

Considering that oilseed rape has an important economic value breeders aim for improving seed yield, enhance meal quality and disease resistance. To achieve this, several QTLs were identified in respect to seed yield (Lu et al. 2019; Luo et al. 2017), disease resistance for key pathogens causing clubroot (*Plasmodiophora brassicae*), blackleg (*Leptosphaeria maculans* and *L. biglobosa*), sclerotinia stem rot (*Sclerotinia sclerotiorum*), and downy mildew (*Hyaloperonospora parasitica*) (reviewed in Neik et al. 2017) as well as meal quality with low glucosinolate contents (He et al. 2018; Nour-Eldin et al. 2017). With the change in climate, increasing pathogen pressures and increase in demand for human needs, all above mentioned goals are a major focus in rapeseed breeding.

1.2. Breeding of oilseed rape to improve seed quality traits

The versatile uses of oilseed rape with its well-balanced amino acid profiles and protein contents made it the third most important oil crop in the world. Tremendous increase of research has been performed

General introduction

for improving oil and meal quality. *B. napus* meal contains 33- 48% proteins after oil extraction (Nesi et al. 2008). However, the negative correlation of oil and protein content made it challenging to breed for both traits simultaneously (Gu et al. 2017; Jasinski et al. 2018; Balalic et al. 2017; Tang et al. 2019). Subsequently, reducing the antinutritive compounds in the meal can enhance the rapeseed protein contents with no loss of oil contents. Several studies showed that by reducing compounds such as tannins, sinapins, glucosinolates or fibers, which hinder the rapeseed meal quality, no negative effects on oil contents were observed. The production of double 00 varieties reduced the aliphatic glucosinolates to less than 18 $\mu\text{mol/g}$ seed and erucic acid to very low contents thereby facilitating for better feed palatability (Downey and Rimmer 1994). Furthermore, cultivars with high oleic acid and low linolenic acid content were developed which increased the oil oxidative stability and hence reducing the formation of deleterious *trans*-fatty acids during frying processes (Long et al. 2018). Currently, the possibility to increase the value of protein in rapeseed is limited by the negative correlation between oil and protein contents. Therefore, new breeding targets should be identified for increasing both contents without affecting each other (Jasinski et al. 2018).

Seed color in *B. napus* varies from light yellow over dark brown to black color which might be due to the amount of tannins present in the respective genotypes. Tannins are a distinctive group of high molecular weight phenolic compounds, which are belonging to three groups referred to as phlorotannins, hydrolysable and condensed tannins (Lipsa et al. 2012). Tannins form insoluble complexes with proteins and reduce their bioavailability in the meal (Naczek et al. 2000). The yellow seed trait was widely desired for its higher oil and protein contents as compared to the brown varieties. The yellow seeds have thinner seed coats owing to less anti-nutritive crude fiber and phenolics. Several QTLs have been identified for yellow seed color, which can be used in further breeding programs (Vollmann and Istvan 2010). Besides this, interspecific crosses have been performed to get yellow seeded oilseed rape. Furthermore, a recent study showed that knockout of two paralogous genes of *BnTT8* by CRISPR-Cas resulted in yellow seeds, which can be of importance in future rapeseed breeding (Zhai et al. 2019b).

In line with reducing bitter taste and increasing digestibility, sinapins were also targeted. Sinapic acid is an alkaloid amine, which is widespread in the plant kingdom and is the most predominant phenolic compound in seeds (Nićiforović and Abramović 2014). It is synthesized by the shikimate/phenyl alanine pathway (Milkowski and Strack 2010). The presence of sinapins in the meal also causes an unpleasant taste and reduces the palatability for animals. Several genes like ferulate acid 5-hydroxylase, UDP-glucose:sinapate glucosyltransferase, sinapoylglucose:choline sinapoyltransferase and reduced epidermal fluorescence 1 were either downregulated by antisense suppression, RNAi or knocked out by random mutagenesis to reduce the sinapine contents in seeds (Nair et al. 2000; Hüsken et al. 2005a; Hüsken et al. 2005b; Bhinu et al. 2009; Emrani et al. 2015; Wolfram et al. 2010). In all these studies, a reduction of 40-90% of sinapine was achieved in the homozygous offspring.

1.3. Phosphate storage in plants

1.3.1 Phosphorus uptake in plants

Phosphorus is an essential mineral for various physiological processes in plants. The primary source of phosphorus for plants is inorganic phosphorus/orthophosphate (Pi) which occurs mainly in the form of phosphate salts in the soil. Maintaining the phosphorus balance is crucial in plants due to the wide range of environmental factors and soil types influencing their growth. Plants have developed various physiological and morphological responses to maintain phosphorus homeostasis by changing the root architecture, exudation of different kinds of acid phosphates, accumulation of anthocyanin pigments in

response to Pi starvation (Misson et al. 2005) and arbuscular mycorrhizal symbiotic mechanisms which are regulated by PHT1 transporters (Młodzińska and Zboińska 2016; Srivastava et al. 2018; Yang et al. 2012). Different kind of transporters act according to the phosphorus availability (Figure 1a,b). In plants, the phosphate transporters are classified into five families namely PHT1, PHT2, PHT3, PHT4 and PHT5 (Zhang et al. 2016a). In Arabidopsis, there are nine PHT1 genes which are highly redundant in function and sequences (PHT1;1- PHT1;9) (Nussaume et al. 2011) along with one PHT2 (Versaw and Harrison 2002), three PHT3 (Rausch and Bucher 2002), six PHT4 (Guo et al. 2008) and three PHT5 genes (Liu et al. 2016; Liu et al. 2018). Another class of transporters is PHO1 that has 11 members in Arabidopsis, which are involved in phosphate homeostasis (Wang et al. 2004).

Each of the phosphate transporters has specific roles in mobilizing phosphorus in an organelle dependent manner based on the Pi availability. In regard to this PHT1 transporters are exceptional and are localized in the plasma membranes where they are responsible for the external Pi acquisition or Pi translocation between cells or tissues (Figure 1a). In Arabidopsis, PHT1;1 and PHT 1;4 were shown to be important for up taking Pi by the root at both high and low Pi conditions (Shin et al. 2004) next to PHT 1;2 and PHT 1;3 (Ayadi et al. 2015). Whilst the other members of PHT1, like PHT1;5, PHT 1;8 and PHT1;9 play a major role in the relocation of Pi from shoots to roots and to reproductive organs (Nagarajan et al. 2011; Ayadi et al. 2015). The PHT2 and PHT3 transporters are specific to plastids and mitochondrial phosphate transport, respectively (Daram et al. 1998; Zhu et al. 2012b). From the PHT4 group five members are located in the Golgi membrane and one is specifically located in the plastid envelope (Guo et al. 2008) where as PHO1 and PHO2 translocate Pi from roots to shoots (Stefanovic et al. 2007). The uptake and distribution of Pi from soil to its final storage in seeds is shown in Figure 1b. Vacuoles are the major storage compartments for Pi and the transport is carried out by PHT5 proteins (Liu et al. 2016). PHT5 belong to the major facility superfamily (MFS) where SPX-MFS1 and SPX-MFS3/PHT5;1 mediate vacuolar Pi influx and efflux, respectively as shown in Figure 1b. Furthermore, the levels of PHT1, PHO1 and PHO2 transporters are regulated by miR399 and *cis*-natural antisense transcripts (*cis*-NAT_{PHO1;2}) in the xylem and miR399 and IPS1/AT4 in roots (Figure 1b) (Srivastava et al. 2018). During phosphate deficient conditions, *cis*-NAT_{PHO1;2} positively regulates the expression of PHO 1;2 to maintain phosphate homeostasis in plants (Jabnourne et al. 2013). Finally, with the help of ABC-MRP type transporters, Pi is transported from leaves to seeds where it is stored in the form of phytic acid.

The first proof that *myo*-inositol monophosphate synthase (MIPS) was the initial enzyme in the PA biosynthesis chain was established in rice (Yoshida et al. 1999). PA started to accumulate in the aleurone layer at 4 days after anthesis (DAA), which coincided with the accumulation of rice Ins(3)P1 synthase gene (*RINO1*; referred as *MIPS* in other species). To examine this further, Mitsuhashi et al (2005), performed studies in suspension-cultured cells of *Catharanthus roseus* (commonly known as periwinkle). They could show that the available levels of Pi influence the synthesis of PA in protoplasts and vacuoles. High levels of Pi resulted in higher PA in vacuoles and lower levels of PA under low Pi conditions (Mitsuhashi et al. 2005).

In addition to the physiological importance in the plants, PA has gained the attention in treating many types of cancers because of its antioxidative properties (Vucenik et al. 2005; Vucenik and Shamsuddin 2006). Furthermore, it has also been shown in some studies that PA can be used as a preservative for many kinds of food (Yang et al. 2015; Du et al. 2012; Silva and Bracarense 2016).

1.4. Strategies to attenuate PA contents in the meal

1.4.1. Use of external phytases

Monogastric animals cannot digest PA due to lack of phytases and thereby leading to reduced bioavailability of phosphorus and other essential minerals. Therefore, external phosphate was supplemented to the meal in the form of dicalcium phosphate to compensate the low availability. However, the decrease in the natural resources of rock phosphate led to commercial production of phytases, which are supplemented to seed meals. It was estimated from laboratory and field experiments that 500–1000 units of phytase can replace 1 g of Pi (in the form of dicalcium phosphate) supplementation, and reduce total phosphorus excretion by 30–60% (Yao et al. 2012; Lei et al. 2013). In addition, it was estimated that supplemental phytase at 1000 units/kg of feed costs approximately 0.5–2US\$/ton of feed, whereas addition of 1% Pi to the meal costs 12US\$/ton (Lei et al. 2013).

Phytases are enzymes which hydrolyze PA into one molecule of inositol and six molecules of Pi (Figure 2a). Phytases can be classified based on the position, where they dephosphorylate the carbon ring. There are three types of phytases: 3-phytases (*myo*-inositol hexakis phosphate-3-phosphohydrolase, EC 3.1.3.8), 4/6-phytases (*myo*-inositol hexakis phosphate 6-phosphohydrolase, EC 3.1.3.26) and 5-phytases (*myo*-inositol hexakis phosphate 5-phosphohydrolase, EC 3.1.3.72) (Yao et al. 2012). 3-phytases are derived from bacteria and fungi (Sajidan et al. 2004), 4/6-phytases were discovered in plants along with 5-phytases, but 5-phytases were also found in the rumen bacterium *Selenomonas ruminantium* (Chu et al. 2004). Furthermore, based on their catalytic mechanism of hydrolysis they are further grouped into four classes, histidine acid phosphatases (HAPhy or acid phytases), β -propeller phosphatase (BPPhy or alkaline phytases), purple acid phosphatase (PAPhy or metalloenzymes), protein tyrosine phosphatase (PTPhy; also referred to as dual-specificity phosphatase (DSP) or cysteine phytase) (Lei et al. 2013; Mullaney and Ullah 2003). Each class of phytases hydrolyzes PA depending on the pH conditions (Suleimanova et al. 2015) (Figure 2b). Initially, phytases from *Aspergillus niger* were used to break down the PA in the meal (Irving and Cosgrove 1972). Later, bacterial phytases from *Escherichia coli*, which belongs to the HAP class of phytases, showed higher specificity for PA and hydrolyzed PA more efficiently into free phosphates and *myo*-inositol (Greiner et al. 1993).

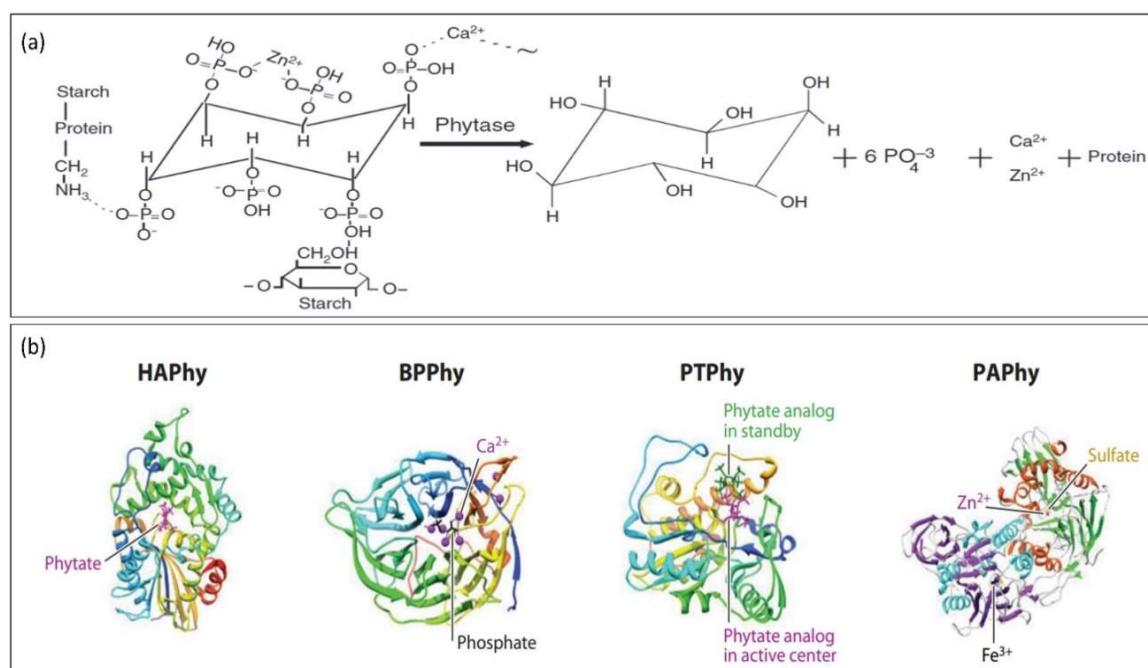


Figure 2: Mechanism of phytases and their crystal structures. (a): Phytic acid (PA), which is bound to starch, protein and other divalent ions is hydrolyzed by phytases into six phosphates, *myo*-inositol, free divalent ions and proteins (Figure taken from Yao et al. (2012)). (b): Types of phytases. Histidine acid phosphatases (HAPhy or acid phytases), β -propeller phosphatase (BPPhy or alkaline phytases), purple acid phosphatase (PAPHy or metalloenzymes), protein tyrosine phosphatase (PTPhy; also referred to as dual-specificity phosphatase (DSP) or cysteine phytase) (Figure taken from Lei et al. (2013)).

In this scenario addition of microbial phytases paved ways in the animal feed industries. More recently, to cut down the costs of production of phytases, especially in developing countries, a study was performed using a fungal phytase gene from *Acremonium zeae* and expressing it in the yeast *Kluyveromyces marxianus* (Pires et al. 2019). This was the first study to express phytases in yeast with high yields. Another interesting way was to express a fungal phytase in the cell wall deficient microalgae *Chlamydomonas reinhardtii*, which can be supplemented to animals as a source of carbohydrates, proteins and other nutrients together with the overexpressed phytase (Erpel et al. 2016; Gantar and Svirčev 2008).

1.4.2. Development of transgenic animals and plants

Albeit the addition of phytases to the meal has solved the problem of increasing free Pi in the animal diets, there are still few drawbacks. Many factors such as protein solubility, pH, temperature for phytate hydrolysis, type of isolate and organism, effect the phytate degrading activity (Vashishth et al. 2017). However, due to the catalytic properties at certain pH and the thermostability of external phytases it was not considered as an ideal option. Alternatively, transgenic animals and plants overexpressing phytases were produced. These included pigs (Enviropig) overexpressing phytases in their salivary glands (Golovan et al. 2001), and medaka fishes constitutively expressing phytases from *A. niger* (Hostetler et al. 2003). Furthermore, the expression of foreign proteins in animals might develop allergenic properties and therefore Cho et al. (2016) expressed avian multiple inositol polyphosphate phosphatase (MINPP) which has phytase like properties in chicken and observed improved digestion of PA (Cho et al. 2006). In addition, phytases from *A. niger* were expressed in tomato, wheat, soybean, alfalfa, Arabidopsis, canola and maize (Li et al. 1997; Brinch-Pedersen et al. 2000; Gutknecht 1997; Mudge et al. 2003; Ponstein et al. 2002; Chen et al. 2008; Ma et al. 2012) and bacterial phytases from *E.coli* and *S. ruminantium* were also expressed in rice (Hong et al. 2004).

All above-mentioned transgenic approaches did not meet consumer's acceptance and therefore, strategies to identify low PA mutants gained more importance. In this regard, several studies have been performed to either identify or induce mutations resulting in low PA contents in cereal crops, legumes and oilseed plants, or identifying natural mutants within the existing germplasms. Noteworthy, due to the importance of PA in plant metabolic processes, null mutants were never a favorable option for biofortification in seeds. In Table 1, a summary of low PA mutants available from various crop species is given. Furthermore, the need for iron and zinc, as major micronutrients essential for human nutrition in developing countries has motivated to breed for low PA crops (Perera et al. 2018).

1.4.3. Phytic acid storage in rapeseed

Studies performed in different canola grade varieties for PA contents revealed 4.0 - 4.7 % in defatted seed meals (Uppström and Svensson 1980). It has been found that the amount of PA varies from 2.0 - 4.0 % in seeds, 2.0 - 5.0 % in defatted meals and 5.0-7.5 % in protein concentrates in oilseed rape (Matthäus et al. 1995). Furthermore, oilseed rape has higher PA contents when compared to the other oil crops like soybean, sunflower and cotton (Matthäus et al. 1995). A latest study in oilseed rape quoted, 1-6% PA contents in matured seeds (Von Der Haar et al. 2014). However, all these values depend on growth conditions and the type of method used for PA measurements.

An external supply of Pi in the soil at different concentrations showed the higher the Pi the more PA is accumulated in seeds of the two tested oilseed rape genotypes (cv. Lirajet and cv. Bristol) (Möllers et al. 1999). Furthermore, Ponstein et al. (2002) have expressed fungal phytase *phyA* from *A. niger* under the control of a seed specific cruciferin promoter. They found that the *phyA* was stably expressed over generations with a slight decrease under field conditions (Ponstein et al. 2002). Subsequently expression of both bacterial and fungal phytases (*phyA* and *appA*) in oilseed rape demonstrated that the transgenic lines not only had lower PA levels in seeds, but also showed an increase in seed yield and shoot biomass, which increased by 14.7% and 36.8%, respectively (Wang et al. 2013). Furthermore, the study from Wang et al. (2013) showed that the phytases were exuded from the roots under PA rich conditions to hydrolyze PA.

The studies mentioned above were either focused on identifying natural variation in PA contents or on transgenic approaches overexpressing bacterial phytases. Until now, the complete biosynthesis pathway of PA in *B. napus* was not explored and the underlying genes were not identified except for the initial enzyme BnMIPS. However, the starting gene of the PA pathway *BnMIPS* was first identified in 2006 and a functional characterization was done by using antisense and co-suppression approach in cv. Westar (Georges et al. 2006). This was the first attempt to identify a low PA mutant and was patented under US 7,148,064 B1. Later the same group had approached a different method of reducing PA contents in seeds. They have introduced a new metabolic step by using the gene *myo*-inositol methyltransferase (*IMT*) which was isolated from *Mesembryanthemum crystallinum* (ice plant) and transformed into *B. napus* under the control of napin and phaseolin promoters. The idea was to divert the free *myo*-inositol as a substrate for the PA biosynthesis and by this approach, a reduction of 19-35% PA was observed in seeds (Dong et al. 2013).

Table 1: Summary of *lpa* mutants in various plant species (Modified after Sparvoli and Cominelli 2015)

Gene	Species	% Phytic acid in dry weight of seeds *	Gene	% phytic acid reduced*	Kind of mutation	Reference
MIPS1	<i>Arabidopsis thaliana</i>	1.41	At4g39800	Nd	T-DNA insertion	(Murphy et al. 2008; Meng et al. 2009; Chen and Xiong 2010; Donahue et al. 2010; Kim and Tai 2011)
MIPS2	<i>Arabidopsis thaliana</i>	1.41	At2g22240	Nd	T-DNA insertion	(Murphy et al. 2008; Kim and Tai 2011; Luo et al. 2011)
MIPS3	<i>Arabidopsis thaliana</i>	1.41	At5g10170	Nd	T-DNA insertion	(Meng et al. 2009; Kim and Tai 2011; Luo et al. 2011)
MIPS1	<i>Glycine max</i>	1.55	Ghma18g02210	50	Gamma rays	(Yuan et al. 2007; Frank et al. 2009; Yuan et al. 2009)
MIPS1	<i>Glycine max</i>	1.55	Ghma18g02210	50	<i>N</i> -nitroso- <i>N</i> methylurea (NMU)	(Hitz et al. 2002; Meis et al. 2003)
MIPS1	<i>Glycine max</i>	1.55	Ghma18g02211	50	-	(Maupin et al. 2011; Gao et al. 2008)
MIPS1	<i>Glycine max</i>	1.55	Ghma18g02212	35-95	RNAi	(Nunes et al. 2006)
MIPS	<i>Solanum tuberosum</i>			Nd	antisense	(Murphy et al. 2008; Keller et al. 1998)
MIPS	<i>Oryza sativa</i>	0.90	Os03g09250	75	antisense	(Kuwano et al. 2009)
MIPS	<i>Gossypium hirsutum</i>	2.94	GhMIPS1D	Nd	-	(Ma et al. 2019)
IMP1	<i>Arabidopsis thaliana</i>	1.41	At3g02870	Nd	T-DNA insertion	(Torabinejad et al. 2009; Kim and Tai 2011)
IMPL1	<i>Arabidopsis thaliana</i>	1.41	At1g31190	Nd	T-DNA insertion	(Kim and Tai 2011)
IMPL2	<i>Arabidopsis thaliana</i>	1.41	At4g39120	Nd	T-DNA insertion	(Kim and Tai 2011)

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IMP2	<i>Arabidopsis thaliana</i>	1.41	At5g05090	Nd	T-DNA insertion	(Kim and Tai 2011)
MIK	<i>Arabidopsis thaliana</i>	1.41	At5g58730	62-66	T-DNA insertion	(Kim and Tai 2011)
MIK	<i>Zea mays</i>	0.86	GRMZM2G361593	45	<i>Mu</i> insertion	(Shi et al. 2005)
MIK+ITPK	<i>Zea mays</i>	0.86	GRMZM2G361593 GRMZM2G456626	66	<i>Mu</i> insertion	(Shi et al. 2005)
MIK	<i>Oryza sativa</i>	0.90	Os03g52760	64	Gamma rays + sodium azide	(Liu et al. 2007; Frank et al. 2007; Zhao et al. 2008)
MIK	<i>Oryza sativa</i>	0.90		75	EMS	(Kim et al. 2008)
2-PGK	<i>Arabidopsis thaliana</i>	1.41	At5g60760	66	T-DNA insertion	(Kim and Tai 2011; Kim and Tai 2010)
2-PGK	<i>Oryza sativa</i>	0.90	Os02g57400	40	Gamma rays	(Zhao et al. 2008)
IPK2 β	<i>Arabidopsis thaliana</i>	1.41	At5g61760	35	T-DNA insertion	
IPK2 β	<i>Arabidopsis thaliana</i>	1.41	At5g61760	48	T-DNA insertion	(Kim and Tai 2011)
ITPK1	<i>Arabidopsis thaliana</i>	1.41	At5g16760	46	T-DNA insertion	(Kim and Tai 2011)
ITPK4	<i>Arabidopsis thaliana</i>	1.41	At2g43980	40-51	T-DNA insertion	(Kim and Tai 2011)
ITPK	<i>Zea mays</i>	0.86	GRMZM2G456626	30	<i>Mu</i> insertion	(Raboy et al. 2000; Shi et al. 2003)
ITPK	<i>Oryza sativa</i>	0.90	Os09g34300	46-68	EMS	(Kim and Tai 2014)
ITPK	<i>Oryza sativa</i>	0.90	Os09g0518700	32	CRISPR-Cas	(Jiang et al. 2019)
IPK1	<i>Arabidopsis thaliana</i>	1.41	At5g42810	49-83	T-DNA insertion	(Kim and Tai 2011; Stevenson-Paulik et al. 2005)
IPK1	<i>Arabidopsis thaliana</i>	1.41	At5g42810	nd	T-DNA insertion	(Murphy et al. 2008; Kuo et al. 2014)
IPK1	<i>Glycine max</i>	1.55	Glyma14g07880	46	Gama rays	(Yuan et al. 2007)
IPK1	<i>Glycine max</i>	1.55	Glyma12G240900	45	RNAi	(Punjabi et al. 2018)
IPK1	<i>Triticum aestivum</i>	1.02	Ta.41955	56	RNAi	(Aggarwal et al. 2018)

General introduction

IPK1	<i>Zea mays</i>	0.86	GRMZM2G067299	50	ZFN-DNA editing	(Shukla et al. 2004)
IPK1	<i>Oryza sativa</i>	0.90	Os04g56580	70	RNAi under Oleosin 18 promoter	(Ali et al. 2013)
MRP	<i>Arabidopsis thaliana</i>	1.41	At1g04120	73-80	T-DNA insertion	(Nagy et al. 2009; Kim and Tai 2011)
MRP	<i>Glycine max</i>	1.55	Glyma03g32500 Glyma19g35230	80	EMS	(Gao et al. 2008; Maupin et al. 2011; Wilcox et al. 2000; Oltmans et al. 2005; Hulke et al. 2004; Pilu et al. 2009; Pandey et al. 2018)
MRP	<i>Phaseolus vulgaris</i>	1.41	Phvul001g165500	90	EMS	(Panzeri et al. 2011)
MRP	<i>Zea mays</i>	0.86	GRMZM5G820122	90	EMS	(Pilu et al. 2003; Pilu et al. 2005; Cerino Badone et al. 2012; Doria et al. 2009; Badone et al. 2010; Landoni et al. 2013)
MRP	<i>Zea mays</i>	0.86	GRMZM5G820123	66	EMS	(Shi et al. 2003)
MRP	<i>Oryza sativa</i>	0.90	Os03g04920	20-100	Gamma rays + sodium azide	(Xu et al. 2009; Naidoo et al. 2012)
MRP	<i>Oryza sativa</i>	0.90	Os03g04920	90	T-DNA insertion	(Liu et al. 2007)
MRP (<i>ABCC13</i>)	<i>Triticum aestivum</i>	1.02	Ta.74676	22-34	RNAi	(Bhati et al. 2016)
Sultr3;3	<i>Hordeum vulgare</i>	1.19		50	sodium azide	(Bregitzer and Raboy 2006; Raboy et al. 2015; Karner et al. 2004)
Sultr3;3	<i>Oryza sativa</i>	0.90	Os04g0652400	44	Gamma rays	(Yamaji et al. 2016; Frank et al. 2007; Liu et al. 2007)

* Phytic acid values are from (Lott et al. 2007). *Nd- not determined

1.5. Mutant screening in crop plants

Mutations are the key to understand the function of a gene. In nature, due to spontaneous mutations many important traits were discovered which had agronomical importance. For example, dwarf gene *Rht* in wheat (Peng et al. 1999), and low glucosinolate and erucic acid contents in oilseed rape, which were influential milestones for plant breeding. However, natural spontaneous mutations are very rare and recessive alleles are not easily noticeable in plants with higher ploidy levels. Therefore, several approaches have been developed to induce mutations or to identify mutations in plants by forward and reverse genetic tools.

1.5.1. Random mutagenesis

Targeted Induced Local Lesions In Genomes (TILLING) is a reverse genetic approach to screen the mutations in a mutagenized population. Ethyl methane sulfate (EMS), N-nitroso-N-methyl urea (NMU) or ethyl nitrosourea (ENU) are most commonly used chemical mutagens for creating point mutations randomly across the genome (McCallum et al. 2000). In classical TILLING, induced mutations are screened by using a mismatch-specific endonuclease (CEL1) that cleaves heteroduplexes formed by the mutated and non-mutated strands of PCR products at the position of a SNP. The cleaved products can be identified on denaturing gels or by High Resolution Melting (HRM) analysis. However, these technologies might be limited because of many technical reasons like efficiency of the CEL1 enzyme to cleave the heteroduplex formations or in the latter case the detection of base changes like C to G or A to T due to the smaller difference in their melting temperature (Tsuda et al. 2015). On the other hand, these methods are labor-intensive and the pooling depth is limited to eight individuals. TILLING by Sequencing (TbyS) allows for an efficient screening of mutations in larger populations in a limited time period and with a high resolution and pooling depths of up to 96 individuals (Tsai et al. 2011). TbyS was used in many crop plants such as wheat, maize, rice, tef, tomato, peanut, quinoa and also in oilseed rape (Krasileva et al. 2017; Tsai et al. 2011; Rigola 2009; Tsuda et al. 2015; Zhu et al. 2012a; Mestanza et al. 2019; Guo et al. 2015; Lu et al. 2018; Wells et al. 2013; Gilchrist et al. 2013). Further improvements in sequencing machines with higher data output facilitated screening of large number of genes or amplicons by stacking them even up to 100. With availability of different barcodes, this method can be used in multi-dimensional pooling studies to identify the mutation along with its type (missense, nonsense or splice site mutations) in a single step (Tsai et al. 2011; Tsuda et al. 2015; Pan et al. 2015). Today bioinformatic tools such as CAMBA and MAPS are available that are exclusively dedicated for TbyS approaches to detect mutations more efficiently in a limited time (Missirian et al. 2011; Rigola 2009; Chi et al. 2014). An overview of TbyS done so far is shown in Table 2 and a more detailed description is given in Chapter 2.

Table 2: TILLING by sequencing (TbyS) studies performed in crop plants.

Species	Population size	Mutation density	Poolin g depth	No. of genes analyzed	Sequencing machine	Approach [§]	Bioinformatics pipeline	Reference
Wheat	768	1/19.7 Mb	64	7(6)	Illumina GA	AS	CAMBA	(Tsai et al. 2011)
Rice	512	1/3.3 Mb	48	17(33)		AS	CAMBA	
Soybean	1536	1/74 kb	96	7	MiSeq	AS	CLC Genomics work bench	(Tsuda et al. 2015)
Canola (DH-line)	384	1/72 kb	96	4	GAIIX	AS	Bowtie, SAM tools	(Gilchrist et al. 2013; Wells et al. 2013)
Rapeseed	4608	10-41/ gene	8	3	GAI	AS	Maq v0.7.1	(Wells et al. 2013)
Wheat (4x)	1535	1/34.8kb	-	-		WGS	MAPS	(Krasileva et al. 2017)
Wheat (6x)	1200	1/39.5kb	-	-		WGS	MAPS	
Barley	20	1/154kb	-	-	Illumina MiSeq	WGS	BWA, GATK	(Schreiber et al. 2019)
	256	30 mutations	8	10		EC		
Maize	1,086	6.1 mutations/ gene		32069		EC	BWA, GATK	(Lu et al. 2018)
Peanut	768	1/344 kb and 1 /3,028 kb	64-96	1	Illumina	AS	BWA, SAM tools	(Guo et al. 2015)
Quinoa	3,072	1/203 kb	96	6	Illumina GA	AS	CODDLE	(Mestanza et al. 2019)
Tef (DZ-Cr-37)	21,210	120 mutations / 4 genes	90-100	4	454 GS FLX	AS	SIFT (sorting intolerant from tolerant)	(Zhu et al. 2012a)
Tomato	4 EMS mutants	1/0.8 Mb	-	-	454 GS FLX	WGS	Bowtie, SAM tools	(Rigola 2009)
Tomato	3000	-	-	3		AS		
Sorghum	256	>1.8 million	-	-	Illumina HiSeq 2000	WGS	Bowtie, SAM tools	(Jiao et al. 2016)

[§] AS: amplicon sequencing, WGS: whole genome sequencing, ECS: exome capture sequencing

1.5.2. Precise genome editing

The discovery of precise genome editing tools has revolutionized the way to create single and multiple knockout mutants of genes in a short time (Belhaj et al. 2013; Jinek et al. 2012). This might play a crucial role in many crop genomes having multiple, redundant genes and extensive gene family networks as a result of polyploidization (Voytas 2013). Genome editing technologies are based on the introduction of double strand breaks (DSBs) by specific nucleases at desired location of a genome. With the benefit of natural DNA repair mechanisms of cells, these induced DSBs can be repaired either by non-homologous end joining (NHEJ) or by homology directed repair (HDR) (Voytas 2013). NHEJ is an error prone process and after DNA repair insertions, deletions or substitutions can be observed at the repaired locations. Whereas in HDR, DNA is repaired by means of a homologous sequence that serves as a template for the sequence to be repaired (Belhaj et al. 2013). Most of the studies aimed at NHEJ repair mechanism as this creates knockouts in targeted genes that are valuable for studying unknown gene functions. Moreover, the induced mutations are not distinguishable from natural mutations. However, HDR mediated editing involves introduction of a DNA sequence which is a homolog to the target DNA and is similar to transgenesis which is considered to be a GMO technique (Voytas and Gao 2014). With the recent European legislation, regardless of type of the method used, all the obtained mutants by gene editing technologies are considered as GMO and are prohibited to be used in practical breeding approaches.

Many CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated) systems from various bacteria like *Streptococcus pyogenes*, *Staphylococcus aureus*, *Streptococcus thermophiles* are codon optimized for precise gene editing in plants. This had led to modify many agronomical traits in plants, which are beneficial in breeding for increased human demands (Jung et al. 2018). At present, a plethora of Cas proteins are available for various purposes of gene editing. Within no time, CRISPR-Cas has evolved as a toolbox for various purposes in plant breeding to induce mutations. In addition, genes can be turned on or turned off with the help of deactivated Cas9 (dCas9) fused with enhancers or repressors, respectively. HDR was shown to be possible in plants with the help of Cas12 and more recently, the use of Cas13 was shown to edit RNAs more efficiently than RNAi technologies (Schmidt et al. 2019; Wolter et al. 2019; Wolter and Puchta 2018).

Successful experiments were shown in oilseed rape using CRISPR-Cas for editing several important agronomic traits. Silique shatter resistance was increased by knocking out multiple paralogs of *BnALC*, *BnIND* and *BnFUL* (Braatz et al. 2017; Zhai et al. 2019a; Yang et al. 2017). Seed yield was increased by modifying meristem identity genes like *BnCLV1* (Yang et al. 2018). By knocking out *BnFANCM*, a meiotic recombination gene, higher crossover frequencies were observed which might be useful to create genetic variation (Blary et al. 2018). Fatty acid composition and oil contents were also improved by knocking out *BnFAD2*, *BnLPAT2* and *BnLPAT5*, respectively (Okuzaki et al. 2018; Zhang et al. 2019). Other breeding targets like increased resistance to *Sclerotinia* was shown by knocking out *BnWRKY70* (Sun et al. 2018) and more recently yellow-seeded oilseed rape was produced for enhancing the nutritional values by knocking out *BnTT8* (Zhai et al. 2019b).

1.6. Scientific hypothesis and objectives

This study was focused on reducing phytic acid contents in oilseed rape by using state of the art methods for the identification and characterization of responsible genes. Polyploids often can tolerate mutations without any visible effects and knocking out multiple copies is essential to observe a noticeable phenotype. Therefore, I hypothesized that multiple knockout mutations in only one gene family can result in reduced PA contents with no pleiotropic effects on agronomical traits. Since it was not known at the beginning of the study which genes might be the best candidates, I have broaden the analysis and concentrated on different gene families during my study. Furthermore, I had the following aims and the following questions to answer:

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- I aimed to identify all genes in rapeseed involved in PA synthesis and intracellular transport and to study their expression in developing seeds.
- Is it possible to identify and recover mutations when sequencing DNA of highly pooled M₂ plants?
- In addition, how efficient is TbyS in terms of time and costs as compared to classical gel based TILLING approaches?
- I aimed to produce double mutants because I expected single mutants might not show a visible phenotype in a polyploid crop like oilseed rape.
- I also aimed to analyze whether the obtained mutants showed pleiotropic effects on yield related traits by a reduction of PA.

2. Identification of phytic acid mutants in oilseed rape (*Brassica napus*) by large scale screening of mutant populations through amplicon sequencing

Niharika Sashidhar, Hans-J Harloff, Christian Jung

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2.1. Summary

- *Brassica napus* L. (oilseed rape) is an important oil crop in temperate regions, which originated from hybridization of *B. oleracea* and *B. rapa*. Due to its polyploidy, the functional study of single genes is cumbersome. Phytic acid is considered as an anti-nutritive compound and we aimed to knockout the underlying synthesis and transporter genes to identify low phytic acid mutants.
- We implemented a high throughput NGS screening protocol for an EMS population of 7680 plants in 6 gene families (*BnMIPS*, *BnMIK*, *Bn2-PGK*, *BnIPK1*, *BnIPK2* and *BnMRP5*) with two paralogs for each gene.
- A total of 1487 mutations were revealed and the vast majority (96%) were confirmed by Sanger sequencing. Furthermore, the characterization of double mutants of *Bn.2-PGK2* showed a significant reduction of phytic acid contents.
- We propose to use three-dimensional pooling combined with amplicon stacking and next generation sequencing to identify mutations in polyploid rapeseed in a fast and cost-effective manner for complex metabolic pathways. Furthermore, the identified mutants in *Bn2-PGK2* might be a very valuable resource for industrial production of rapeseed protein for human consumption.

2.2. Introduction

Brassica napus L. (oilseed rape) is a recent polyploid that emerged ~7500 years ago and is gaining more importance for its versatile uses as a crop for oil, bioenergy and livestock feed (Fu et al. 2019; Gacek et al. 2018). As a result, breeding of oilseed rape has become an essential task in the recent years. Dry mature seeds from oilseed rape accumulate oil (45-50%) and proteins (20-25%), while the meal remaining after oil extraction contains about 40% proteins (Nesi et al. 2008). Often, the meal is considered as anti-nutritive due to the presence of glucosinolates, tannins, phenolic acids and phytic acid that cannot be metabolized (Campbell et al. 2016). Phytic acid is the major phosphorus storage compound in seeds with levels from 2-4 % in oilseed rape seeds, 2-5% in the defatted meal and 5-7% for the protein concentrates (Thompson 1990). Many low phytic acid mutants have been identified in barley, rice, wheat, soybean, maize, peas and sesame (Sparvoli and Cominelli 2015). In oilseed rape no stable mutants have been reported, but a downregulation of *MIPS* genes by RNAi has been described in a patent with a reduced phytate content (Georges et al. 2006). Phytic acid is synthesized via two different pathways which include seven biosynthetic enzymes. These are *myo*-inositol phosphate synthase (*MIPS*), *myo*-inositol kinase (*MIK*), *myo*-inositol monophosphatase (*IMP*), inositol tetrakis-phosphate kinase (*ITPK*), inositol multi phosphate kinase (*IPK2*), 2-phosphoglyceric acid kinase (*2-PGK*), inositol pentakis-phosphate 2-kinase (*IPK1*) and one transporter protein, a multidrug resistance-associated protein (*MRP5*) (Figure 3) (Raboy 2009). The model plant *Arabidopsis thaliana* has multiple loci for the encoding genes, namely three *AtMIPS*, three *AtIMP*, one *AtMIK*, four *AtITPK*, two

IPK2, two *At2-PGK*, two *AtIPK1* and one *AtMRP5* (Kim and Tai 2011). We expected even more copies in polyploid *B. napus* ($2n=4x=38$), which complicates the search for low phytic acid mutants.

Screening of metabolic mutants is difficult if the metabolite is formed in a complex biochemical pathway involving many genes. In oilseed rape, due to its amphidiploid genome, a forward genetic approach is not very promising because single gene mutations did not result in a visible phenotype (Uauy et al. 2017; Emrani et al. 2015). To date no low phytic acid oilseed rape cultivars are available for commercialization of rapeseed meal as a protein source. Therefore, external phytases are applied to the meal for improving the meal quality (Adem et al. 2014). In this scenario, mutational breeding is an alternative for identification of low phytic acid mutants. So far, high throughput mutation screening techniques are lacking in oilseed rape. Among the reverse genetic tools, TILLING (Targeted Induced Local Lesions IN Genomes) is a valuable platform for characterization of point mutations across the whole genome. However, the classical screening methods based on mismatch-specific endonucleases is laborious and time consuming, especially when mutations for multiple genes are needed. Therefore, studies performed until now were limited to one or two genes (Blary et al. 2018; Braatz et al. 2018; Wang et al. 2008). In this study, a TILLING population of 7680 M₂ plants derived from 1902 EMS (ethylmethane sulfonate) treated M₁ plants was used. This population had been proven to be an excellent resource due to its high mutation frequencies (Harloff et al 2012).

Genome editing mediated by CRISPR-Cas has been successfully implemented to induce mutations in oilseed rape (Sun et al. 2018; Yang et al. 2018; Braatz et al. 2017). This method has the advantage of precise modification of the targeted genes unlike random mutagenesis in TILLING. Unfortunately, any kind of genome editing is legally classified as genetic modification especially in the EU which prevents its application in plant breeding. Today, with the availability of advanced bioinformatics and reduced sequencing costs, TILLING by Sequencing (TbyS) can be implemented in every crop in various approaches like whole genome, exome capture or amplicon sequencing (Rigola et al. 2009; Tsai et al. 2011; Tsuda et al. 2015; Krasileva et al. 2017; Kim et al. 2018). The selection of a suitable method depends on the size of the genome and on underlying costs for library preparation and sequencing. With these advancements, TbyS was performed in oilseed rape as a proof of concept at a small scale with one to three genes in small populations by amplicon sequencing (Gilchrist et al. 2013; Wells et al. 2013).

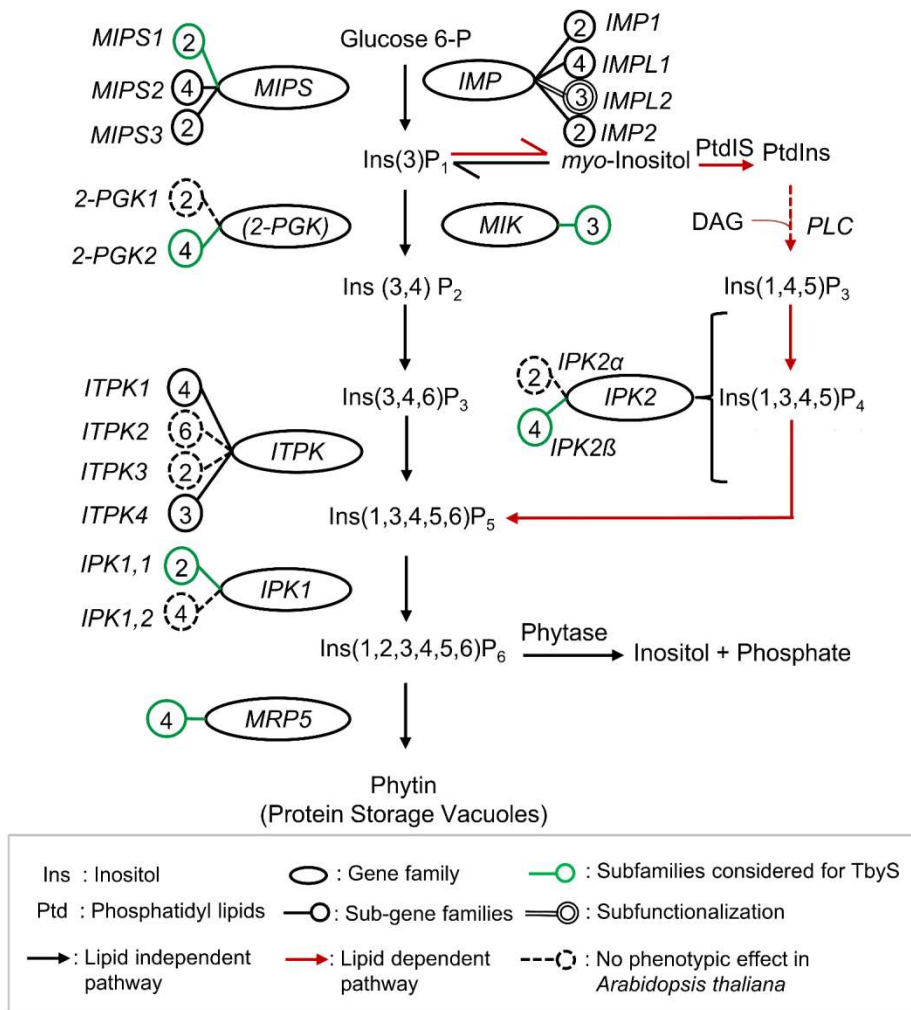


Figure 3: Proposed phytic acid pathway in rapeseed with all *Brassica napus*. L genes identified in this study (after Raboy, 2009). Targeted paralogs from the respective families are shown in green circles. MIPS: *myo*-inositol phosphate synthase, MIK: *myo*-inositol kinase, IMP: *myo*-inositol mono phosphatase, 2-PGK: 2-phosphoglyceric acid kinase, ITPK: inositol tetrakis-phosphate kinase, IPK2 β : inositol multi phosphate kinase, IPK: inositol pentakis-phosphate 2-kinase, MRP5: multi drug resistance protein, DAG: Diacyl glycerol, PLC: phospholipase C, PtdIS: phosphatidyl inositol synthase. The paralog number of each subgene family is given in the circles.

It has been demonstrated for several crops that a knockout of phytic acid synthesis genes results in low phytic acid accumulation in the seed (Larson et al. 2000; Raboy 2000; Guttieri et al. 2004; Campion et al. 2009). In this study, we identified all paralogs of phytic acid pathway genes known from *Arabidopsis* in oilseed rape. We hypothesized that nonsense mutations in these genes lead to a reduction of phytic acid. We describe in detail the screening of a winter type EMS population of *B. napus* with 7680 plants by using a 3D-pooling strategy and amplicon stacking. Single nucleotide polymorphisms identified by NGS were verified by Sanger sequencing, with low number of false positives. We propose to use NGS for future screening of rapeseed mutant populations for large number of genes in an effortless manner. We have identified knockout mutants in five key genes of the phytic acid biosynthetic pathway (*MIPS*, *MIK*, *IPK2*, *2-PGK*, *IPK1*) and another gene encoding the transporter *MRP5*. Furthermore, we found that *2-PGK2* double mutants had significantly reduced phytic acid contents.

2.3. Materials and Methods

2.3.1. Plant material

An EMS population of the inbred line Express 617 (F₁₁) derived from the German cultivar Express was used for mutation screening (Harloff et al. 2012). A total of 7680 M₂ DNA samples, normalized to 5 ng µl⁻¹ and stored in eighty 96-well microtiter plates were used for a 3D-pooling strategy.

2.3.2. *In silico* analysis of phytic acid pathway genes

Genes from the phytic acid pathway in *Arabidopsis thaliana* were used for identifying the respective homologs in the *B. napus* reference genome (Kim and Tai 2011) (<http://www.genoscope.cns.fr/brassicanapus/>). All possible hits were retrieved and compared for the exon intron structure and conserved domains (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Protein domains were identified by searching the pFam database (<http://pfam.xfam.org/>). All hits were confirmed using TAIR (https://www.arabidopsis.org/servlets/Search?type=gene&action=new_search) and NCBI annotations (<https://www.ncbi.nlm.nih.gov/gene>) and the *Brassica* database (BRAD) (<http://brassicadb.org/brad/>).

2.3.3. Designing paralog specific primers

Five genes encoding enzymes of the phytic acid synthesis pathway and one phytic acid transporter were chosen for screening an EMS population by using paralog specific primers. For this purpose, paralogs of each family were aligned separately using the CLC Main work bench 7.9.1 (CLC, Aarhus, Denmark) software. Then, an existing SNP within the paralogs was placed at the 3' end of a primer and the 3rd base from the 3' region was modified to another base to increase allele specificity (Liu et al. 2012) (Figure S1). The modified base was preferred to be C or G due to its high bonding. Primer properties were checked by Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). Only primers with GC content above 45%, a melting temperature above or equal to 58°C were chosen for amplification. The primer combinations covered 60-90% of the coding sequences and the specificity of primers was tested by Sanger sequencing of all targeted amplicons from non-mutated Express 617.

2.3.4. Expression analysis

Five biological replicates of Express 617 were used for qPCR analysis. Plants were grown under controlled conditions in a growth chamber at 22°C under long day conditions for three weeks and subsequently vernalized at 4°C for eight weeks (16h: 8h, light: dark). Later, the plants were transferred back to 22°C, (16h: 8h, light: dark). The opening of the flower was marked and the following days were recorded as days after pollination (DAP). Seeds from developing siliques from 15 DAP, 25 DAP, 35 DAP and 45 DAP were harvested and frozen in liquid nitrogen. mRNA was isolated from the homogenized seed samples using buffer P from the PeqGold Plant RNA isolation kit (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. DNase digestion was performed after RNA isolation using the peqGOLD DNase I Digest Kit (PEQLAB, Erlangen, Germany) to remove genomic DNA. cDNA first strand synthesis was carried out using a first strand cDNA synthesis kit (ThermoFisher Scientific Inc., Waltham, United States) according to the manufacturer's protocol. RT-qPCR was performed with three technical repeats using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Karlsruhe, Germany) on a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, USA) in a volume of 20µl. *BnActin2* was used as a house keeping gene for normalization. Relative expression was calculated using the ΔC_t method.

2.3.5. Construction of 3D pools

For 3D pooling, 7680 M₂ DNA samples, which were distributed in 80 microtiter plates, were used. The microtiter plates were arranged in 10 stacks and 96 individual DNA samples were pooled in 80 plate, 80 row and 80 column pools (Figure 4). Each pool was prepared by pipetting 2 μ l of DNA from each M₂ individual. The plate pools were prepared by combining all 96 samples of each plate into individual pools. Row pools were prepared by mixing eight rows, i.e. for the first sample A1 to A12 from plate one to plate eight, into one pool of stack one. Column pools were prepared by mixing corresponding columns of each plate, i.e. for the first sample A1 to H1 from plate one to eight and A2 to H2 from plate one to four were mixed (Figure 4).

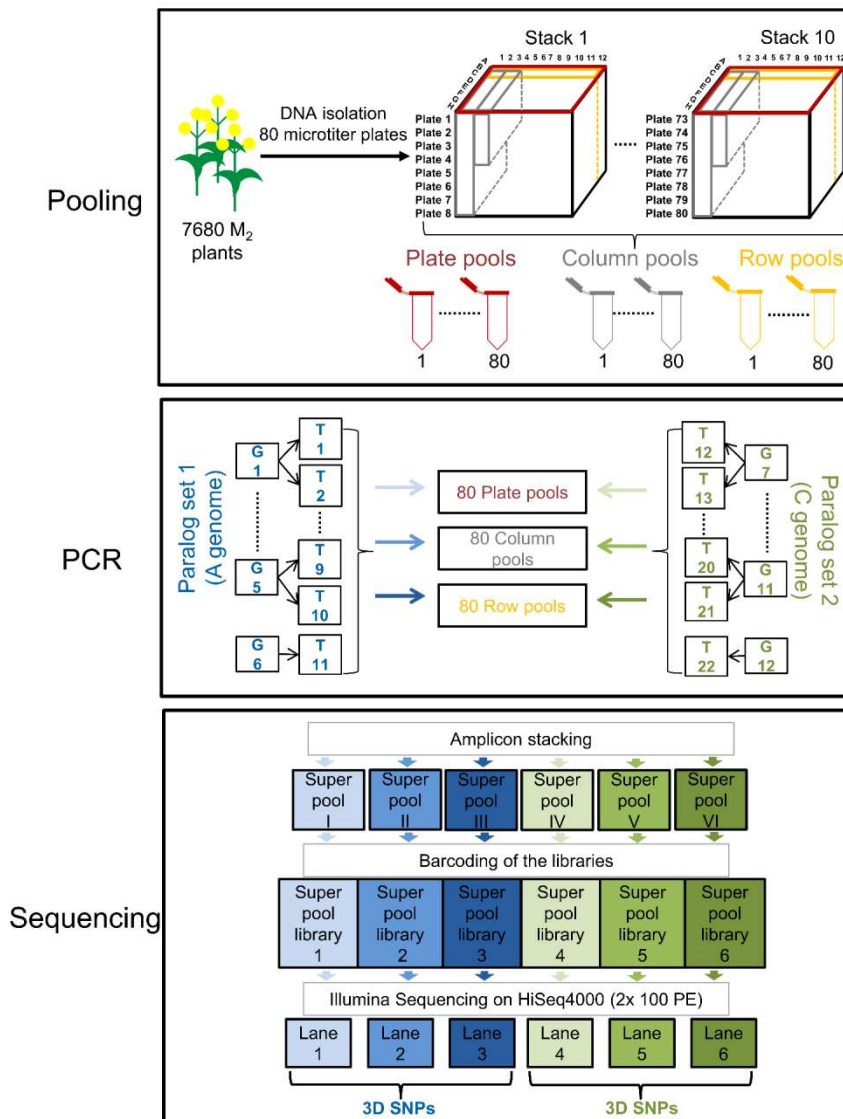


Figure 4: Set up of the TILLING by sequencing (TbyS) platform: DNA from 7680 M₂ plants was stored in 80 microtiter plates, which were arranged into 10 stacks of 8 plates each. 3D pools were prepared as shown in maroon, yellow and grey markings. Plate pools were prepared by combining the 96 samples of each plate into individual pools. Column pools were prepared by pipetting the samples from A1 to H1 from plate 1 to 8 and A2 to H2 from plate 1 to 4 for the first pool and accordingly for the next pools. Row pools were prepared by pipetting the samples from A1 to A12 from plate 1 to 8. In total, out of 80 individual M₂ microtiter plates, 80 plate pools, 80 row pools, and 80 column pools were formed. Six genes were selected as candidate genes for TbyS, with two paralogs from each gene family, resulting in 12 paralogs (G1 through G 12). A- and C-genome copies were grouped into paralog set 1 and 2, respectively. Ten long genes were divided into two

Chapter 2

TILLING amplicons each, and the remaining two short genes were represented by one TILLING amplicon each (T1 to T22). All TILLING amplicons were amplified from 3D pools as shown in shades of blue and green arrows. Each super pool was formed by combining the amplicons of 11 PCR of the respective dimension from each paralog library as shown in shades of blue and green. 80 unique barcodes were added to each super pool forming super pool libraries, which were sequenced on individual lanes. Combining the data from lane 1, 2 and 3 showed mutations as 3D SNPs for paralog set 1 while lanes 4, 5 and 6 showed mutations as 3D SNPs for paralog set 2.

2.3.6. Candidate gene amplification, library preparation and sequencing

Locus specific primers were used to amplify each paralog specifically. Primers were designed to span a minimum of 900 bp to a maximum of 1.5 kb of the respective gene (Table S). DNA aliquots of 2 μ l of the prepared 3D pools were used as a PCR template for amplifying the candidate genes (92°C: 3min, [92°C: 30 sec, 58°C to 63°C: 30 sec, 72°C: 65 sec] 36x, 72°C: 5 min). The amplicons from A and C sub genomes were combined separately to form two paralog sets as shown in the blue and green colors in Figure 4. PCR products were purified using the magnetic bead MagSi-NGS^{PREP} Plus kit (Version 1.0|03/09/2015) (Steinbrenner Labor Systeme GmbH, Wiesenbach, Germany). After purification, the quality of samples was checked with the Quant iT dsDNA HS Kit (Thermo Fischer, Germany) prior to library preparation and all samples met the required conditions of $>1\text{ng } \mu\text{l}^{-1}$. The 80 plate, row and column pools of the paralog sets were tagged, respectively, with 80 unique barcodes consisting of ten i7 and eight i5 adaptors forming six super pool libraries. A Nextera XT kit was used for library preparation according to the manufacturer's protocol (Illumina document # 15031942 v01). Sequencing was done on an Illumina HiSeq4000 at CeGaT (Tübingen, Germany) using 2x 100 bp paired-end reads (Figure 4).

2.3.7. Parameters used for the data analysis

Raw data were obtained from CeGaT GmbH (Tübingen, Germany) in a FastQ format. The specifications for FastQ files were read out using the program FASTQC (version 0.10.1) (Cock et al. 2010; Andrews 2014). Illumina CASAVA (1.8.2) was used to de-multiplex the sequenced reads. Adapter sequences were removed from the sequence files using skewer (version 0.1.116) (Jiang et al. 2014). The obtained reads were analyzed with CLC Genomics Workbench 8.1 (Aarhus, Denmark). After de-multiplexing the reads, the 100 bp paired-end reads were imported into CLC Genomic Workbench 8.1 using the NCBI/Sanger or Illumina 1.8 and later tool with insert sizes 50 to 350 bp. The chosen trimming parameters in CLC were: ambiguous limit - two, quality limit - 0.01 and removal of three 5' and 3' terminal nucleotides - yes, discard read pairs with minimum a number of nucleotides - less than 60 bp. The trimmed paired end reads were mapped using Darmor-bzh as a reference sequence (Chalhoub et al. 2014). All the reads were mapped using the following parameters: no global alignment, auto detect paired distances- yes, insertion open cost -5, deletion open cost -5, insertion extension cost and deletion extension cost - 1, similarity fraction - 0.99. Mapped reads were called for single nucleotide variation (SNV, also referred to as SNP) using the basic variant detection tool to identify the amino acid changes. The parameters were as follows: ignore nonspecific matches and broken read pairs, minimum read coverage -10, minimum count -2, minimum frequency (%) -0.1. The obtained data were combined from the respective stacks and sorted according to the reference position using Microsoft excel worksheets. The final 3D SNPs were recovered by using count value from CLC, which is calculated as $(\text{coverage} \times \text{frequency}) / 100$ of the SNP. Coverage per lane was calculated using the following formula: $\text{number of reads} \times \text{read length} / (\text{library ratio} \times \text{pool depth} \times \text{total input DNA})$ (Tsai et al. 2011).

2.3.8. Phytic acid measurements

Plants from an F₂ population segregating for the mutant alleles and the donor variety Express 617 were grown in 11 cm x 11 cm pots in the glasshouse (16h: 8h, light: dark) without P fertilizer. Five plants

from each homozygous F₂ genotype were used as biological replicates (Figure S2). Dry matured seeds (200 mg) were ground into fine powder and phytic acid was extracted in three replicates of 50 mg according to Matthäus et al. (1995) with minor modifications. Phytic acid was measured by HPLC according to Rounds and Nielsen (1993) calibrating with a phytic acid standard (Sigma P-8810).

2.3.9. Availability of sequencing data

All the raw sequence reads from amplicon sequencing are available at NCBI under Bio-project Id: PRJNA578364 with Sequence Read Archive (SRA) accession number 'SAMN13058248 to SAMN13058647' (<http://www.ncbi.nlm.nih.gov/bioproject/578364>).

2.4. Results

2.4.1. Target gene selection

As a first step, we identified putatively functional paralogs of the *A. thaliana* genes encoding the key enzymes of the phytic acid synthesis pathway. In *B. napus*, these enzymes are encoded by 19 subgene families comprising a total of 59 genes (Figure 3, Table S1). To identify the functional gene copies, transcript abundance was measured in developing seeds of Express 617. Upon normalization with *BnActin2*, one (*BnMIK*, *BnIPK2β*), two (*BnMIPS1*, *Bn2-PGK2*, *BnIPK1,1*), or three paralogs (*BnMRP5*) were found to be highly expressed at all developmental stages (Figure 5). For the TbyS analysis, paralogs from the subgene families that had no effect on phytic acid reduction in *A. thaliana* were excluded from the analysis with the exception of *MIPS1* (Kim and Tai 2011; Kim and Tai 2010) (Figure 3), because a *MIPS1* soybean knockout mutant and also a *B. napus* RNAi mutant displayed a low phytic acid phenotype (Kuwano et al. 2009; Georges et al. 2006; Meis et al. 2003). Paralogs were selected that showed a constitutive expression over the whole seed filling period (Figure 5), thereby, we chose the 12 most highly expressed genes for TbyS from six gene families (Table S2).

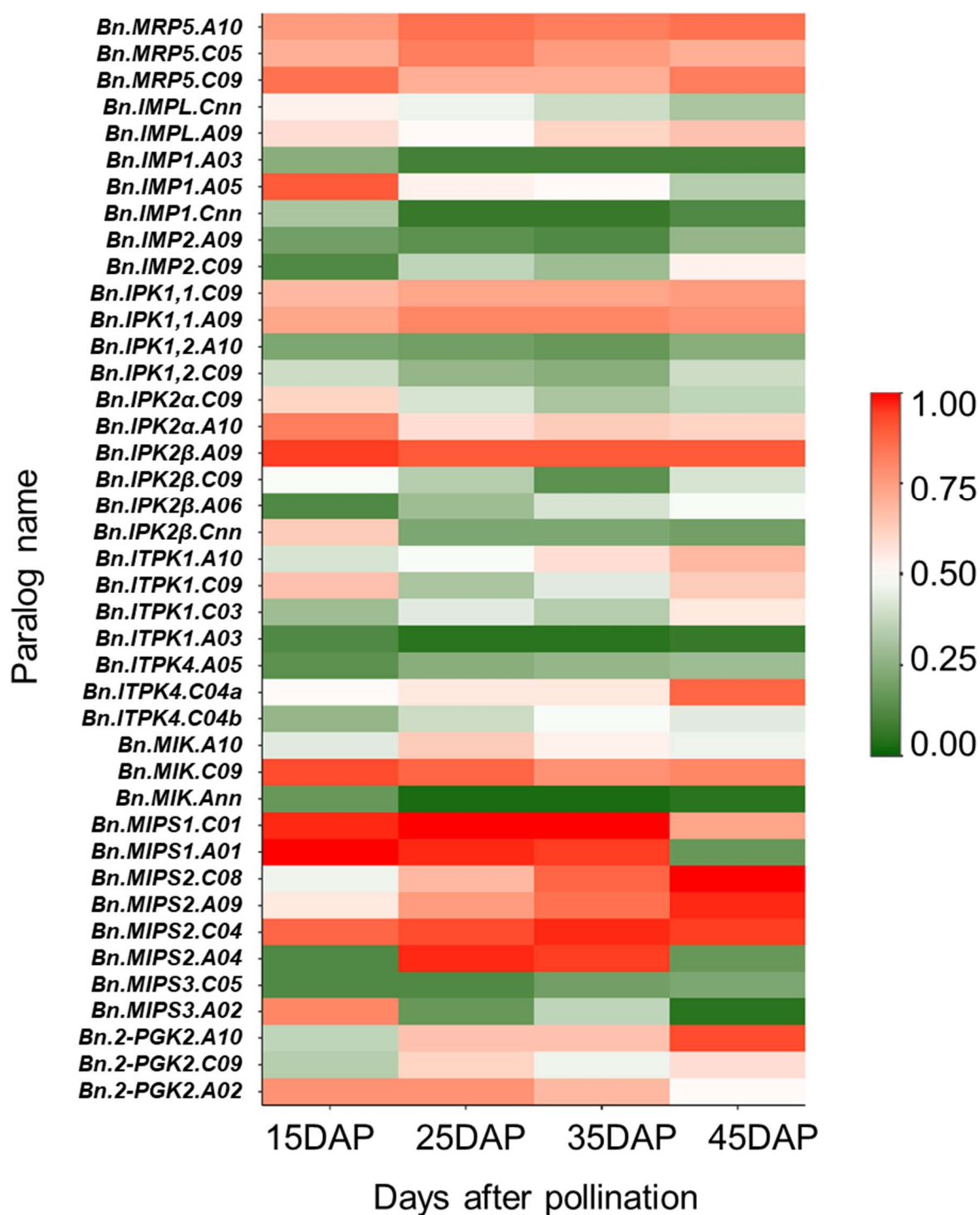


Figure 5: A heatmap showing the relative expression of phytic acid pathway genes in developing seeds of Express 617. The normalized C_t values of the paralogs from 15, 25, 35, and 45 days after pollination (DAP) were used for calculation of the heatmap. For construction of the heatmap, the Heatmaply package (Galili et al. 2017) was used in R 3.6.1. High and low expressed genes are shown in red and green colors, respectively.

2.4.2. Library preparation

Due to the high sequence homology between the paralogs and the limited number of indexing tags, a strategy was chosen to combine pools paralog-wise. The paralogs of the twelve genes were divided into two groups according to their origin from the A and C genomes, with the exception of the *Bn2-PGK2* gene, for which the two highest expressed A genome paralogs have been chosen (Figure 4, Table S2). Larger genes (> 1 kb transcript length) were amplified with two primer pairs to screen 60-90 % of the total exon sequence, whereas for the smaller gene *BnIPK2 β* (transcript length ~860 bp), only one primer pair was used. The amplicons of *BnMIPS*, *BnMIK*, *Bn2-PGK2*, *BnIPK2 β* and *BnIPK1,1* covered 90 % to 100 % of the open reading frame and the amplicons for *BnMRP5* comprised 60 % of the coding region including the transmembrane domains. The targeted regions contained most of the predicted stop codons that might occur by EMS treatment (G/C to A/T transitions).

2.4.3. Illumina sequencing and data analysis

A total number of 22 amplicons derived from 12 genes were sequenced on an Illumina HiSeq 4000 (Figure 4). The raw data in FastQ format were obtained by FASTQC (version 0.10.1) from CeGat, which resulted in an average lane yield of 60 Gb. The Phred score for most of the reads was Q30 with a base call accuracy of 92.67 %. In order to reduce the mapping errors, reads from the imported raw data that had lengths below 100 bp and also ambiguous SNPs (typically N) were trimmed. After trimming an average of 55 million reads were retained for a total of 22 amplicons. Trimmed reads passing the quality parameters were mapped to the Darmor-bzh reference sequence. High stringent mapping parameters were used in CLC Genomics work bench 8.1, which resulted in only few to no gaps in the mapped sequences. The total number of mapped reads for the 12 analyzed genes comprising 22 amplicons ranged from 10 million to 100 million reads (Figure S3a). The average coverage of the aligned reads to the reference in all plate, row and column pools of the 12 genes ranged from 5×10^5 to 6×10^6 (Figure S3b). The total number of reads from the overall pools obtained at each step is shown in Figure S3c. SNPs identified using the CLC Genomics work bench 8.1 were filtered for C to T and G to A transitions for all 240 pools. After retrieval of the SNP data, mutations that appeared in corresponding 3D pools were selected from the processed data according to the reference position. A 3D mutation is characterized by the presence of SNPs in all three dimensions, 2D SNPs present in either of the two dimension pools and any SNPs that could not be clearly assigned to two or three corresponding pools were considered as noise. On combining the data from lanes one to three for paralog library 1 and from the lanes four to six for paralog library 2, 3D SNPs could be identified. We obtained an overall coverage per allele of 143. However, based on 'coverage', we could not select true SNPs, as 'noise' attained similar coverage values. Therefore, this was not an appropriate parameter to determine a detection limit. Instead, we considered 'frequency' as a second parameter which in combination with 'coverage' allowed detecting true SNPs. Hence, a threshold value based on the count parameter, which is calculated as $(\text{coverage} * \text{frequency}) / 100$, was used. Any 3D SNP which had at least two values ≥ 30 , was considered as a putative 3D SNP. SNPs that had a threshold value below 30 and also SNPs that could not be narrowed down to single plant even though their count values were higher than the threshold were considered as noise and were thus eliminated (Figure 6, Figure S4). Furthermore, the SNPs from the modified base from the 3' end of the primers occurred with very high frequency and they were present in most of the pools. We eliminated the SNPs from the primer mismatches along with the ubiquitous SNPs that were regarded as genotype variations to the reference genome (Figure S3d). We only focused on C>T and G>A transitions, as these are the causal mutations from EMS treatments. Putative stop codon mutations were found for all targeted genes except for *Bn.MIK.A10*. The total number of mutations identified in the whole population is given in Table 3. The overall mutation frequencies (F) are calculated according to the formula $1 / [(\text{number of}$

M_1 plants* amplicon length)/ (number of mutations*1000)] and ranged from 1/15 kb to 1/64 kb from all screened amplicons.

Table 3: Three dimensional (3D) mutations identified by Illumina sequencing of 7680 M_2 DNA samples in 12 genes involved in the phytic acid metabolic pathway.

Paralog	Number of Mutations					Total No.	Mutation frequency (F) (1/kb) [§]
	Nonsense	Missense	Silent	Intronic	Splice site		
<i>Bn.MIPS1.A01</i>	3	45	29	38	3	118	1/22
<i>Bn.MIPS1.C01</i>	3	48	31	21	1	104	1/39
<i>Bn.MIK.A10</i>	0	53	39	4	0	96	1/22
<i>Bn.MIK.C09</i>	1	64	42	31	0	138	1/24
<i>Bn.2-PGK2.A02</i>	2	39	21	14	4	80	1/36
<i>Bn.2-PGK2.A10</i>	3	136	63	42	4	248	1/18
<i>Bn.IPK1,1.A09</i>	4	38	18	19	0	79	1/30
<i>Bn.IPK1,1.C09</i>	7	30	27	3	0	67	1/34
<i>Bn.IPK2β.A09</i>	6	62	52	0	0	120	1/15
<i>Bn.IPK2β.C09</i>	2	45	38	0	0	85	1/64
<i>Bn.MRP5.A10</i>	8	109	54	5	3	179	1/20
<i>Bn.MRP5.C05</i>	10	103	48	11	1	173	1/32
Total	49	772	462	188	16	1487	1/27

[§] F is calculated as $1 / [\text{Number of } M_1 \text{ plants* amplicon length}] / (\text{Number of mutations*1000})$

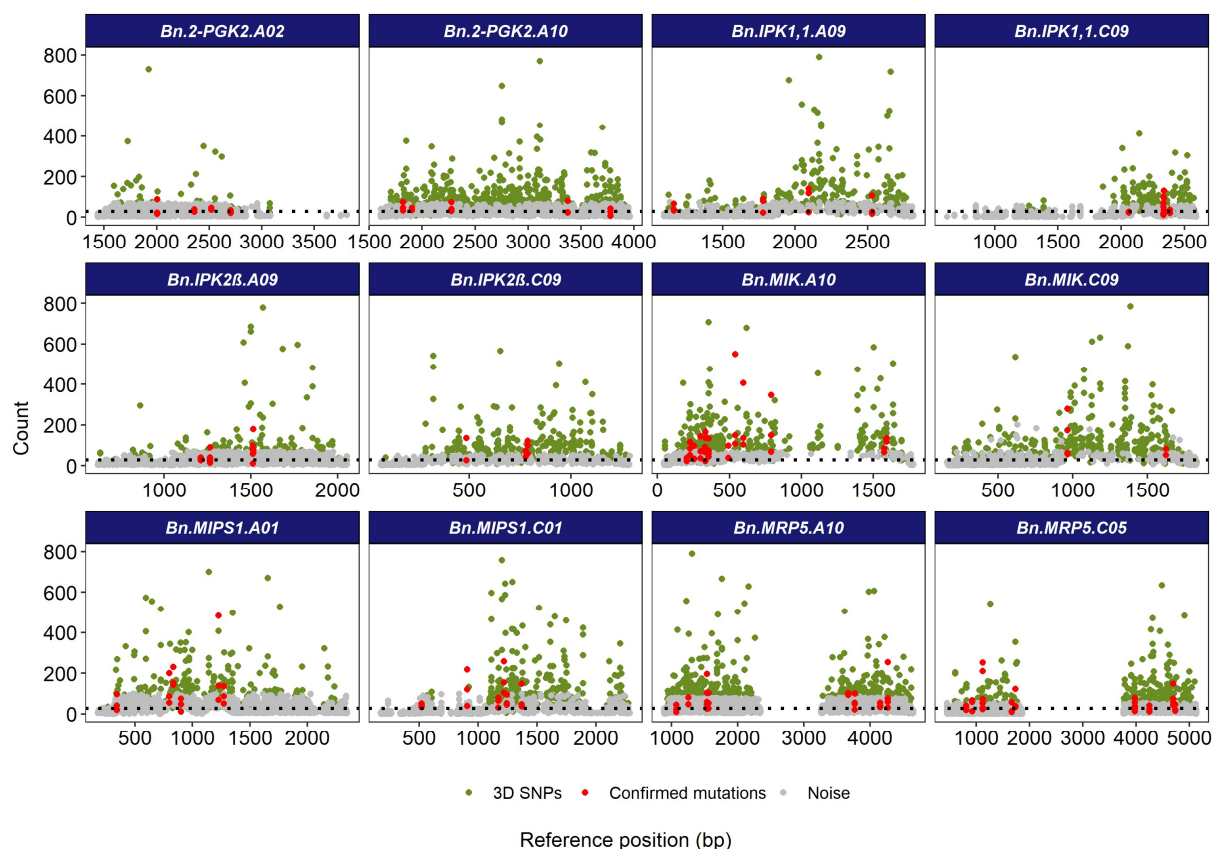


Figure 6: Classification of mutation types from the NGS data set by Sanger sequencing. The total number of SNPs obtained after basic variant detection calling. Each dot represents one SNP at the particular reference position. A true 3D SNP, shown in green is represented by three dots corresponding to the three-dimensional pooling strategy. ‘Noise’ was referred to as SNPs which could not be assigned to single plants or SNPs with a threshold value <30 . These SNPs are displayed as grey dots. Red dots are mutations confirmed by Sanger sequencing. ‘Count’ was calculated as $(\text{coverage} \times \text{frequency}) / 100$ for every SNP. The threshold ($\text{count} = 30$) is shown in black dotted lines.

2.4.4. Confirmation of mutations by Sanger sequencing

A subset of the 3D mutations obtained by NGS was verified by Sanger sequencing in order to determine a possible false positive rate. This comprised 71 stop codon, splice site and putative functional missense mutations, as these were considered for further analysis. The amplification of single M_2 DNA samples by paralog specific primers confirmed 70 out of 71 mutations in all genes targeted in this study. For future phenotypic analyses and combination of single mutations by crossing, out of 70 confirmed M_2 mutations 19 putative loss of function mutations were analyzed and confirmed also in the M_3 generation. All mutant alleles segregated in M_2 according to Mendelian inheritance (Table S3).

2.4.5. Analysis of phytic acid contents in single and double mutants

For measuring the effect of knockout mutations, we focused on the 2-phosphoglyceric acid kinase (*Bn2-PGK2*), which is involved at the early stage of the lipid independent pathway (Figure 3). It was hypothesized that this enzyme in addition to producing 2,3-bisphosphoglycerate (2,3-BPG) also uses *myo*-inositol monophosphate as a substrate resulting in the synthesis of inositol bisphosphate (Raboy 2007; Lu et al. 2016). The enzyme was initially identified in rice and knockout mutants are available from rice and Arabidopsis which showed a phytic acid reduction of 45% and 57%, respectively (Kim et al. 2008; Kim and Tai 2011; Kishor et al. 2019). Paralogs of *Bn2-PGK2* showed a conserved P-loop

kinase domain with two conserved motifs (Walker A and Walker B) (Figure 7a, b) (Snider et al. 2008). The two targeted paralogs of *Bn2-PGK2*, *Bn.2-PGK2.A02* and *Bn.2-PGK2.A10*, contained two and three stop codon mutations and three splice site mutations, respectively (Figure 7a). For ease of understanding, the alleles from *Bn.2-PGK2.A02* and *Bn.2-PGK2.A10* are named with a letter *H* or *I*, respectively, where the suffix ‘e’ describes the allele of the non-mutagenized donor Express 617. For the phenotyping analysis, stop codon mutations (*H₁* and *I₁*) preceding the Walker B motif were chosen (Table 4, Figure 7a).

Since single mutants have not shown significant phenotypic effects in previous studies (Emrani et al. 2015), a segregating F₂ population was created by combining the mutant alleles (*H₁H₁* and *I₁I₁*) from M₃ parents to form a double mutant for *Bn2-PGK2* (Figure S2). *H₁* contained a C1511T and *I₁* a C1689T transition, resulting in premature stop codons at Q269* and Q283*, respectively (Figure 7a). Homozygous double mutants (*H₁H₁I₁I₁*) and single mutants (*H₁H₁I_eI_e* and *H_eH_eI₁I₁*) were selected from the segregating F₂ population and the phytic acid contents were measured in matured F₃ seeds. Mutants and control plants were grown under 16h: 8h, light: dark conditions under controlled glasshouse conditions. Plants were randomized every week and no external phosphorus was added to the plants. During harvest, plant height and thousand kernel weights (TKW) were recorded (Table S4). Both were slightly but not significantly reduced in the segregating F₂ as compared to Express 617. The phytic acid content in single mutants and internal wild type controls (*H_eH_eI_eI_e*) did not vary significantly (4.76 % – 4.73 %) and was in the range of the non-mutated Express 617 control (4.89 %). However, the phytic acid content in the double mutant (3.65 %) was significantly lower (Figure 7c) which constitutes a 25.4 % phytic acid reduction compared to the non-mutagenized EMS donor Express 617. In contrast, single mutants did not show any reduction in phytic acid contents.

Table 4: All *Bn2-PGK2* mutant alleles detected in this study.

Gene	Mutation type	Mutation site (bp) [§]	Allele*	Mutation
<i>Bn.2-PGK2A02</i>	Nonsense	1511	<i>H₁</i>	C to T
<i>Bn.2-PGK2.A02</i>	Splice site	1866	<i>H₂</i>	G to A
<i>Bn.2-PGK2.A02</i>	Nonsense	2212	<i>H₃</i>	C to T
<i>Bn.2-PGK2.A02</i>	Splice site	1139	<i>H₄</i>	G to A
<i>Bn.2-PGK2.A10</i>	Nonsense	1689	<i>I₁</i>	C to T
<i>Bn.2-PGK2.A10</i>	Splice site	1231	<i>I₂</i>	G to A
<i>Bn.2-PGK2.A10</i>	Splice site	1821	<i>I₃</i>	G to A
<i>Bn.2-PGK2.A10</i>	Nonsense	2783	<i>I₄</i>	C to T
<i>Bn.2-PGK2.A10</i>	Splice site	3024	<i>I₅</i>	G to A
<i>Bn.2-PGK2.A10</i>	Nonsense	3190	<i>I₆</i>	C to T
<i>Bn.2-PGK2.A02</i>	-	-	<i>H_e</i>	-
<i>Bn.2-PGK2.A10</i>	-	-	<i>I_e</i>	-

[§] distance from the Start codon

* A single letter code was used to display M₂ genotypes where ‘e’ denotes the allele of non-mutagenized Express 617 donor variety.

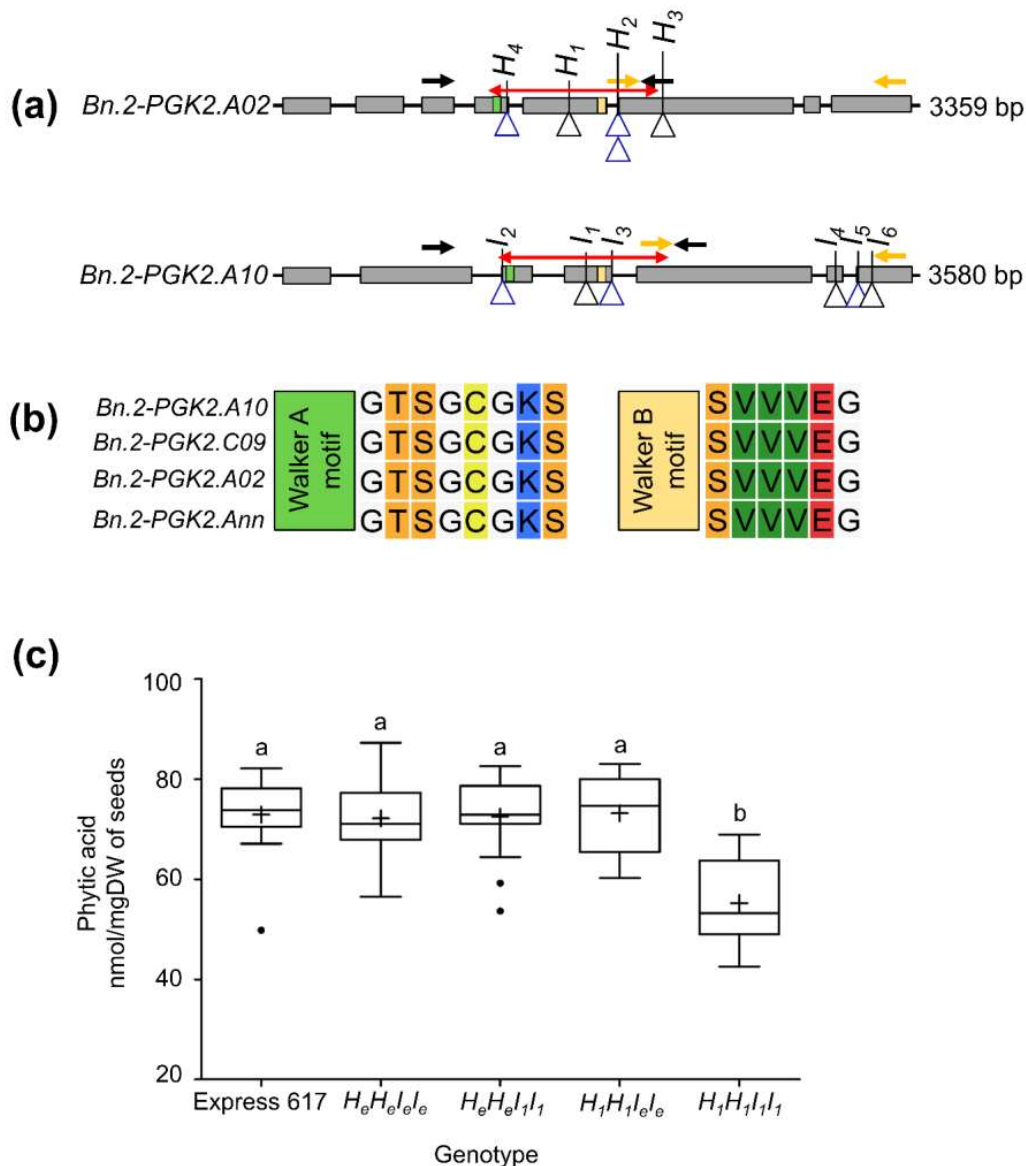


Figure 7: Phenotypic analysis of *bn2-pgk2* mutants. (a): Gene structure showing the positions of the identified mutations. Exons are shown in grey bars. Primer pairs used for amplicon sequencing are shown in black and yellow arrows. The double-headed red arrow indicates the P loop kinase domain and green and yellow boxes show the Walker A and B motifs, respectively. Stop codons are shown as black triangles and splice site mutations as blue triangles. The mutant alleles are indicated with respective numbers (b): Conserved Walker A and Walker B motifs in different *Bn2-PGK2* enzymes (c): Box plot of phytic acid contents in seeds of *bn2-pgk2* mutants. Statistical analysis was performed using Anova and a posthoc test using Tukey's Multiple Comparison Test; $p=0.05$. Same letters indicate no significant differences. The bottom and top boxes represent the upper and lower quartile, respectively, middle line indicates the median, and the whiskers represent the maximum and minimum values. Means of 15 samples are indicated by '+' and dots indicate the outliers. The non-mutated Express 617 alleles are indicated by 'e'.

2.5. Discussion

Alike oilseed rape, many economically important crops are polyploids such as wheat, potato, soybean, cotton, sugarcane, coffee and sunflower. Polyploids are often better adapted to adverse environments, show improved growth vigor and increased stress tolerance. However, due to gene redundancy identification of mutant alleles is more difficult in polyploids because mutations are mostly recessive impeding a direct forward genetics approach (Krasileva et al. 2017). This is the reason why a sequence based mutant detection pipeline is very attractive for polyploid species. In addition, intensive selection

breeding has narrowed genetic diversity. As an example, for low phytic acid content in the seeds, which is a desired trait in rapeseed meal as an animal feed, so far, no low phytic acid rapeseed cultivars are available (Lickfett et al. 1999). Therefore, new strategies to increase genetic variation are needed. While transgenic technologies are not available to breeders in many parts of the world due to legal constraints, mutation breeding became an interesting alternative. Moreover, polyploid *B. napus* tolerates a high mutation load which gave rise to numerous mutants with altered agronomical traits (Harloff et al. 2012; Uauy et al. 2017).

We had established an EMS-TILLING platform for rapeseed which gave rise to many desired knockout mutants for important agronomic traits (Guo et al. 2014; Emrani et al. 2015; Braatz et al. 2018). Although whole genome sequencing would be the gold standard for identification of mutations, at present high coverage sequencing of all 7,680 plants from our EMS population with a high coverage is still not feasible due to the underlying costs. Exome capture approaches would offer an alternative and have been successfully used in wheat and maize (Krasileva et al. 2017; Lu et al. 2018). Unfortunately, for oilseed rape an exome capture kit is not yet available. In contrast, amplicon sequencing offers high coverage for screening of large populations by implementing multidimensional pooling and amplicon stacking. Furthermore, as TILLING experiments mainly aim at the identification of knockout mutations, screening of coding regions is in most cases sufficient. In our lab, the TbyS approach took four months and cost about 50% less than the classical gel-based TILLING.

In previous studies, the mutation frequencies in the Express 617 mutant population ranged from 1/15 kb to 1/54 kb (Guo et al. 2014; Emrani et al. 2015; Braatz et al. 2018). In this study, a mutation frequency from 1/15 kb to 1/64 kb across the targeted genes was observed when only 3D SNPs were considered. In addition to 3D SNPs, we found that 2D SNPs also occur at mutation frequencies between 1/16 kb to 1/120 kb. Upon verification of a subset of 2D SNPs, all the tested 15 2D SNPs encompassing 10 stop codon and 5 splice site mutations were proven to be positive (Table S5). With this, we could show that screening of our population by NGS outperformed our previous studies because we could identify more mutations. Moreover, we clearly demonstrated the advantage of amplicon sequencing to the previously used method based on CEL1 digestion followed by polyacrylamide gel electrophoresis. We reason that the 2D SNPs mainly occur due to either loss of samples during the preparation which is quite often observed in bead based library preparations or bias of *Taq* polymerase during PCR amplification (Marroni et al. 2012; Van Dijk et al. 2014). In addition, the coverage values varied between pools and in some cases two dimensions showed sufficient coverage, whereas the third dimension was less covered, which could have led to loss of the third dimension (Figure S5). Nonetheless, we conclude that amplicon sequencing could recover higher mutation frequencies than the traditional screening procedures, showcasing the efficiency of our EMS population to screen for higher number of genes.

In the following, we will compare our data with those from previously published TbyS studies. One of the problems with polyploid crops is to develop gene specific primers, which is often a backbreaker. In comparison to previous studies in oilseed rape (Gilchrist et al. 2013; Wells et al. 2013), the availability of a published reference genome of the *B. napus* variety Darmor-*bzh* helped to specifically amplify all the underlying gene copies (Figure S1). This was only possible by introducing mismatches close to the 3' end of primers to generate paralog specific amplicons for qPCR and TILLING by NGS. Another problem in data analysis after sequencing is to avoid occurrence of false positives. Most of the studies lack to discuss how to overcome the occurrence of false positive SNPs. In a previous study in allotetraploid *Eragrostis tef* only 60% of true SNPs could be recovered, indicating the complexity of assigning a true SNP in a polyploid genome when using higher pool depths (Zhu et al. 2012). As of now, there is no hard end rule to avoid the false positive rates. Today, only few bespoke bioinformatic pipelines are available for the identification of mutations in polyploids, namely MAPS (Henry et al. 2014) and CAMBA (Missirian et al. 2011; Tsai et al. 2013). A recent study in tomato discussed all the

existing bioinformatics pipelines for reducing the occurrence of false positives (Gupta et al. 2017). The authors of this study claimed the need for establishing robust pipelines for studies involving multidimensional pooling and amplicon stacking. In our study, we used a correlation of coverage and frequency values for the obtained NGS SNPs, which allowed a clear differentiation between noise and 3D SNPs (Figure S4). Using the parameter count, derived from this plot and calculated as $(\text{coverage} \times \text{frequency}) / 100$ for each SNP, we could reduce the occurrence of false positives to less than 5%. The noise levels obtained during sequencing could be the result of errors during the initial cycles of PCR. By our classification of data, we can safely assess mutations *in silico* in the future without ample Sanger sequencing saving additional costs.

The pipeline proposed here can be easily scaled up for higher numbers of genes with the availability of more advanced sequencing machines and at reduced costs. Although CRISPR-Cas mediated mutagenesis is thriving technology for creating mutations in multiple copies of genes and without off-target effects, the availability of this technology is restricted due to legal constraints. In particular, the recent decision of the European court made genome edited plants inaccessible to breeders in Europe. Moreover, the limited transformation ability of many cultivars and unpredictable editing efficiencies at desired target sites might impede a general use of CRISPR-Cas (Wang et al. 2008; LeBlanc et al. 2018; Wang et al. 2018). Therefore, we will further select EMS mutants from our population which is also available to the scientific community.

In this study we could identify novel mutations in genes involved in the phytic acid synthesis pathway. We propose a further reduction of phytic acid contents by pyramiding more mutated paralogs in one genotype, which could be very valuable for further oilseed rape breeding. The double mutants that have been produced in this study could be crossed with elite cultivars to breed low phytic acid varieties. One of the major aims of oilseed rape breeding is to improve the meal quality, which is rich in favorable proteins and amino acid profiles for human and mono-gastric animal nutrition (Campbell et al. 2016; Rodrigues et al. 2017). Hitherto, external phytases are added to the meal to decrease phytic acid contents and to increase the bio-availability of minerals (Al-Asheh and Duvnjak 1995; Campbell et al. 2016). Although, studies have shown the benefits of the usage of external phytases, there are still many drawbacks regarding the activity of phytases in the crude meal to digest phytic acid. These include also the type of organism used as a source for phytases, the used pH and the method of phytase extraction (Dersjant-Li et al. 2015). Therefore, the use of low phytic acid mutants that are identified in this study is a first step to increase the quality of the meal for food and feed production. Moreover, high quality rapeseed meal can also be an alternative for soybean meal for human and animal nutrition in regions where soybean cannot be grown. Finally, the use of low phytic acid varieties will have also an important impact on less water eutrophication, because the P content in the manure will be lower (Raboy 2000; Ockenden et al. 2004).

Since the biochemical function of the 2-PGK2 protein is largely unknown in plant species (Lu et al. 2016; Kishor et al. 2019), the novel *bn2-pgk2* mutants can be used to investigate its biochemical properties in the phytic acid pathway in *B. napus*. Since we have not seen pleiotropic effects on plant growth and thousand kernel weights between the mutants and the controls there are no adverse effects of the induced mutations (Table S4). Albeit the experiments were conducted under controlled conditions, evaluation of the mutant's field trails will be an essential task for the future. Furthermore, the high mutation load of EMS mutants prevents the direct use of these mutants in breeding due to sometimes observed low vigor and poor yielding capacities and therefore we initiated a program for a fast elimination of background mutations by backcrossing with distantly related early flowering spring type parents under speed breeding conditions (Watson et al. 2018).

2.6. Supporting information

Figure S1: Designing paralog specific primers.

Figure S2: Crossing scheme for production of *bn2-pgk2* double mutants.

Figure S3: Overview of reads, coverage across the pools and eliminated reads.

Figure S4: Threshold for selecting the putative three-dimensional (3D) SNPs.

Figure S5: Coverage across the total number of pools.

Table S1: *In silico* analysis of phytic acid pathway genes in *Brassica napus*. L

Table S2: Primers used in this study for TILLING by Sequencing (TbyS).

Table S3: M₃ generation genotyping to select homozygous mutant lines.

Table S4: Evaluation of plant height and thousand-kernel weights (TKW) of *bn2-pgk2* mutants.

Table S5: Total number of two-dimensional (2D) mutations obtained by TILLING by Sequencing (TbyS).

2.7. Acknowledgements

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3. Gene editing of three *BnITPK* genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds

Niharika Sashidhar, Hans-J Harloff, Lizel Potgieter, Christian Jung
(Under review)

3.1. Summary

Commercialization of *Brassica napus*. L (oilseed rape) meal as protein diet is gaining more attention due to its well-balanced amino acid and protein contents. Phytic acid (PA) is a major source of phosphorus in plants but is considered as anti-nutritive for mono-gastric animals including humans due to its adverse effects on essential mineral absorption. The undigested PA causes eutrophication, which potentially threatens aquatic life. PA accounts to 2-5% in mature seeds of oilseed rape and is synthesized by complex pathways involving multiple enzymes. Breeding polyploids for recessive traits is challenging as gene functions are encoded by several paralogs. Gene redundancy often requires to knockout several gene copies to study their underlying effects. Therefore, we adopted CRISPR-Cas mutagenesis to knock out three functional paralogs of *BnITPK*. We obtained low PA mutants with an increase of phosphorus in the canola grade spring cultivar Haydn. These mutants could mark an important milestone in rapeseed breeding with an increase in protein value and no adverse effects on oil contents.

3.2. Key words

Brassica napus, *BnITPK*, CRISPR-Cas, inositol (1, 3, 4) P₃ 5/6 Kinase, inorganic phosphorus, *lpa* mutants, phytic acid, polyploidy, rapeseed meal.

3.3. Introduction

Seeds are major reserves for nutritional elements. Inositol-hexakis phosphate, also known as phytic acid (PA), contributes to 65-90% of total phosphorus in seeds across a wide range of plant species from cereals to oilseeds (Raboy 2000). PA is a negatively charged molecule and chelates essential minerals, thereby leading to the so-called hidden hunger (Perera et al. 2018; Gibson et al. 2018). Furthermore, due to lack of phytases, mono-gastric animals including humans cannot digest PA and the undigested PA is causing eutrophication (Raboy et al. 2000). In plants, PA is synthesized in two different ways, via a lipid dependent and a lipid independent pathway (Raboy 2003). The PA pathway is initiated by oxidative cyclization of glucose 6-phosphate to *myo*-inositol monophosphate via *myo*-inositol phosphate synthase. Subsequently, *myo*-inositol monophosphate is converted to free *myo*-inositol via de-phosphorylation by *myo*-inositol monophosphatase. Concurrently, a reverse reaction occurs by *myo*-inositol kinase, where the free *myo*-inositol is phosphorylated again to *myo*-inositol monophosphate. These reactions are considered to be the housekeeping pathway for various *myo*-inositol dependent pathways and commonly known as Loewus pathway (Loewus and Murthy 2000; Donahue et al. 2010). Furthermore, interaction of *myo*-inositol with phosphatidyl lipids leads to the lipid dependent, whereas subsequent phosphorylation by respective kinases leads to the lipid independent pathway (Figure 8) (Raboy 2009). Although, both pathways contribute to PA biosynthesis, the lipid independent pathway is considered as the predominant pathway for PA accumulation in seeds (Raboy 2000). Finally, the synthesized PA is transported by a multi-drug resistant protein into vacuoles where it is stored as calcium, magnesium and potassium salts referred to as phytins (Figure 8) (Otegui et al. 2002). Upon seed germination, endogenous phytases are activated and PA is hydrolyzed to free inorganic phosphate (P_i) and *myo*-inositol (Yao et al. 2012). The released

P_i and partially phosphorylated inositols are utilized in various physiological processes during plant development including DNA repair, mRNA export, cellular signaling, and biotic and abiotic stress tolerance (Sparvoli and Cominelli 2015; Dieck et al. 2012). In oilseed rape, PA contents vary from 2-5%. This includes quality type ('canola') varieties with reduced erucic acid and glucosinolate contents (Tan et al. 2011; Thompson 1990; Uppström and Svensson 1980; Zhao et al. 2008). So far, approaches to lower the PA content in extracted rapeseed meal focused either on adding phytases to the meal or to remove PA by technical processes (Adem et al. 2014; El-Batal and Abdel Karem 2001). In spite of these strategies to reduce PA, there are still several obstacles for an efficient purification of the meal (Dersjant-Li et al. 2015). In this study we aimed to knockout the key enzyme ITPK (inositol tetrakis-phosphate kinase), which is catalyzing the penultimate step for the synthesis of PA in plants (Raboy 2009). ITPK is a highly conserved enzyme across various species from plants to humans and has been shown to have diverse functions (Jiang et al. 2019). It belongs to the super family of ATP-grasp fold proteins and is involved in photomorphogenesis by interacting via the COP9 signalosome, in stress responses, in signal transduction and in seed coat development (Du et al. 2011; Qin et al. 2005; Sweetman et al. 2007; Tang et al. 2013). In our previous study, we have identified EMS (ethyl methane sulfonate) induced loss-of-function mutations in six genes of the PA biosynthesis pathway (Sashidhar et al. 2019). However, due to gene redundancy in polyploid rapeseed noticeable phenotypic effects could only be observed in plants carrying multiple mutations which required time consuming crossing experiments. Thereby, functional analysis of traits that are encoded by several paralogs need a fast forward approach for simultaneous knockouts. In this study we aimed to knockout multiple copies of the key enzyme ITPK (inositol tetrakis-phosphate kinase), which is catalyzing the synthesis of inositol pentakis phosphate by implementing CRISPR-Cas mediated mutagenesis (Figure 8) (Zhang et al. 2012), to obtain *lpa* mutants with no pleiotropic effects. Therefore, our study offers new perspectives for breeding low PA rapeseed.

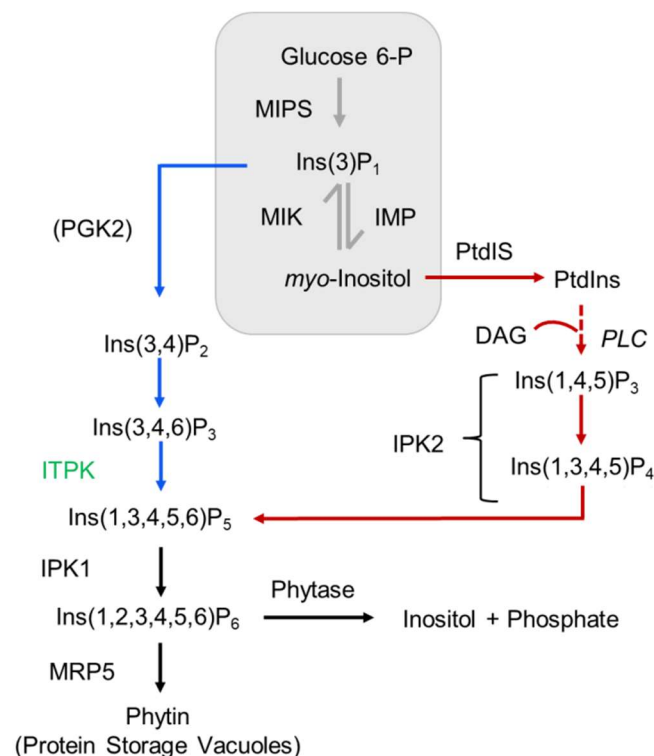


Figure 8: Proposed phytic acid pathway in plants (modified after Raboy et al (2009)). The grey color box indicates the Loewus pathway, which is a housekeeping pathway for various *myo*-inositol dependent pathways. Blue and red arrows indicate the lipid independent pathway and lipid dependent pathway, respectively. The targeted gene (*ITPK*) for CRISPR-Cas9 mutagenesis is highlighted in green. MIPS: *myo*-inositol phosphate synthase, MIPK: *myo*-inositol kinase, IMP: *myo*-inositol mono phosphatase, 2-PGK: 2-phosphoglyceric acid kinase, ITPK: inositol tetrakis-phosphate kinase, IPK2 β :

inositol multi phosphate kinase, IPK: inositol pentakis-phosphate 2-kinase, MRP5: multi drug resistance protein, DAG: Diacyl glycerol, PLC: phospholipase C, PtdIS: phosphatidyl inositol synthase.

3.4.Results

3.4.1. Identification of *ITPK* genes and selection of paralogs for mutagenesis

So far, no oilseed rape orthologs of *Arabidopsis thaliana* *ITPK* genes have been identified. We have used four known *AtITPK* sequences for a BLAST query in the *B. napus* database (<http://www.genoscope.cns.fr/brassicnapus/>) which resulted in 15 paralogs (Table 5). The gene families in *B. napus* were referred to as *BnITPK1* to *BnITPK4* and the homology between members of the same gene family ranged between 89-99%. *BnITPK1* shared approx. 80% similarity with *BnITPK2* and *BnITPK3*, but less than 20% similarity with *BnITPK4* (Table 5). In order to find suitable target genes for a CRISPR-Cas9 knockout experiments, a phylogenetic tree was constructed between *BnITPKs* and *ITPK* orthologs from other plants. The analysis resulted in three distinct clustered α , β and γ groups (Figure 9). Typically, *ITPK* orthologs which had been successfully targeted for mutations in previous studies belonged to the α and γ groups (Sparvoli and Cominelli 2015). We expected that knocking out the orthologs in rapeseed would give comparable results and therefore focused on the α and γ group genes *BnITPK1* and *BnITPK4* (Figure 9). The *BnITPK1* and *BnITPK4* genes could be amplified by PCR in the cv. Haydn, with the exception of *Bn.ITPK4.C04a* (Table S6). However, several primer combinations failed to amplify the putative *Bn.ITPK4.C04a* ortholog from five different rapeseed genotypes (cv. Haydn, cv. Express 617, cv. Mozart, cv. Tapidor and RS 306) which was therefore excluded from further studies. For CRISPR-Cas9 knockout studies, we choose to knockout paralogs of *BnITPK1* and *BnITPK4* gene families in a single plant.

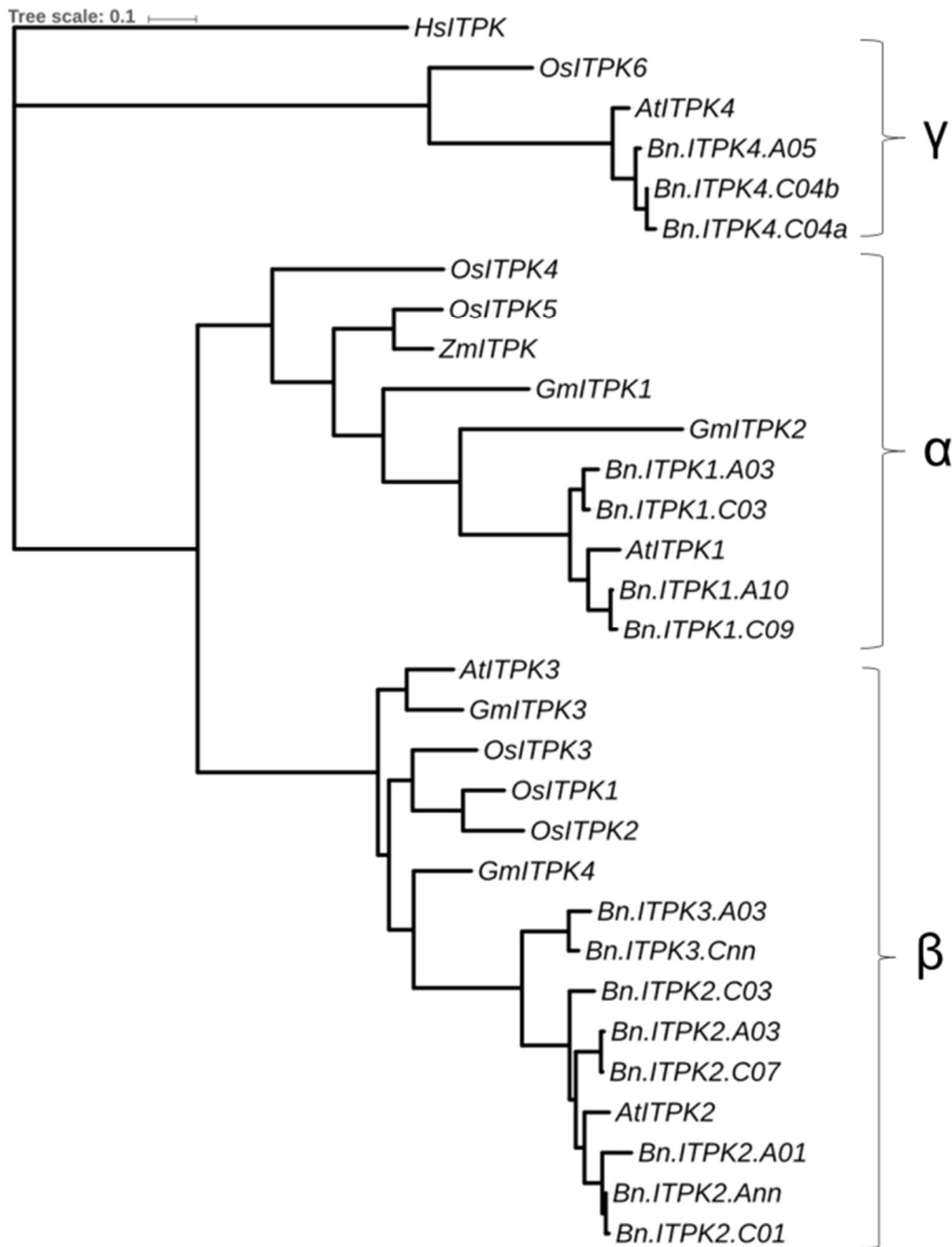


Figure 9: Phylogenetic tree of *ITPK* genes in different plant families. The tree was constructed using the Maximum Likelihood method with Nucleotide Substitution Model as General Time reversible. Bootstrapping using 500 replicates. *At-A. thaliana*, *Bn-B. napus*, *Os-Oryza sativa*, *Gm-Glycine max*, *Zm-Zea mays*, *Hs-Homo sapiens*, *Eh-Entamoeba histolytica*. Accession numbers of rice ITP5/6Ks are as follows: *OsITPK5/6-1* (AK106544), *OsITPK5/6-2* (AK100971), *OsITPK5/6-3* (AK067068), *OsITPK5/6-4* (AK071209), *OsITPK5/6-5* (AK059148) and *OsITPK5/6-6* (AK102571) (Kim and Tai 2014). Arabidopsis and *B. napus* genes are mentioned in the Table 1. *Eh*: AF118848, *Hs*: NP055031, *Gm*: type 1- EU033958, type 2- EU033959, type 3- EU03396h, type 4-EU033961, *Zm*: AY172635 (Stiles et al. 2008). The groups of ITPKs are shown as α , β and γ

Table 5: Features of *BnITPK* genes used in this study.

Arabidopsis gene	<i>B. napus</i> sequence annotation	<i>B. napus</i> paralogs	Genomic sequence length (bp)	Exon/Intron structure		Coding region (bp)	Protein size (aa)	Genome sequence identity with <i>AtITPK</i> (%)	Amino acid sequence identity with <i>AtITPK</i> (%)
				Exons	Introns				
<i>AtITPK1</i>	<i>BnaA10g17710D</i>	<i>Bn.ITPK1.A10</i>	960	1	0	960	320	86.7	89.3
	<i>BnaC09g41080D</i>	<i>Bn.ITPK1.C09</i>	960	1	0	960	320	86.1	89.3
	<i>BnaC03g07940D</i>	<i>Bn.ITPK1.C03</i>	975	1	0	975	325	84.8	88.4
	<i>BnaA03g06170D</i>	<i>Bn.ITPK1.A03</i>	981	1	0	981	327	84.9	88.4
<i>AtITPK2</i>	<i>BnaAnng34680D</i>	<i>Bn.ITPK2.Ann</i>	2454	9	8	1164	388	89.2	84.5
	<i>BnaC01g04480D</i>	<i>Bn.ITPK2.C01</i>	2456	9	8	1155	385	89.3	85.4
	<i>BnaA03g50630D</i>	<i>Bn.ITPK2.A03</i>	2316	9	8	1158	386	88.5	84.9
	<i>BnaC07g44490D</i>	<i>Bn.ITPK2.C07</i>	2391	9	8	1185	395	88.5	82.1
	<i>BnaC03g66400D</i>	<i>Bn.ITPK2.C03</i>	2539	10	9	1041	347	89.7	82.3
	<i>BnaA01g03220D</i>	<i>Bn.ITPK2.A01</i>	969	5	4	654	218	88.8	83.1
<i>AtITPK3</i>	<i>BnaCnng47190D</i>	<i>Bn.ITPK3.Cnn</i>	1964	9	8	1077	359	88.8	80.3
	<i>BnaA03g38640D</i>	<i>Bn.ITPK3.A03</i>	1451	8	7	930	310	82.6	80.5
<i>AtITPK4</i>	<i>BnaA05g03660D</i>	<i>Bn.ITPK4.A05</i>	2634	12	11	1467	489	88	86.9
	<i>BnaC04g03240D</i>	<i>Bn.ITPK4.C04a</i>	2455	12	11	1374	458	88.7	86.7
	<i>BnaC04g03250D</i>	<i>Bn.ITPK4.C04b</i>	2751	12	11	1452	484	89	86.7

3.4.2. CRISPR-Cas9 mutagenesis of *BnITPK* genes

Due to different gene structures between *BnITPK1* and *BnITPK4*, two different target sites, referred to as target site 1 and 2, respectively were chosen (Figure 10). Within each family, the target sequences differed by one nucleotide between the respective members of the family (position 10 upstream of the PAM sequence) (Figure 10). Since the BLAST search against the rapeseed reference genome did not show any off-targets, neither in *BnITPK2* and *BnITPK3* genes nor in any other coding sequence of the rapeseed genome, these target sites were used for subsequent gene editing. Moreover, the genes containing the SNPs were lowly expressed in developing seeds of Express 617 and leaves of a Chinese semi-winter line compared to the other members of the respective family (Shah et al. 2018) (Figure S6), indicating that they might play a minor role in PA biosynthesis in oilseed rape.

Hypocotyl transformation was performed to introduce the CRISPR-Cas9 constructs into the spring cv. Haydn. A total of 321 hypocotyls were co-cultivated with *Agrobacterium tumefaciens* containing the Cas9-sgRNA cassette with both target sites. From the treated hypocotyls, 23 shoots from 10 independent transgenic events (termed T₁ generation) were regenerated to whole plants (itpk_C1 to itpk_C10) on BASTA selection media (Table S7). All T₁ plants were tested positive for the presence of the transgene using primers flanking the *Cas9* gene (Table S6), which is corresponding to a transformation efficiency of 3.1%. PCR amplicons from leaf DNA of each transformant were sequenced to search for mutations within the targeted regions. All T₁ plants showed gene editing in up to four genes. Three mutants were selected for further studies (itpk_C2.1, itpk_C3.5, and itpk_C6.1). Sanger sequencing revealed an overlay of more than two different sequences, suggesting that these were chimeric plants and that some mutations might have occurred somatically. Therefore, amplicons containing the target regions were cloned into plasmids and were subsequently sequenced. We observed presence of three to eight different mutant alleles (single letter code *A*, *B*, *E*, *G*) for each gene along with non-edited alleles for each of the genes targeted thus confirming the chimeric nature of the T₁ plants (Figure 11).

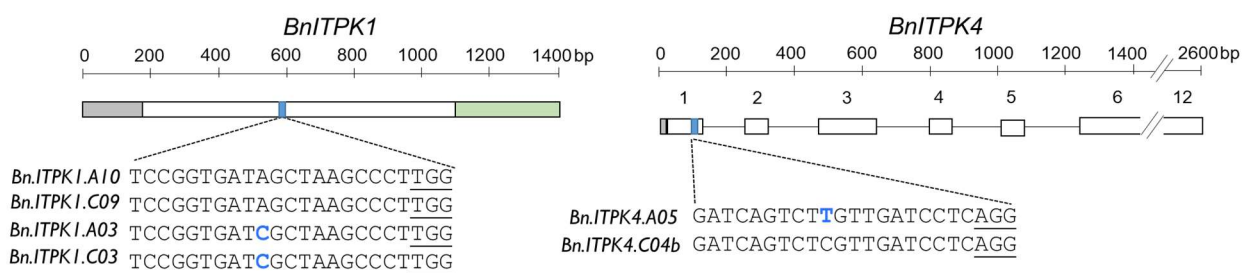


Figure 10: Gene structure and CRISPR-Cas9 target sites in six *BnITPK* genes. Transcribed sequences of *BnITPK1* and *BnITPK4* gene families. Grey and green boxes indicate the 5' UTR and 3' UTR, respectively. Open boxes indicate exons and the black lines indicate the introns. The *Bn.ITPK1.A10* sequence represents target site 1 whereas the *Bn.ITPK4.C04b* sequence represents target site 2 (Express 617). SNPs are indicated in blue letters. The *Streptococcus pyogenes* PAM sequence is underlined.

3.4.3. Generating homozygous double and triple mutants

We expected that chimeric T₁ plants give rise to complex segregation patterns in the T₂ generation and that homozygous mutants can only be selected from later generations. Of each T₂ populations 10 (itpk_C2.1 and itpk_C6.1) and 55 plants (itpk_C3.5), respectively were genotyped with locus specific primers (Figure 11). All T₁ mutant alleles were found in the T₂ generations except in the itpk_C3.5 offspring where not any of the *Bn.ITPK4.A05* mutations (*E*₁, *E*₂, *E*₃ and *E*₄ alleles) was inherited to the

next generation. This was also consistent with the T₁ plasmid sequencing results where the ratio of edited to non-edited alleles was considerably low. Interestingly, new mutant alleles appeared in two T₂ populations (offspring of itpk_C2.1 and itpk_C6.1) which had not been detected in their T₁ parents (Figure 11). Furthermore, complex editing patterns were found even in T₂ plants illustrating that Cas9 activity giving rise to *de novo* mutations in this generation. However, no editing was observed in *Bn.ITPK1.A03* and *Bn.ITPK1.C03* (*C_h* and *D_h*). The presence of a transgene in all analyzed T₂ plants suggested that more than one copy of the transgene was inserted which explains that Cas9 was still active in all T₂ plants analyzed. Since the mutant alleles were not fixed in T₂ plants, three genotypes from each family (seed codes: 180716, 180663 and 180705) were randomly chosen and selfed to get homozygous non-transgenic mutants in the T₃ generation. Firstly, the segregation ratios for the transgene loci were determined. T₃ offspring of itpk_C2.1, itpk_C3.5 and itpk_C6.1 showed a 3:1 segregation (transgenic: non-transgenic) indicating a single insertion event (Table S9). Secondly, homozygous double mutants (*BnITPK1*) and homozygous triple mutants (*BnITPK1* and *BnITPK4*) were identified in the T₃ generation. Heterozygous triple mutations for *BnITPK1* and *BnITPK4* genes were found in the itpk_C3.5 progenies (Figure 11, Table S8). Finally, T₄ populations were produced where non-transgenic homozygous plants for the *ITPK* mutations were selected for phenotypic analysis.

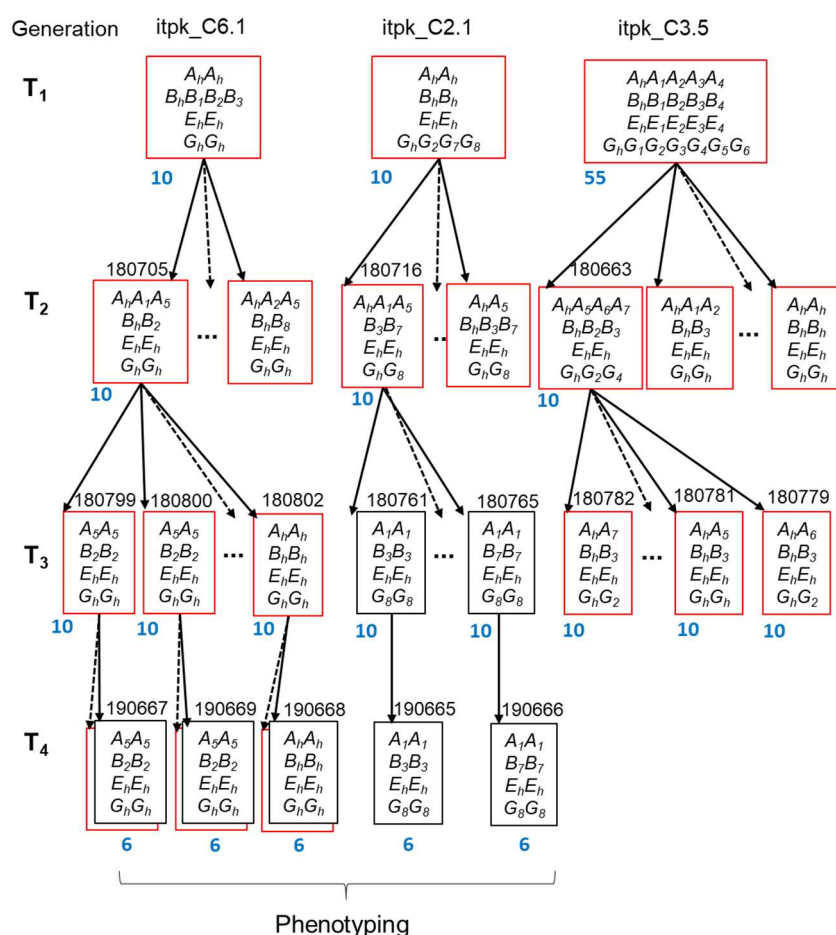


Figure 11: Pedigree of three *BnITPK* mutant generations derived from three different T₁ plants. Target sites and T-DNA insertion sites were genotyped with primer combinations given in Table S6. Red boxes indicate transgenic and black boxes indicate non-transgenic plants, respectively. Homozygous plants used for phenotyping were obtained in the T₄ generation. Seed codes are written on top of each genotype. A non-edited Haydn allele is indicated by a suffix ‘h’ whereas edited alleles are numbered according to Table S8. Dotted arrows indicate progenies that were not further analyzed in this study. The number of plants used for genotyping is given in blue color.

3.4.4. Triple mutants showed reduced PA contents and increased Pi levels

Four T₄ mutant lines homozygous for the mutations were chosen to examine the effect of the edited alleles. Two triple mutants (No. 190665, 190666) with different *Bn.ITPK1.C09* mutant alleles and two double mutants (No. 190667, 190669) with the same mutated *Bn.ITPK1* alleles were analyzed. A T₄ line homozygous for the Haydn alleles (190668) and the donor line Haydn were used as controls (Figure 12 a). Only the two triple mutants displayed significantly reduced (27.2% - 35.3%) seed PA contents while the reduction in double mutants was not significant as compared to the control plants (Figure 12 b). Furthermore, no partially phosphorylated inositol phosphate intermediates could be detected. To find out whether a decreased amount of PA led to an increase of P_i contents in the seeds, P_i contents were measured in all the genotypes with the colorimetric ammonium molybdate test. As expected, the two triple mutants showed higher P_i contents as compared to the control. These results demonstrate that in oilseed rape, a decrease of PA is highly correlated with an increase of free phosphorus (Figure 12 b). However, this increase is not molar equivalent to the reduced PA contents, which might indicate that less phosphorus is taken up by the plants.

We questioned whether the CRISPR-Cas9 mutations could have pleiotropic effects on yield related traits and seed vigor. We observed that mutant plants grew normally and did not show any obvious phenotype. Only minor differences were observed between mutant and control lines. Plant height and thousand kernel weight varied slightly between mutants. The vigor of T₅ seedlings was evaluated five days after germination. Root length and hypocotyl length did not vary significantly as compared to the controls. The seed oil content, a major yield component of *B. napus*, was between 40-50%, which is within the range of modern rapeseed cultivars (Figure 13). Although the experiments were conducted under controlled conditions, the data suggest that altered PA and P_i contents might not have a negative impact on important agronomic traits in rapeseed.

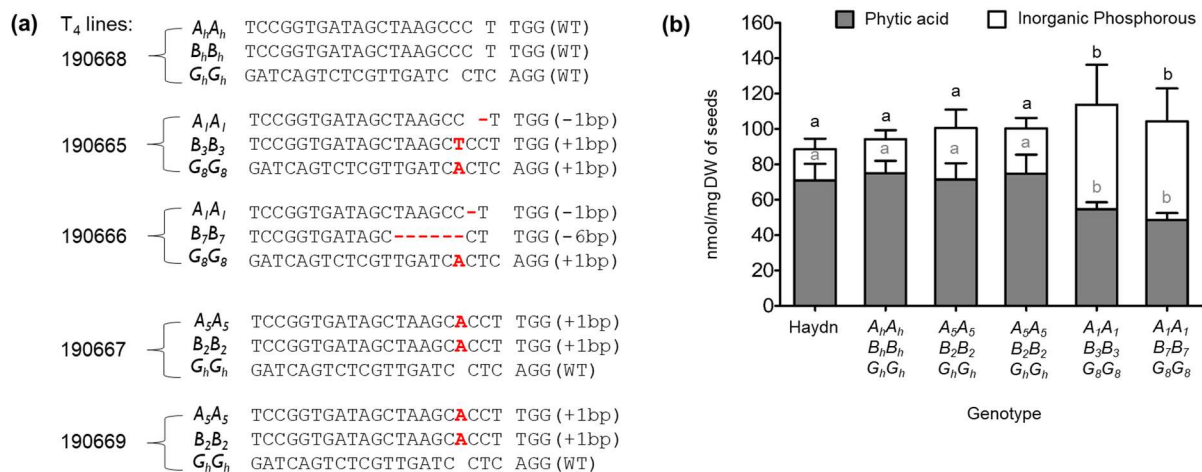


Figure 12: Genotypes and phenotypes of *Bn.ITPK* mutants. (a): A non-edited allele is indicated by a suffix 'h' while edited alleles carry numbers (see Table S8). WT stands for the Haydn allele (wild type). CRISPR-Cas9 mutations are indicated by red letters (insertions) and by '-' (deletions). (b): Phytic acid and free inorganic P were measured in homozygous T₅ seeds. ANOVA and posthoc test using Tukey's Multiple Comparison Test; $p=0.05$ were performed for statistical significance. Similar letters indicate no significant differences.

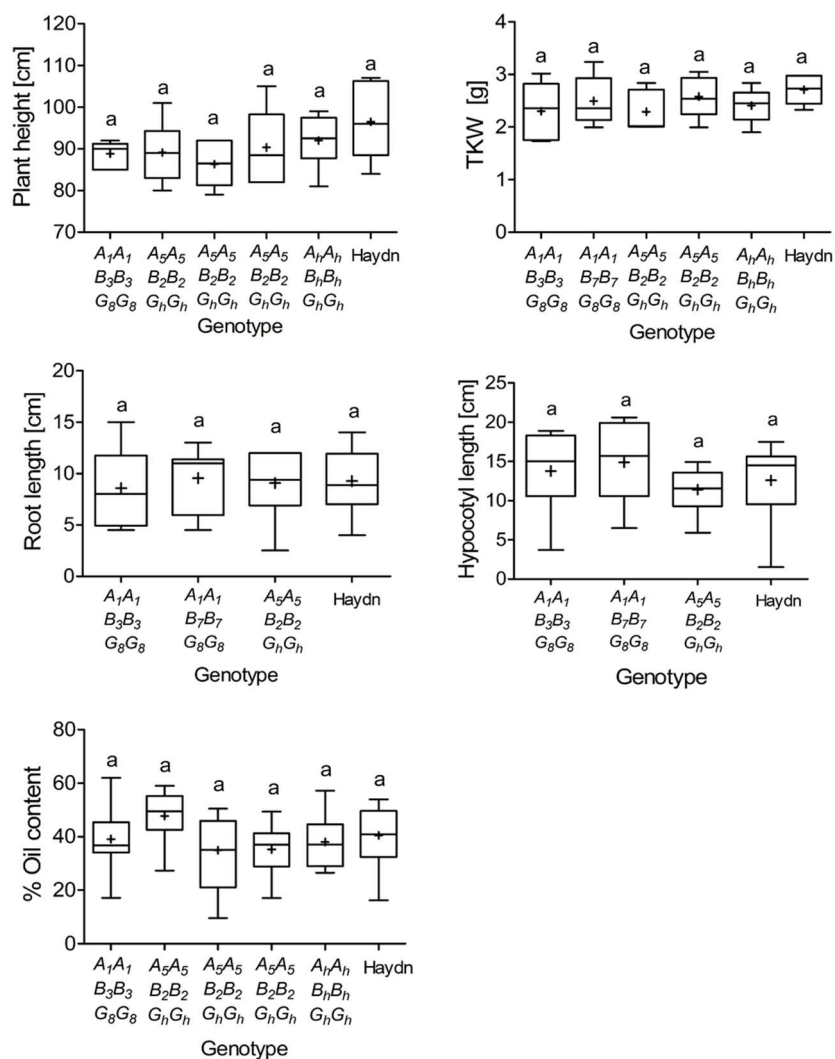


Figure 13: Yield related traits of *BnITPK* double and triple mutants. Plants were grown in the greenhouse under long day conditions (16 light/ 8h dark) at 22°C and T₅ seeds were analyzed. Line 180800 is a mutant offspring carrying the Haydn alleles. Root and hypocotyl length were measured five days after germination. The oil content was measured using the n-hexane method. Statistical significance was calculated using ANOVA followed by a post hoc test (Tuckey) in R. 3.6.1. Same letters indicate no significant effect. + indicates the mean of the samples.

3.5.Discussion

This study describes low phytic acid mutants in oilseed rape by targeting genes from the PA biosynthesis pathway. We selected the *BnITPK* gene family as a suitable target for knockout analysis by CRISPR-Cas in the canola grade cv. Haydn. We found a reduction of about 35% of PA with a simultaneous increase of Pi by knocking out three essential *BnITPK* genes. Interestingly, a significant reduction in PA content was only observed in triple mutants. This clearly demonstrates that knockout of multiple paralogs is necessary to observe a noticeable phenotype which can only be achieved by CRISPR-Cas mutagenesis. Our data are in line with previous studies in wheat and potato where only multiple knockouts of different paralogs resulted in a desired phenotype (Wang et al. 2014; Zhang et al. 2019; Andersson et al. 2017). In rapeseed, the situation is even more complicated because during evolution the genome underwent several whole genome duplication and triplication events resulting in an average copy number of two to eight for each Arabidopsis ortholog (Chalhoub et al. 2014). Another important aspect of our study adds to our understanding of CRISPR-Cas mutations during plant development and between generations. We observed mutations in T₂ generation that had not been

found in their T₁ parents. One reason could be that only a small sector of the T₁ plant carried the mutation, which was not detected by sequencing. However, we favor another explanation that de novo mutations appeared in the T₂ generation. This could be due to the low activity of the Cas9 gene which is influenced by the position of the T-DNA in the genome and by multiple T-DNA insertions which can result in low transcriptional activities or even gene silencing (De Buck et al. 2009; Tang et al. 2006). In conclusion, future studies should carefully examine also later generations even if T₁ plants were lacking mutations.

Our study adds to our understanding of PA biosynthesis and storage in rapeseed. We demonstrate that ITPKs play an important role in PA metabolism and thus are a suitable target for reducing PA amounts. The *ITPK* gene was first identified in maize *lpa2* mutants (*zmitpk*) which showed a 30% reduction in PA without any effect on plant performance. Subsequently, knockout mutants with reduced PA contents in seeds have also been described in rice, Arabidopsis and maize (Shi et al. 2003; Raboy 2009). A study in rice identified two knockout mutants of *OsITPK6* showing 46% reduction of PA seed content caused by a missense mutation and 68% caused by a splice site mutation without any adverse effects on seedling performance (Kim and Tai 2011). Another study in rice showed contradictory result. While the PA content was reduced by 32% due to the loss of two amino acids in the *OsITPK6* protein, negative effects on seed set, seed weight and germination were reported (Jiang et al. 2019). Mutants in Arabidopsis *atitpk1* and *atipk4* showed a 46% and 51% reduction, respectively with no pleiotropic effects on seed germination and seedling performances (Kim and Tai 2011). We also did not observe any negative effects on germination rate and seedling vigor which suggests that PA reduction in our mutants is below a critical threshold. We observed that the triple mutant with a 6 bp deletion (line No. 190666) had the lowest PA content. According to protein modelling, this mutation results in the loss of two amino acids within an ATP-binding site (see Figure S7, Figure S8) which has also been proposed for a *StITPK1* ortholog in potato (Caddick et al. 2008). Although the contribution of each gene to the synthesis of PA in *B. napus* is unknown, we reason that *BnITPK* genes act in an additive manner and that an even higher reduction could be achieved by a complete knockout of all *BnITPK1* and *BnITPK4* genes. It is important to know what happens to inorganic seed Pi if it is not bound in PA. Due to the loss of PA, the triple mutants showed a threefold increase of Pi. Similar results were obtained with a maize *lpa2* mutant where the Pi content was increased by three fold (Shi et al. 2003). The lower increase of Pi was also shown previously as a general pattern of *itpk* mutants in rice and Arabidopsis with decreased contents of PA accompanied by a minor increase of Pi and lower inositol phosphates (Sparvoli and Cominelli 2015). It is noteworthy, that in our study the increase in seed Pi was also not a molar equivalent to the reduced PA contents, which might indicate that less phosphorus is needed by the mutants.

Lowering the PA amounts in seeds is an important milestone for enhancing rapeseed meal quality for human and animal diets. Moreover, the mutants offer new perspectives for oilseed rape breeding. 1) Reduced PA content will be beneficial to combat the mineral deficiencies of the meal. 2) Recovery of rapeseed protein will be more efficient due to lower the amount of tightly bound PA. 3) Protein contents will be improved with no effect on oil contents and thus overruling their negative correlation (Jasinski et al. 2018). 4) Increase of Pi contents might pave ways to reduced dependency on phosphate fertilizers, which is a non-renewable resource (Cordell et al. 2009). 5) Increased bioavailability of Pi attenuates eutrophication of waters (Conley et al. 2009). *B. napus* is primarily an oil crop but can also be used as a nutritional source due to its rich protein and well-balanced amino acid contents for human and animal diets (Campbell et al. 2016; Wanasundara et al. 2016). There is increasing effort to improve the nutritional value of *B. napus* meal in order to overcome the dependence on the expensive soybean imports in regions where soybean cannot be grown (Jasinski et al. 2018). So far, rapeseed meal is used for aquatic and poultry industries as a feed, but it can also be used as a valuable protein source for humans and non-ruminants (Gacek et al. 2018). However, the presence of various anti-

nutritive compounds like phytic acid, sinapine, fiber and glucosinolates impedes its commercialization as a major protein source. Whilst rapeseed cultivars and lines exist which are low in glucosinolates, sinapic acid and erucic acid, no reduced PA lines are available. Furthermore, the protein extraction from seeds is impaired by the presence of tightly bound phytins and tannins (Wanasundara et al. 2016), which reduces the digestion by enzymes like trypsin and pepsin but also inhibits α -amylases at physiological pH in monogastric animals (Reddy et al. 1996). Therefore, the established mutants pave the way to breed rapeseed with improved meal quality and reduced phosphorus demand.

3.6. Experimental procedures

3.6.1. Plant material and growth conditions

The German spring cultivar Haydn (double low '00' or 'canola' quality) was used for transformation experiments. Greenhouse experiments were performed under long day conditions (16h light and 8h dark) at 22°C. Plants were grown in 11x 11cm pots and they were not fertilized to avoid phosphorus contamination. Upon flowering, the inflorescences were bagged, and dry mature seeds were harvested. For phenotyping experiments, five biological replicates for each genotype were grown. Plants were randomized every week and plant height (length between base of the stem to the tip of the inflorescence) and thousand kernel weight (TKW) were measured at the time of harvesting. Seed oil content was measured using the n-hexane method. To measure the seedling's vigor, 20 seeds of each genotype were sown on ½ Murashige-Skoog (MS) medium and germinated in the dark under long day conditions. Five days after germination the root and hypocotyl lengths were measured.

3.6.2. Phylogenetic analysis

Published *ITPK* sequences were retrieved from respective crop's databases. Sequences from rice were obtained from <http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search>, *Arabidopsis* from TAIR (https://www.arabidopsis.org/servlets/Search?action=new_search&type=gene), soybean, maize and human sequences were retrieved from NCBI (<https://www.ncbi.nlm.nih.gov/>). The amino acid sequences of the ITPK proteins were aligned using MAFFT v7.313 with the G-INS-i strategy (Katoh and Standley 2013). The alignment was manually curated. The substitution model for the maximum likelihood analysis of the curated alignment was determined by ProtTest v3.4.2 (Abascal et al. 2005). The maximum likelihood phylogenetic analysis was conducted with MEGA X (Kumar et al. 2018). A JTT model was applied, and 500 bootstrap replicates were performed. The resulting phylogenetic tree was visualized with iTOL (Letunic and Bork 2007; Letunic and Bork 2019).

3.6.3. Target site design and plasmid vector construction

The vectors pChimera and pCas9_TPC were obtained from Prof. Holger Puchta (Karlsruhe institute of technology, Germany). pChimera contains the sgRNA sequence driven by an *Arabidopsis thaliana* U6-26 promoter and pCas9_TPC vector has the Cas9 gene from *Streptococcus pyogenes* driven by an Ubi4-2 promoter from parsley. These vectors have ampicillin and spectinomycin resistance genes respectively, as selective markers for bacteria and BASTA resistance to select transgenic shoots (Fauser et al. 2014).

The *BnITPK* CRISPR-Cas9 cassette was constructed according to published protocols (Fauser et al. 2014) with minor modifications. sgRNAs targets were cloned into the pCas9-TPC vector by using *AvrII* and *BcuI* restriction enzymes, as these are isocaudomeres. The final sgRNA-target vector was transformed into the *Agrobacterium tumefaciens* strain GV3101 pMP90RK, which was then used for rapeseed hypocotyl transformation.

We used four *Arabidopsis* sequences encoding *ITPK* genes to BLAST against the rapeseed reference genome (<http://www.genoscope.cns.fr/brassicnapus/>) (Kim and Tai 2011). Retrieved sequences were annotated according to the Genoscope browser using the CLC Main Work Bench 7.9.1 (CLC, Aarhus, Denmark) software. For the target design, all paralogs of each *ITPK* gene family were aligned and regions of 20bp length and a GC content >40% were chosen which were next to a PAM sequence (NGG). Conserved regions with a maximum of one SNP upstream of 10th position of the PAM site were considered as suitable targets because they can be tolerated by Cas9 (Braatz et al. 2017). All target sites chosen were blasted against the *B. napus* genome database using CLC Main Work Bench 7.0.3 for verification of any off targets. Sequences lacking BLAST hits were selected for further analysis. Oligonucleotides were ordered from Eurofins Genomics Company (Ebersberg, Germany) and purified by a high purity salt free (HPSF) method with a purity of >70 %. (<http://www.eurofins.de/de-de.aspx>).

3.6.4. Rapeseed hypocotyl transformation

We used a protocol kindly provided by Dr. José Orsini (Saaten-Union Biotec GmbH, Germany) with minor adaptations from a published protocol (Zarhloul et al. 1999). Seeds from cultivar Haydn were surface sterilized with 70% EtOH for two minutes followed by 3% sodium hypochlorite and 0.01% Tween 20 for 10 min. Seeds were washed thoroughly with autoclaved water and sown on MS medium. Five days old hypocotyls were used for transformation. The hypocotyls were cut into 1cm explants and incubated for 45 to 60 min with an *A. tumefaciens* overnight culture adjusted to OD₆₀₀ 1.2. The hypocotyls were co-cultivated for two days and transferred to shoot-inducing medium containing 500 mg L⁻¹ ticarcillin disodium / clavulanate potassium (Duchefa-T0190.0002). After 4 weeks on shoot inducing medium, the regenerated shoots were transferred to shoot inducing medium containing 5 mg L⁻¹ BASTA (phosphinotricin) (Duchefa: P0159.0250). Surviving shoots were transferred to root inducing medium for regeneration of complete plants.

3.6.5. Verification of the mutations by molecular marker analysis

Paralog specific primers were designed using the Darmor-*bzh* reference sequence to amplify the individual copies according to the protocol (Table S6) (Liu et al. 2012). DNA was isolated from the leaf samples using the CTAB method. Sanger sequencing was performed from the paralog specific amplicons to verify the putative editing events. Furthermore, PCR amplicons from chimeric T₁ plants were cloned into the pGEM-T vector (Promega, Germany) and plasmids were Sanger sequenced. For verification of the underlying alleles in the chimeric plants in the T₂ generation we used an online software tool called Tracking of InDels by Decomposition (TIDE) (<https://tide.deskgen.com/>) (Brinkman et al. 2014). Raw Sanger sequencing reads of individual paralogs (.abi files) were used as query, which were then compared to the provided reference sequences (non-edited sequences). The analysis resulted in a plot for each sequence of a gene showing the probable nucleotide changes caused by the NHEJ repair mechanism.

3.6.6. Measuring seed phytic acid and inorganic phosphorus contents

Phytic acid and inorganic phosphorus (Pi) were extracted following the procedure of (Matthäus et al. 1995) with slight modifications. Dry matured seeds of 200 mg were ground into fine powder and three technical replicates of ~50 mg were made from each sample. Defatting was done by extraction with 1 ml n-hexane overnight at room temperature on an overhead shaker. After centrifugation (10 min at 20,800 g), the defatted seed cake was extracted with 1 ml of 0.5 M HCl by vigorous shaking on a Bioer mixing block (MB-102) at 30°C for 4h. After centrifugation at 20,800 g, the supernatant was freeze-dried, re-dissolved in 1 ml double distilled water. A 0.5 ml aliquot was applied on a 2 ml

column of Dowex 1x2 (Acros Organics, A0346424), washed with 2x10 ml water and eluted with 25 ml 2 M HCl. The eluates were freeze-dried and re-dissolved in 1 ml double distilled water. Phosphoinositols (IP1 to IP6) were analyzed by HPLC following the procedure published by (Rounds and Nielsen 1993) with minor modifications. Fifty microliter samples were injected onto a PL-SAX 1000 HPLC column (50 x 4.6 mm, 5 μ , Agilent), and IP1 to IP6 were eluted at 30°C with a flow rate of 1 ml min⁻¹. A 25-min linear gradient from 0.01 M 1-methylpiperazine (pH 4.0) to 0.5 M NaNO₃ dissolved in 0.01 M 1-methylpiperazine (pH 4.0) with a further elution for 2 min, 1 min returning to starting conditions and 10 min equilibration was used. Analytes were detected at 500 nm using a Pinnacle PCX device (LCTech, Germany) with a post-column reagent consisting of 0.015% (w/v) FeCl₃ x 6H₂O and 0.15% (w/v) sulfosalicylic acid. A calibration curve was plotted using 10-50 μ g of PA sodium salt hydrate (Sigma: P8810). For determining Pi contents, 50 μ l of the purified column extracts were mixed with 500 μ l of coloring reagent (10% w/v of ascorbic acid (Roth- Art.Nr.3666.1) and 5% w/v of ammonium molybdate (Roth, Art. No.3525.2), adjusted to 1.5 ml with double distilled water and incubated at 40°C for one hour. The samples were measured against a reagent blank in a spectrophotometer at 655 nm. A calibration curve 0 to 100 nmol Pi was used.

3.6.7. Statistical Analysis

For greenhouse experiment, five plants of each line were grown (biological replicates). Of each plant, three technical replicates were taken for PA, inorganic P, and oil measurements. An ANOVA was performed using R 3.6.0 and the MulticomView package and post hoc test was done by using Tukey's Multiple Comparison Test; $p=0.05$.

3.7. Acknowledgements

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3.8. Supporting information

Figure S6: Expression analysis of *BnITPK* paralogs in seeds and leaves.

Figure S7: Protein alignment of ITPK showing the inositol, ATP and Mg²⁺ binding sites.

Figure S8: Predicted protein structure of *Bn.ITPK1.C09* in Haydn and in the mutant line 190666.

Table S6: Primer sequences used in this study.

Table S7: Editing status in regenerated T₁ plants

Table S8: Gene acronyms for four *BnITPK* genes used in this study.

Table S9: Segregation analysis in T₃ generations.

3.9. References

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4. Knockout of *MULTI-DRUG RESISTANT PROTEIN 5* genes lead to low phytic acid contents in *Brassica napus* L.

4.1. Abstract

Understanding phosphate uptake and storage is interesting in optimizing the plant's performances to the phosphorus fluctuations. Phytic acid (PA) is the major source of inorganic phosphorus (Pi) storage in plants. Genetic analyses of PA pathway transporter genes (*BnMRP5*) and their functional characterization might provide clues in better utilizing the available phosphate resources. Furthermore, the failure to assimilate PA by monogastric animals results in an excess accumulation in soils, which ultimately causes eutrophication of waters. As a first step towards breeding low PA mutants in *Brassica napus* L. (oilseed rape), I identified knockout mutants in PA biosynthesis and transporter genes (as described in Chapter 2). The obtained M₃ single mutants of *Bn.MRP5.A10* and *Bn.MRP5.C09* were combined by crossing to produce double mutants. Simultaneously, crosses were performed with EMS donor plants Express 617 to reduce background mutation load. Double mutants identified from the F₂ progeny of M₃ crosses and once backcrossed plants showed 15% reduction in PA contents with no significant differences in Pi. In this chapter, I will discuss in detail the functional characterization of *BnMRP5* single and double mutants and the benefits for breeding with *bnmrp5* mutants in respect to low PA, yield and stress tolerances.

4.2. Introduction

Phosphorus is an essential macronutrient required for plant development. In plants, numerous transporters such as PHT, SPX-MFS and phosphate antiporters facilitate the uptake of phosphorus from the soil and its mobilization across various tissues (Kopriva and Chu 2018). The up taken phosphorus is utilized in various metabolic processes and is finally stored in seed vacuoles in the form of phytic acid (PA; also referred as inositol hexakis phosphate), which is readily available for seedling development. The stored PA in vacuoles is referred to as phytin, which is a complex salt of divalent ions. Dry mature seeds contain PA up to 80% of total phosphorus across various plant species (Raboy et al. 2000). PA storage in seeds is correlated to the amount of phosphorus available in soil. Therefore, low PA mutants are desirable for a reduced application of external phosphorus in the form of fertilizers. Furthermore, they could help to decrease the dependency on non-renewable rock phosphates (Lott et al. 2007). In this regard, many PA biosynthetic genes were knocked out in plants to obtain low PA mutants with simultaneous increase of inorganic phosphorus (Pi) (Sparvoli and Cominelli 2015). In some cases, the knockout of biosynthetic genes involved in PA has proven to have adverse effects on plant performances such as poor seed set, delayed germination, low yield (Shi et al. 2007). Therefore, targeting the transporters that are accumulating PA in seeds gained more attention.

A multi- drug resistant protein (MRP5), acting as a tonoplast transporter was shown to have high affinity towards PA (Nagy et al. 2009). MRP5 is a member of the ATP binding cassette (ATP) super family and belongs to the ABCC subfamily (Kang et al. 2011). The protein has two transmembrane domains and two nucleotide-binding domains encasing the walker A, B and C motifs (Verrier et al. 2008) and it has been shown that the transport of PA is strictly dependent on ATP (Nagy et al. 2009). However, in rice knockout of SULTR-like phosphorus distribution transporter (SPDT) also leads to 25-32% reduced PA contents in seeds (Yamaji et al. 2016). This indicates that the loading of phosphorus in seeds is not only limited to MRP5 protein but also to other transporters. Furthermore, the accumulation of PA in the vacuoles depends on the amount of Pi availability, as it is highly important to maintain the ratio of vacuolar to cytoplasmic Pi for overall phosphorus homeostasis (Pratt et al. 2009).

Brassica napus. L (oilseed rape) is the third major oil crop in the world and its seed meal is rich in proteins and amino acid contents (Gacek et al. 2018). However, the presence of anti-nutritive compounds in *B. napus* impedes its use as a valuable protein source for human and animal feed. The usage of the meal is limited by the presence of compounds such as glucosinolates, tannins, fiber and high PA contents. PA is a strong chelator of positive ions like magnesium, potassium, zinc and iron, thereby reducing their bioavailability in monogastric animals. Several knockout and knockdown mutants of PA transporter genes were identified in cereal crops such as maize, rice, wheat and barley with reduced PA contents from 32% to 90% in seeds and a simultaneous increase of Pi (Shi et al. 2007; Panzeri et al. 2011; Dorsch et al. 2003; Bhati et al. 2016; Xu et al. 2009). In addition, MRP mutants were also identified from common bean and soybean, which also showed a reduction of up to 90% (Wilcox et al. 2000; Champion et al. 2009; Shi et al. 2007).

In this chapter along with obtained EMS mutants by a TILLING by Sequencing (TbyS) approach (described in Chapter 2), I further aimed to select mutants by CRISPR-Cas9. Since, multiple knockouts are necessary to observe a noticeable phenotype in polyploids, I reasoned that CRISPR-Cas9 mutants might have higher reductions in PA contents than EMS double mutants. I will discuss the generation of EMS double mutants and their phenotypic effects. Since, to my surprise, I could not obtain CRISPR-Cas9 mutants I will also discuss possible reasons for the failed experiments in the following paragraphs.

4.3. Materials and Methods

4.3.1. Plant Material and growth conditions

For accumulation analysis of PA and Pi in developing stages of seed, five plants from Express 617 were used for each time point. During flowering, young buds were emasculated and were hand pollinated with Express 617 pollen and the day of pollination is recorded. For PA and Pi extractions developing seeds at 15, 25, 35, 45 and 55 days after pollination (DAP) were collected and frozen in liquid nitrogen. Samples were kept at -80°C until extraction.

M₃ seeds of the identified mutants were obtained from the breeding company Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Hohenlieth. Homozygous M₃ plants of *Bn.MRP5.A10* (M₃ seed code-170482) were crossed with *Bn.MRP5.C05* (M₃ seed code-170481) to produce F₁ plants with mutant alleles for both genes. Simultaneously, M₃ homozygous plants from each of the families were further crossed with EMS donor Express 617 to obtain F₁ plants with reduced background mutation load. All obtained F₁ plants were selfed to obtain F₂ seeds (Figure 14).

Unless mentioned, all the plants for the experiments were grown in 11cm x 11 cm pots under 16h of light and 8h of dark at 22°C in greenhouse. For plant growth, no external phosphorus was added to the soil. Three week old plants were vernalized for eight weeks at 4°C (16h light and 8h dark) and after eight weeks plants were transferred back to 22°C (16h light and 8h dark) until harvest. For phenotyping analysis, Express 617 controls were grown along with the mutants for every experiment.

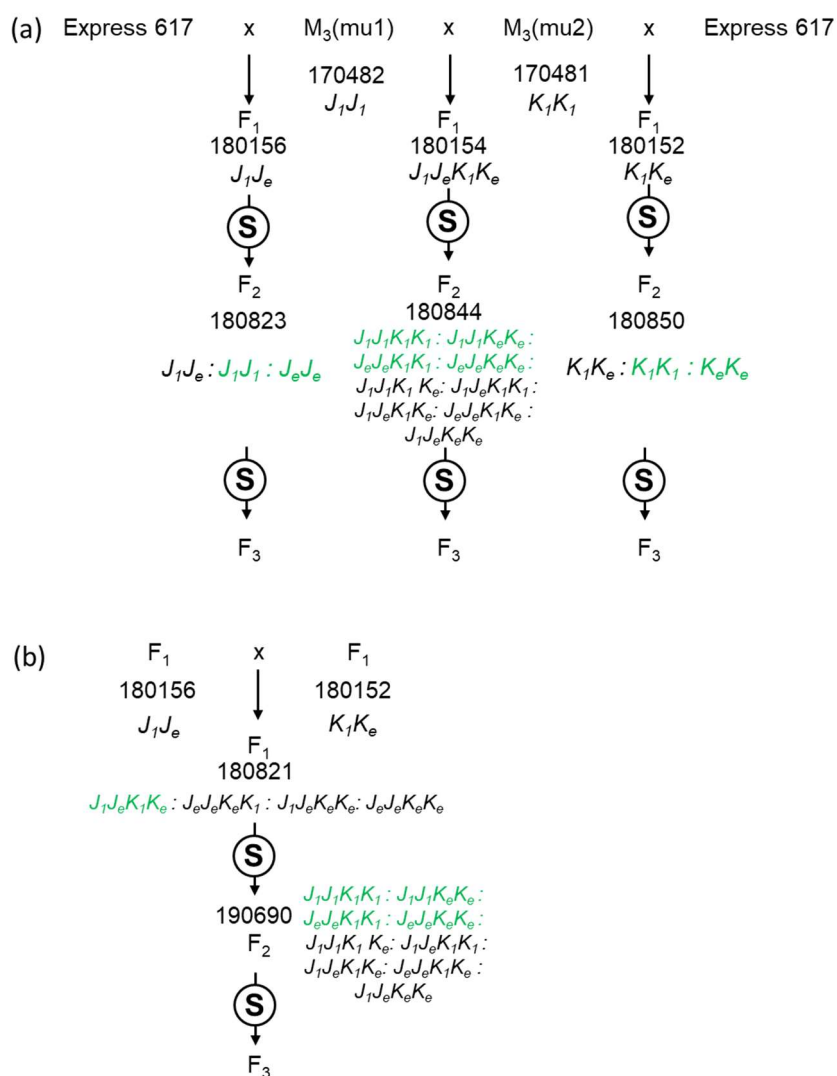


Figure 14: Crossing scheme for production of *BnMRP5* single and double mutants. The seed codes are written above each genotype. The alleles are explained in Table 2. The genotypes highlighted in green were selfed to produce F_3 seeds, which were used for phenotyping; ‘S’: selfing. Mu stands for mutation and 1, 2 are for *Bn.MRP5.A10* and *Bn.MRP5.C05* genes, respectively. (a) and (b) shows crossing schemes used for M_3 crosses and once backcrossed plant material for production of double mutants, respectively.

4.3.2. Identification of *MRP5* genes and mutant screening

In Arabidopsis, the MRP5 protein is encoded by one gene (*At1g0420*), which was used for retrieving all paralogous genes from *B. napus* using the Genoscope browser (<http://www.genoscope.cns.fr/brassicanapus/>). Genes having high sequence identity to the Arabidopsis genes were considered as true paralogous genes. Protein domains were identified using the pFam database search (<http://pfam.xfam.org/>). All hits were confirmed using TAIR (https://www.arabidopsis.org/servlets/Search?type=gene&action=new_search), NCBI annotations (<https://www.ncbi.nlm.nih.gov/gene>) and BRAD databases (<http://brassicadb.org/brad/>).

I have used TILLING by sequencing (Tbys) for screening of the mutations as described in Chapter 2 (Sashidhar et al. 2019). Two *BnMRP5* paralogs were chosen for identifying the putative loss of function mutations. Four primer pairs were used to screen amplicons of a length of 1414 bp to 1509 bp (Table S6). Sanger sequencing was used to confirm the mutations in M_2 generation with the help of paralog specific primers.

Mutation frequencies (F) were calculated based on the mutations per M_1 plant (Harloff et al. 2012):

$$F [1/kb] = 1 / \left(\frac{(\text{amplicon size [bp]} * (\text{number of } M_1 \text{ plants}))}{(\text{number of mutations}) * 1,000} \right)$$

For CRISPR-Cas9 experiments, two conserved target sites were used for knocking out four paralogs of the gene family (Table S10). Design of the target sites and their cloning was performed according to the published protocol (Fauser et al. 2014). Rapeseed hypocotyl transformation was done according to the described protocol in Chapter 3 under Section 3.6.4.

4.3.3. Expression analysis

Five Express 617 plants were used for expression analysis. Immature flower buds were emasculated, and hand pollination was done to ensure the day of pollination. Seed samples of 50 mg were harvested at 15, 25, 35 and 45 days after pollination (DAP) and were shock frozen in liquid nitrogen. RNA was isolated using PeqGOLD RNA isolation kit (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. DNase digestion was performed after RNA isolation using the peqGOLD DNase I Digest Kit (PEQLAB, Erlangen, Germany) to remove genomic DNA. cDNA first strand synthesis was carried out using a first strand cDNA synthesis kit (ThermoFisher Scientific Inc., Waltham, United States) according to the manufacturer's protocol. RT-qPCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Karlsruhe, Germany) on a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, USA) in a volume of 20 µl. *BnActin2* was used as a reference gene for normalization. Relative expression was calculated using the ΔC_t method.

4.3.4. PA and Pi measurements

Dry seeds of 200 mg were ground into fine powder and phytic acid was extracted in three replicates of 50 mg according to Matthäus et al. (1995) with minor modifications. Phytic acid was measured by HPLC according to Rounds and Nielsen (1993) calibrating with a phytic acid standard (Sigma P-8810). For determining Pi contents, 50 µl of the purified column extracts were mixed with 500 µl of coloring reagent (10% w/v of ascorbic acid (Roth- Art.Nr.3666.1) and 5% w/v of ammonium molybdate (Roth, Art. No.3525.2), adjusted to 1.5 ml with double distilled water and incubated at 40°C for one hour. The samples were measured against a reagent blank in a spectrophotometer at 655 nm. A calibration curve ranging from 0 to 100 nmol was used for both PA and Pi measurements with standards for Pi and PA, respectively.

4.3.5. Statistical Analysis

Data obtained from PA and Pi measurements were used for statistical analysis. Significance was calculated using ANOVA in R 3.6.0 and the MulticomView package and Post hoc test was performed by using Tukey's Multiple Comparison Test; $p=0.05$. Standard deviation was measured between five biological samples along with their three technical repeats.

4.4. Results

4.4.1. Dynamics of phytic acid (PA) and inorganic phosphorous (Pi) accumulation in Express 617

To determine the dynamic changes of PA and Pi in developing seeds of rapeseed, I have collected seed samples at 15,25,35,45 and 55 days after pollination (DAP). The obtained values from HPLC analysis for PA and Pi values by an ammonium molybdate spectrophotometric test are plotted in Figure 15. PA was not observed at 15 DAP but it accumulated significantly from 15 to 35 DAP. In contrast to PA, Pi

was decreased significantly from 15 to 35 DAP. This coordinated decrease of Pi and increased PA might indicate that most of the Pi is assimilated to PA at 35 DAP.

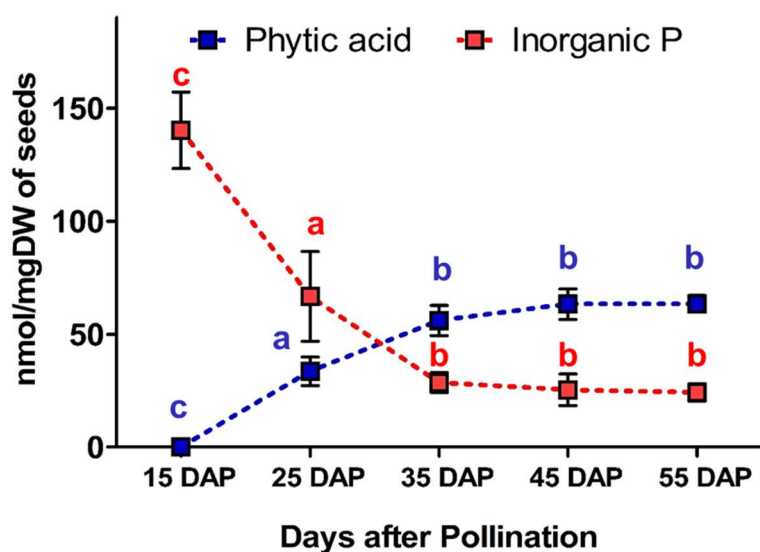


Figure 15: Dynamics of phytic acid (PA) and inorganic phosphorus (Pi) accumulation during seed maturation in winter cv. Express 617. measurements. Plants were grown in green house under 16h of light and 8h of dark at 22°C. Five plants were used as biological replicates for PA and Pi measurements. Error bars denote the SD among five biological samples along with three technical repeats. Statistical significance was calculated using ANOVA and grouping was done using Tuckey test. Different letters indicate statistical significance.

4.4.2. Bioinformatic analysis

Four genes were identified as putative paralogous genes for the Arabidopsis *AtMRP5* from the Brassica Genoscope database (Table 6). However, according to the gene models from a recent RNAseq study in Ningyou7 (Shah et al. 2018), *Bn.MRP5.A09* was annotated to *BnMRP15*. Exon-intron structure and protein similarity of the identified genes is given in Table 6. Indeed, this paralog had less protein similarity to the MRP5 proteins and a low expression from the available RNAseq data of Ningyou7. Therefore, this gene was not considered for TbyS analysis but was considered for CRISPR-Cas9 experiments. All identified genes showed Walker A, B and C motifs, which were encased by the transmembrane domain (Figure 17).

Table 6: *In silico* analysis of MRP5 genes in *Brassica napus* L.

Arabidopsis gene	<i>B. napus</i> sequence annotation	<i>B. napus</i> paralogs	CDS length (bp)	Exon/Intron structure		Protein size (aa)	Amino acid sequence identity with <i>AtMRP5</i> (%)
				Exons	Introns		
	<i>BnaA10g02400D</i>	<i>Bn.MRP5.A10</i>	5284	9	8	1509	93.0
At1g0420	<i>BnaC05g02300D</i>	<i>Bn.MRP5.C05</i>	5278	9	8	1510	93.6
	<i>BnaC09g10390D</i>	<i>Bn.MRP5.C09</i>	5399	12	11	1436	85.3
At3g60970 [§] / At1g0420*	<i>BnaA09g10230D</i>	<i>Bn.MRP5.A09</i>	4168	13	12	1071	84.7

[§]Annotation according to (Shah et al. 2018). * Annotation according to published *Brassica napus* genome (Darmor-bzh) (Chalhoub et al. 2014). CDS length is from start to stop codon.

4.4.3. Expression analysis

Messenger RNA abundance was measured in developing seeds of Express617 and obtained Ct values were normalized to the house keeping gene *BnActin2*. The three most homologous genes of *BnMRP5* were considered for the expression analysis. All genes showed an increase of transcript accumulation from 15 to 25 DAP, where all showed a peak of expression. The expression levels stayed constant or decreased until 45DAP (Figure 16). For the TbyS strategy, the two highly expressed paralogs were used for knockout analysis. In the CRISPR-Cas9 experiments, a conserved target site sequence for all four paralogs was used to identify complete knockouts.

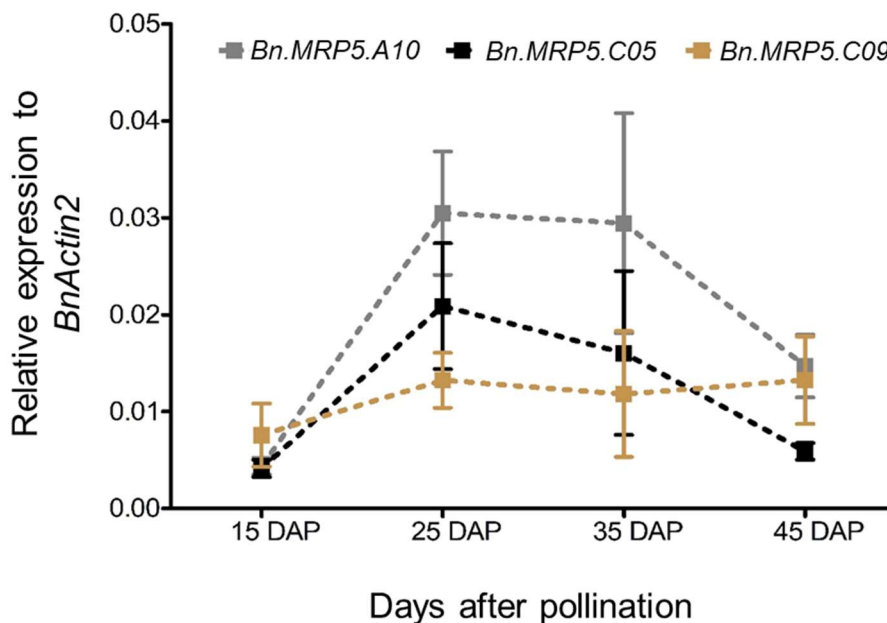


Figure 16: Expression profiles of three *BnMRP5* genes in Express 617 at seed filling stages. Plants were grown at 16h of light and 8h of dark at 22°C. Seed samples from four developmental stages of Express 617 which were harvested at 15, 25, 35 and 45 DAP were used. The expression was normalized to the housekeeping gene *BnActin2*. Error bars indicate SD of five biological samples along with three technical repeats.

4.4.4. Identification of EMS mutants

The coding regions of the targeted genes *Bn.MRP5.A10* and *Bn.MRP5.C05* from start site to stop site are 5284 bp and 5278 bp long respectively. Both genes have the functional Walker A, B and C motifs (also known as the signature motif of ABC genes). The amplicons used for TbyS approach covered approx. 60% of the coding region including the Walker A and C motifs of the genes (see Chapter 2 for TbyS strategy) (Figure 17a, b). By implementing TbyS, eight stop codon and three splice site mutations for *Bn.MRP5.A10* and 10 stop codon and one splice site mutations for *Bn.MRP5.C05* were obtained (Table 7), which were also confirmed by Sanger sequencing. Mutations in *Bn.MRP5.A10* and *Bn.MRP5.C05* were assigned with the allele code *J* and *K*, respectively. For a phenotyping analysis, I have chosen the alleles *J₁* and *K₁* as these mutations were in beginning of the gene and the translation should result in a loss of all the important domains of the BnMRP5 protein (Table 7, Figure 17). Homozygous M₃ mutants were combined according to the crossing scheme shown in Figure 14. All obtained F₁ plants were selfed to generate segregating F₂ plants from which seeds were used for PA measurements. An expected segregation ratio of 1:15 was observed in F₂ generation for double mutants and 1:3 for single mutants (Chi square analysis is shown in Table 8).

Table 7: All *Brassica napus* *BnMRP5*. mutant alleles detected by TILLING by sequencing (TbyS).

Gene	Mutation type	Mutation site (bp) [§]	Allele ^a	Mutation type	Effect on protein sequence
<i>Bn.MRP5.A10</i>	Nonsense	1077 [§]	<i>J</i> ₁	G to A	W379*
<i>Bn.MRP5.A10</i>	Nonsense	1072	<i>J</i> ₂	C to T	Q378*
<i>Bn.MRP5.A10</i>	Nonsense	1242	<i>J</i> ₃	G to A	W414*
<i>Bn.MRP5.A10</i>	Nonsense	1260	<i>J</i> ₄	G to A	W420*
<i>Bn.MRP5.A10</i>	Nonsense	1561	<i>J</i> ₅	C to T	Q521*
<i>Bn.MRP5.A10</i>	Splice site	3557	<i>J</i> ₆	-	-
<i>Bn.MRP5.A10</i>	Splice site	4093	<i>J</i> ₇	-	-
<i>Bn.MRP5.A10</i>	Nonsense	4152	<i>J</i> ₈	G to A	W1216*
<i>Bn.MRP5.A10</i>	Nonsense	4271	<i>J</i> ₉	G to A	W1255*
<i>Bn.MRP5.A10</i>	Splice site	4390	<i>J</i> ₁₀	-	-
<i>Bn.MRP5.C05</i>	Nonsense	432	<i>K</i> ₂	G to A	W144*
<i>Bn.MRP5.C05</i>	Nonsense	628 [§]	<i>K</i> ₁	C to T	Q210*
<i>Bn.MRP5.C05</i>	Nonsense	1174	<i>K</i> ₃	C to T	Q392*
<i>Bn.MRP5.C05</i>	Nonsense	1244	<i>K</i> ₄	G to A	W415*
<i>Bn.MRP5.C05</i>	Nonsense	3487 [§]	<i>K</i> ₅	C to T	R1044*
<i>Bn.MRP5.C05</i>	Nonsense	3762	<i>K</i> ₆	C to T	Q1116*
<i>Bn.MRP5.C05</i>	Nonsense	4206	<i>K</i> ₇	C to T	Q1239*
<i>Bn.MRP5.C05</i>	Nonsense	4245	<i>K</i> ₈	C to T	Q1252*
<i>Bn.MRP5.C05</i>	Splice site	4299	<i>K</i> ₉	-	-
<i>Bn.MRP5.A10</i>	-	-	<i>J</i> _e	-	-
<i>Bn.MRP5.C05</i>	-	-	<i>K</i> _e	-	-

[§] Mutation site from the start codon.

^a A single letter code was used to display M₂ genotypes where 'e' denotes the allele of non-mutagenized Express 617 donor variety.

[§] Two plants from the EMS population had similar mutation.

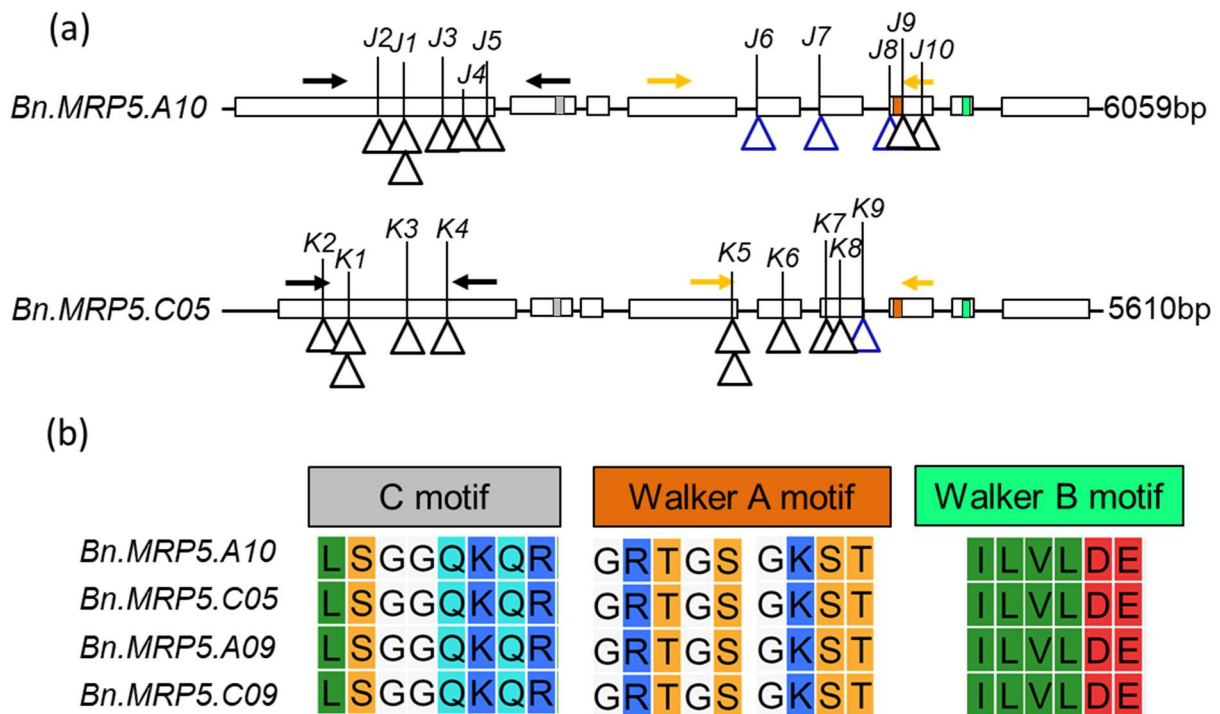


Figure 17: Gene structure of *BnMRP5*. (a) Exon-intron structure showing the positions of the identified mutations. Exons are shown in white bars. Primer pairs used for amplicon sequencing are shown in black solid and dotted arrows. C motif, Walker A and Walker B motif are shown in grey, black and dotted boxes, respectively. The C motif is also known as ABC signature motif. Stop codon mutations are shown as black triangles and splice site mutations as grey triangles. The mutant alleles are indicated by respective numbers (See table 7). (b): Conserved C motif, Walker A and Walker B motifs in different *BnMRP5* enzymes.

Table 8: Genotypic segregation for *Bn.MRP5.A10* and *Bn.MRP5.C05* in 4 F₂ populations. Genotypes shown in green were used for phenotyping experiments.

F ₂ seed code	Expected segregation pattern	Gene	Number of plants analyzed	Chi square value (χ^2)
180850	<i>J₁J₁: J_eJ_e: J₁J_e</i>	<i>Bn.MRP5.A10</i>	50	4.2 ^a
180823	<i>K₁K₁: K_eK_e: K₁K_e</i>	<i>Bn.MRP5.C05</i>	50	3.45 ^a
180844	<i>J₁J₁K₁K₁: J₁J₁K_eK_e: J_eJ_eK₁K₁: J_eJ_eK_eK_e: J₁J₁K₁K_e: J₁J_eK₁K₁: J₁J_eK₁K_e: J_eJ_eK₁K_e: J₁J_eK_eK_e</i>	<i>Bn.MRP5.A10</i> ; <i>Bn.MRP5.C05</i>	230	3.66 ^b
190690	<i>J₁J₁K₁K₁: J₁J₁K_eK_e: J_eJ_eK₁K₁: J_eJ_eK_eK_e: J₁J₁K₁K_e: J₁J_eK₁K₁: J₁J_eK₁K_e: J_eJ_eK₁K_e: J₁J_eK_eK_e</i>	<i>Bn.MRP5.A10</i> ; <i>Bn.MRP5.C05</i>	200	4.4 ^b

^a 3:1 segregation, $\chi^2 < 5.99$, at $p=0.05$, $df=2$

^b 15:1 segregation, $\chi^2 < 7.82$, at $p=0.05$, $df=3$

4.4.5. CRISPR-Cas9 knockout analysis

Despite the annotation of the fourth paralog was not clear, I aimed to knockout all paralogs by using a conserved sequence as a target site. Upon rapeseed hypocotyl transformation using 640 hypocotyls, 17

shoots from a total of seven independent transgenic events (termed T₁ generation) were regenerated to whole plants (MRP5_TS1_C1 to MRP5_TS1_C7, where TS= target site 1) on BASTA selection media. Out of seven independent events, shoots from only three events were transgenic. The presence of the transgene was confirmed by using primers flanking the *Cas9* region. PCR amplicons of the targeted sites from leaf DNA of each transformant were sequenced to search for mutations within genes of interest. None of the regenerated plants showed editing in the targeted genes. To verify whether the transgenic plants showed any editing patterns in T₂ generation, ten seeds from two of the independent events were sown and leaf samples of T₂ plants were tested for the presence of transgene. Although the regenerated plants were transgenic, no editing was observed. Therefore, a second target site (TS-2) was identified and the corresponding sgRNA was cloned into the transformation vector and subsequently was transformed by using rapeseed hypocotyl transformation. From this second experiment, five plants were obtained. Upon evaluation for presence of the transgene and for editing, all plants were neither transgenic nor edited in the targeted genes (Table S10).

4.4.6. Phenotypic analysis of *bnmrp5* mutants obtained by EMS

Plants were grown after vernalization at 16h of light and 8h of dark at 22°C for phenotyping analyses. Single mutants from the F₂ families 180823 (*J₁J₁*) and 180850 (*K₁K₁*), showed no significant reduction in PA contents when compared to Express 617 (Figure 18). Therefore, double mutants were produced as shown in Figure 14a, for a phenotyping analysis. However, the double mutants obtained from an F₂ family (180844; *J₁J₁ K₁K₁*) showed significant reduction only to the internal control plants carrying homozygous alleles for Express 617 (*J_eJ_e K_eK_e*) and not with non-treated Express 617 (Figure 18). Furthermore, I expected clear phenotypic effects in the double mutants of F₂ progeny, which were performed between two F₁ mutants once backcrossed with Express 617 (crossing scheme shown in Figure 14b). Upon HPLC measurements in the seeds of the segregating F₂ family (190690), I observed that the double mutants showed PA reduction to the internal control plants (*JeJeKeKe*) as well to the Express 617 plants. A reduction of 15% PA contents was observed indicating that the PA reduction was due to the underlying mutant alleles and not due to the background mutation load. Indeed, a similar reduction was observed in the double mutants of the 180844 family when compared to internal control plants (*JeJeKeKe*), indicating that the observed reduction of 15% PA is based on the two mutated alleles. In addition, I have measured the Pi contents in matured seeds of the 190690 progeny to see if the reduced PA had any effects on Pi accumulation. I did not find any significant increase in double mutants as compared to control plants (Figure 19). Besides this, the plant height, silique number per plant and thousand kernel weights were also not significantly different, indicating that the low PA was not effecting the overall plant performances (Table 9).

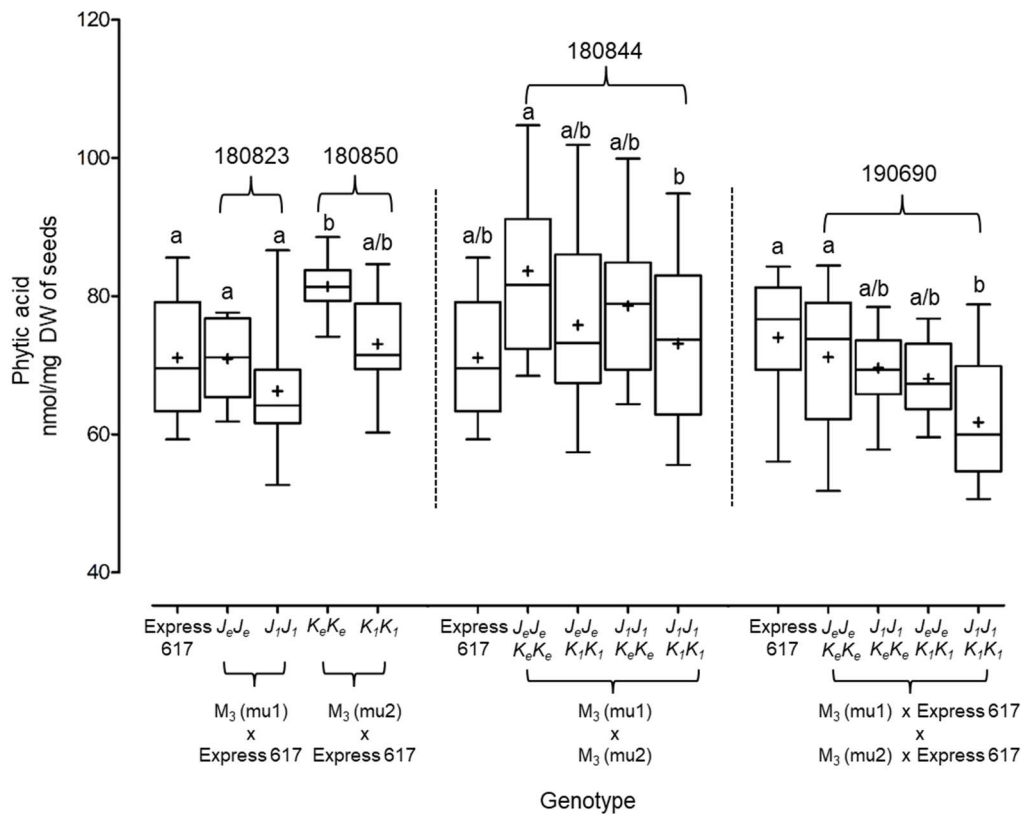


Figure 18: Phenotypic analysis of *bnmrp5* mutants. Box plot of phytic acid contents in seeds of *bn2-pgk2* mutants. Statistical analysis was performed using Anova and a posthoc test using Tukey's Multiple Comparison Test; $p=0.05$. Same letters indicate no significant differences. The non-mutated Express 617 alleles are indicated by 'e'. Mu stands for mutation and 1, 2 are for *Bn.MRP5.A10* and *Bn.MRP5.CO5* genes, respectively. Seed codes of F₂ families are given above the flower brackets. Mean is represented by +. See Table 7 for respective allele codes.

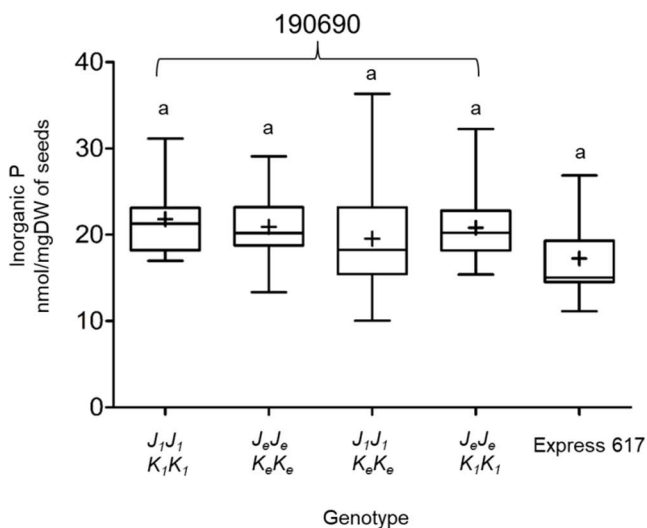


Figure 19: Inorganic P (Pi) measurements in *bnmrp5* double mutants of F₂ family (190690). Five plants were grown under 16h of light and 8h of dark at 22°C. The boxplot represents Pi measurements in five plants for each genotype from matured seeds. Of each plant, three technical repeats were taken. Mean is represented by +. Statistical analysis was performed using ANOVA and posthoc test by Tuckey at $p = 0.05$. Same letters indicate no significant differences between the samples. The non-mutated Express 617 alleles are indicated by 'e'. See Table 7 for respective allele codes.

Table 9: Evaluation of pleiotropic effects in *bnmrp5* mutants in F₃ seeds. Plants were grown under 16h light and 8h dark in 11 x 11 cm pots at 22°C for all measurements. Five plants for each genotype were

used for measuring TKW, plant height and silique number. Mu stands for mutation and 1, 2 are for *Bn.MRP5.A10* and *Bn.MRP5.C05*, respectively.

F ₂ Seed code	Generation	Genotype	TKW (g)	Plant height (cm)	Silique number/plant
180844	M ₃ (mu1) x M ₃ (mu2)	<i>J₁J₁K₁K₁</i>	3.29± 0.33	93.06± 9.57	39 ±11.98
		<i>J₁J₁K_eK_e</i>	3.37± 0.10	101.80± 9.12	42 ±7.08
		<i>J_eJ_eK₁K₁</i>	3.32± 0.38	105.00±9.33	37 ±10.30
		<i>J_eJ_eK_eK_e</i>	3.28± 0.14	103.20±3.56	39 ±9.80
		Express 617	3.30 ±0.22	118.02± 6.12	42 ±6.08
180823	M ₃ (mu1) x Express 617	<i>J₁J₁</i>	2.61± 0.16	91.53± 8.56	46 ±5.08
		<i>J_eJ_e</i>	2.37± 0.20	98.88± 4.60	52 ±7.10
180850	M ₃ (mu2) x Express 617	<i>K₁K₁</i>	3.24± 0.32	97.50± 8.35	39 ±9.08
		<i>K_eK_e</i>	3.09± 0.60	97.45± 8.40	46 ±6.50
190690	(M ₃ (mu1) x Express 617) x (M ₃ (mu2) x Express 617)	<i>J₁J₁K₁K₁</i>	2.50± 0.15	84.80± 3.65	40 ±4.60
		<i>J₁J₁K_eK_e</i>	2.15± 0.14	86.20± 2.13	45 ±12.20
		<i>J_eJ_eK₁K₁</i>	2.67± 0.40	90.20± 8.25	40 ±8.79
		<i>J_eJ_eK_eK_e</i>	2.38± 0.15	83.66± 3.44	47 ±8.28
		Express 617	2.59± 0.49	89.45± 5.40	49 ±5.23

4.5. Discussion

In this chapter, I described low phytic acid mutants in oilseed rape by targeting *BnMRP5*, PA transporter genes. Three paralogs were increasingly expressed during seed development until 35DAP. The expression profiles of *AtMRP5* from siliques (Kim and Tai 2011) and *PvMRP1* from common bean seeds (measured at 6 to 24 days after flowering) (Panzeri et al. 2011) showed similar expression profiles as *BnMRP5* paralogs. At 25 DAP the expression of the paralogs peaked and this coincided with the PA and Pi measurements from Express 617. At 35 DAP, I observed only low amounts of Pi and no further increase in PA indicating that the major incorporation of Pi into PA occurred until 35 DAP. These results might also suggest that a knockout of all three paralogs might be essential for obtaining low PA contents in seeds.

The double mutants obtained by combining the mutations of *Bn.MRP5.A10* and *Bn.MRP5.C05* resulted in reduced PA contents of 15%. One of the major concerns regarding EMS mutations is background mutation load. The wild type plants (*J_eJ_eK_eK_e*) from the M₃ mutant crosses (180844) and once backcrossed progeny (190690) did not show any significant differences to Express 617 PA contents. Interestingly, in one of the M₃ single mutant's families (180850), the wild type plants with the alleles *K_eK_e* showed a significant difference to Express 617 plants and had accumulated more PA in the seeds (Figure 18). Therefore, I hypothesize that crossing of M₃ mutants perhaps might be the cause for no significant differences between double mutants (*J₁J₁K₁K₁*) and Express 617. However, the double mutants obtained from a backcross with Express 617 (F₁: 180821, Figure 14b), could clearly rule out the background noise coming from the M₃ families. A significant reduction was observed when compared to the segregating wild type plants (*J_eJ_eK_eK_e*) as well as to Express 617.

Efficient CRISPR-Cas9 editing depends on three parameters. 1) Checking for a complementary sequence on the genome by the sgRNA guided Cas9 nuclease to find the complementary DNA sequence, 2) inducing a DNA double strand break (DSB) by Cas9 endonuclease, and 3) efficient repair of the DSBs by the endogenous DNA repair machinery (Chakrabarti et al. 2019). The study from Chakrabarti *et al.* showed that upon testing 1000 human genomic loci, the fourth position from the PAM site is most important for efficient editing. The choice of T or A in this position resulted in higher gene editing in human cells (Chakrabarti et al. 2019). Interestingly, out of two selected target sites for *BnMRP5*, one target site (TS1) had nucleotide ‘T’ at position 4 (Table S10). The regenerated transgenic plants containing this target site sequence for a knockout did not show any editing. Therefore, the conclusions from the human study might not be true for plants and specifically not for rapeseed. Another explanation could be that the T-DNA was inserted in a region, which is not transcriptionally active or that multiple T-DNA insertions might have resulted in silencing of the transgene (De Buck et al. 2009; Tang et al. 2007). Furthermore, the chromatin accessibility at the position of the chosen target site in the genomic DNA might also influence an efficient binding and subsequent induction of DSB (Uusi-Mäkelä et al. 2018). In conclusion, the failure to edit target genes remains elusive and further experiments with other target sites should allow to find the gene editing in *BnMRP5* paralogs. In addition, the expression analysis of *Cas9* in the obtained mutants might provide additional information regarding the transgene silencing.

Knocking out mutants of MRP transporters showed significant reductions in PA contents in maize, rice, wheat, barley, Arabidopsis, soybean and common bean of up to 90% (Sparvoli and Cominelli 2015). Demonstrating that PA transporters are potential targets for low PA breeding across different crops without pleiotropic effects. I expected that the two knockout mutants resulted in a truncated protein, where the Walker A, B and C motifs were completely lost, thereby leading to a failure to transport PA. In comparison to knockout mutants from other crop species which ranged from 32% to 90%, I observed only 15% reduction of PA. One of the reasons for a smaller reduction could be due to the relative high expression of three paralogs in seeds, and the knockout effects of two genes might be compensated by the third functional paralog (Figure 16). Moreover, it was shown in several studies simultaneous knockout of multiple genes is required for loss of function in targeted genes in polyploids (Wang et al. 2014; Del Pozo and Ramirez-Parra 2015). Regardless, in wheat a reduction of 22% PA was observed when all three paralogous genes of *TaABCC5* were downregulated by using RNAi (Bhati et al. 2016). Taken together the results from wheat (Bhati et al. 2016) and from this study might indicate that probably in polyploids alternative transporters exist which might compensate for the loss of knockout mutations in MRP5 proteins.

It was shown in common bean, where *PvMRP2* compensates for the loss of *PvMRP1* function. However, compensation of the loss of *PvMRP1* was not observed in seeds but in other tissues (Cominelli et al. 2018). In addition, knockout mutants of *OsSPDT* in rice, a sulphur transporter has shown reduced PA contents in seeds (Yamaji et al. 2016), which might also contribute to PA loading in seeds. Since, loss of function mutants in PA transporters were never identified before in oilseed rape, the hypothesis that other MRP transporters compensate the knockout effects need to be further analyzed. In future to observe higher reductions, an additional mutation in the third paralog *Bn.MRP5.C09* could be an alternative in oilseed rape. Besides this, the obtained *bnmrp5* mutants can also be used for crossing with previously identified *bn2-pgk2* mutants to have higher PA reduction. Since in both the cases no negative effects on plant performances such as TKW, plant height, silique number/plant were observed, this could be a promising alternative.

Loss of *BnMRP5* function is attractive in three different aspects. 1) Knockout mutants of *BnMRP5* reduced the PA contents in seeds, which is beneficial for monogastric animals to improve their dietary requirements. 2) It is interesting to question, what happens with the PA if the transport function is

knocked out. PA transporter mutants in various species do have higher inositol kinases (Vip/VIH) referred to as inositol hexakis phosphate kinases (Desai et al. 2014), which synthesize InsP7 (also referred as PP-InsP₅) and InsP8 (also referred as 1,5PP₂-InsP₄). For instance, the knockout mutants of *atmrp5* and *zmmrp4* showed an increased accumulation of InsP7. Therefore, one can conclude that loss of function in MRP5 might lead also to an increased accumulation of InsP6 (or PA), which can be used as a substrate to produce InsP7 or InsP8. Indirectly, a reduction of PA can be observed as the substrate is used up by the inositol hexakis phosphate kinases. Accumulating InsP7 and InsP8 might be beneficial to the plants as these are energy rich molecules having energy rich pyrophosphate moieties, which are comparable to free energy of hydrolysis by ATP (Desai et al. 2014; Hand and Honek 2007). By an increased accumulation of such molecules, the yield capacity can be improved, and fertilizer demands might be reduced (Williams et al. 2015). Finally, inositol pyrophosphates are regulating the jasmonic acid based defense mechanisms, which might be beneficial for an improved growth under stress conditions (Laha et al. 2015). 3) Mutants of PA transporter, *AtMRP5* have been shown to have an effect on drought tolerance with a reduced ABA sensitivity resulting in stomatal closure (Gaedeke et al. 2001; Klein et al. 2003). In *atmrp5* mutants, failure to transport PA into vacuoles resulted in reduced K⁺ uptake into the guard cells by inhibiting K⁺ inward rectifying channels (Nagy et al. 2009). The stomatal closure might reduce water loss from leaves and might foster plants to cope with drought stress and it was shown that *atmrp5* mutants showed reduced water uptake and survived longer than the wild type plants under low water levels (Klein et al. 2003).

4.6. References

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5. Closing discussion

The two main objectives of my research were 1) to develop mutant detection protocols for obtaining knockout mutations of complex pathways in polyploid crops in an efficient manner and 2) to identify loss of function mutants in the phytic acid (PA) pathway to enrich oilseed rape protein quality for monogastric livestock. To fulfill the aimed objectives, I have targeted all genes of PA biosynthesis and a transporter by a transgenic CRISPR-Cas based and a non-transgenic (TILLING by sequencing, TbyS) approach. I could obtain knockout mutants for all targeted genes of the pathway by either of the used approaches (Figure 20, Table 10). This study was the first to identify low PA mutants from the different steps of the pathway in oilseed rape, which is an essential trait for practical breeding purposes. In the following paragraphs, I will critically discuss the obtained results from successful and failed experiments.

My first aim was to work out a protocol for TbyS using amplicon sequencing for 12 highly expressed genes of the PA pathway in a population of 7680 EMS mutants. By implementing TbyS, mutations in all targeted genes were obtained at an average mutation frequency of 1/27 kb. The use of an Illumina HiSeq 4000 sequencing machine resulted a coverage of 143x per allele for each of 22 amplicons, which was high enough to find loss of function mutations for every gene except for *Bn.MIK.A10*. Failure to identify the mutation in *Bn.MIK.A10* might not be due to limitations of the detection method, but more likely due to vital function of this gene for plant development and a loss of function mutation might be lethal. Furthermore, mutations were identified without the need of bespoke bioinformatic pipelines (described in Chapter 2). Altogether, the mutation detection method was efficient when compared to previous studies as shown in Table 2, where a list of all TbyS studies performed so far are given.

Today, with the advancements in sequencing technologies, higher amplicon stacking could be used with the help of dual indexing barcodes. Besides this, an increased pool depth to 192 or 265, as shown in a recent proof of concept study (Tramontano et al. 2018) can be beneficial to screen for larger populations. This not only will reduce the costs of TbyS but will also allow the screening for higher number of genes, especially when multiple genes are responsible for a single trait such as in polyploids. Likewise, development of WGS or exome capture kits for oilseed rape can provide a long-term database for identifying mutants for any kind of genes. By developing the mutant database, a one-step identification of mutants can be achieved where the type of mutation and its effects on protein structure can be easily identified (Henry et al. 2014). Studies in wheat showed that with exome capture sequencing not only SNPs but also larger InDels could be identified, which can be a valuable resource for fast marker development for genotyping approaches. The usage of high EMS concentrations was a reason for the occurrence of InDels in wheat (Krasileva et al. 2017). The available oilseed rape TILLING population from our lab was also established by using high EMS concentrations and therefore, WGS or exome capture sequencing can be beneficial to detect InDels in this population. Moreover, in my amplicon sequencing data, I observed small InDels (data not shown) but these were not tested using Sanger sequencing.

One of the major drawbacks of mutants obtained by random mutagenesis is the high mutational background load, which limits the use of these mutants directly for practical breeding purposes. To solve this problem, the obtained mutants of *Bn2-PGK2* and *BnMRP5* were crossed with an early flowering spring genotype (cv. Peace) and a BC₁ progeny was produced. By using a 15K SNP array the background mutation load can be reduced in a fast way by selecting genotypes with enriched Peace backgrounds. Further rounds of backcrossing with this early flowering variety can reduce the EMS background in few generations drastically. Thereby, the established varieties will contain mostly the desired mutations, and can be used immediately for further physiological experiments. Likewise, they

can be used for fish and poultry feed experiments to analyze whether a lowered PA content has a direct consequence on their growth performances. A crossing scheme used to generate the BC1 mutants is shown in Figure S10, Table S11.

Gene editing by CRISPR-Cas9 offers a solution to obtain mutants without mutated backgrounds easily and it provides also an elegant solution for simultaneous knockout of multiple genes in polyploids, which is often required to overcome gene redundancy (Wang et al. 2014). Therefore, my second aim was to identify mutants for genes of the PA pathway by using the CRISPR-Cas9 approach. This should have allowed me to obtain multiple knockout mutants for a gene family in a single plant, unlike in the TbyS approach where only single mutants were obtained. Among the CRISPR-Cas9 experiments performed, stable transgenic plants could be only obtained for *BnITPK* and *BnMRP5* genes. However, editing was observed only in *BnITPK* paralogs, but not in *BnMRP5* genes (Table 10, Table S10). A detailed description of the used CRISPR-Cas9 strategy is discussed for *BnITPK* in Chapter 3 and for *BnMRP5* in Chapter 4.

One of the reasons for the failure of CRISPR-Cas9 experiments in the targeted genes, except *BnITPK*, could be due to the design of inefficient target sites for the cloning of sgRNA that have been used for rapeseed transformations. The design of efficient target sites that are specific to homologous regions of different targeted paralogs is a challenging step. There are different tools available for the design of sgRNAs, but all these tools generate different results for the same query (Uniyal et al. 2019). Previous studies using CRISPR-Cas9 approaches have shown that the use of multiple sgRNAs is often necessary to edit the genes of interest. For instance in tomato, out of eight used tandem sgRNAs for one promoter region, editing was possible only by six of them (Rodríguez-Leal et al. 2017). In this context it seems desirable to design different target sites for any gene to be knocked out efficiently, and to use multiplexing sgRNAs vector systems that have been developed (Zhang et al. 2016b; Minkenberg et al. 2017). For rapeseed this infers that multiple target sites for several paralogs should be identified, which is challenging and for some genes even might not be feasible, as I have shown for the *BnITPK* genes. It was possible to design conserved target sites for two *BnITPK* paralogs, but for the other paralogs a SNP had to be accepted in the end of the sgRNA (see Chapter 3). Therefore, for future experiments designing multiple sgRNAs for efficient knockout of genes of interest is necessary. Besides this, knocking out a complete gene family might also result in lethal phenotypes as intermediate products of the PA pathway are involved in basic plant developmental processes.

Nonetheless, for future experiments using CRISPR-Cas, I would propose to use multiple target sites for targeting different paralogs at once. Moreover, the use of other CRISPR vector systems such as those from *Staphylococcus aureus* or *Streptococcus thermophilus* could increase the chance of obtaining mutants by gene editing (Steinert et al. 2017). It was shown in Arabidopsis that use of *SaCas9* increased the editing efficiency to about 90% when compared to the SpCas9 system (Wolter et al. 2018). In addition, these vectors harbor a kanamycin resistance gene, which was better performing in the regeneration of Brassica tissues when compared to the BASTA resistance which is encoded on the here used pCas9_TPC vector system from *Streptococcus pyogenes* (De Block et al. 1989; Fauser et al. 2014).

Promoters driving the Cas9 gene are highly important for an efficient editing. The SpCas9 system was mainly designed for Arabidopsis floral dip experiments for which a strong expression of the Cas9 gene in egg cells is desirable, which is conferred by the used ubiquitin promoter. However, this promoter might not be the best for a use in tissue culture experiments. A study in *B. napus* stated that use of tissue specific promoters rather than constitutive promoters might increase the editing efficiency by the increased expression level of *Cas9* and *sgRNAs* during plant regeneration (Yang et al. 2017).

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Therefore, this system might not be suitable for an efficient gene editing in *B. napus* through hypocotyl transformations.

A further aim of this study was to knockout paralogs of the PA transporter gene *MULTI-DRUG RESISTANT PROTEIN5* (*BnMRP5*). Lowering the PA content in seeds by knocking out the biosynthetic genes is often impeded by negative agronomic traits such as poor yield, reduced stress tolerance and delayed germination (Shi et al. 2007; Sparvoli and Cominelli 2015). Knockout and functional analysis of *bnmrp5* mutants is described in Chapter 4 and an overview of PA reduction is given in Table 10. Studying PA transporters is essential to understand the source and sink mechanisms of phosphorus from soil uptake to seed storage. In addition, knowing how P homeostasis is established in plants could be useful to improve crop yields, especially for regions where Pi availability is low. It has been shown that winter oilseed rape at flowering needs phosphorus for high yield (Grzebisz et al. 2018). Therefore, the available mutants from this study could be used to decipher phosphorus requirements in oilseed rape.

The next question to answer was whether the obtained mutants showed any significant reductions in PA contents in seeds. Since I hypothesized that single mutants will not show an effect and that more paralogs have to be mutated to see a visible phenotype, single mutants obtained by TbyS were combined to form double mutants, where by CRISPR-Cas9 double and triple mutants were obtained in T₄ generation.

I questioned also about the function of early and later steps of the PA pathway in rapeseed. Interestingly, segregating single mutants ($R_2R_2S_eS_e$ and $R_eR_eS_1S_1$) of the *bnmips1* F₂ family (182085) showed a significant PA reduction, whereas double mutants ($R_2R_2S_1S_1$) showed increased PA levels (Figure S9). However, this increase in double mutants was not significant in comparison to Express 617 and internal control plants carrying homozygous alleles for Express 617 ($R_eR_eS_eS_e$). The observed reduction in single knockout mutants cannot be explained to date, as in most cases single mutants did not show any effect in *B. napus* (Emrani et al. 2015; Braatz et al. 2018). Henceforth, I take these results at the moment with great care and this experiment should be repeated to confirm the results. Contradictory to this in Arabidopsis, single T-DNA mutants of *atmips1*, *atmips2* and *atmips3* did not show any reduced PA contents in seeds (Kim and Tai 2011). However, when RNAi was used in rice and oilseed rape low PA contents have been observed in seeds (Kuwano et al. 2009; Georges et al. 2006). This showed indeed that knockouts have a lower PA content, but since with RNAi more than one gene might be targeted, this situation is not directly comparable to what I have observed. In fact, it further indicates that RNAi cannot suppress the genes completely and therefore PA could be reduced in moderate amounts of up to 30% as in *B. napus*. Since, knockout mutations might have severe consequences in *bnmips* mutants as it is the initial step of PA synthesis as well as many other *myo*-inositol dependent products, which are needed for the development of plants. More speculative, the increased PA levels in double mutants could be a result of compensation by other paralogs of the *MIPS* gene family to ensure plant viability.

Besides *bnmips1*, the double mutants of *BnMIK* (F₂ seed code: 183301; $P_1P_1Q_1Q_1$) also did not show any reduced PA contents in seeds (Figure S9). *BnMIK* had two exons separating a single intron for both paralogs (*Bn.MIK.A10* and *Bn.MIK.C09*). Stop codon mutations could be recovered only for the *C09*, but not for the *A10* gene. In addition, I could also not find a splice site mutation as the chance of getting them was only at two positions. Therefore, a missense mutation ($Q_1 - D69N$) was used which changed the protein charge from negative to neutral. Unfortunately, this mutation might have only subtle consequence on the protein function and hence might be the reason for the observed phenotype. Another explanation would be that *BnMIK* is involved in maintaining the *myo*-inositol pools and has a crucial role in different developmental processes. Disturbing this pool might trigger feedback mechanisms to maintain the *myo*-inositol homeostasis. However, these hypotheses need additional

experiments in measuring the *myo*-inositol contents and other sugar compounds such as raffinose, UDP-galactose and UDP-glucose that are upstream of the PA pathway, and which might be responsible for the initiation of a possible feedback mechanism.

Knockout mutants of later steps of the PA pathway (*BnIPK2β* and *BnIPK1*) showed pleiotropic effects on plant performance. The double mutants of F₂ family (182087) of *BnIPK2β* (*N₁N₁O₂O₂*) had poor seed set resulting in only 5-15 seeds per plant. However, seeds from single mutants (*N₁N₁O_eO_e* and *N_eN_eO₂O₂*) and mutant plants carrying homozygous Express 617 alleles (*N_eN_eO_eO_e*) could be obtained. Initially, I expected that *BnIPK2β* has only one highly expressed paralog (*Bn.IPK2β.A09*) in seeds and knockout of this paralog might show a greater reduction of PA contents during seed development, which might be the reason for poor seed set. Interestingly, no significant differences were observed between single mutants and control plants (Figure S9). Therefore, I reason that the poor seed set might not be due to low PA contents. Noteworthy, this is the only gene I targeted from lipid dependent pathway and knockout of *BnIPK2β* might have affected the lipid metabolism which might be crucial for seed formation or the knockout might directly affect the function of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). IPK2β uses the substrates from PtdIns(4,5)P₂ which is involved in a multitude of cellular functions and also serving as the precursors of secondary messengers such as diacylglycerol (Heilmann et al. 2001). Although, these data are first observations, they might already indicate that *BnIPK2β* might not be a suitable target for low PA breeding in oilseed rape.

Surprisingly, in the segregating F₂ family (182086_1) of *BnIPK1;1*, no double mutants (*L₂L₂M₁M₁*) were found. To confirm these results, I have sown 200 seeds from a F₁ sister plant (182086_2) from which two double homozygous mutants carrying the alleles *L₂L₂M₁M₁* were found. Nonetheless, the plants did not survive, and no seeds were obtained for double homozygous mutant plants. The obtained single mutants (*L₂L₂M_eM_e* and *L_eL_eM₁M₁*) from the F₂ generation did not show any significant differences when compared to the control plants (*L_eL_eM_eM_e*) (Figure S9). By sowing the seeds of the segregating F₂ families, it was remarkable that about 8 to 12 seedlings died two days after germination in both experiments. The number of seedlings that were dead after germination is close to the number of expected plants in a digenic segregation, indicating that the double knockouts might be lethal. In addition, *IPK1;1* is encoded by only two paralogs, further indicating that the double knockout mutants might have a severe effect on plant growth in initial stages.

In conclusion, from the obtained results in this study, so far only few genes can be emphasized as targets for breeding low PA mutants in oilseed rape. This has also been observed in other crop species (Sparvoli and Cominelli 2015; Kim and Tai 2011). Besides this, knockout mutants of later steps of PA synthesis would be risky for breeding due to the observed pleiotropic effects. Moreover, it is still not known how much PA can be reduced until critical thresholds will be reached that will influence plant development. The obtained double mutants of *bn2-pgk2*, *bnmrp5* and triple mutants of *bnitpk* showed 15-35 % reduction without any pleiotropic effects. I suggest crossing the obtained mutants from *Bn2-PGK2*, *BnITPK* and *BnMRP5* with each other to stack the mutant alleles for further reduction of PA contents in seeds. By obtaining triple, quadruple mutants of these genes, the critical thresholds for PA contents can be defined for long-term breeding of low PA mutants without any pleiotropic effects. Considering that the mutant phenotypes obtained in this study were from greenhouse experiments, further field trials will be essential to confirm the present data.

Taken together, with the increasing knowledge on PA synthesis and PA transport mechanisms, new enzymes such as PHT transporters and sulfate transporters have been shown to have an effect on PA accumulation and could also be possible targets for low PA breeding (Figure 20). PA contents in seeds are highly correlated to available phosphorus levels in soil, which was shown in several studies, including one in oilseed rape (Möllers et al. 1999; Kopriva and Chu 2018; Lott et al. 2007). Breeding

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for low PA crops has also been encouraged regarding phosphorus efficiency in plants (Wang et al. 2017). In a review of Kopriva and Chu (2018), it was shown that manipulating phosphorus transporters effects the PA and Pi contents in leaves and seeds of the studied mutants. Furthermore, they stated, “P homeostasis and control of PA content are important targets in approaches for improvement of sustainability of agricultural production” (the text in “” is from Kopriva and Chu (2018)). Additionally, the depletion of phosphate resources reinforces to modulate plant’s phosphorus uptake systems for maximizing yields without any external fertilizer applications (Lott et al. 2007). Therefore, new breeding targets which are shown in Figure 20 could be studied further for deeper insights into phosphorus mechanisms in relation to PA accumulation in seeds of oilseed rape. Knockout of PHT transporters were shown to reduce PA contents in rice seeds. Therefore, this class of transporters might not only pave ways to modulate phosphate mobilization in plants but also might have effects on reducing PA contents as observed in rice (Yamaji et al. 2016; Wild et al. 2016; Liu et al. 2016; Zhang et al. 2015). *BnPHT1*, a phosphorus transporter involved in phosphate uptake has been exploited and was shown as a potential target for manipulating phosphate mechanisms in oilseed rape (Ren et al. 2014; Li et al. 2019; Huang et al. 2019). This might be a good candidate gene for future experiments to study PA contents by knockout analysis. As an outlook for future experiments, I propose genes as putative targets for low PA contents other than the ones obtained from this study in Figure 20. Moreover, genetic analysis of these genes might broaden our basic understanding of P efficiencies in oilseed rape, which will be indispensable for oilseed breeding. Finally, my study provided a framework of mutants that can be used in future to add more to our understanding of PA biosynthesis and transport in rapeseed and their impact on phosphate homeostasis in this crop.

Table 10: Overview of the mutants obtained from this study and their phytic acid (PA) reduction in seeds

Paralog	Mutant detection approach	Mutation type	Allele name	Mutant genotype observed	% phytic acid reduction in seeds [§]
<i>Bn.ITPK1.A10</i>	CRISPR-Cas9	1bp deletion	<i>A</i> ₁	<i>A</i> ₅ <i>A</i> ₅ <i>B</i> ₂ <i>B</i> ₂ <i>G</i> _h <i>G</i> _h	Nd
		A insertion	<i>A</i> ₅		
<i>Bn.ITPK1.C09</i>		A insertion	<i>B</i> ₂	<i>A</i> ₁ <i>A</i> ₁ <i>B</i> ₃ <i>B</i> ₃ <i>G</i> ₈ <i>G</i> ₈	27*
		T insertion	<i>B</i> ₃		
<i>Bn.ITPK4.C04b</i>		6bp deletion	<i>B</i> ₇	<i>A</i> ₁ <i>A</i> ₁ <i>B</i> ₇ <i>B</i> ₇ <i>G</i> ₈ <i>G</i> ₈	35*
		A insertion	<i>G</i> ₈		
<i>Bn.2-PGK2.A02</i>	TILLING by sequencing	Q 269 stop	<i>H</i> ₁	<i>H</i> ₁ <i>H</i> ₁ <i>I</i> ₁ <i>I</i> ₁	25*
		splice site	<i>H</i> ₂	<i>H</i> ₁ <i>H</i> ₁ <i>I</i> _e <i>I</i> _e	Nd
<i>Bn.2-PGK2.A10</i>		Q 283 stop	<i>I</i> ₁	<i>H</i> _e <i>H</i> _e <i>I</i> ₁ <i>I</i> ₁	Nd
		splice site	<i>I</i> ₂	<i>H</i> _e <i>H</i> _e <i>I</i> _e <i>I</i> _e	Nd
<i>Bn.MRP5.A10</i>	TILLING by sequencing	W 359 stop	<i>J</i> ₁	<i>J</i> ₁ <i>J</i> ₁ <i>K</i> ₁ <i>K</i> ₁	15*
				<i>J</i> ₁ <i>J</i> ₁ <i>K</i> _e <i>K</i> _e	5
<i>Bn.MRP5.C05</i>		Q 210 stop	<i>K</i> ₁	<i>J</i> _e <i>J</i> _e <i>K</i> ₁ <i>K</i> ₁	8
		W 144 stop	<i>K</i> ₂	<i>J</i> _e <i>J</i> _e <i>K</i> _e <i>K</i> _e	3
<i>Bn.IPK1,1.A09</i>	TILLING by sequencing	Q 234 stop	<i>L</i> ₁	<i>L</i> ₂ <i>L</i> ₂ <i>M</i> _e <i>M</i> _e	Nd
		W 379 stop	<i>L</i> ₂	<i>L</i> _e <i>L</i> _e <i>M</i> ₁ <i>M</i> ₁	Nd
<i>Bn.IPK1,1.C09</i>		Q 288 stop	<i>M</i> ₁	<i>L</i> _e <i>L</i> _e <i>M</i> _e <i>M</i> _e	Nd
<i>Bn.IPK2β.A09</i>	TILLING by sequencing	Q 26 stop	<i>N</i> ₁	<i>N</i> ₁ <i>N</i> ₁ <i>O</i> _e <i>O</i> _e	Nd
		S105F	<i>O</i> ₁	<i>N</i> _e <i>N</i> _e <i>O</i> ₂ <i>O</i> ₂	Nd
<i>Bn.IPK2β.C09</i>		Q 8 stop	<i>O</i> ₂	<i>N</i> _e <i>N</i> _e <i>O</i> _e <i>O</i> _e	Nd

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<i>Bn.MIK.C09</i>	TILLING by sequencing	Q 284 stop	P_1	$P_1P_1Q_1Q_1$	Nd
		D 69 N	Q_1	$P_1P_1Q_eQ_e$	Nd
<i>Bn.MIK.A10</i>	TILLING by sequencing	C110Y	Q_2	$P_eP_eQ_1Q_1$	Nd
		W 229 stop	R_1	$P_eP_eQ_eQ_e$	Nd
<i>Bn.MIPSI.A01</i>	TILLING by sequencing	W 142 stop	R_2	$R_2R_2S_1S_1$	2
		Q 164 stop	S_1	$R_2R_2S_eS_e$	14*
<i>Bn.MIPSI.C01</i>	TILLING by sequencing	Q 164 stop	S_1	$R_eR_eS_1S_1$	12*
				$R_eR_eS_eS_e$	9

Nd represents significant PA reduction was not detectable.

§ The reduction of PA is in comparison to non-mutated Haydn in case of CRISPR-Cas9 mutants and to Express 617 for EMS mutants.

* indicates significant reductions to the control plants. Statistical analysis was performed by using Anova at $p=0.05$.

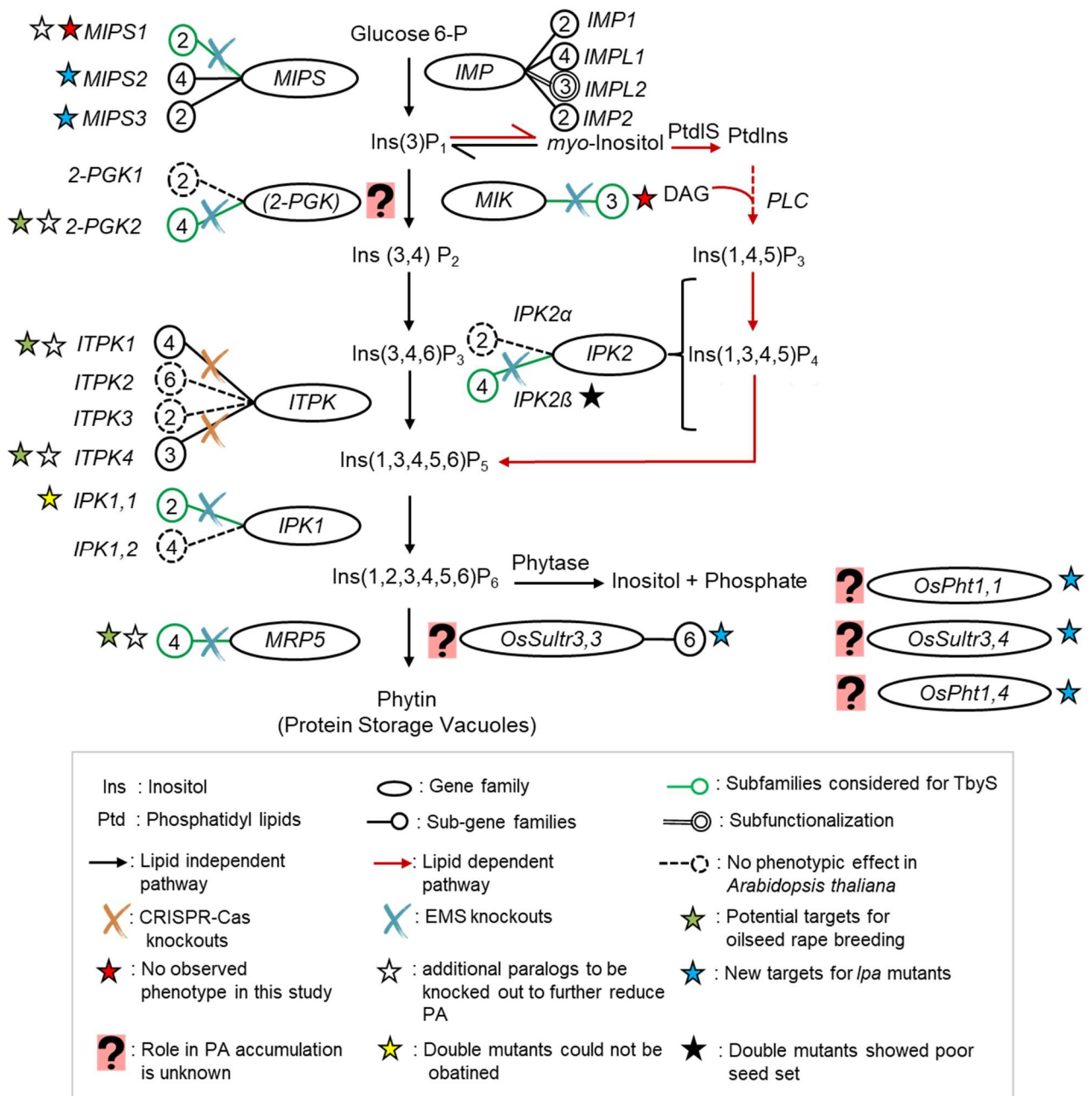


Figure 20: Overview of PA mutants obtained in this study with additional targets for future knockout analysis for identifying low PA mutants in oil seed rape (modified after Sashidhar et al. 2019). MIPS: *myo*-inositol phosphate synthase, MIK: *myo*-inositol kinase, IMP: *myo*-inositol mono phosphatase, 2-PGK: 2-phosphoglyceric acid kinase, ITPK: inositol tetrakis-phosphate kinase, IPK2β: inositol multi phosphate kinase, IPK: inositol pentakis-phosphate 2-kinase, MRP5: multi drug resistance protein, DAG: Diacyl glycerol, PLC: phospholipase C, PtdIS: phosphatidyl inositol synthase. The paralogue number of each subgene family is given in the circles.

6. Summary

Brassica napus L. is an important oil crop in the temperate regions where it is grown for oil production and feed for animals. Breeders aim to reduce the anti-nutritive compounds in rapeseed meal, which is causing serious problems in monogastric livestock. Since the natural variation of phytic acid (PA) contents in oilseed rape is low, the aim of this study was to reduce PA by a mutational approach. Using gene annotations in Arabidopsis, I identified seven genes for key enzymes and one transporter gene. The genes in oilseed rape were named as *BnMIPS*, *BnMIK*, *BnIMP*, *Bn2-PGK*, *BnITPK*, *BnIPK2 β* , *BnIPK1* and *BnMRP5*. Since many PA synthesis intermediates are also substrates for other important metabolic processes, a careful selection of genes was essential in order not to disturb physiological processes. Furthermore, inositol monophosphate is involved in different signaling processes and the basis for purine metabolism and therefore I have not analyzed *BnIMP* in this study.

To identify the total number of genes encoding the seven key enzymes, I performed *in silico* analysis and found in total 59 genes in oilseed rape that corresponded to eight Arabidopsis genes. To narrow down to the candidate genes for knockout approaches, mRNA expression analysis was performed in developing seed stages. For mutational approach gene editing by CRISPR-Cas9 and TILLING by sequencing (TbyS) in an EMS mutagenized rapeseed population were used. Paralog specific primers for all the genes were developed, which became later also very useful in distinguishing the paralog specific mutations. Finally, twelve highly expressed paralogs were selected for TbyS approach and more than 90% of the coding regions were analyzed to obtain putative knockout mutants. I could identify 1437 putative EMS mutations, out of which 49 were stop codon mutations that resulted in loss of function of the encoded proteins. All identified stop codon mutations along with splice site, missense mutations were confirmed by Sanger sequencing. Since, the *BnITPK* gene family comprised 14 paralogs, I have chosen these genes for the CRISPR-Cas9 approach and found knockout mutants in three paralogs of *BnITPK1* and *BnITPK4*. I could also show the inheritance of edited alleles from T₁ to T₄ generation, which was a valuable information for selecting the additional mutant alleles in later generations.

Single EMS mutants were combined to produce double mutants of the corresponding gene families. Upon phenotyping, single mutants did not show reduced PA contents supporting the idea of gene redundancy in polyploids. However, in double mutants of *bn2-pgk2* I observed a PA reduction of 25.4%. The reduction of *bnitpk* was even higher and showed 27.2% - 35.3% less PA in two T₄ plants having different mutants alleles. Finally, in double mutants of the PA transporter *BnMRP5* 15% less PA was observed. EMS knockout mutations in other genes need to be further investigated. In addition to PA reduction, *bnitpk* mutants also showed an increased Pi contents, which might be an added value for its use as animal feed. Finally, with this study promising candidates for breeding of low PA in oilseed rape could be identified, which will be beneficial for fish and poultry feeding as well as for human diets and could also serve as a substitute for soybean proteins in future.

7. Zusammenfassung

Brassica napus L. ist eine bedeutende Ölfrucht in gemäßigten Breiten und wird zur Ölproduktion und zur Nutzung als Tierfutter angebaut. Züchter bemühen sich um eine Verringerung von antinutritiven Substanzen wie Phytinsäure (PS) im Rapsextraktionsschrot, da diese ernsthafte Probleme bei der Fütterung von monogastrischen Nutztieren verursachen.

Weil die natürliche Variation des PS-Gehalts in Raps gering ist, war das Ziel dieser Studie die Verringerung von PS in einem Mutationsansatz. Aufgrund von Gen-Annotationen in Arabidopsis wählte ich sieben Gene, die für Schlüsselenzyme der PS-Biosynthese kodieren, und ein Transportergen aus. Die entsprechenden Rapsgene wurden als *BnMIPS*, *BnMIK*, *BnIMP*, *Bn2-PGK*, *BnITPK*, *BnIPK2 β* , *BnIPK1* und *BnMRP5* bezeichnet. Da viele Zwischenprodukte der PS-Synthese auch eine Rolle als Substrate in anderen Stoffwechselprozessen spielen, war ein sorgfältige Auswahl der Zielgene nötig, um keine Störungen in wesentlichen physiologischen Prozessen zu verursachen. Ich habe in dieser Studie das Gen *BnIMP* nicht verwendet, da sein Produkt Inositol-Monophosphat an verschiedenen Signalprozessen und im Purinstoffwechsel beteiligt ist.

Um die Gesamtzahl der Gene zu identifizieren, die für die sieben Schlüsselenzyme kodieren, führte ich eine *in silico* Analyse durch und fand insgesamt 59 Gene in Raps, die den sieben Arabidopsis-Genen entsprachen. Um die Zahl von Kandidatengenen für Knockout-Studien weiter einzugrenzen, wurde eine Genexpressionsanalyse durchgeführt. Für den Mutationsansatz wurde sowohl Genom-Editing mittels CRISPR-Cas9 sowie TILLING by Sequencing (TbyS) in einer EMS-mutagenisierten Rapspopulation verwendet. Für alle Gene wurden paralog-spezifische Primer entwickelt, die sich auch nachher bei der Unterscheidung paralog-spezifischer Mutanten als nützlich erwiesen. Abschließend wurden 12 hochexprimierte Paraloge für den TbyS-Ansatz ausgewählt und mehr als 90% ihrer kodierenden Regionen untersucht, um mutmaßliche Knockout-Mutanten zu erhalten. Ich konnte insgesamt 1437 putative EMS-Mutationen identifizieren. Für alle untersuchten Gene mit Ausnahme eines *BnMIK* Paralogs wurden Stopcodon-Mutationen gefunden, die einen Funktionsverlust des kodierten Proteins verursachen. Da die Genfamilie von *BnITPK* 14 Paraloge umfaßte, habe ich dafür einen CRISPR-Cas9-Ansatz gewählt und fand Knockout-Mutanten in drei Paralogen von *BnITPK1* und *BnITPK4*. Ich konnte die Vererbung der editierten Allele von der T₁ bis zur T₄ Generation aufzeigen und damit wertvolle Information zur Selektion zusätzlicher Mutantenallele in fortgeschrittenen Generationen gewinnen.

EMS Einzelmutanten wurden durch Kreuzung zu Doppelmutanten kombiniert. Bei der Phänotypisierung zeigten die Einzelmutanten keine reduzierten PS-Gehalte, was die Vorstellung redundanter Gene in Polyploiden unterstützt. In Doppelmutanten von *bn2-pgk2* beobachtete ich jedoch eine PS-Reduktion von 25,4% und in Doppelmutanten des PS-Transportergens *BnMRP5* 15% weniger PS. Weitere Untersuchungen von Doppelmutanten der übrigen EMS-Mutanten stehen noch aus. Die Reduktion in den CRISPR-Cas *bnitpk* Dreifachmutanten war noch größer und betrug 27,2%. Darüberhinaus zeigten sie auch eine Erhöhung der Gehalte an anorganischem Phosphat, was einen zusätzlichen wirtschaftlichen Nutzen für die Verwendung als Tierfutter bedeuten könnte.

Abschließend läßt sich sagen, daß in dieser Studie vielversprechende Kandidaten für die Züchtung von Niedrig-Phytinsäure-Raps gefunden wurden, sowohl für die Nutzung als Tierfutter für Geflügel und Fische als auch für die menschliche Ernährung. Dies könnte ein Ersatz für Sojaprotein in der Zukunft sein.

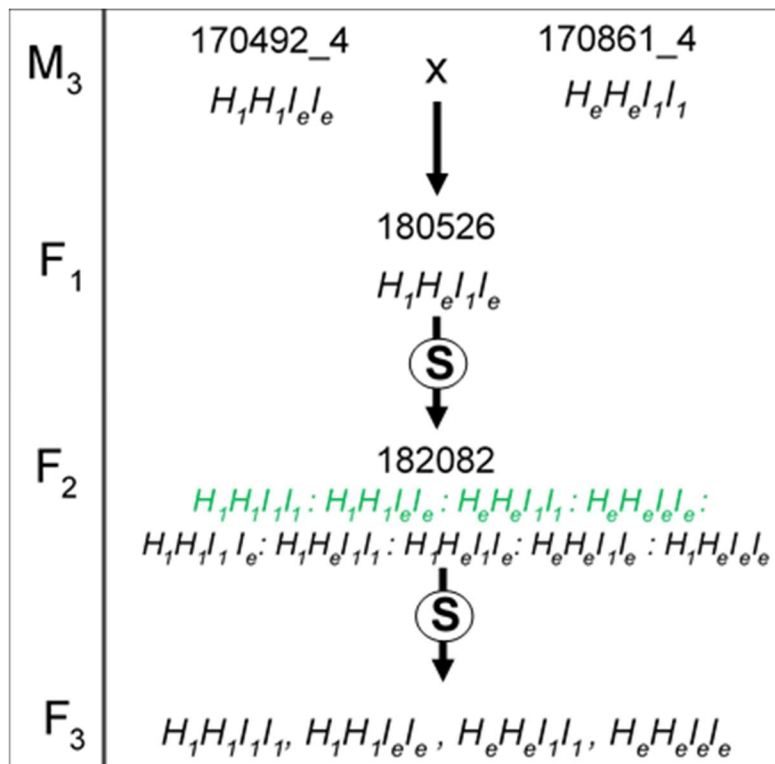


Figure S2: Crossing scheme for production of *bn2-pgk2* double mutants. The seed codes are written above each genotype. The alleles are explained in Table 2. The genotypes highlighted in green were selfed to produce F_3 seeds which were used for phenotyping; 'S': selfing

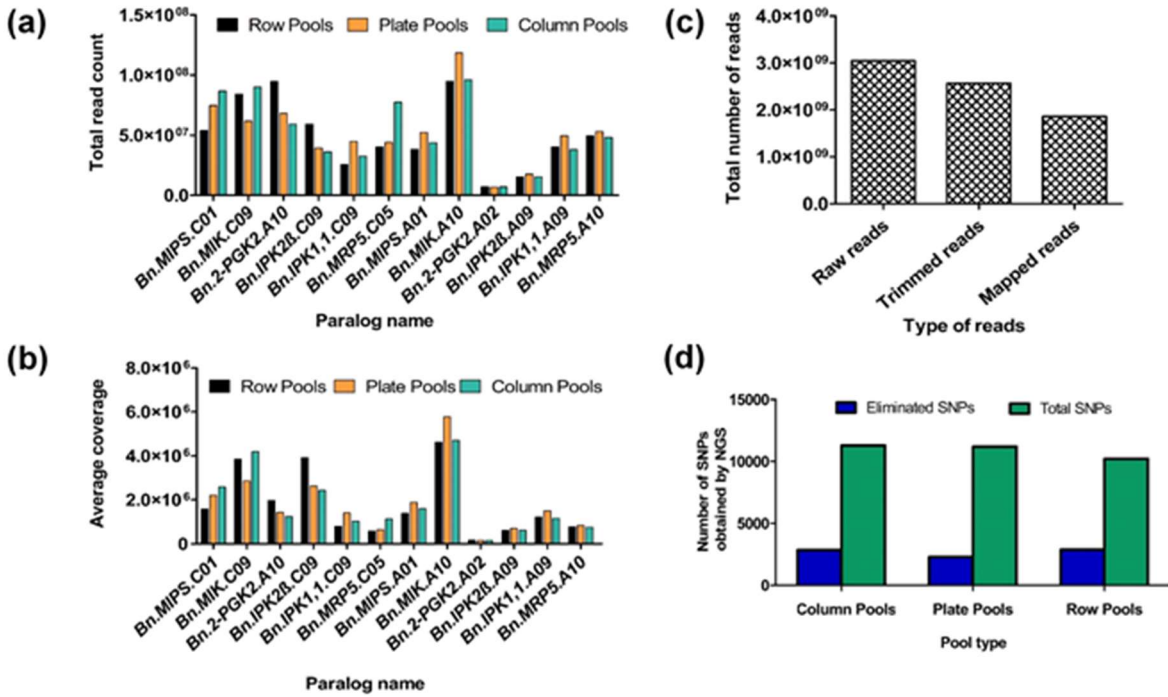


Figure S3: Overview of reads, coverage across the pools and eliminated reads. (a): Total number of reads mapped to the reference sequence Darmor-bzh for paralog library 1 and 2 across 80 row, 80 column and 80 plate pools. *Bn.2-PGK2.A02* showed a poor amplification, therefore, this paralog had lower number of reads compared to the other amplicons. (b): Obtained average coverage across the pools for all the 22 amplicons. The 'average coverage' was calculated with the CLC Genomic workbench 8.1 by considering the mean of all the calculated coverages in all the positions in all target regions. (c): Number of reads obtained at each stage of the data analysis. (d): The green bars show the total number of SNPs that were called during basic variant detection. Few SNPs were present in all the pools irrespective of stacks and these reads were eliminated during the analysis, shown in blue bars.

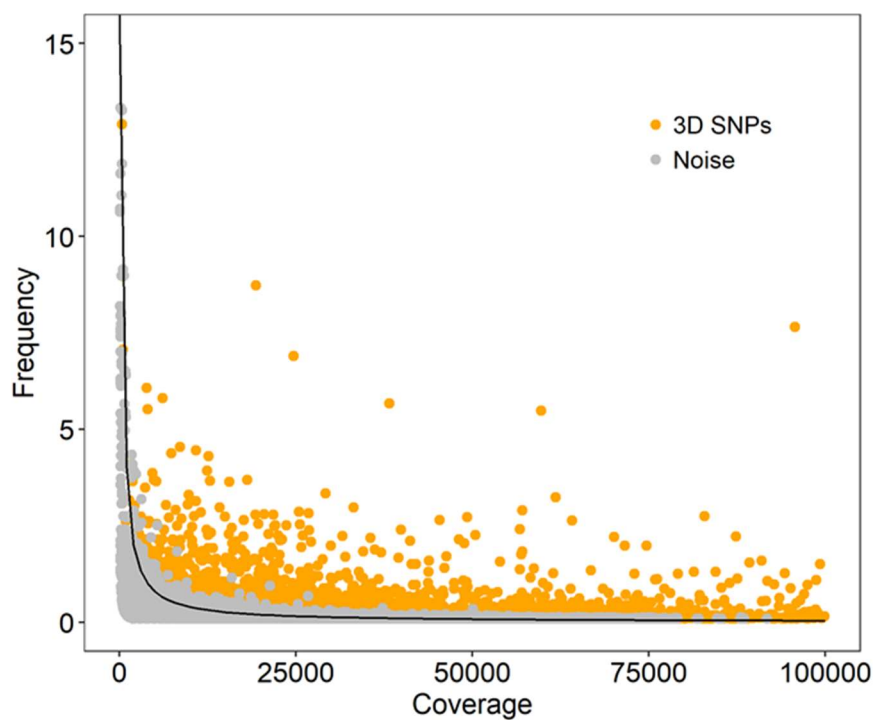


Figure S4: Threshold for selecting the putative three-dimensional (3D) SNPs. The black line shows the threshold value of 30, which was plotted using the formula $\text{frequency} = (\text{count} * 100) / \text{coverage}$. The 3D SNPs above the threshold were considered as putative SNPs for Sanger sequencing.

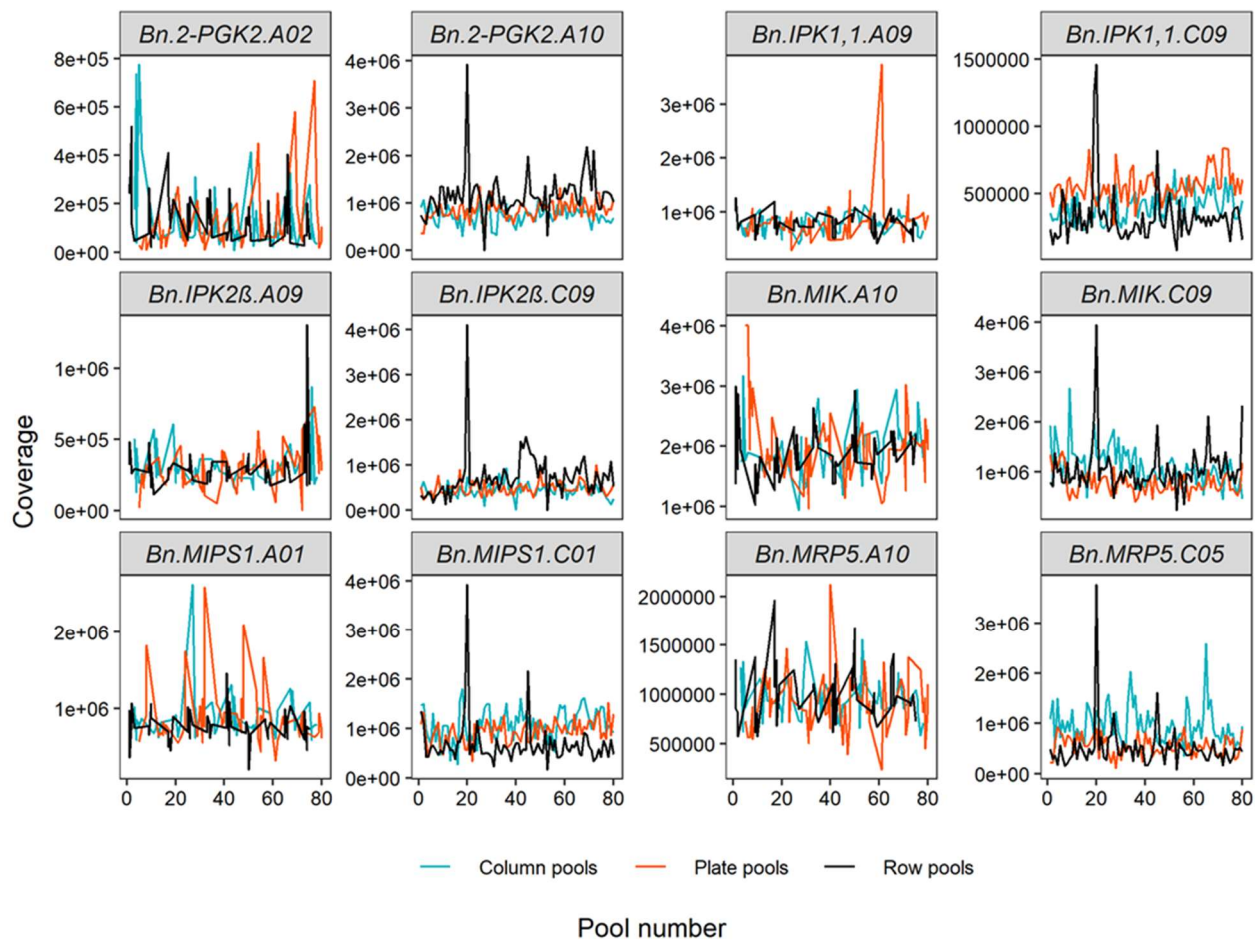


Figure S5: Coverage across the total number of pools. The obtained coverage per pool is given on the y axis. The distribution of coverage was unequal across the pools, suggesting that certain SNPs in the low coverage pools could be underrepresented.

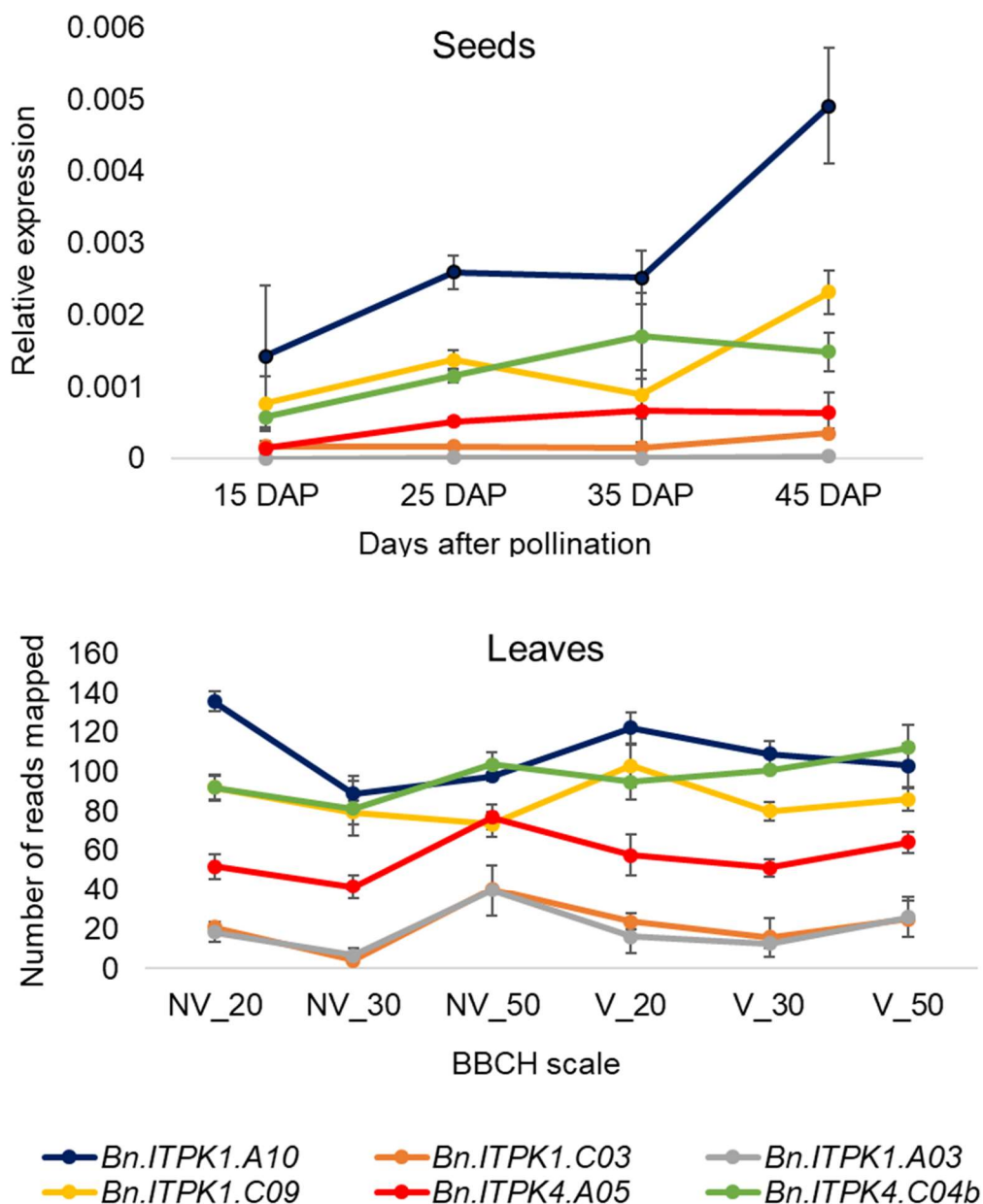


Figure S6: Expression analysis of *BnITPK* paralogs in seeds and leaves. Seed samples were collected from Express 617 at different time points. Normalization was done using *BnActin2* as a reference gene. Leaf data were taken from an RNAseq study Shah et al. (2018). Y-axis represents the number of reads mapped to the reference sequence *Darmor-bzh*. NV, non-vernalized; V, vernalized. The BBCH scale stands for different developmental stages of the plant (<https://www.canolacouncil.org/canola-encyclopedia/crop-development/growth-stages/>).

Appendix

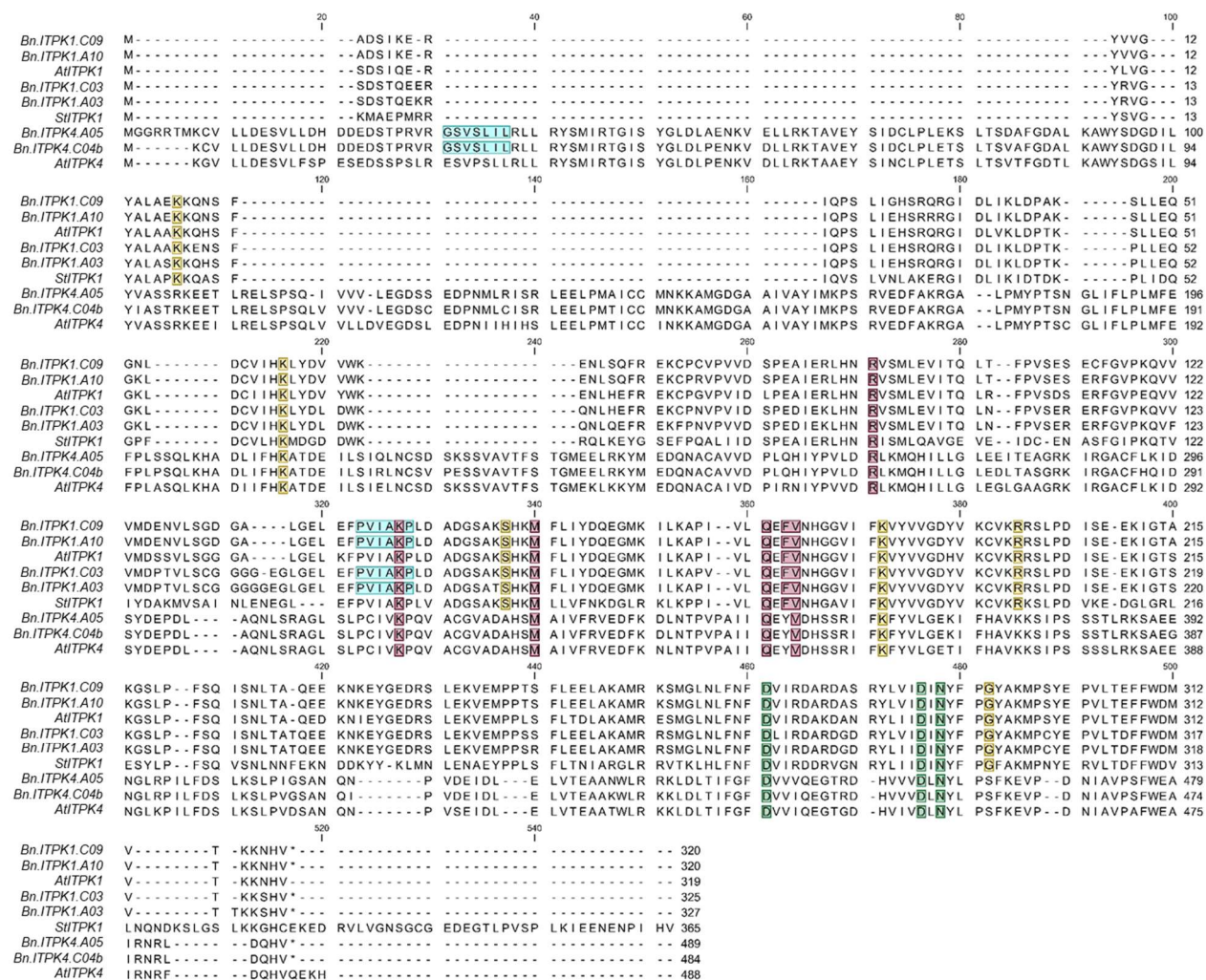


Figure S7: Protein alignment of ITPK showing the inositol, ATP and Mg²⁺ binding sites. Inositol binding residues are shown yellow; ATP binding sites are shown in maroon and Mg²⁺ residues are shown in green. The target sites of *BnITPK1* and *BnITPK4* are shown in blue, where target site 1 contains one of the ATP binding sites (PVIAKP).

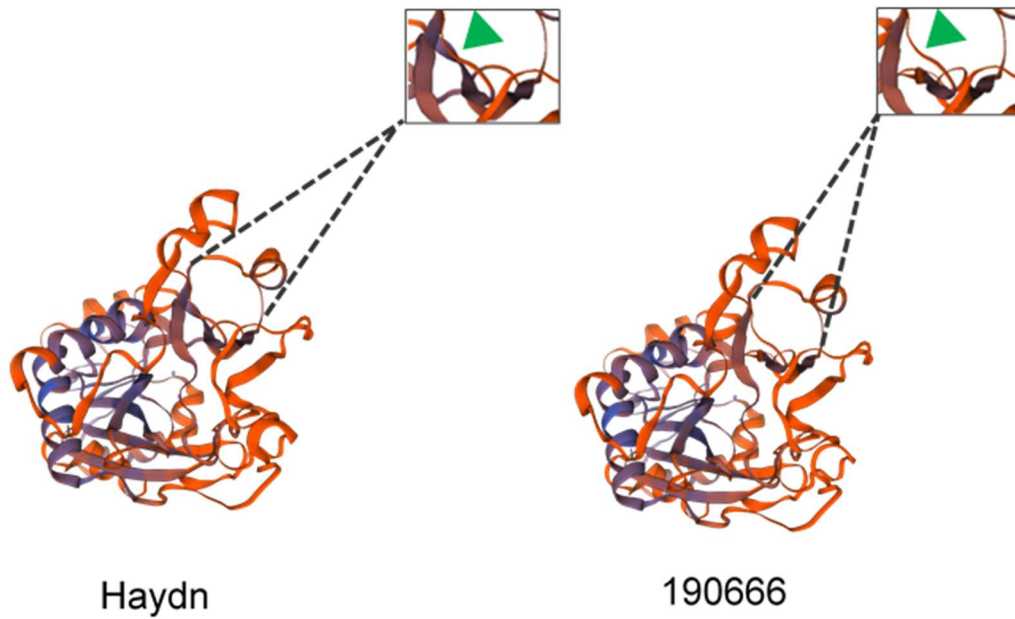


Figure S8: Predicted protein structure of *Bn.ITPK1.C09* in Haydn and in the mutant line 190666. Protein sequences from Haydn and from the triple mutant line 190666 carrying the B_5 allele were used as query in Expasy- SWISS-Model (<https://swissmodel.expasy.org/interactive>). The mutant has a different protein folding structure (marked with green triangles) at one of the ATP binding sites (amino acid 'K').

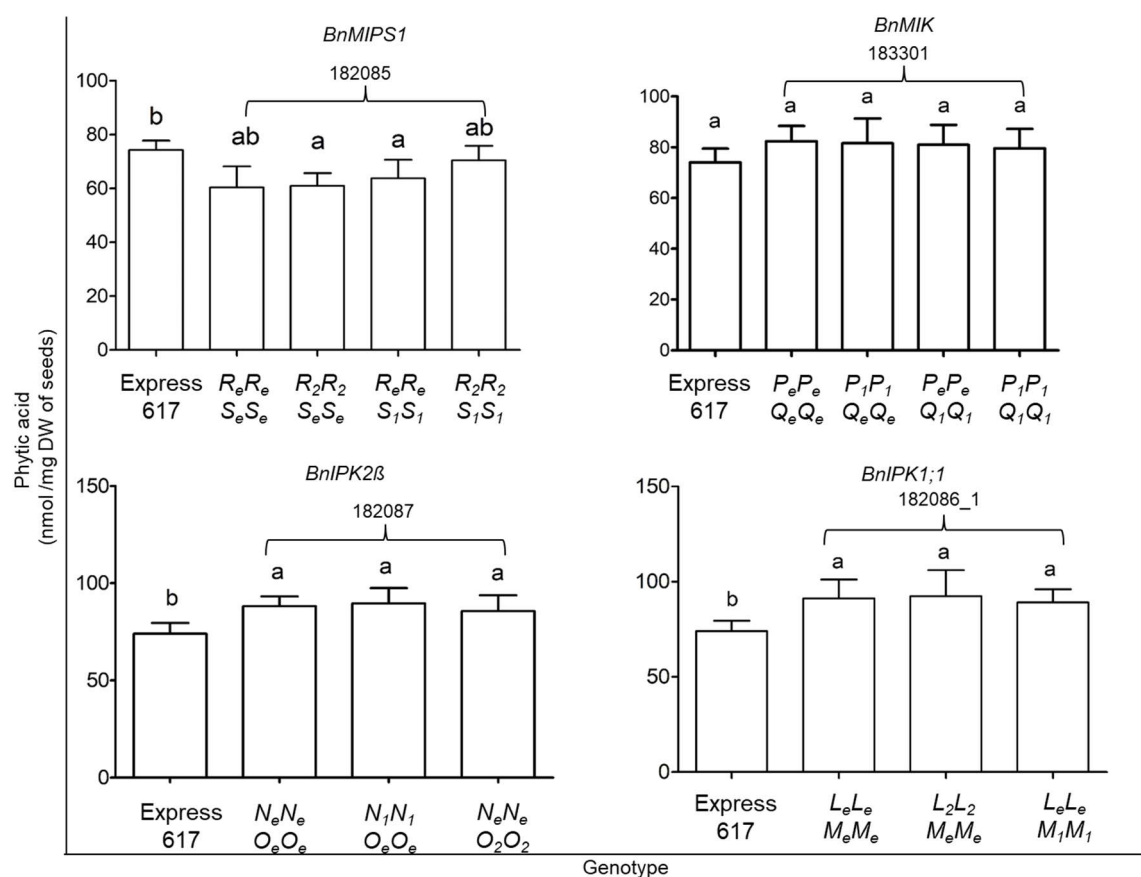


Figure S9: Phenotyping results of single and double mutants of F₂ segregating families of *BnMIPS1*, *BnMIK*, *BnIPK2β* and *BnIPK1*. Each gene is represented by allele codes indicating the genotype. For mutant allele codes see Table S3. Each bar represents five biological replicates along with three technical replicates. Anova was performed for statistical significance and grouping was done using Tuckey multiple comparison test. Same letters indicate no significant differences.

8.3. Supplementary Tables

Table S1: *In silico* analysis of phytic acid pathway genes in *Brassica napus* L.

Gene	Gene product	Enzyme product	TAIR/ Genoscope Id	Transcript size (bp)	Protein length (aa)	Exon intron structure	
						No. of exons	No. of introns
<i>MIPS</i>	<i>myo</i> -inositol 1-phosphate synthase	<i>myo</i> -inositol (3)P ₁	At4g39800	2235	512	9	8
			BnaC01g00680D	2542	511	8	7
			BnaA01g34710D	2330	451	7	6
			At2g22240	2039	511	7	6
			BnaA09g42760D	2058	511	7	6
			BnaC08g35240D	2045	511	7	6
			BnaC04g34650D	2060	511	7	6
			BnaA04g12720D	2056	511	7	6
			At5g10170	2624	511	9	8
			BnaC02g00460D	3195	511	10	9
BnaA02g00400D	2911	511	10	9			
<i>IMPL1</i>			At1g31190	2028	371	9	8
			BnaCnng21130D	1842	372	9	8
			BnaA09g25360D	1858	373	9	8
			At3g02870	1941	272	12	11
<i>IMP</i>	<i>myo</i> -inositol monophosphatase	<i>myo</i> -inositol	BnaA03g28170D	645	137	4	3
			BnaA05g33260D	1841	272	11	10
			BnaC03g33220D	1107	164	5	4
			BnaCnng06030D	1784	272	12	11
<i>IMPL2</i>			At4g39120	2411	376	9	8
			BnaC03g60380D	2348	347	9	8
			BnaA08g16710D	2293	342	9	8

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			BnaA01g05350D	1061	154	5	4
			At4g05090	2384	398	9	8
<i>IMP2</i>			BnaA09g19960D	2074	389	8	7
			BnaC09g22270D	2125	389	8	7
			At5g58730	1698	353	2	1
<i>MIK</i>	<i>myo</i> -inositol kinase	<i>myo</i> -inositol (3)P ₁	BnaA10g29530D	1641	344	2	1
			BnaC09g34030D	1556	339	2	1
			BnaAnng17330D	2116	214	4	3
			At5g07370	861	286	1	0
<i>IPK2α</i>			BnaC09g48570D	852	284	1	0
			BnaA10g23820D	852	284	1	0
	Inositol (1,4,5) P _{3_3/6} kinase	inositol (1,4,5)P ₃	At5g61760	903	301	1	0
<i>IPK2β</i>			BnaA09g05790D	861	287	1	0
			BnaC09g05390D	858	286	1	0
			BnaA06g31750D	846	282	1	0
			BnaCnng33290D	846	282	1	0
			At5g16760	960	320	1	0
<i>ITPK1</i>			BnaA10g17710D	960	320	1	0
			BnaC09g41080D	960	320	1	0
			BnaC03g07940D	975	325	1	0
			BnaA03g06170D	981	327	1	0
	Inositol (1,3,4) P _{3_5/6} kinase	inositol (1,3,4,5,6)P ₅	At4g33770	2654	392	9	8
			BnaAnng34680D	2454	388	9	8
<i>ITPK2</i>			BnaC01g04480D	2456	385	9	8
			BnaC03g66400D	2539	347	10	9
			BnaC07g44490D	2391	395	9	8
			BnaA03g50630D	2316	386	9	8
			BnaA01g03220D	969	218	5	4

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<i>ITPK3</i>			At4g08170	3294	354	10	9
			BnaCnng47190D	1964	359	9	8
			BnaA03g38640D	1451	310	8	7
<i>ITPK4</i>			At2g43980	3168	489	12	11
			BnaA05g03660D	2634	489	12	11
			BnaC04g03240D	2455	458	12	11
			BnaC04g03250D	2751	484	12	11
<i>IPK1</i>	Inositol pentakis-phosphate 2-kinase	inositol (1,2,3,4,5,6)P ₆	At5g42810	2265	451	7	6
			BnaA09g15720D	2072	451	7	6
			BnaC09g17180D	2059	448	7	6
			At5g59900	2721	907	1	0
			BnaA10g12860D	2697	506	4	3
			BnaC09g35270D	2688	504	4	3
			BnaAnng24160D	528	81	4	3
BnaC04g32880D	785	111	6	5			
<i>MRP5</i>	multi-drug resistant protein (transporter)		At1g04120	6474	1514	12	11
			BnaA10g02400D	5284	1509	9	8
			BnaC05g02300D	5278	1510	9	8
			BnaA09g10230D	4168	1071	13	12
			BnaC09g10390D	5399	1436	12	11
<i>(2-PGK)</i>	2-phosphoglycerate	2,3- bisphosphoglycerate or inositol bisphosphate	At3g45090	4163	717	8	7
			BnaA06g18580D	3386	715	7	6
			BnaC03g74690D	3530	718	7	6
			At5g60760	4017	738	8	7
			BnaA10g13560D	3393	726	7	6
			BnaC09g35980D	3619	706	8	7
			BnaA02g06110D	3359	704	8	7
BnaAnng35980D	2593	544	6	5			

Appendix

Table S2: Primers used in this study for TILLING by Sequencing (TbyS).

Amplicon Number	Paralog	Primer combination	Primer Sequence [§]	Temp (°C)	Amplicon length (bp)	ORF coverage (%)	Predicted stop codon sites	Paralog set
1	<i>Bn.MIPS.A01</i>	NS_P112+236	GCTTATCAGTGGATCGTGAAGGCT + GCGTGGAAGGAGAGATCTCG	62.0	1225	88.60	26	
2	<i>Bn.MIPS.A01</i>	NS_P255+115	CACGATGGAGAATCTTATGAACGCC + CTCTTCTCGGATTTGAACTGAATCC	62.3	1049		18	
3	<i>Bn.MIK.A10</i>	NS_P187+143	GCCTAACCCACCTCATCGAGTC + CTTCTCTGCACCTCATCCTTTAGTC	63.0	1479	97.77	7	
4	<i>Bn.MIK.A10</i>	NS_P216+217	GATTTGACGATGCTTCAGGTCCG + AAACTCTGCATTGGCGGCTAT	62.0	955		7	
5	<i>Bn.2-PGK2.A02</i>	NS_P248+205	TCTCGCTGAATATAAGTACGTCTGC + CGGATGCACCATTCCATTGCC	61.5	1463	81.62	15	
6	<i>Bn.2-PGK2.A02</i>	NS_P214+215	GCATCTAATGGCTCTTCTCAAGAA + GGCACCCAAGACAATCGTAGCT	61.0	1074		12	1
7	<i>Bn.IPK2β.A09</i>	NS_P159+167	GTCGTGAATATTTGTGTACATCGGG + TATTTGAAGGGACACACACAGTCAG	61.3	1489	100.00	12	
8	<i>Bn.IPK1,1.A09</i>	NS_P179+194	GATTCGTATCCAGAAGGCTCCC + CTAATTTATCCAGCTTCTGAACTGCC	62.5	1484	93.21	14	
9	<i>Bn.IPK1,1.A09</i>	NS_P241+242	CCTTAAAGAACAGGGTCGGAACAAC + TGAGACAATCCATACATGACTTCGG	62.0	1015		11	
10	<i>Bn.MRP5.A10</i>	NS_P208+209	TGGCTTGGGCGAATCCTTAG + GAATGTTCTTCAAGAGGGTCAAGACTC	62.0	1434	60.12	36	
11	<i>Bn.MRP5.A10</i>	NS_P252+253	ACGTGCGATTCTCAAGTCCCTT + GGATCGTCTAGTAAATAAATGTCAACCG	61.0	1509		17	
12	<i>Bn.MIPS.C01</i>	NS_P116+234	CGAGAACGTCAACGGTGCTAAC + CCCTAGGTGATTGTAGCTCGCA	62.0	1467	81.25	34	2

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13	<i>Bn.MIPS.C01</i>	NS_P256+237	GACACTATGGAGAATCTTATGAACGCT + TTCTCGGATTTGAACTGAATCCTGG	61.9	1049		18
14	<i>Bn.MIK.C09</i>	NS_P188+249	GCAGCAAACGCCTAATTCGACG + GTTGTGGCTGCTTGAGAGACTG	62.1	1428	90.28	8
15	<i>Bn.MIK.C09</i>	NS_P218+219	GGGTTTGGACGTTCCCGATTCA + TCAAATTTAAAACGGAATTTGCTAGCG	62.0	1034		5
16	<i>Bn.2-PGK2.A10</i>	NS_P45+172	TGAGATGTATAAAAAGCGCACACCA + CGGCCAAGTTCTTGCAAAAAGTC	60.0	1360	89.07	22
17	<i>Bn.2-PGK2.A10</i>	NS_P212+293	GGAACCAGTGTACAGCTAACTTGC + GCACCCAAGAGAATCGGACCA	62.7	1154		15
18	<i>Bn.IPK2β.C09</i>	NS_P168+207	CTCATTGTTTGATTCTCTCTCTGTC + CAGCGGTTGTCTGAGCTTTC	59.8	1259	98.21	12
19	<i>Bn.IPK1,1.C09</i>	NS_P257+258	CCTGAATCATAACAGTATCAGTTGCT+ GCGAAGGAGGTGCCAATGTC	61.4	1491	95.47	13
20	<i>Bn.IPK1,1.C09</i>	NS_P261+292	GCACATAACTTGTACCTTAAAGAAGAA + TTTGGGATAGCCGACTTGTTCTTG	60.0	975		14
21	<i>Bn.MRP5.C05</i>	NS_P210+211	CTTGGGCGAATCCTCAAGCT + GAATGTTCTTCAAGAGGGTCAAGACTA	61.0	1414	60.00	27
22	<i>Bn.MRP5.C05</i>	NS_P250+251	CGTCGTTATAGCTTGTAGAATCCACC + CTCTTCTGAACCTTAGCCGAC	63.0	1458		59

[§]Green letters indicate variations among the paralogs and red letters indicate modified bases to increase the specificity of the primer (see Fig.S1).

Appendix

Table S3: M₃ generation genotyping to select homozygous mutant lines.

Paralog	Mutation type	Allele name	M ₂ Zygosity	Seed code	Germination rate	M ₃ Zygosity (Total number of plants) [§]		
						Homozygous (mutant allele)	Heterozygous	Homozygous (Express allele)
<i>Bn.2-PGK2.A02</i>	Q269* splice site	<i>H</i> ₁	Heterozygous	170561	50.00%	4	7	4
		<i>H</i> ₂	Heterozygous	170562	20.00%	0	2	4
<i>Bn.2-PGK2.A10</i>	Q283* splice site	<i>I</i> ₁	Heterozygous	170492	46.70%	3	6	5
		<i>I</i> ₂	Heterozygous	170495	26.70%	0	0	8
<i>Bn.MRP5.A10</i>	W359*	<i>J</i> ₁	Homozygous	170482	80.00%	8	0	0
<i>Bn.MRP5.C05</i>	Q210*	<i>K</i> ₁	Heterozygous	170481	66.60%	4	11	5
	W144*	<i>K</i> ₂	Heterozygous	170483	16.70%	1	4	0
<i>Bn.IPK1,1.A09</i>	Q234*	<i>L</i> ₁	Heterozygous	170484	26.70%	1	4	3
	W379*	<i>L</i> ₂	Heterozygous	170485	40.00%	1	7	4
<i>Bn.IPK1,1.C09</i>	Q288*	<i>M</i> ₁	Heterozygous	170858	46.70%	2	9	3
<i>Bn.IPK2β.A09</i>	Q26*	<i>N</i> ₁	Heterozygous	170487	30.00%	1	8	0
<i>Bn.IPK2β.C09</i>	S105F	<i>O</i> ₁	Heterozygous	170863	66.70%	5	8	7
	Q8*	<i>O</i> ₂	Homozygous	170860	46.70%	14	0	0
<i>Bn.MIK.C09</i>	Q284*	<i>P</i> ₁	Heterozygous	170489	46.70%	2	6	6
<i>Bn.MIK.A10</i>	D69N	<i>Q</i> ₁	Heterozygous	170490	33.30%	3	5	2
	C110Y	<i>Q</i> ₂	Homozygous	170491	43.30%	13	0	0
<i>Bn.MIPS1.A01</i>	W229*	<i>R</i> ₁	Heterozygous	170497	30.00%	5	4	0
	W142*	<i>R</i> ₂	Heterozygous	170496	30.00%	0	7	2
<i>Bn.MIPS1.C01</i>	Q164*	<i>S</i> ₁	Homozygous	170857	50.00%	15	0	0

[§]A maximum of 30 seeds was available from each M₃ population. Stop codons are shown in *

Table S4: Evaluation of plant height and thousand-kernel weights (TKW) of *bn2-pgk2* mutants.

Genotype	TKW [g]	Plant height [cm]
<i>H₁H₁I₁I₁</i>	2.56 ± 0.2 ^a	90.2 ± 4.7 ^a
<i>H₁H₁I₁e_e</i>	1.92 ± 0.3 ^a	95.4 ± 2.8 ^a
<i>H_eH_eI₁I₁</i>	2.61 ± 0.3 ^a	92.8 ± 5.4 ^a
<i>H_eH_eI₁e_e</i>	2.49 ± 0.2 ^a	95.8 ± 4.0 ^a
Express 617	3.30 ± 0.2 ^a	102 ± 3.5 ^a

^a Mutants were statistically insignificant to the controls. Anova was performed using Tukey's Multiple Comparison Test; $p=0.05$. Standard deviation (SD) is indicated as ±

Table S5: Total number of two-dimensional (2D) mutations obtained by TILLING by Sequencing (TbyS)

Paralog	Number of mutations						Total number	Mutation frequency (F) (1/kb) [§]
	Nonsense	Missense	Silent	Intronic	Splice site	outside coding sequence		
<i>Bn.MIPS.A01</i>	2	39	17	18	2	0	78	1/34
<i>Bn.MIPS.C01</i>	2	44	25	7	1	0	79	1/32
<i>Bn.MIK.A10</i>	0	54	37	13	0	0	104	1/20
<i>Bn.MIK.C09</i>	3	28	17	28	1	4	81	1/25
<i>Bn.2-PGK2.A02</i>	0	11	7	6	0	0	24	1/120
<i>Bn.2-PGK2.A10</i>	5	82	47	15	3	0	152	1/19
<i>Bn.IPK1,1.A09</i>	2	28	17	19	0	0	66	1/36
<i>Bn.IPK1,1.C09</i>	1	18	10	2	0	0	31	1/48
<i>Bn.IPK2β.A09</i>	4	59	45	3	1	0	112	1/16
<i>Bn.IPK2β.C09</i>	1	47	40	0	0	0	88	1/39
<i>Bn.MRP5.A10</i>	5	132	68	13	2	0	220	1/16
<i>Bn.MRP5.C05</i>	4	45	22	9	0	0	80	1/42
Total	29	587	352	133	10	4	1115	1/36

[§] F is calculated as $1 / [\text{number of } M_1 \text{ plants} * \text{amplicon length}] / (\text{number of mutations} * 1000)$

Table S6: Primer sequences used in this study for TILLING by sequencing

Gene family	Gene/target	Gene nomenclature	Primer name	Primer type	Primer sequence*	Temp (°C)	Amplicon length (bp)
<i>BnITPK1</i>	BnaA10g17710D	<i>Bn.ITPK1.A10</i>	NS_P138	Forward	GTTAACGTTTCCTGTATCGGAA GGT	60	259
			NS_P147	Reverse	GTAGACCTTAAAGATCACACCG TCA		
	BnaC09g41080D	<i>Bn.ITPK1.C09</i>	NS_P222	Forward	GCATGAATGTTAACCTAACTTC CTC	60	599
			NS_P274	Reverse	CGTGATTACAAACTCCTG GAG		
	BnaC03g07940D	<i>Bn.ITPK1.C03</i>	NS_P290	Forward	GTGCCCTAACGTCCCTG CCA	63	295
			NS_P275	Reverse	CTTGAGTATCTTCATCCCTTCT CGG		
	BnaA03g06170D	<i>Bn.ITPK1.A03</i>	NS_P226	Forward	GCTCTCGCATCCAAGAAAC GAC	61	753
			NS_P146	Reverse	CTTCGCCAACTCCTCCAAG TAC		
<i>BnITPK4</i>	BnaA05g03660D	<i>Bn.ITPK4.A05</i>	NS_P277	Forward	TCGATCACGATGATGAAGAT GCA	60	428
			NS_P135	Reverse	CTTCTCCAAAGGTAAGCAATCT CTG		
	BnaC04g03250D	<i>Bn.ITPK4.C04b</i>	NS_P279	Forward	AATGTGTTTTACTCGACGAGT GTG	60	321
			NS_P278	Reverse	TGTTTTCCGGAAGATCAAG GCC		
Chimera	-	pChimera_f	Forward	AGTGAGCGCGACGTAATACG	58	702	
		pChimera_r	Reverse	GTTAATGCAGCTGGCACGAC			
Cas9	<i>SpCas9</i>	pCas9_f	Forward	CAGTCTTTCACCTCTCTTTGG	58	1500	
		pCas9_r	Reverse	CCATCTTTGGGACCACTGTC			
Spectinomycin	-	Spec_for	Forward	TGCCGACTACCTTGGTGATC	58	910	
		Spec_rev	Reverse	GTACAGTCTATGCCTCGGGC			
<i>BnACTIN2</i>	XM_013786210.1	-	Act1	Forward	TCTGGTGATGGTGTGTCTCA	60	141
	XM_013888645.1	-					
	XM_013893921.1	-	Act2	Reverse	GGTGAACATGTACCCTCTCTCG		
	XM_013829442.1	-					
	XM_013830703.1	-					

*Green letters indicate SNPs between the paralogs, red letters indicate the modified base to increase the primer specificity and blue letters indicate SNPs between reference sequence Darmor-*bzh* and Haydn.

Appendix

Table S7: Editing status in regenerated T₁ plants

Seed code	T ₁ plants	Genotype [§]
180906	<i>itpk_C1.1</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
180624	<i>itpk_C2.1</i>	<i>A_hB_hC_hD_hE_hG_{2,7,8}</i>
180625	<i>itpk_C2.2</i>	<i>A_hB_{ch}C_hD_hE_hG_{ch}</i>
180626	<i>itpk_C2.3</i>	<i>A_hB_hC_hD_hE_hG_h</i>
180616	<i>itpk_C3.1</i>	<i>A_hB_hC_hD_hE_hG_h</i>
180617	<i>itpk_C3.2</i>	<i>A_hB_hC_hD_hE_hG_h</i>
180618	<i>itpk_C3.3</i>	<i>A_hB_hC_hD_hE_hG_h</i>
180619	<i>itpk_C3.4</i>	<i>A_hB_hC_hD_hE_hG_h</i>
180620	<i>itpk_C3.5</i>	<i>A_{1,2,3,4}B_{1,2,3,4}C_hD_hE_{1,2,3,4}G_{1,2,3,4,5,6}</i>
180621	<i>itpk_C3.6</i>	<i>A_{ch}B_{ch}C_hD_hE_{ch}G_{ch}</i>
180622	<i>itpk_C3.7</i>	<i>A_{ch}B_{ch}C_hD_hE_{ch}G_{ch}</i>
180623	<i>itpk_C3.8</i>	<i>A_{ch}B_{ch}C_hD_hE_{ch}G_{ch}</i>
180907	<i>itpk_C4.1</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
190662	<i>itpk_C4.2</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
180908	<i>itpk_C5.1</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
180627	<i>itpk_C6.1</i>	<i>A_hB_{1,2,3}C_hD_hE_hG_h</i>
180628	<i>itpk_C6.2</i>	<i>A_hB_{ch}C_hD_hE_hG_h</i>
180629	<i>itpk_C6.3</i>	<i>A_hB_{ch}C_hD_hE_hG_h</i>
180630	<i>itpk_C6.4</i>	<i>A_hB_{ch}C_hD_hE_hG_h</i>
180909	<i>itpk_C7.1</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
190663	<i>itpk_C8.1</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
190664	<i>itpk_C9.1</i>	<i>A_{ch}B_hC_hD_hE_hG_h</i>
190844	<i>itpk_C10.1</i>	<i>A_hB_{ch}C_hD_hE_hG_h</i>

[§] *ch* indicates that the plants had more than two alleles, indicating the chimeric nature of the plant. *h* indicates the donor allele from cv. Haydn. The genotypes indicated in bold were used for further propagation of T₂ generation and only these plant DNAs were cloned into plasmids for analyzing the type of mutant alleles. See Table S8 for allele nomenclature codes.

Appendix

Table S8: Gene acronyms for four *BnITPK* genes used in this study. All mutant alleles are listed with their effects on their protein sequence

Gene	Acronym	Allele	Type of mutation	Mutation position (upstream of PAM site)	Type of change in protein structure*
<i>Bn.ITPK1.A10</i>	<i>A</i>	<i>A_h</i>	Wild type (Haydn)	-	no change
		<i>A₁</i>	1bp deletion	3	aa change at 146 and premature stop at 160
		<i>A₂</i>	2bp deletion	3,4	aa change at 146 and premature stop at 148
		<i>A₃</i>	3bp deletion	3	P146T
		<i>A₄</i>	GTTT insertion	from 3 rd base	aa change at 146 and premature stop at 150
		<i>A₅</i>	A insertion	3	premature stop codon at 150
		<i>A₆</i>	T insertion	3	premature stop codon at 150
		<i>A₇</i>	10bp deletion	from 3 rd base	premature stop codon at 156
<i>Bn.ITPK1.C09</i>	<i>B</i>	<i>B_h</i>	Wild type	-	no change
		<i>B₁</i>	C insertion	5	aa change at 146 and premature stop at 150
		<i>B₂</i>	A insertion	4	aa change at 146 and premature stop at 150
		<i>B₃</i>	T insertion	4	aa change at 146 and premature stop at 150
		<i>B₄</i>	2bp deletion	3,4	aa change at 146 and premature stop at 148
		<i>B₅</i>	GTT insertion	5	aa change at 146 and premature stop at 150
		<i>B₆</i>	G insertion	3	aa change at 146 and premature stop at 148
		<i>B₇</i>	6bp deletion	from 3 rd base	loss of two aa (KP at 144, 145)
<i>B₈</i>	1bp deletion	3	aa change at 146 and premature stop at 150		
<i>Bn.ITPK4.A05</i>	<i>E</i>	<i>E_h</i>	Wild type (Haydn)	-	no change

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	<i>E</i> ₁	A insertion	3	aa change at 36 and premature stop codon at 138	
	<i>E</i> ₂	AA insertion	3,4	premature stop codon at 44	
	<i>E</i> ₃	T insertion	3	aa change at 35 and premature at 149 position	
	<i>E</i> ₄	T insertion	6	aa change at 35 and premature at 149 position	
	<i>G</i> _h	Wild type (Haydn)	-	no change	
	<i>G</i> ₁	AA insertion	3,4	aa change at 31 and premature stop codon at 38	
	<i>G</i> ₂	T insertion	3	aa change at 31 and premature stop codon at 38	
	<i>G</i> ₃	TT insertion	3,4	aa change at 31 and premature stop codon at 38	
<i>Bn.ITPK4.C04b</i>	<i>G</i>	<i>G</i> ₄	T insertion	5	aa change at 31 and premature stop codon at 38
		<i>G</i> ₅	4bp deletion	from 4th base	aa change at 29 and premature stop at 36
		<i>G</i> ₆	3bp deletion	from 3rd base	loss of one aa (I at 29)
		<i>G</i> ₇	6bp deletion	from 4th base	loss of two aa (LI at 28,29)
		<i>G</i> ₈	A insertion	4	aa change at 31 and premature stop codon at 38

*aa stands for amino acids and deletion of respective amino acids are shown by single letter amino acid code

Appendix

Table S9: Segregation analysis in T₃ generations.

seed code	No. of plants	Transgene genotype		χ^2 (3:1)	<i>ITPK</i> genotypes		
		Transgenic	Non-transgenic				
180705	10	8	2	0.13	<i>A₅A₅B₂B₂E_hE_hG_hG_h</i> 2	<i>A₅A₅B₂B_hE_hE_hG_hG_h</i> 5	<i>A₅A₅B_hB_hE_hE_hG_hG_h</i> 3
180716	10	7	3	0.13	<i>A₁A₁B₃B₃E_hE_hG₈G₈</i> 4	<i>A₁A₁B₃B₇E_hE_hG₈G₈</i> 2	<i>A₅A₅B₇B₇E_hE_hG_hG₈</i> 4
180663	10	9	1	1.2	<i>A_hA₇B_hB₃E_hE_hG_hG₂</i> 7	<i>A_hA₅B_hB₃E_hE_hG_hG_h</i> 1	<i>A_hA₆B_hB₃E_hE_hG_hG₂</i> 2

Three populations were genotyped with locus specific primer combinations. The significance of the chi-square test was validated at $p=0.05$.

Table S10: Overview of CRISPR-Cas9 experiments performed in this study

Gene family	Oligo name	Target site sequence	No. of hypocotyles used for transformation	No. of regenerated plants obtained	No. of transgenic plants obtained	No. of plants with editing
<i>BnMIPS1</i> [§]	NS_C1	ATTGGTGGGTTGGGGAGGAAACAA	-	-	-	-
	NS_C2	AAACTTGTTTCCTCCCCACCCAC				
<i>BnMIK</i>	NS_C3	ATTGTCATGGATGTTGAAGAGATG	677	11	-	-
	NS_C4	AAACCATCTCTTCAACATCCATGA				
<i>Bn2-PGK2</i>	NS_C5	ATTGAAGAAGACGGAAGCGGGAAT	652	8	-	-
	NS_C6	AAACATTCCCGCTTCCGTCTTCTT				
<i>BnMRP5</i>	NS_C13	ATTGGATTGCTAGTAACTGGTGGG (TS1)	640	17	6	-
	NS_C14	AAACTCCACCAGTTACTAGCAATC (TS1)	531	5	-	-
	NS_C15	ATTGTAGATCAGCTCTAACGGCTA (TS2)				
	NS_C16	AAACTAGCCGTTAGAGCTGATCTA (TS2)				
<i>BnIPK1</i>	NS_C11	ATTGAGGAGAAAGATGCATCCGAT	475	15	-	-
	NS_C12	AAACATCGGATGCATCTTTCTCCT				
<i>Os-Sultr3-3</i> [§]	NS_C17	ATTGTTGTTTCCTATATTCACATG	-	-	-	-
	NS_C18	AAACCATGTGAATATAGGAAACAA				

[§] Rapeseed hypocotyl transformations were not performed but CRISPR-Cas9 vectors are available for these genes. All the experiments were performed using cv. Haydn.

Table S11: Seed material used for 15K SNP chip assay for selecting higher peace background from EMS mutants of *BnMRP5* and *Bn2-PGK2* genes

Kiel code	Gene / Paralog name	crossing type	Generation	Expected segregation
180858	<i>Bn.MRP5.A10</i>	F ₁ x Peace	BC ₁	$J_1J_e:J_eJ_e$ (1:1)
180837	<i>Bn.MRP5.C05</i>	F ₁ x Peace	BC ₁	$K_1K_e:K_eK_e$ (1:1)
180859	<i>BnMRP5</i>	F ₁ x F ₁	F ₁	$J_1J_eK_eK_e: J_eJ_e K_eK_e: J_1J_e K_1K_e: J_eJ_e K_1K_e$ (1:1:1:1)
183295	<i>Bn.PGK2.A10</i>	F ₁ x Peace	BC ₁	$H_1H_e:H_eH_e$ (1:1)
183296	<i>Bn.PGK2.A02</i>	F ₁ x Peace	BC ₁	$I_1I_e:I_eI_e$ (1:1)
183297	<i>BnPGK2</i>	F ₁ x F ₁	F ₁	$H_1H_eI_eI_e: H_eH_e I_eI_e: H_1H_e I_1I_e: H_eH_e I_1I_e$ (1:1:1:1)

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10. Supplementary data on CD/DVD

The following supplemental data are available on CD/DVD and can be distributed upon request (contact: Prof. Dr.Christian Jung, c.jung@plantbreeding.uni-kiel.de).

File name	Content	Format
List of primers.xlsx	List of primers developed during the study	xlsx, Microsoft Excel file
NGS SNPs identified by TbyS.csv	List of total number of NGS SNPs identified by TbyS	csv, comma separated values
Sanger sequencing of confirmed M ₂	Screenshots of Sanger sequencing results of NGS SNPs in M ₂ DNA samples	.docx
Seed material.xlsx	Seed material produced during this study	xlsx, Microsoft Excel file
PA and Pi measurements of all the genes.xlsx	Phytic acid and inorganic phosphorus measurements of all the genes	xlsx, Microsoft Excel file
Chromatograms	Chromatograms of HPLC measurements	.zip
Sequences	Annotated gene sequences of phytic acid genes	.fa

11. Curriculum Vitae and Publications

11.1. Curriculum Vitae

Personal data	
Name	Niharika Sashidhar
Date of birth	17. 11. 1990
Place of birth	Kurnool, Andhra Pradesh
Nationality	Indian
Education	
2016 - 2019	Christian-Albrechts-University of Kiel, Germany Doctoral candidate in Plant Breeding and Genetics Doctoral Thesis: Random and targeted (CRISPR-Cas) mutagenesis to create oilseed rape with reduced seed phytic acid content
2013 - 2016	Christian-Albrechts-University of Kiel, Germany M.Sc. in Agricultural Genomics Master's Thesis: Establishing a CRISPR/Cas9 system for genome editing in crops with a case study in <i>Arabidopsis thaliana</i> .
2012 – 2013	TATA Consultancy Services Pvt Ltd. (MNC) Designation: Assistance systems Engineer Domain: Java
2008 - 2012	GITAM University, India B.Tech in Biotechnology Bachelor's Thesis: Bioreactor design and tissue culture for extraction of anti-cancerous drug from <i>Ophiorrhiza pumila</i>
2006 - 2008	Masters Junior college, India Intermediate (equivalent to High school) Focus subjects: Botany, Zoology, Physics and Chemistry

11.2. Own publications

Sashidhar N, Harloff H-J, Potgieter L, Jung C. Gene editing of three *BnITPK* genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds (Manuscript under review)

Sashidhar N, Harloff H-J, Jung C (2019). Identification of phytic acid mutants in oilseed rape (*Brassica napus* L.) by large scale screening of mutant populations through amplicon sequencing. New Phytologist, <https://doi.org/10.1111/nph.16281>

Jung C, Capistrano-Gossmann G, Braatz J, Sashidhar N, Melzer S (2017). Recent developments in genome editing and applications in plant breeding. Plant Breeding, 00:1-9. doi:10.1111/pbr.12526

11.2.1. Oral presentations and posters

- Sashidhar, N. (2019). Discovering novel phytic acid mutants in Oilseed rape for future breeding, International Rapeseed Conference, Berlin.-16.06.2019. Oral Presentation.
- Sashidhar, N. (2019). Breeding towards low phytic acid content in *Brassica napus* , 2019, Genome Research for Plant Breeding, University of Hohenheim, Stuttgart.-28.03.2019. Oral Presentation.
 - Sashidhar, N. (2019) ., Harloff, H.-J., Jung, C. High throughput reverse genetic tools for knocking out several genes of the phytic acid pathway in *Brassica napus* , International Plant & Animal Genome XXVII, San Diego, 12.-16.01.2019- Poster Presentation.
 - Sashidhar, N. (2018). High throughput reverse genetic tools for knocking out several genes of the phytic acid pathway in *Brassica napus* , Young Scientists Meeting 2018, JKI, Braunschweig.-14.11.2018. Oral Presentation.
 - Sashidhar, N. (2018) Genome editing strategies for multiple knock out of phytic acid pathway gene families in *Brassica napus* , PGSC 2018, IPK, Gatersleben .-06.06.2018. Oral Presentation.
 - Sashidhar, N. (2018). Reverse genetic tools for mutational analyses of all genes of a complex metabolic pathway in *Brassica napus*. Kiel Plant Science symposium 2018, Kiel.-31.05.2018. Oral Presentation.

12. Declaration of co-authorship

Chapters 2 and 3 were prepared as manuscripts for publication. The respective declarations of co-authorship are presented on the following pages. Meanwhile, chapter 4 includes work of the doctoral candidate alone.

13. Publications and declaration of own contribution

C A U	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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Declaration of co-authorship

If a dissertation is based on already published or submitted co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

1. Doctoral candidate

Name: Niharika Sashidhar

2. This co-author declaration applies to the following article:

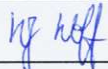

Identification of phytic acid mutants in oilseed rape (*Brassica napus* L.) by large scale screening of mutant populations through amplicon sequencing

The extent of the doctoral candidat's contribution to the article is assessed on the following scale:

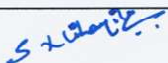
- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
Concept: Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	B
Planning: Planning of experiments/analyses and formulation of investigative methodology, including choice of method and independent methodological development, in such a way that the scientific questions asked can be expected to be answered	C
Execution: Involvement in the analysis or the concrete experiments/investigation	C
Manuscript preparation: Presentation, interpretation and discussion of the results obtained in article form	C

4. Signature of all co-authors

Date	Name	Signature
27.11.19	Hans-Joachim Harloff	
	Christian Jung	
25.11.19		

5. Signature of doctoral candidate

Date	Name	Signature
27.11.19	Niharika Sashidhar	

C A U	Christian-Albrechts-Universität zu Kiel	Agrar- und Ernährungs- wissenschaftliche Fakultät
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Declaration of co-authorship

If a dissertation is based on already published or submitted co-authored articles, a declaration from each of the authors regarding the part of the work done by the doctoral candidate must be enclosed when submitting the dissertation.

1. Doctoral candidate

Name: Niharika Sashidhar

2. This co-author declaration applies to the following article:




Gene editing of three *BnITPK* genes in tetraploid oilseed rape leads to significant reduction of phytic acid in seeds

The extent of the doctoral candidat's contribution to the article is assessed on the following scale:

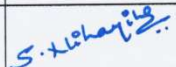
- A. Has contributed to the work (0-33%)
- B. Has made a substantial contribution (34-66%)
- C. Did the majority of the work independently (67-100%)

3. Declaration on the individual phases of the scientific work (A,B,C)	Extent
Concept: Formulation of the basic scientific problem based on theoretical questions which require clarification, including a summary of the general questions which, it is assumed, will be answerable via analyses or concrete experiments/investigations	B
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Execution: Involvement in the analysis or the concrete experiments/investgation	C
Manuscript preparation: Presentation, interpretation and discussion of the results obtained in article form	C

4. Signature of all co-authors

Date	Name	Signature
27.11.19	Hans-Joachim Harloff	
27.11.19	Lizel Potgieter	
27-11-19	Christian Jung	

5. Signature of doctoral candidate

Date	Name	Signature
27.11.19	Niharika Sashidhar	

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