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Biofortification of spring barley

Ph.D. thesis

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Abstract: Barley (*Hordeum vulgare*) is one of the most important cereal crops in agriculture. Barley grains contain nutrients such as starch, micro- and macro- elements. They are used as animal feed, in brewing industry and in the production of world renowned whiskeys. One of the essential and abundant elements in barley grains is phosphorus. In cereals generally including barley the majority of grain phosphorus is stored in an organic form as phytic acid which is poorly digested in most barley-based feeders – swine, and possibly poultry and fish. Phosphorus biofortification strategies are hence desirable and these are mainly based on alteration of the form, in which phosphorus is present in grains. A recently adapted genome editing technique called CRISPR/Cas is being exploited to generate mutation in the *HvITPK1* gene which takes part in phytic acid synthesis. Generated mutant plants have been characterized on the genomic level and selected lines have also been characterized for abiotic stress response during germination. Moreover, expression profile of *ITPK* genes has been evaluated in wild type and *itpk1* mutants cultivated under control and stress conditions.

Keywords	barley, biofortification, CRISPR, genome-editing, phosphate
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Declaration

Hereby I declare that the thesis summarizes original results obtained during my Ph.D. under the supervision of Prof. RNDr. Martin Fellner, Ph.D. and Ing. Ludmila Ohnoutková Ph.D. using the literature sources listed below.

In Olomouc

Tomáš Vlčko

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Content

List of papers

This thesis summarizes and links the following papers that are referred to within the text by Roman numerals I-II and attached at the end of the thesis in the Supplementary section.

I. Vlčko T., Ohnoutková L. (2019). Recent advances and perspectives in crop biofortification. *Biologia Plantarum* 63:586–593. doi: 10.32615/bp.2019.056. IF: 1,384

II. Vlčko T., Ohnoutková L. (2020). Allelic Variants of CRISPR/Cas9 Induced Mutation in an Inositol Trisphosphate 5/6 Kinase Gene Manifest Different Phenotypes in Barley. *Plants* 9:1–20. doi: 10.3390/plants9020195. IF: 2,632

Contribution report

I. As the first author, TV carried out the literature review, evaluated the literature sources and wrote the manuscript.

II. As the first author, TV designed the experiment, performed *in silico* analyses of gene sequences and protospacer design, cloning of the expression vector, plant transformation, genotyping and characterization of regenerated plants, characterization of phosphate content in mature grains, and abiotic stress assays. TV evaluated the data and wrote the manuscript.

Abbreviations

AA	amino acid
ABC	ATP-binding cassette transporter
ACP	acyl carrier protein
ATP	adenosine trisphosphate
Cas	CRISPR associated protein
CasPDB	Cas Protein Data Bank
Cas9	CRISPR associated protein 9
CKX	cytokinin oxidase/dehydrogenase
CMF7	CCT Motif Family gene 7
CoA	Coenzyme A
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR-derived RNA
CRTI	*Pantoea ananatis* phytoene desaturase
DEP1	dense and erect panicle 1
DXS	1-deoxyxylulose 5-phosphate synthase
EDR1	enhanced disease resistance 1
ENG	endo-N-acetyl-β-D- glucosaminidase
FA	fatty acid
FAD2	fatty acid desaturase 2
FAE1	fatty acid elongase 1
FT1	flowering time 1
GASR7	Snakin/GASA protein 7
GE	genome editing
GMO	genetically modified organism
GS-MAS	genomic selection based MAS
GW2	grain weight 2
HDR	homology-directed repair
HGO1	homogentisate dioxygenase 1
HvPAPhy_1	purple acid phytase
HvPM19	gene for ABA-inducible plasma membrane protein
IMP	*myo*-Inositolmonophosphatase
InsP	inositol phosphate
InsP3	inositol trisphosphate
InsP4	inositol tetrakisphosphate
InsP6	inositol hexakisphosphate
Ins(3)P1	*myo*-Inositol-3-phosphate
IPK	inositol phosphate kinase
ITPK	inositol trisphosphate 5/6 kinase
LOX1	lipoxygenase 1

LCY	lycopene β-cyclase
LKR	lysine ketoglutarate reductase
LOX3	lipoxygenase 3
LPA	low phytic acid
MAS	marker-assisted selection
MIPS	*myo*-Inositolphosphatesynthase
MLO	mildew-resistance locus
MORC1	CRT1-like GHKL ATPase
Ms1	male sterility gene 1
Ms45	male sterility 45
NA	nicotianamine
NAS	nicotianamine synthase
NHEJ	non-homologous end joining
PA	phytic acid
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
PHT	phosphate transporters
PLC	phospholipase C
PP-InsP	inositol pyrophosphate
PSD	phytoene desaturase
PSV	protein storage vacuoles
PSY	phytoene synthase
PtdInsP	phosphatidylinositol phosphate
PUFA	polyunsaturated FA
QSD1	quantitative trait locus on seed dormancy 1
QTL	quantitative trait locus
RNAi	RNA interference
RISC	RNA-induced silencing complex
RNP	ribonucleoprotein
TAL	transcription activator-like
TALEN	transcription activator-like effector nuclease
TAG	triacylglycerol
T-DNA	transfer DNA
SAD1	stearoyl-acyl carrier protein desaturase-1
SDH	saccharopine dehydrogenase
SDP1	sugar dependent 1
sgRNA	single guide RNA
SPX	SYG1/PHO81/XPR1 phosphate sensing domain
VIH	diphosphoinositol penta*kis*phosphate kinase
WT	wild type
ZIP4	synaptonemal complex protein
ZNF	zinc finger nuclease

1. Introduction

Barley is annually sown on thousands of hectares and is the fourth most important cereal crop in the world. The majority of barley production is used as animal feed and smaller part in the production of malt used in the brewing of beer and in whiskey production. The most valuable part of the barley plant is its grains which are full of starch, vitamins, and microelements. Of these, phosphorus is an essential element with a high value for nutrition. It is also essential for plant growth and the second most limiting element to plant development. Therefore, in order to sustain high yield and especially in soils poor in nutrients, phosphorus is being added into the soil as a fertilizer to provide a sufficient amount of nutrients. However, global deposits of rock phosphate are not infinite. An alarming state was described by Cordell et al. (2009) who reported that the application of rock phosphate steeply increased in the second half of the twentieth century. The remaining deposits could be depleted within this century. Requirements for agricultural production are high, regarding fuel consumption, application of fertilizers and technology, so that maximal effectiveness in the utilization of agricultural products is demanding. Maintenance or increase of yield under sub-optimal environmental conditions is of great importance. In this view, application of modern breeding techniques and biotechnology could assist in the breeding of modern cultivars.

Generally, agricultural production is energetically expensive. Additionally, cereals are very demanding crops that requires crop rotation and also rich fertilization for optimal yield. In an effort to achieve maximal effectiveness in the utilization of agricultural products for food and feed, it is of great interest to fully exploit their nutritional and energetic value. For instance, phosphate and microelement utilization from cereal grains depend strongly on the form in which phosphate and microelements are stored. Therefore, sophisticated approaches aiming at more efficient phosphate utilization are needed. The currently available tools of genome editing (GE) technology facilitate genetic manipulation. The major advantage is the ability to induce mutations at predefined sites in the genome by targeting one gene or even the whole gene family. In combination with available genome sequences of all major crops through free access databases, it has never been more realizable to identify an exact gene or gene family and elucidate its function.

In this work, application of clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein (Cas) system for development of mutant barley lines with altered phosphate content is described. Genes for inositol trisphosphate 5/6 kinases (ITPK) were selected as targets for GE. ITPKs play a role in sequential phosphorylation of *myo*-Inositol phosphates thus participating in the storage of phosphate in developing grains.

2. Aims and scope

The scope of the work is the improvement of phosphate utilization in the barley-based feed. Since phosphate is stored in barley grains in an organic form of phytic acid, which is difficult to digest by non-ruminants, phosphate is not fully bio-available and a substantial part of the total content is undigested. Alteration of phosphate content in barley grains can improve phosphate utilization and increase the nutritional value of barley-based feed. GE techniques enable extraordinarily precise manipulation with genetic information including induction of mutations at predefined sites within the genome. Emphasis was placed on application modern techniques of GE in the preparation of biofortified barley with improved digestible phosphate content.

Aims of this thesis were

- Evaluation of biofortification strategies focused on improved phosphate utilization.
- Analysis of barley enzymes participating in phytic acid synthesis, selection of potential candidate genes and design and cloning of expression vector for genome-editing.
- Preparation of transgenic plants expressing programmable nuclease Cas9, their genotypic characterization, identification of mutations.
- Phenotypic characterization of mutant barley lines - phosphate content, germination rate.

3. Literature review

3.1. Biofortification

Food quality and environmental balance are becoming of great importance for modern consumers, pressing farmers into sustainable, ecological-friendly farming that produces high-quality products. Biofortification represents a modern strategy aiming at an increase in the nutritional value of food, mostly by enrichment of plant seeds with nutritionally important substances (Bouis, 2002). A list of substances subjected to biofortification consists mainly of microelements but also covers unsaturated fatty acids (FA), vitamins, and essential amino acids (AA). Many staple cereal crops such as wheat (Singh et al., 2017), barley (Sikdar et al., 2016), corn (Zhu et al., 2008; Du et al., 2016), rice (Johnson et al., 2011; Lee et al., 2011; Ma et al., 2015a; Yang et al., 2016), sorghum (Che et al., 2016), and banana (Paul et al., 2017) have been subjected to biofortification. Multiple techniques have been applied to develop a biofortified product. There are two main strategies for crop biofortification. A fertilization-based strategy historically preceded biotechnological strategies, which are acquiring more significance with progress in technology. Both strategies strive for an increased level of the desired substance in a final product, but they differ in terms of financial cost and technological requirements.

Conventional biofortification strategies can be defined as approaches which apply fertilizer directly to the growth substrate or on the plant in the case of foliar application. The use of fertilizers commenced the epoch of practical plant biofortification. The simplicity of the approach, sophisticating no technological equipment, is characteristic of this strategy.

For foliar fertilization, nutrients are applied as a liquid solution to the plant. However, the form in which the nutrient is applied is often crucial for maximal results. Wei et al. (2012) reported that of four different forms of Zn compounds, the Zn-AA form resulted in the highest overall increase in zinc levels in rice grains. Combined application of selenite and selenate negatively affected microelement accumulation, but use of selenate alone led to magnesium, zinc, and manganese accumulation in corn (Longchamp et al., 2016). Moreover, Ducsay et al. (2016) reported that the absorption efficiency of selenium was up to 15 % when applied as selenate, whereas selenite did not lead to substantial selenium accumulation in winter wheat. As well as the form of the applied nutrient, the timing of fertilizer application with regard to plant physiological processes contributes to optimal absorption. The optimal timing can even impair the accumulation of heavy metals (Saifullah et al., 2016). Well-known information is addition of a surfactant into the mixture with

nutrients that increases nutrient permeability via stomata (Field and Bishop, 1988). Generally, there are two ways of absorption of the foliar-applied fertilizer into a plant, particularly via cuticle or stomata (Fernández and Eichert, 2009; Fernández and Brown, 2013). Mobility of the compound within the plant tissues should be also taken into account when considering the target nutrient and the means of its delivery to the plant. Doolette et al. (2018) showed that zinc has very low mobility within a leaf, migrating less than 25 mm from the site of application after one day. Interestingly, advances in nanoparticle technology have been successfully transferred to plant biofortification. Zinc complexed chitosan nanoparticles used for foliar application as a "micronutrient nanocarrier" in wheat resulted in an increase in zinc by 42% in grains (Deshpande et al., 2017). Cost-effectiveness and sustainability of the foliar micronutrient fertilization were proven in field conditions, provided application of the fertilizer was combined with pesticide treatment (Wang et al., 2016). However, efforts to increase micronutrient content can have unintended consequences. For instance, lower food quality of strawberries, due to over-accumulation of nitrate in the fruits, was a consequence of IO_3^- treatment (Li et al., 2017). It was shown in one field trial that proper phosphate management was important for optimal zinc enhancement (Zhang et al., 2015).

A possible alternative to fertilizing with micronutrients is focusing on improving the plant growth and micronutrient uptake from the soil. This could be achieved by the treatment of plants with chemical substances possessing biological activity or by the improvement of root and soil microbiome interaction. Enhanced root formation after treatment with two substances called AZAL5 and HA7, which were derived from *Algae* and humic acid was observed by Billard et al. (2014). Interestingly, both substances promoted not only improved uptake of N, K, S, and P via a more branched root system but also increased plant concentrations of Mg, Mn, Cu, and Na. Derivatives of amino acids are another class of substances with a beneficial effect on microelement acquisition (Kałużewicz et al., 2018; Popko et al., 2018). Promising substances with diverse biological functions are derivatives of plant growth regulators. In field conditions, it was shown that cytokinin derivative 2-Cl-3-MeOBAP promoted tillering and grain filling resulting in a more homogenous spike (Koprna et al., 2016).

In recent years, the intricate interaction between plant roots and soil microbiome has been extensively studied (Fitzpatrick et al., 2018; Hu et al., 2018). Soil bacteria and symbiotic fungi can substantially increase the availability of nutrients from the soil through the transformation of insoluble compounds to soluble forms that plant roots can absorb. Notably, two isolates of rhizobacterium *Exiguobacterium aurantiacum* were proposed as a low-cost and sustainable option

for wheat biofortification and described having a beneficial effect on zinc and iron levels in grains (Shaikh and Saraf, 2017). Interaction between plants and soil microorganisms also involves the production of hormones to support mutual cohabitation. Plants are capable of exuding strigolactones, hormones that promote mycorrhizal symbiosis (Genre et al., 2013). In turn, soil bacteria have been reported to produce plant hormones that induce root plant growth (Patel and Saraf, 2017; Rohini et al., 2018).

Conventional biofortification approaches have focused on the development of micro-carriers of nutrients and growth regulators, or the exploitation of beneficial soil microorganisms, which are applied directly to the plants. In comparison, biotechnological approaches exploited in biofortification, aim at the engineering of metabolic pathways, transport and storage systems at the genetic level. The biofortification strategy which exploits biotechnological techniques has advanced hand in hand with progress in technology. This strategy can be divided onto approaches using solely various genetic markers and those exploiting recombinant technology and producing genetically modified organisms (GMO). Specific genomic (DNA) markers derived from repetitive sequences (Morgante and Olivieri, 1993), transposons (Kalendar et al., 1999) or after digestion of a genomic DNA by restriction enzymes (Paterson et al., 1988), are used in the so-called marker-assisted selection (MAS) or molecular breeding. Subsequently, linkage mapping associating a DNA marker with a phenotypic trait or desirable quantitative trait locus (QTL) utilized promptly MAS, which has become a reliable technique in breeding programs, accelerating the selection of lines carrying desirable traits associated with specific DNA markers. Additionally, traits with minor effects on a phenotype could be identified using MAS based on genomic selection (GS-MAS) (Meuwissen et al., 2001). MAS represents a handful of techniques that has become established among breeders.

Concerning plant biofortification, DNA markers have been successfully applied in the selection of lines with increased microelement accumulation. In short, the acquisition of microelements by plants naturally occurs via root uptake from the soil with subsequent loading of the nutrient into the xylem. Then, the xylem sap transports the mineral solution to the sink where the microelements are further processed. During the grain-filling period, the majority of the minerals are transported into the grains. Minerals are transported not only from the roots but also translocated from the leaves. Importantly, a substantial proportion of microelements in the grain can originate in remobilization from other plant tissues such as leaves. The grain ratio between microelements translocated within a plant and transported directly from the roots can differ for distinct genotypes

(Johnson-Beebout et al., 2016). The exploitation of natural variability in a remobilization capacity may lead to an increase in the grain mineral content. Identification and mapping of QTL associated with microelement accumulation could contribute to the development of biofortified cultivars. Indeed, there is variability in the remobilization capacity among cultivars as reported by Hussain et al. (2016) who found that in barley cv. Sahara nearly 40% of Zn in the grain was translocated from the plant. Generally, genotype has a substantial effect on the accumulation of microelements in the grains. Remarkably, a single QTL could be responsible for one-fifth of the Zn content in grain (Crespo-Herrera et al., 2016).

Techniques utilizing recombinant technology are dependent on the development of efficient protocols for plant transformation, *in vitro* propagation and plant regeneration. Once established, these enabled the practical application of genetic modifications in a gradually increasing range of plant species. For biofortification purposes, generating transgenic plants opened possibilities that could not be attained by conventional breeding.

For instance, enhanced microelement content can be achieved by modeling of transport pathways. When microelements are transported in the xylem sap, they are bound to molecules such as nicotianamine (NA), which have chelating properties. Having microelements bound in complexes, plants acquire control over their loading and transport. *Nicotianamine synthase* genes (*NAS*) encode proteins responsible for the synthesis of NA. It has been confirmed that either specific over-expression of the *NAS* gene in the rice (Johnson et al., 2011), and wheat (Singh et al., 2017) endosperm or *NAS* nonspecific over-expression (Lee et al., 2011) lead to a significant rise in zinc concentration in grains. Moreover, grain localized expression of *NAS* also positively affected iron content (Singh et al., 2017). Additionally, combining over-expression of NAS with transmembrane transporters and storage proteins can further support microelement accumulation (Boonyaves et al., 2017). It is noteworthy that manipulation of the transport pathways and the storage of microelements in seeds could have a negative effect such as the unintended accumulation of cadmium. Hence, it is crucial to carefully manipulate the plant's metabolism to avoid detracting from the positive intended effects with negative side-effects. Strategies for preventing cadmium acquisition without affecting Fe and Zn accumulation have been summarized by Slamet-Loedin et al. (2015).

These examples of plant biofortification concern primarily the enhancement of microelement content, mostly zinc. There are other important nutrients, to which biofortification efforts are directed. FAs are essential for the proper function of all cells. They serve as the central core of cell

membranes, carbon and energy storage, and their derivatives could act as signal or hormone molecules (Ohlrogge and Jaworski, 1997). It is known that they are synthesized from acetyl-coenzyme A (CoA) in plastids. Regulation of FA synthesis is a complex process, in which biochemical, transcriptional and posttranscriptional factors are involved. In an abundance of FA, these are used for synthesis and further storage as triacylglycerol (TAG). Xu and Shanklin (2016) concluded that to increase TAG accumulation, a combination of downregulation or upregulation of two or more synthesis controlling factors is desirable. A fraction of the total amount of FAs in plants is unsaturated FA. Unsaturated FAs, which are characterized by possessing at least one double bond in the carbon chain, have beneficial effects on the cardiovascular system and a preventive function in some brain disorders (Connor, 2000). Hence, modification of the ratio between saturated and unsaturated FAs in plant seeds would be attractive. Knowledge of a biosynthetic pathway of a desired product including entering precursors is a favorable precondition for successful biofortification.

Genetic manipulations include both gene over-expression, and the opposite – gene silencing. A technique of gene silencing, which is also known as RNA interference or shortly RNAi, is based on the post-transcriptional regulation of gene expression via nuclease activity of an RNA-induced silencing complex (RISC) against mRNA transcripts (Fire et al., 1998). Stearoyl-acyl carrier protein desaturase-1 (SAD1) catalyzes the conversion of a stearoyl-acyl carrier protein (ACP) to oleoyl-ACP, which predominantly serves as a precursor of polyunsaturated FA. Knock-down of *sad1* using an RNAi technology was applied to support the conversion of saturated to unsaturated FA. This resulted in elevated levels of stearic acid and other long FAs accompanied by a decrease in the oleic acid level (Du et al., 2016). Another approach for induced accumulation of TAG in seeds suggested was by restricting the metabolization of TAG *in planta* (Kim et al., 2014). The authors developed plants exhibiting up to 30 % higher total lipid content through knock-down of sugar dependent 1 (SDP1) lipase, which participates in catabolizing FA. RNAi was also used to induce the reduction of grain storage protein C-hordein in barley. The drop in C-hordein was compensated by an increase in B-hordein. Remarkably, the change in the main storage protein was followed by an increase the essential amino acids leucine, methionine, and threonine (Sikdar et al., 2016).

With ongoing progress in transformation protocols which have enabled the transformation of large constructs carrying multiple genes, modification or even *de novo* establishing of biosynthetic pathways through heterologous expression became available. A preminent example of *de novo* pathway construction was the development of Golden Rice by Ye and Beyer (2000), who succeeded

in the development of rice with dramatically increased carotenoid content by introducing plant phytoene synthase (*PSY*), bacterial phytoene desaturase (*CRTI*), and lycopene β-cyclase (*LCY*). Continuing in the research work, Paine et al. (2005) substantially improved carotenoid accumulation, up to 23-fold in comparison to the original Golden Rice, by selecting more effective enzymes. Similarly, Che et al. (2016) prepared transgenic sorghum by introducing the same set of genes as described (Paine et al., 2005) and added 1-deoxyxylulose 5-phosphate synthase (DXS), which facilitates carotenoid biosynthesis by providing the precursor. Analogously, production of polyunsaturated FAs (PUFA), eicosapentaenoic acid (20:5, n-3) and docosahexaenoic acid (22:6, n-3), in *Camelina sativa* was achieved by introducing up to seven genes (Ruiz-Lopez et al., 2014). The enzymes for PUFA biosynthesis were thoroughly selected for optimal production as in the case of acyl CoA-dependent Δ6-desaturase from *Ostrococcus tauri* (Domergue et al., 2005). This restricted unwanted accumulation of C18 intermediates (Sayanova et al., 2012). Notably, not only biosynthetic intermediates but also a final product could be further metabolized. In order to avoid subsequent metabolization of lysine, an essential amino acid, down-regulation of lysine metabolizing enzymes, lysine ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH), was done in the lysine biofortified rice, which consequently possessed up to 25-fold higher content of lysine in comparison to wild type (WT) (Long et al., 2013; Yang et al., 2016).

3.2. Genome-editing techniques

The above described biotechnological approaches share the feature of introducing a transgene that affects the content of desired nutrient in the final product. Biofortification objectives can also be accomplished by the generation of desirable mutations. Indeed, classical mutagenesis contributed significantly to the second green revolution. Breeding of new varieties very soon encompassed mutagenesis, which dramatically increased production of new traits exploitable in breeding programs that significantly contributed to the higher yield and pathogen resistance of modern cultivars. Many elite cultivars still carry traits generated by radiation or chemical mutagenesis. Mutagenesis has undergone an evolution from uncontrolled chromosomal fragmentations, deletions, and fusions induced by radiation, through multiple indirect small mutations resulting from the application of chemical mutagens, to precisely created mutations.

Biotechnological progress has facilitated the production of mutants without the use of dangerous radioactive material or chemical mutagens. Initially, libraries of transfer DNA (T-DNA) mutants were assembled from plant material obtained from extensive *Agrobacterium*-mediated transformation

experiments and this generated thousands of T-DNA mutants. However, integration of T-DNA was still random and a large number of mutant plants was needed to cover a significant portion of genes. Additionally, T-DNA mutated plant material is considered to be genetically modified so that it came in useful mainly in basic research as in case of *Arabidopsis thaliana* (O'Malley and Ecker, 2010) and rice (*Oryza sativa*) (Jeon et al., 2000). The resulting collections of insertion mutants is being widely utilized by the scientific community.

Fortunately, progress in biotechnology has facilitated the preparation of mutant lines with extreme precision. A known drawback of classical mutagenesis – multiple mutations within a genome that had to be crossed-out to select the desired trait was overcome by the so-called GE techniques, which have spread across all fields of biotechnology. GE enables precise manipulation of genetic information, mostly generation of mutations at predefined positions in a genome using enzymes called programmable nucleases. Hence, in plants, the application of GE was initially aimed at generating mutations. However, the spectrum of applications of programmable nucleases is broader, including fine regulation of gene expression and design of chromosome markers. The techniques of classical mutagenesis and modern GE can all be included in the group of approaches of reverse genetics, which are used in basic research to characterize gene function. Of these, GE has the greatest potential to accelerate the generation of new traits that can be transferred to agricultural practice.

Programmable nucleases represent enzymes used mainly for inducing mutations in the genomic DNA of host organisms. There are three main classes of programmable nucleases: i) zinc finger nucleases (ZNF), ii) transcription activator-like effector nuclease (TALEN) and iii) CRISPR/Cas.

Zinc fingers are a group of enzymes, originally described as DNA binding proteins that participate in transcription regulation (Frankel et al., 1987; Gibson et al., 1988; Vrana et al., 1988). ZNF was engineered by fusing zinc finger transcription factor with the cleavage domain of DNA nuclease *FokI*, which is a type IIS restriction endonuclease, to gain function as hybrid restriction enzymes that induce a double-strand break in a sequence-specific manner (Kim et al., 1996). Analogously, a transcription activator-like (TAL) effector was demonstrated to be a transcription regulator of a plant pathogen of the genus *Xanthomonas* (Gu et al., 2005; Sugio et al., 2007). Fusing TAL with *FokI* endonuclease, another instrument of generic engineering was developed (Christian et al., 2010). Both ZNF and TALEN are programmable nucleases that are characterized by a protein: DNA interaction, which defines specificity for the effector complex. Genomic sites that could be targeted by ZNF were limited by the specificity of the DNA binding domain. Synthetic ZNF recognizes

a genomic region up to 18 bp in length where each zinc finger binds 3 bp (Liu et al., 1997). In comparison to ZNF, TALEN offered a significantly larger range of genomic loci that could be selected for cleavage because recognition of the target DNA site when using TALEN relies on repeat-variable residues which each bind to a single bp (Deng et al., 2012).

The last member in the group of programmable nucleases called CRISPR/Cas. CRISPR/Cas system is characterized by a universal effector nuclease whose specificity to the target site is based on an RNA: DNA interaction. The RNA molecule that determines specificity to the target site is called a guide RNA (Jinek et al., 2012). The specificity of the programmable nuclease due to the short RNA molecule greatly simplified the cloning step and was a prime reason for the overwhelming acceptance of CRISPR over the other programmable nucleases.

GE techniques soon found their way not only into basic research but also into crop biofortification. For example, the CRISPR/Cas system was employed to induce mutations in a gene for fatty acid elongase 1 (*FAE1*) of an allohexaploid crop, *Camelina sativa*. Remarkably, this goal was achieved in a single generation resulting in approximately complete restriction of production of very-long-chain FA (Ozseyhan et al., 2018). Another example, alteration of the content of oleic acid, was effectively accomplished by knocking out the fatty acid desaturase 2 (*FAD2*) gene in both rice (Abe et al., 2018) and *Camelina sativa* (Jiang et al., 2017), where it caused 3-fold increase in production of monounsaturated FA. Nevertheless, the popularity of the CRISPR system did not result in abandonment of the other programmable nucleases. For instance, TALEN was used to develop a rice mutant in lipoxygenase 3 (*LOX3*). Knock out of *LOX3* reduced the oxidation of FA during long-term storage thus improving one of the most important yield parameters (Ma et al., 2015a). Despite this progress, classical mutagenesis remains a powerful and reliable instrument for the development of desirable mutants. Proving a point, a soy mutant with disrupted homogentisate dioxygenase (*HGO1*), which showed elevated levels of lipid-soluble antioxidants, such as vitamin E, was found in a population produced from neutron mutagenesis (Stacey et al., 2016). All the techniques of mutagenesis proved well in both reverse genetics and in biofortification efforts. Classical mutagenesis is still a viable technique. However, programmable nucleases possess indisputable advantages especially regarding use in plant species with polyploidy genomes. Taken together, various nutrients have been biofortified via various strategies and tested in experimental or field conditions.

3.3. CRISPR/Cas technology

CRISPR/Cas has evolved in unicellular organisms of clade *Bacteria* and *Archaea* as an adaptive immune system against phages (Barrangou et al., 2007). Additionally, the CRISPR/Cas immune system was reported to be able to destroy foreign RNA molecules too (Hale et al., 2009). In general, the CRISPR-Cas system comprises a CRISPR locus and CRISPR associated proteins (Cas), (Figure 1).

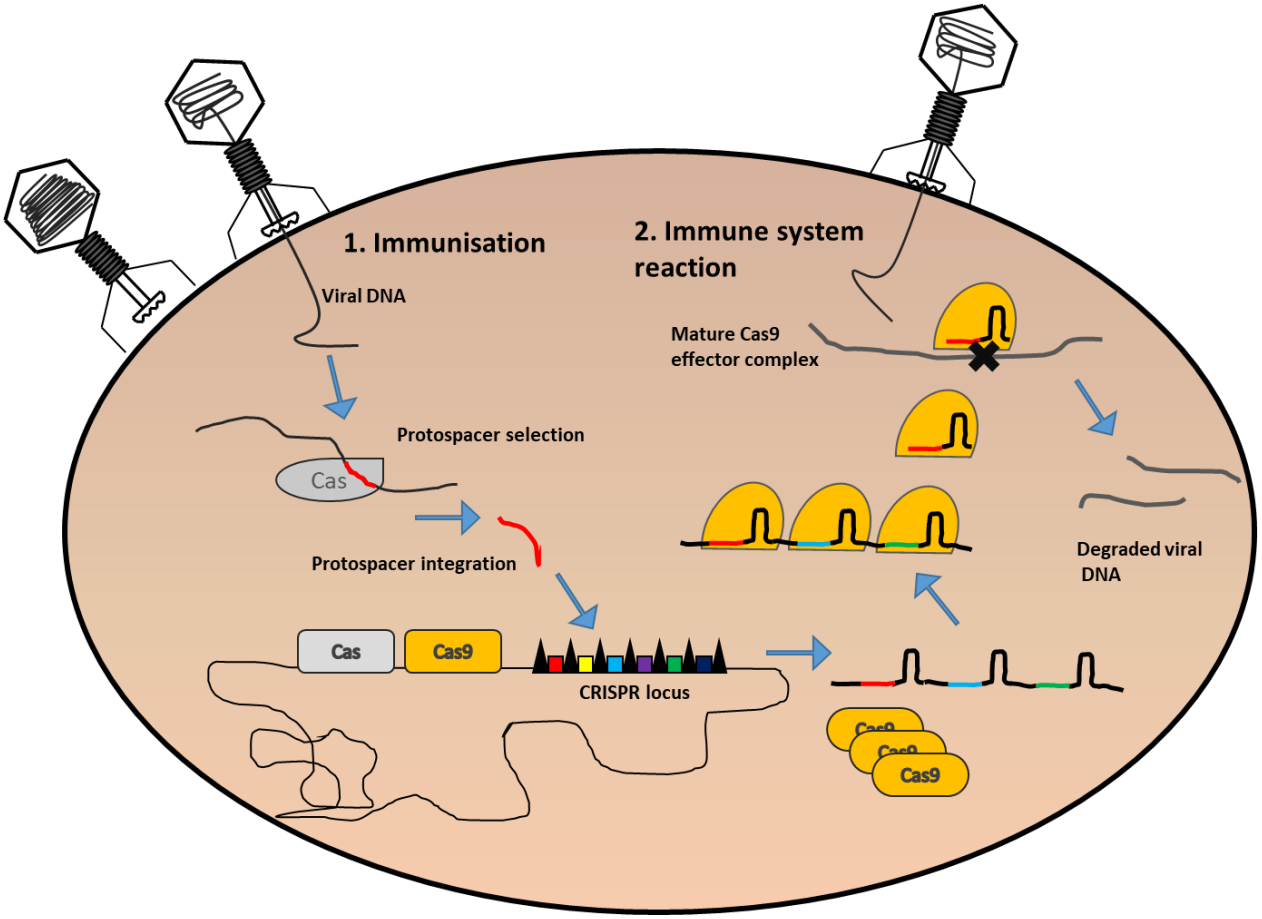

Figure 1. The concept of CRISPR/Cas bacterial adaptive immunity. This consists of two phases. In the first phase, a bacterial cell infected by bacteriophage acquires fragments of viral DNA. Provided survival of the bacterium, Cas proteins process viral DNA and integrate it into the CRISPR locus. Then, in the second phase, if the bacterium is infected repeatedly by the same bacteriophage, Cas nuclease proteins are expressed and integrated spacers are used to specify the target site in the invading viral DNA to be cleaved (adapted and modified from Vlčko, 2018).

Originally, direct repeats with 32-bp spacers were identified in the genome of *Escherichia coli* (Ishino et al., 1987). CRISPR locus assembles short fragments of foreign DNA called spacers, which are

interspaced with short repeats. There may be more than one CRISPR loci in the genome, differing significantly in size and comprising a variable number of integrated spacers. This has major effects on resistance to phages (Barrangou et al., 2007). Upon infection, CRISPR genomic loci are transcribed in the long precursor RNA containing multiple spacers, which are further processed into short CRISPR-derived RNAs (crRNA) by the activity of the Cas6 enzyme (Brouns et al., 2008; Carte et al., 2008). It was further suggested that Cas6 also participates in the selection of spacers to be integrated into the CRISPR locus (Sternberg et al., 2012). Each crRNA gives specificity to the effector complex for cleavage of foreign DNA or RNA. Cas proteins are encoded by genes whose operon is closely associated with the CRISPR locus. The current classification of Koonin et al. (2017) divides CRISPR systems into two classes each consisting of three subtypes. The classes are distinguished by the effector complex assemblies, where a multiple protein complex is formed in class I, whereas class II is characteristic by a large single-protein module. Recently constructed is the Cas Protein Data Bank (CasPDB), which harbors over two hundred thousand reviewed and putative Cas proteins identified mostly in *Bacteria* and also in *Archaea* species (Tang et al., 2019). Cas1 is the most conservative enzyme of the Cas proteins and has been identified in all the systems. It was demonstrated that Cas1 together with Cas2 are capable of integrating foreign DNA into the CRISPR locus in *E. coli*. The described process of spacer integration showed resemblance to the activity of retroviral integrases or transposases (Nuñez et al., 2015). Another well-described protein is Cas9 which belongs to the Class II system. Primarily, Cas9 represents a well-documented example of the effector module which cleaves invading DNA at a predefined site determined by crRNA and the protospacer adjacent motif (PAM) (Jinek et al., 2012). Protein domains RuvC and HNH of the Cas9 enzyme are necessary for cleavage in double-strand DNA, where each domain generates site-specific nicks on opposite DNA strands (Gasiunas et al., 2012). Moreover, it was reported that Cas9 plays a role during the immunization phase by association with Cas1 and Cas2 proteins thus giving specificity to a spacer acquisition complex (Heler et al., 2015).

In theory, established *Streptococcus pyogenes* Cas9 enzyme can cut a DNA strand at any given site under the condition of the presence of a protospacer adjacent motif (PAM) that is essential for nuclease activity. For the *Sp*Cas9, this motif is defined as NGG. Hence, *Sp*Cas9 cannot in practice target a particular designated sequence, as not all genomic sites offer the NGG motif neighboring the desired target site. It was demonstrated that Cas9 nuclease can be applied to induce mutations in genomic sites of both dicot and monocot plant species (Jiang et al., 2013; Shan et al., 2013). GE using Cas9 has already been implemented for inducing mutations in different plant species (Ma et

al., 2015b; Li et al., 2018a; Wang et al., 2018c). Notably, several Cas enzymes which require distinct PAM motifs, have been described thus broadening the range of genomic loci that can be edited (Murovec et al., 2017).

Yet, it is necessary to mention that programmable nucleases including Cas9 mediate the only cleavage of genomic DNA in host cells and mutations themselves emerge subsequently during the reparation process. There are two main ways of reparation of double-strand DNA breaks, which are generated by programmable nucleases non-homologous end joining (NHEJ) (Wilson et al., 1982) and homology-directed repair (HDR) (Puchta and Hohn, 1991; Puchta et al., 1996). Typically, NHEJ reparation is error-prone causing indels or substitutions thus leading to mutation.

To achieve genetic manipulation using the CRISPR/Cas system, there has to be an assembled effector nucleoprotein enzymatic complex such as Cas9 with a guide RNA in the host cells. To achieve this, either standard stable genetic transformation or transient transformation has to be performed. The gene for Cas9 enzyme can be introduced into plant cells as DNA in the standard expression vector by *Agrobacterium*-mediated or biolistic transformation (Schiml et al., 2014). The DNA encoding Cas9 enzyme could be accompanied by one or multiple single guide RNAs (sgRNA) that can be cloned into expression vector either simultaneously as a set of U6 promoter-sgRNA cassettes (Ma et al., 2015b), or as a chimeric tRNA-gRNA under regular promoter (Xie et al., 2015). A feasible way of inducing defined fragment deletions is delivery of the two constructs each possessing the distinct sgRNA cassette, in a mixture of two *Agrobacterium* inoculums, which are simultaneously transformed as confirmed by Kapusi et al. (2017).

Alternatively, a purified Cas9 enzyme in complex with an *in vitro* transcribed guide RNA can be coated on gold microparticles and biolistically delivered into mature embryos that had grown spontaneously into plants. Such a method elegantly circumvents demanding *in vitro* cultures and produced a transgene-free plant directely (Liang et al., 2017; Hamada et al., 2018). Transformation of protoplasts with either plasmid DNA constructs (Wang et al., 2018a) or Cas ribonucleoprotein (RNP) complex also generates mutated plants (Kim et al., 2018). However, the process of *in vitro* regeneration from protoplasts is less effective than immature embryos-derived tissue cultures. Recently, a sophisticated method of delivery of CRISPR/Cas RNP complex into plant cells was shown by Toda et al. (2019) who described the transfection of plasmid or RNP into rice zygote thus producing efficiently mutant plants.

In practice, other factors influencing GE have to be considered. For example, a propensity for the same type of mutation, either deletion or insertion of a single base-pair, at the same target site

among different transgenic events has been observed. Further, the target genomic site can have an impact on the double-strand break (DSB) repair thus affecting not only the type of induced mutation (single or multiple bp insertion, deletion, substitution) but also directly the preference for either an insertion or a deletion (Pathak et al., 2019). Additionally, the expression level of Cas9 correlates with GE frequency (Jang et al., 2016). It has been shown that the nuclease Cas9 possesses off-targeting activity (Fu et al., 2013; Xie and Yang, 2013). Off-targeting is the result of imprecise pairing between the protospacer sequence and the non-target genomic sequence resembling the target sequence, which results in unspecific cleavage. In turn, off-targeting can be alleviated by using either paired nickases (Fauser et al., 2014; Shen et al., 2014), or by an engineered Cas9 enzyme with reduced off-target activity (Slaymaker et al., 2015; Kleinstiver et al., 2016). Overall, the most important factor reducing unintended off-targeting remains careful design of sgRNA. Tang et al. (2018) recommended selecting those protospacers that have at least two mismatches in their seed sequence (i.e. in 10 nucleotides proximal to PAM). Additionally, Young et al. (2019) reported that bioinformatically designed sgRNAs, which differ from the other genomic location by at least three mismatches with one in seed sequence, can significantly reduce the risk of off-targeting. Besides off-targeting, both studies proved Cas9 to be efficient in on-target editing.

Chimerism is a state when there are different genotypes within one organism. Primary plant *in vitro* regenerants, which express programmed Cas nuclease, exhibit frequent chimeric mutational effects (Jang et al., 2016; Pathak et al., 2019). For instance, plants carry typically two different mutations at the same target site in each chromosome set. Therefore, analysis of the T1 generation is necessary to confirm the stability and transmission of mutation to the next generation. In the case of stable transformation of plant material, the transgenerational activity of Cas nuclease, on the one hand, facilitates the development of mutant lines, especially in allopolyploid species such as wheat with multiple gene copy number (Wang et al., 2018a). On the other hand, the chance of generating chimeric plants as well as off-targeting increases provided that programmable nuclease remains active in transgenic lines over several generations. A step following GE is the genotyping of obtained plant material. For genotyping of the target locus, it is advisable to amplify a larger genomic sequence due to the unpredictable character of mutations induced by programmable nucleases. Some authors have reported, mostly short or even only 1-bp indels (Singh et al., 2018). However, deletions in tens or hundreds of bp are not exceptional (Holme et al., 2017; Sánchez-León et al., 2018).

Apart from CRISPR/Cas-mediated directed mutagenesis, GE technology allows directed replacement of genomic DNA for designed DNA templates, so-called knock-in. If there is induced DNA DSB, this leads to activation of reparation mechanisms, either NHEJ or HDR. It has been shown that if a homologous template is accessible, HDR mediated reparation can occur (Budhagatapalli et al., 2015; Watanabe et al., 2016). The rate of knock-in event was unfortunately too low to be acceptable for practical application. Hence, to improve the ratio of knock-in events in transgenic plants, several technical platforms have been tested. These were designed to support HDR by increasing the accessibility of artificial templates intended for integration. Gil-Humanes et al. (2017) in wheat and later Wang et al. (2017) likewise introduced in rice a pioneering idea utilizing geminiviral replicons for amplification of Cas9 and repair template. This approach enabled the integration of the repair template as long as several hundreds of bp with relatively high efficiency. Less efficient was the preparation of chimeric sgRNAs encoding protospacer-guide RNA and RNA repair template. The RNA guide was either designed as a single bifunctional molecule or guide RNA and the repair templates were interspaced by tRNA that is assumed to be processed to generate two RNA molecules. The Cas enzyme thus acquired another function, namely to serve as a scaffold for an RNA template used in RNA-mediated DNA repair (Butt et al., 2017). Large expectations are put into prime editing (Anzalone et al., 2019). New technique enabling genome edits without generation of DSB, while enabling precisely designed deletions, insertions, transitions, or transversions.

3.4. Application of CRISPR/Cas in barley and wheat

Programmable nucleases are showing very high applicability for staple crops. Moreover, there is an established barley transformation protocol (Harwood, 2014). Historically, barley was used as a suitable plant material at the dawn of classical mutagenesis thanks to the diploid state of its genome. Yet, in comparison to wheat, a comparable number of articles have been published describing applications of CRISPR technology. This fact could be easily explained by the importance of wheat for agricultural production. The first publication on mutagenesis of the barley genome using CRISPR appeared in 2015 (Lawrenson et al.2015). The authors showed that like other plant species, on-target mutations could be induced by Cas9 in the barley genome and that off-targets among close gene homologs can emerge. GE has enormous value in basic research. For example, barley mutants for cytokinin oxidase/dehydrogenase (CKX) genes, *HvCKX1* and *HvCKX3*, which participate in the fine regulation of cytokinin levels, were prepared to study hormonal regulation (Gasparis et al., 2018; Holubová et al., 2018).

Programmable nucleases do not have to be necessarily aimed at gene coding sequences causing mutants by disrupting the open reading frame but can also target regions surrounding the genes, eg. promoter regions, 5' and 3' end untranslated regions. Promoter modifications are interesting applications for fine tuning gene activity. Holme et al. (2017) introduced mutations into the promoter region of *HvPaPhy_a* gene coding mature grain phytase, which degrades phytic acid during germination thus providing phosphate for the seedling. Consequently, the elimination of the GCN4/Skn1/RY motif led to a decrease in mature grain phytase activity. As already mentioned, barley is used in the brewing industry, where it is the main component in the preparation of malt. It is not surprising that GE targets in barley include traits for improving the brewing process, knock-down of barley lipoxygenase 1 (*LOX1*) (Hensel et al., 2018b) and reduction of D-hordein content to improve malt extract quality (Yang et al., 2020). GE can be efficiently used to induce pathogen resistance in plants. Appropriately designed target sites could be of great value in the mutant phenotype and boost pathogen resistance as shown by Kumar et al. (2018) who created *morc1* mutant barley lines. In barley, this gene is described as a likely participant in regulating the expression of pathogen response proteins and is also involved in the process of genome stabilization. In the above study, *morc1* mutant lines revealed higher resistance to powdery mildew and *Fusarium graminearum*. The list of barley CRISPR edited lines to date is shown in Table 1.

Table 1 Application of CRISPR/Cas technology in barley

Gene	Function	Editing efficiency (%)	Reference
HvPM19	Positive regulator of dormancy	3/13 (**23%**) 1/10 (**10%**)	*(Lawrenson et al., 2015)*
ENG	Modification of N-glycans in grains	18/23 (**78%**)	*(Kapusi et al., 2017)*
HvPAPhy_a (promoter)	Mature grain phytase activity	28/64 (**44%**)	*(Holme et al., 2017)*
HvMORC1	Pathogen resistance, nuclear genome stabilization		*(Kumar et al., 2018)*
CKX1	Plant growth regulators - signalling	15/23 (**65%**)	*(Holubová et al., 2018)*
CKX1 *CKX3*	Plant growth regulators - signalling	47/71 (**66%**) 13/72 (**18%**)	*(Gasparis et al., 2018)*
PSD	Carotenoid biosynthesis,Albinism	6/40 (**15%**)	*(Howells et al., 2018)*
LOX1	Dioxygenation of FA, Brewery		*(Hensel et al., 2018b)*
FT1	Flowering time - yield	>12/24 (>**50%**)	*(Hensel et al., 2018a)*
CMF7	Albinism	1/21 (**5%**)	*(Li et al., 2019)*
D-hordein	Brewery	3/29 (**10%**)	*(Yang et al., 2020)*

Wheat may be considered a difficult plant species for gene editing due to its allohexaploid genome and generally lower transformation efficiency in comparison to barley. On the other hand, the complex allohexaploid wheat genome could serve as a model for the demonstration of mutual interaction among A, B, and D sub-genomes. In this regard, programmable nucleases which can induce mutations in multiple combinations in all three subgenomes, provide an advantage over classical mutagenesis techniques. The simple fact of assembling multiple mutations in one line that can take generations with classical breeding could be done in one or two generations using GE techniques. Notably, disruption of the *TaGW2* gene is an example of a Cas9 application. *GRAIN WEIGH 2* (*GW2*) is a negative regulator of grain development. The effect of *GW2* on the size and weight of the grain was studied in single and double mutants. Double mutants revealed increased thousand-grain weight by 12% in comparison to WT, while the average increase in single mutants was around 5.5%. (Wang et al., 2018b). Investigation of *gw2* mutant lines is an illustrative example of the application of the programmable nucleases to elucidate gene function via the rapid production of homozygous mutant lines in plant species with the polyploid genome, typically wheat or rapeseed. Such mutant lines could be effectively used in the study of homeolog gene interactions.

Targets aiming at generating plants with resistance to powdery mildew have been under investigation since the dawn of the expansion of new breeding techniques. As in barley, one of the first studies applying Cas9 *in planta* by Wang et al. (2014) described the simultaneous editing of three homeoalleles of wheat *MLO* genes for powdery mildew resistance. Additionally, Zhang et al. (2017) enhanced powdery mildew resistance by modification of *TaEDR1*, which encodes Raf-like mitogen-activated protein. This protein negatively influences plant response to powdery mildew infection (Frye et al., 2001).

The production of hybrid seed would be valuable in breeding programs. Male sterility was achieved in mutant wheat lines after the *Agrobacterium*-mediated transformation of Cas9 targeting *Ms45* (Singh et al., 2018) or Ms1 (Okada et al., 2019). An overview of works utilizing CRISPR/Cas editing in wheat is shown in Table 2. Clearly, GE techniques and particularly CRISPR/Cas, are a powerful tool in the preparation of plant material that is being used in basic research and could be potentially transferred into practice.

Table 2 Application of CRISPR/Cas technology in wheat

Gene	Function	Editing efficiency	Reference
MLO	Patogen resistance	4/72 (**5.5%**)	*(Wang et al., 2014)*
EDR1	Pathogen resistance	5/?	*(Zhang et al., 2017)*
LOX2	Dioxygenation of FA, Brewing	2/160 (**1.25%**)	*(Zong et al., 2017)*
α-gliadin	Storage protein	21/21 (**100%**)	*(Sánchez-León et al., 2018)*
ZIP4	Crossing-over	4/81 (**5%**)	*(Rey et al., 2018)*
GW2	Grain size, yield	6/17 (**35%**)	*(Wang et al., 2018b)*
DEP1	Grain number and size, erect	5/460 (**1.1%**)	*(Li et al., 2018b)*
GW2	panicle, nitrate uptake yield	2/480 (**0.5%**)	
GASR7	Grain length and weight	11/210 (**5%**)	*(Hamada et al., 2018)*
PSD	Carotenoid biosynthesis, Albinism	2/38 (**5.3%**);6/52 (**11%**); 4/35 (**11%**); 5/30 (**16.7%**); 13/73 (**17.8%**)	*(Howells et al., 2018)*
Ms45	Male sterility	25/181 (**13.8%**)	*(Singh et al., 2018)*
Ms1	Male sterility		*(Okada et al., 2019)*
Qsd1	Dormancy	3/8 (**37.5%**)	*(Abe et al., 2019)*

3.5. Phosphorus

Phosphorus is also a biofortification target. It is characterized by high reactivity and hence mostly occurs as a compound. For plants and living organisms generally, phosphorus enters cells and biochemical reactions in cells in the form of orthophosphate ($H_2PO_4^-$; hereafter phosphate). As an integral part of basic cell biomolecules such as nucleic acids, adenosine triphosphate (ATP), cell membranes, etc., phosphorus is an essential element for living organisms. The importance of phosphorus for plants is illustrated by the fact that it is one of the most growth-limiting factors (Raghothama and Karthikeyan, 2005) and its low availability provokes changes in root architecture (Lopez-Bucio et al., 2002). The form of phosphorus, which plants preferentially take up from the soil, is phosphate (Bieleski, 1973). Abundance and accessibility of phosphates in soils strongly correlate with plant growth and development. Phosphate accessibility is one of the driving forces of plant growth.

The most abundant phosphate-containing molecule in the soil is inositol hexakisphosphate (InsP6), commonly known as phytic acid (PA) (Turner et al., 2002). Plants are considered the most potent PA producers in the world. Phosphate present in PA or other organic forms cannot be directly utilized by plants. However, soil microorganisms such as *Ascomycete* and *Basidiomycete* fungi produce and exudate specific hydrolases – phytases, which are capable of InsP6 degradation (George et al., 2007). Apart from soil microorganisms, released phosphate can be utilized by plants either directly or indirectly through mycorrhizal symbiosis. Sufficiency of phosphate in the soil is necessary for optimal plant development. In the soil, if applied as a fertilizer, phosphate is quickly immobilized in complexes with Ca cations or ferric oxides and becomes unavailable to plants (Sample et al., 1980).

The phosphate route in plants commences with the root uptake system, which mediates the acquisition and transport of water and nutrients. Transmembrane phosphate transporters (PHT) play a key role in phosphate transport. These transporters provide active transport against the concentration gradient between plant cells and soil. There are several groups within this protein family with a specific function in phosphate transport. For instance, members of the PHT1 group are mainly responsible for the uptake in roots, transport to shoots and redistribution as reviewed by (Srivastava et al., 2018). On the other hand, there are transporters of the PHT4 group that drive specifically transport into sub-cellular compartments (Guo et al., 2008). Moreover, Versaw and Harrison (2002) showed that PHT2;1 transporter not only transports phosphate into chloroplast

but also modulates response to phosphate starvation thus giving these transporters a more complex role in the context of plant physiology.

Phosphate uptake is driven by the physiological needs of a plant. Previous hypotheses presumed that it had to be a complex process, where sugars act as a systemic signal conveying information about basal cellular requirements for phosphate (Chiou and Lin, 2011). Multiple ways were proposed to be participating in phosphate regulation including plant hormone signaling, which can modulate root growth and architecture depending on the phosphate availability (Lopez-Bucio et al., 2002). In long-distance signaling, there is production of active miRNA399 which targets PHO2 – E2 ubiquitin ligase involved in protein degradation (Pant et al., 2008). Recently, proteins possessing an SPX domain or SPX proteins themselves were proposed as key regulators of phosphate uptake and cell homeostasis. Inositol pyrophosphates (PP-InsPs) are suggested to be signaling molecules binding to the SPX domain (Wild et al., 2016). Huang et al. (2019) provided evidence that the SPX domain exhibits low affinity for phosphate but high affinity for InsP6. Binding of PP-InsPs into the SPX domain serves as a systemic signal for regulation of phosphate homeostasis. Zhu et al. (2019) described how VIH1 and VIH2 that exhibit kinase and phosphatase activity regulate phosphate homeostasis by relaying changes in cellular ATP and phosphate concentrations to altered levels of PP-InsPs. To maintain phosphate homeostasis in the cytosol, plant cells balance the optimal cytosolic concentration of phosphate via its influx and efflux to/from vacuole (Liu et al., 2015; Wang et al., 2015; Xu et al., 2019). In plant vegetative tissues, up to 90% of the cellular phosphate can be stored in the vacuole and remobilized in case of increased metabolic consumption (Bieleski, 1973). During the vegetative period, phosphate is exploited in cellular and physiological processes associated with growth and development. Change occurs when the plant reorients its efforts to the production of generative organs. Especially, during the grain filling phase, available phosphate in a plant is transported from senescent tissues to developing grain as well as the majority of assimilated phosphate from the soil. In grains of numerous staple crops (barley, wheat, corn, pea, soy, rice), phosphate is converted into organic form – PA, which is the major storage phosphate molecule. PA can account for up to three-quarters of the phosphate pool. However, not all plant species store phosphate in PA as reported by Ryan et al. (2019).

There are two main PA biosynthetic pathways: lipid-dependent and lipid-independent. Both pathways share an initial phase, in which *myo*-Inositol is produced. This phase consists of three steps beginning with phosphorylation of D-glucose by hexokinase (EC 2.7.1.1). The product D-glucose-6-phosphate is subsequently converted by isomerase activity of 1-L-*myo*-inositolphosphatesynthase

(EC 5.5.1.4, MIPS), which plays a pivotal role in *myo*-Inositol synthesis, to *myo*-Inositol-3-phosphate (Ins(3)P_1). Ins(3)P1 is then dephosphorylated by specific *myo*-InositolmonophosphataSe (IMP, EC 3.1.3.25) into the final product of the initial phase, *myo*-Inositol. *De novo* synthesis of *myo*-Inositol from D-glucose-6-phosphate is common to all organisms (Loewus and Murthy, 2000; Fileppi et al., 2010). MIPS enzyme is crucial for the synthesis of PA. It is localized in the cytosol of many organisms of the kingdoms of *Plantae*, *Animalia,* and *Fungi*. Higher organisms do have two forms localized either in the cytosol or cell compartments – chloroplasts (Abid et al., 2009). MIPS enzyme is so far the only known enzyme catalyzing the conversion of D-glucose-6-P to Ins(3)P1 and it appears to be conservative within eukarya (Majumder et al., 2003). It was proved that the *A. thaliana* genome contains three *MIPS* genes (Mitsuhashi et al., 2008), which cooperate to maintain inositol homeostasis (Fleet et al., 2018). In contrast, there is only a single *MIPS* gene in the genome of barley while there are seven in corn (Larson and Raboy, 1999). Lackey et al. (2003) reported that MIPS activity was localized in cell compartments possessing membrane-bound organelles, cell walls, and cytosol thus confirming that the initial phase of PA synthesis begins in the cytosol. In the following phase, sequential phosphorylation of inositol phosphate by multiple inositol kinases occurs.

As indicated, two pathways producing different intermediates contribute to the production of PA. In the lipid-dependent pathway, *myo*-inositol is linked to cytidine diphosphate diacylglycerol to form phosphatidylinositol phosphate (PtdInsP). PtdInsPs are incorporated into double-layered membranes, where they fulfill multiple physiologically important tasks as reviewed by Hirano and Sato (2019). Despite being only a minor part of membranes, PtdInsP homeostasis is necessary for processes of vesicular trafficking and cytoskeletal dynamics (Gerth et al., 2017). InsP3, which is a secondary messenger playing an important role in cell signaling, is produced from PtdInsP by the activity of Phospholipase C (PLC). PLC is a membrane-bounded enzyme catalyzing the hydrolysis of phosphatidylinositol-4,5-bisphosphate to glycerol and InsP3. Under stress conditions, PLC serves as a transductor of external stimuli to cell signal (Wang 2001). InsP3 is then phosphorylated by InsP kinases to PA. The lipid-dependent pathway in plants serves to mostly produce signaling and functional molecules that participate in physiological processes.

In contrast, the lipid-independent pathway seems to be involved mostly in straightforward production of PA by sequential phosphorylation of *myo*-Inositol by various InsP kinases during grain development (Suzuki et al., 2007). For instance, ITPKs catalyze phosphorylation InsP3 and InsP4 (Shi et al., 2003; Stiles et al., 2008). Notably, *A. thaliana* ITPK kinases were described as participating in the maintenance of cellular phosphate levels (Kuo et al., 2018). The last step of the PA synthesis

is provided by inositol pentakisphosphate kinase (Sweetman et al., 2006; Sun et al., 2007; Bhati et al., 2014). A small fraction accounting for approximately 1% of total PA content in plants is further phosphorylated to PP-InsPs. However, this subsequent phosphorylation is not tissue-dependent, as PP-InsPs have been detected in various plant tissues (Desai et al., 2014). At the cellular level, synthesized PA is stored predominantly in vacuoles. Transport of PA into a vacuole is mediated by ATP-binding cassette (ABC) transporter (Shi et al., 2007; Panzeri et al., 2011).

In mature grains, the central vacuole transforms into a number of smaller protein storage vacuoles (PSV). Feeney et al. (2018) demonstrated that PSVs are formed from the pre-existing embryonic vacuoles by a remodeling mechanism. Inside PSV, PA is sequestered in small spherical particles called globoids which are approximately 1-2 µm in diameter (Liu et al., 2004; Regvar et al., 2011). Since the molecule of PA has a strong negative charge due to six phosphate moieties, it occurs often in a form of its salt –phytate that is a result of the interaction between PA and cations of various metal elements, mostly Na, K, Ca, but also As, Cs, or Pb. Phytate comprises approximately 40% of globoid content (Bohn et al., 2007). It has been demonstrated that these particles are likely surrounded by a lipid membrane. Crystals of calcium oxalate and transmembrane transporters have been identified in the globoid membrane (Jiang et al., 2001).

Ionomics, a recently evolving field of research, focuses on ion spatial and temporal distribution in plant tissues (Huang and Salt, 2016). For example, exploration of the transport of microelements can be conducted precisely using stable isotopes (Di Tullo et al., 2015; Xue et al., 2015; Yamunarani et al., 2016). Characterization of the distribution and mutual interactions between ions in plant species can help define the plant's physiological nutritional status, and consequently, the biofortification strategies aimed at an increase in microelement content (Pii et al., 2015). Spatial analysis of phosphorus in a barley grain confirmed co-localization with iron, zinc, and manganese in the aleurone cells in the coat layers of a grain (Detterbeck et al., 2016). This followsthe localization of the main PA synthesis site in aleurone cells and embryo in barley (Tanaka et al., 1974).

Up to 60% of the assimilated phosphate is transported and stored in phytate globoids from the 16th day after flowering. Later, in the period of grain-filling almost all assimilated phosphate is stored in phytate globoids (Coelho et al., 2005). In barley grains, phytate begins to accumulate over seven days. reaching a stable level seven weeks after flowering (Hatzack et al., 2001). The activity of MIPS enzymes regulates the rate at which phosphate is converted into its organic storage form making *MIPS* potential candidate genes for genetic manipulations to alter total grain phosphate content. The highest expression of MIPS in wheat was detected one week after flowering followed

by a gradual decrease of MIPS enzymatic activity (Ma et al., 2013). Following the MIPS, the activity of ITPK and IPK enzymes increases to support PA synthesis during grain development (Bhati et al., 2014).

Plants and plant products constitute a significant source of phosphate in animal and human diets so that it is not surprising that phosphate is subjected to biofortification as well as many other elements. Regarding phosphate, several handicaps limit simple biofortification. As an essential element, the regulation of phosphate total content and homeostasis is highly complex (Chiou and Lin, 2011). In comparison to microelements, phosphate is relatively abundant in plant tissues and primarily in seeds, which are often used as food or feed. Unfortunately, seed phosphate in major staple crops (e.g. wheat, rice, maize, barley, and soy) is mostly stored in an organic form – PA that is relatively indigestible for non-ruminants (Lott et al., 2000). Implementing this knowledge into phosphate biofortification strategies has resulted in approaches that alter the phosphate form rather than increase the total phosphate content in plant seeds. This is in contrast to the biofortification of nutrients such as FA, carotenes and microelements, which mostly rely on transgene over-expression. Therefore, the most straightforward approach to increasing phosphate content was found to be a simple reduction of InsP6.

There are at least two ways for reducing effectively InsP6 content in grains: i) disruption of the InsP6 synthesis pathway and ii) expression of the phytate hydrolyzing enzyme - phytase. Development of *low phytic acid* (*lpa*) mutants historically preceded due to the extensive experience of breeders and researchers with the methods of classical mutagenesis, which was exploited to prepare the first *lpa* mutants (Larson et al., 1998; Raboy et al., 2000; Shi et al., 2007). This approach affected directly the process of phosphate storage by limiting the synthesis of InsP6. The latter became relevant with the optimization of transformation protocols for staple crops and transgenic lines expressing heterologous phytases were prepared. Phytase was of either microbial (Brinch-Pedersen et al., 2006; Peng et al., 2006; Bilyeu et al., 2008; Liu et al., 2011) or of endogenous origin (Holme et al., 2012). Notably, the high-phytase producing wheat line HighPhy was developed (Brinch-Pedersen et al., 2012) and evaluated in a feed trial. It has been reported that higher endogenous phytase activity enhances the digestibility of microelements present in the feed (Scholey et al., 2017). Unfortunately, despite the declared increased phosphate availability via increased mature grain phytase activity or reduced PA content in the prepared lines, the application of neither of the aforementioned approaches has resulted in transferring such biofortified crops to the market.

4. Materials and methods

4.1. Chemicals

List of critical chemicals below, all the other chemicals used in the experiments were of molecular biology grade.

BsaI (cat. no. R0535S) and HF-*MluI* (cat. no. R3198S) (New England Biolab, Ipswich, MA, USA)
T4 DNA ligase (cat. no. M0202S, New England Biolab, Ipswich, MA, USA)
Platinum Pfx DNA polymerase (cat. no. 11708-013, Carlsbad, CA, USA)
MS medium (cat. no. M0221, Duchefa Biochemie, Haarlem, Netherland)
MS medium (cat. no., M0238, Duchefa Biochemie, Haarlem, Netherland)
Phyto agar (cat. no. P1003, Duchefa Biochemie, Haarlem, Netherland)
LB broth (cat. no. L1703, Duchefa Biochemie, Haarlem, Netherland)
Phyto Agar (cat. no. P1003, Duchefa Biochemie, Haarlem, Netherland)
Hygromycin B (cat. no. 10843555001 Roche, Merck, Mannheim, Germany)
Total RNAqueous Kit (cat. no. AM1912, Thermo Fisher, Vilnius, Lithuania)
Turbo DNA-freeTM Kit (cat. no. AM1907, Thermo Fisher, Vilnius, Lithuania)
RevertAid H minus Reverse Transcriptase (cat. no. EP0451, Thermo Fisher, Vilnius, Lithuania)
Oligonucleotides (Generi Biotech a.s., Hradec Kralove, Czech Rep.)
ReadyMix™ PCR Reaction Mix (cat. no. R2523, Sigma-Aldrich, St. Louis, MO, USA)
SsoAdvanced™ Universal SYBR® Green Supermix (cat. no. 1725270, BioRad, Hercules, CA, USA)

4.2. Biological material

Spring barley (*Hordeum vulgare*) cultivar Golden Promise
Chemically competent *Escherichia coli* cells, strain DH5α
Electrocompetent *Agrobacterium tumafaciens* cells, strain AGL1

4.3. Equipment

Environmental Shaker-Incubator ES-20 (Biosan, Riga, Latvia)
Electroporator CelljecT Duo (Thermo Electron Corporation, Milford, MA, USA]
Flowbox HeraGuard (Thermo Scientific, Germany)
Centrifuge BR4i (Jouan, France)
Cultivation box Adaptis A1000 (Conviron, Winnipeg, MB, Canada)

Spectrophotometer DS-11 (DeNovix, Wilmington, Delaware, USA)

MM 400 mixer mill (Retsch, Haan, Germany)

Synergy Mx plate reader (BioTek Instruments, Winooski, VT, USA)

T100^{TM} Thermal Cycler (Biorad, Singapore)

CFX96 Touch^{TM} Real-Time PCR Detection System (BioRad, Singapore)

MEGA software version 6.06

A plasmid Editor (M. Wayne Davis)

Chromas version 2.6.6 (Technelysium, South Brisbane, Australia)

4.4. Methods

Design and cloning of protospacer

Barley inositol trisphosphate 5/6-kinase (HORVU7Hr1G033170 and HORVU1Hr1G050760, here referred to as *HvITPK1* and *HvITPK5* respectively) were chosen as target genes. The protein-coding sequence of *ZmIPK* was used to blast the barley genomic DNA database (EnsemblPlants) to identify homologous barley *ITPK* genes and rice or wheat orthologs. To evaluate phylogenetic relations between *ITPK* genes described in rice, wheat and barley orthologs, a phylogenetic tree was constructed using AA sequences due to its higher conservation across the plant species. The protospacers were designed based on the Golden Promise sequence. Protospacer sequences were sub-cloned into pYLsgRNA-OsU6 and then the U6 promoter-sgRNA cassette was cloned into the expression vector pYLCRISPR/Cas9P$_{ubi}$-H according to the protocol described by Ma et al. (Ma et al., 2015b). The expression vector was verified by digestion with *Mlu*I, and by Sanger sequencing. The vector harboring sgRNA with the protospacer sequence was transformed into *Agrobacterium tumefaciens* strain AGL.1 by electroporation.

Barley transformation

The barley cultivar Golden Promise was used for transformation according to the protocol by Harwood (2014). Two expression vectors coding Cas9 nuclease targeting either *HvITPK1* or *HvITPK5* were transformed independently. Immature barley embryos were used for *Agrobacterium*-mediated transformation. Tissue cultures were derived and callus cultures were selected on Hygromycin. Subsequently, regenerating plantlets were transferred onto regeneration medium in flasks. Regenerating plants were transferred into pots with soil substrate and grown in the greenhouse at 16°C with 16-h light photoperiod until maturity. For the identification of transgenic plants, genomic DNA from young leaves was extracted according to Edwards et al.

(1991). DNA samples were used for analyses of the presence of Cas9 transgene by polymerase chain reaction (PCR) using a premix REDTaq® ReadyMix™ PCR Reaction Mix. The PCR products were separated in 1% agarose gel stained with ethidium bromide. Separated PCR products were evaluated in UV transilluminator.

Detection of the Cas9 and sgRNA transcripts

Transgenic plants with confirmed presence of Cas9 transgene were picked for the analysis of transgene expression. Total RNA was extracted from leaves using a Total RNAqueous Kit. Extracted RNA was treated with DNase. The concentration of RNA was assessed spectrophotometrically. 1 µg of RNA was used for reverse transcription using poly-T primers and RevertAid H minus Reverse Transcriptase. cDNA samples were diluted ten times with nuclease-free water. RT-PCR was performed using specific primers for Cas9 and sgRNA transcripts. PCR products were analyzed using electrophoresis as described in the section Barley transformation.

Genotyping of primary regenerants and their progeny.

To evaluate the induction of target and off-target mutations, primer pairs for amplification of the target and off-target loci in the closest homologs of *HvITPK1* within the group of *ITPK* were designed, verified and used for genotyping. The specificity of the primers was verified by gradient PCR for each primer pair. The optimal annealing temperature for each primer pair was selected. Then, the target and off-target loci in T0 transgenic plants were PCR-amplified. The PCR products were Sanger sequenced. Visual evaluation of sequencing files was conducted in the chromatogram viewer Chromas. For decoding and evaluation of the sequencing chromatograms, the DSDecode online tool (Liu et al., 2018) was used. For the analysis of heritability of the target mutations and induction of off-target mutations in the next generation, the progeny of the bi-allelic mutant plant 3B was genotyped in the T1 and T2 generations.

Seed phosphate analysis

For phosphate analysis in mature grains, progenitor lines originating from bi-allelic mutant plant T0 *itpk1* 3B were selected. T1 progeny of 3B plant was grown in the greenhouse to maturity. Mature barley grains (T2 generation) of mutant T1 3B plants were harvested. 2 g of grains were homogenized to a fine flour. Phosphate analysis was done according to the method of Vaculova et al. (2011). Technical and biological measurements were made in triplicate. The mean values with standard deviations were plotted on a graph.

Abiotic stress experiments

The homozygous T2 mutant lines, deletion mutant *itpk1-2* and insertion mutant *itpk1-33* were selected for *in vitro* testing of their response to abiotic stress factors (NaCl and D-mannitol). Non-transgenic Golden Promise referred to as WT was used as a control. Mature grains of WT and *itpk1* mutants were surface sterilized and soaked in sterile water overnight in the dark. The embryos were dissected, sterilized for 1 min in 1% sodium hypochlorite solution with agitation and then washed twice in sterile water. Embryos were placed on the surface of solid half Murashige-Skoog medium with 10 g/L sucrose, phytoagar 6 g/L, with the pH adjusted to 5.8 was prepared in flasks. The media were supplemented with NaCl (*Supplement II*) or D-mannitol in concentrations 100, 200, and 300 mM. Five embryos of each mutant line and WT per treatment. The flasks with embryos were placed in a growing cabinet and cultivated at 24°C and a day/night regime of 18/6 hours. After two weeks, the phenotype of the seedlings was evaluated by measuring the height of the seedlings.

Germination assay

Grains of *itpk1-2*, *itpk1-33* mutants and WT were assayed for salinity stress during germination according to the protocol of Daszkowska-Golec et al. (2019). Grains were sown 30 per plate. Salt levels were determined at 100 and 200 mM.

Expression profiling of barley ITPK genes under salinity stress

Total RNA from young seedlings of WT and homozygous mutant lines *itpk1-2* and *itpk1-33* cultivated *in vitro* on pure half MS medium and medium supplemented with 200 mM NaCl was extracted as described above. Samples of leaves and roots which were additionally washed in deionized water to remove residues of medium, were immediately after collecting frozen in liquid nitrogen. Three biological samples per treatment were taken. Extracted RNA was treated with DNase. Then, the concentration of RNA was assessed spectrophotometrically. RNA was reverse transcribed as described above. cDNA samples were diluted with nuclease-free water. Diluted cDNA samples were used for qRT-PCR analysis. *ITPK* transcript levels were determined by qRT-PCR.

5. Survey of results

Biofortification of staple crops is a complex process that requires substantial knowledge of plant physiology. Multiple plant species have been subjected to biofortification using various methods and sophisticated approaches as reviewed in *Supplement I*. Simple techniques characterized by direct application of nutrients evolved into the use of encapsulated nutrients in small particles that can be applied to the soil under the seed. Dramatic progress is also reviewed for biotechnological approaches. The portfolio of biotechnological techniques has expanded mainly by a group of GE systems, of which CRISPR/Cas is overwhelmingly superior. Precise and effective manipulation with gene sequences facilitates identification of interesting candidate genes, which can be eventually integrated into breeding programs directed to crop biofortification. On the other hand, developing *de novo* synthetic pathways will still rely on the preparation of classic transgene over-expression and cannot be easily circumvented. All things considered, GE technology shows great potential for preparing plant material with valuable traits as documented by many barley and wheat mutant lines that have been developed using the GE technology to date.

The CRISPR/Cas system was used to develop mutant barley plants in candidate *ITPK* genes (*Supplement II*). Initially*, in silico* analysis of *ITPK* genes revealed there are six members of the *ITPK* group in barley. Two *ITPK* genes, *HvITPK1* and *HvITPK5,* were selected as targets for knock-out. Plasmid vectors were prepared and used for *Agrobacterium*-mediated transformation. No transgenic plants were obtained in the transformation experiment with Cas9 designed for *HvITPK5*. On the other hand, transgenic plants were obtained for the *HvITPK1* gene, Table 3.

Table 3 Transformation of spring barley cv. Golden Promise

Target gene	No. of immature embryos	No of. regenerated plants	No. of T0 transgenic plants	Transformation efficiency (%)	No. of edited plants	Editing efficiency (%)
HvITPK1	100	17	13	13	6	46
HvITPK5	143	3	0	0	–	–

Expression analysis confirmed the transcription of both CRISPR/Cas components Cas9 and sgRNA (*Supplement II*, Figure 2B). No detection of Cas9 at the protein level in transgenic plants was conducted. The presence of the Cas9 protein and, importantly, the activity of the Cas9 effector complex would be confirmed by the detection of mutation at a defined position in a target gene. Indeed, sequencing of target locus in transgenic plants revealed mutated allelic variants of the *HvITPK1* gene in several primary regenerants (*Supplement II*, Figure S2). Chimeric as well as uniform mutant plants were detected in primary regenerants. Sequencing of potential off-target sites among *ITPK* genes revealed no unintended mutation, proving the on-target editing of Cas9.

Analysis of progeny of selected bi-allelic mutant plant 3B revealed stable transmission of mutation into the next generation and segregation of mutant alleles, which resulted in the occurrence of two variants of homozygous mutant plants. These plants showed a homozygous state in single bp deletion or insertion. A substantial portion of progeny maintained the bi-allelic constitution.

Mutation in the *HvITPK1* gene did not result in over-all increased phosphate content, but the phosphate levels varied among the obtained progeny of mutant plants. Among tested mutant lines, those showing a substantial increase in mature grain phosphate content were obtained (*Supplement II*, Figure 3). Deletion and insertion mutant lines in *HvITPK1* differed phenotypically in reaction to abiotic stress during germination and seedling growth under salinity stress. For instance, the germination rate was more affected in *itpk1-33* under salt stress conditions. (*Supplement II*, Figure 4, 5).

Expression profiling revealed the up-regulation of most *ITPK* genes in roots in reaction to salinity stress, which further supported the hypothesis of involvement of *ITPK* genes in the regulation of reaction to salinity in young seedlings (*Supplement II*, Figure 6). Expression profile of *ITPK* genes in *itpk1-2* and *itpk1-33* varied between the two mutant lines under salinity stress conditions.

Unpublished data

Extended *in silico* analysis of barley, rice and additionally wheat *ITPK* genes was performed, Figure 2. Protein coding sequences were mined from the Ensembl Plant database. It was confirmed that three intronless *ITPK* genes identified in barley were confirmed also in wheat, suggesting that divergence of *ITPK* groups between *Oryzoideae* and *Pooideae* subfamilies had occurred after these two subfamilies split phylogenetically.

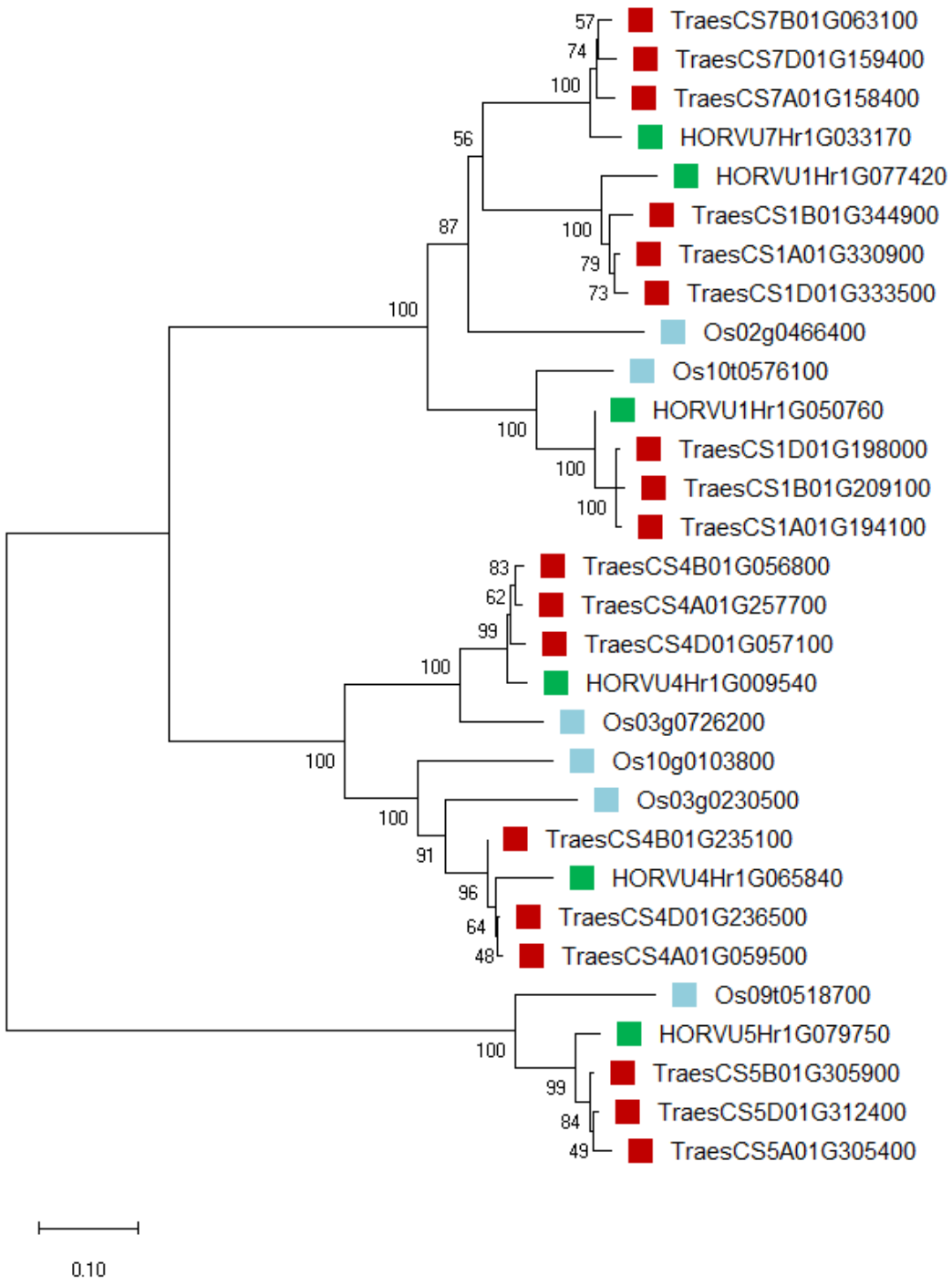

Figure 2 Phylogenetic tree of rice, barley, and wheat *ITPK* coding sequences. Rice orthologs are marked in blue squares, wheat in red and barley in green.

Additionally, to study abiotic stress response in young seedlings more extensively, a similar experiment as described in *Supplement II* was performed. Here, abiotic stress in young seedlings was induced by adding D-mannitol into cultivation medium, Figure 3. D-mannitol affected seedling growth more with increasing concentration. There were no substantial differences between wild type and mutant lines. It can be assumed that both types of mutations have no visible impact on the phenotype of mutant lines in response to the form of abiotic stress induced by D-mannitol in young seedlings.

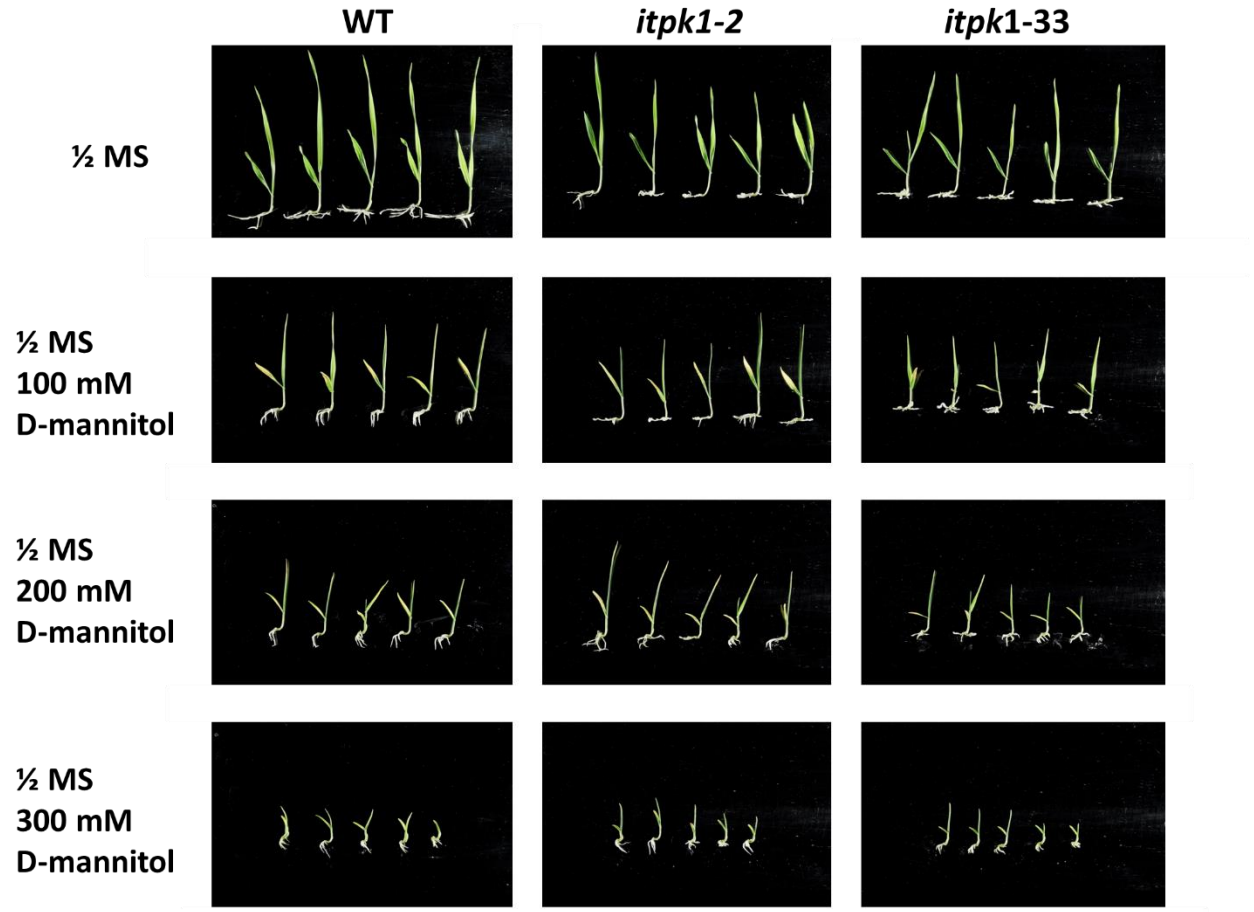

Figure 3 The effect of D-mannitol on *in vitro* cultivated barley *itpk1* mutants.

Moreover, to demonstrate phenotypic performance in more mutant lines, homozygous insertion mutant *itpk1-10* was included in both variants of abiotic stress, NaCl and D-mannitol, Figure 4. Following the published results (*Supplement II*), mutant line *itpk1-10* showed better growth at the

highest salt concentration in comparison to WT. Treatment with D-mannitol induced stress caused no distinguishable phenotypic differences between the mutant and WT.

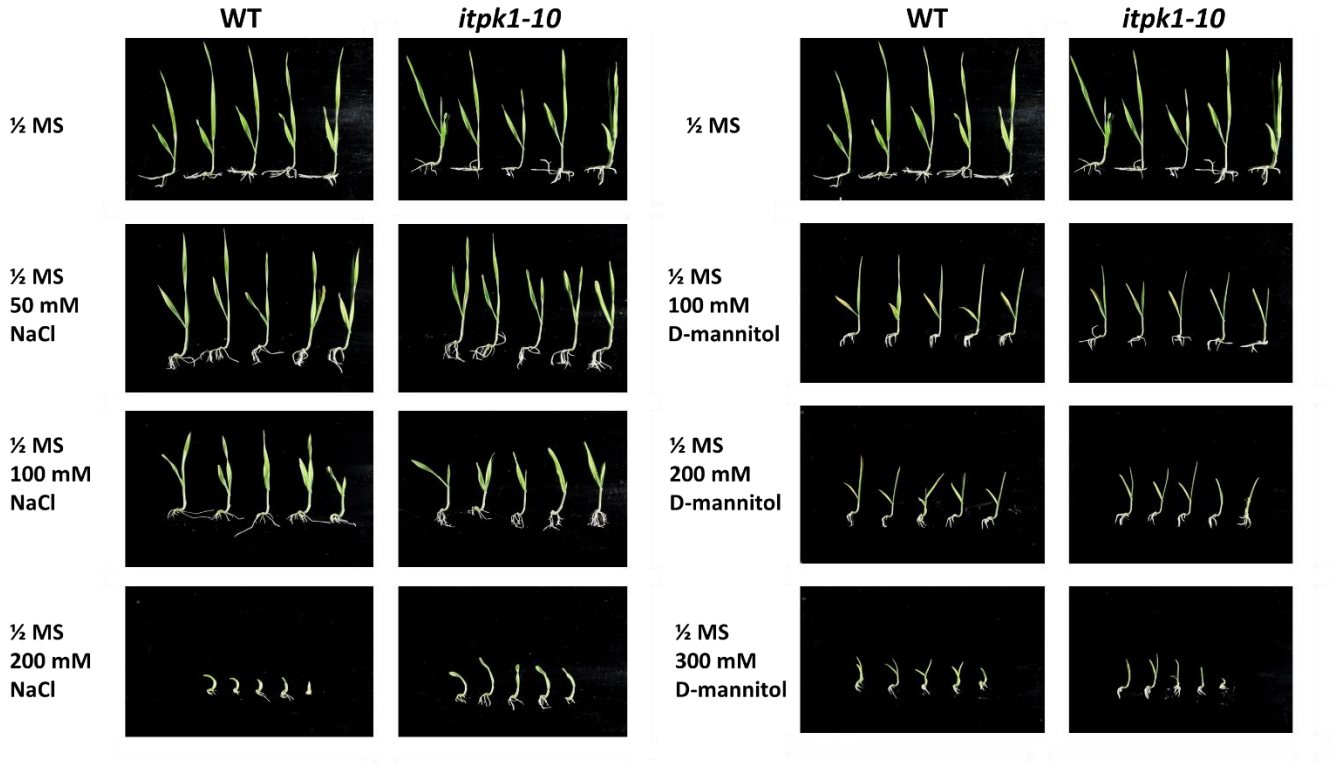

Figure 4 The effect of NaCl and D-mannitol on *in vitro* cultivated barley *itpk1-10* mutants.

Genotyping of T2 generation homozygous mutant lines revealed mutant lines, in which Cas9 transgene was not detected, Figure 5. These lines were considered transgene-free.

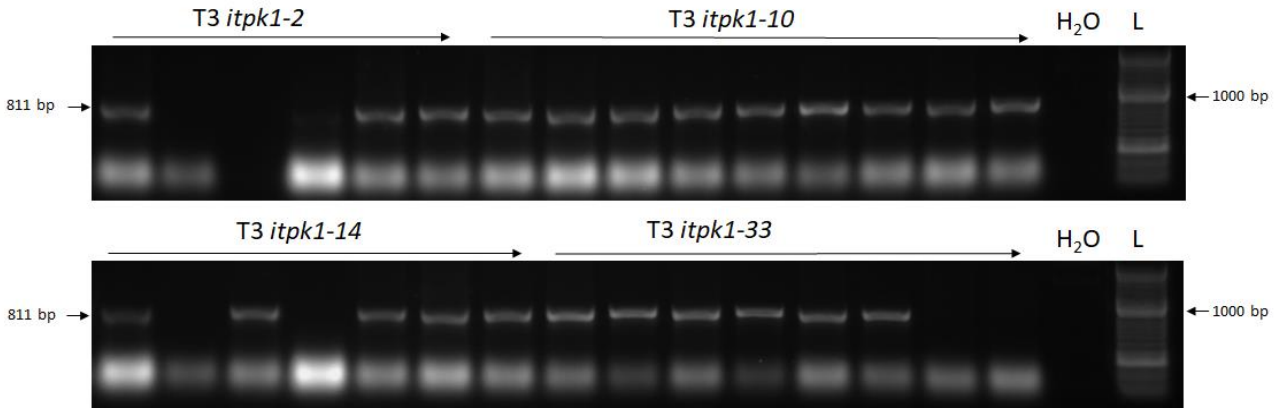

Figure 5 Genotyping of T2 generation *itpk1* mutant lines. Detection of Cas9 transgene in homozygous mutant lines *itpk1-2*, *itpk1-10*, *itpk1-14*, *itpk1-33*; PCR product size 811 bp. L – DNA ladder 50 bp (Bioline).

The phenotype of mutant plants in regard to height was not remarkably different from the WT, Figure 6.

Figure 6 Comparison of *itpk1* mutant with WT. WT on the left, *itpk1* mutant on the right.

6. Conclusion and perspectives

This thesis focuses on phosphate biofortification of the spring barley cultivar Golden Promise. Modern techniques of GE were used to modify phytic acid biosynthesis in order to increase the phosphate content in mature barley grains. Several experiments were performed to further unravel the potential link between *ITPK* genes and reaction to abiotic stress.

The most important outcomes of the work are the following:

- An overview of the published literature on the topic of crop biofortification using modern breeding techniques including genome-editing technology that can be successfully implemented in the process of crop biofortification.
- Barley *ITPK* genes were phylogenetically clustered and ordered.
- It was verified that CRISPR/Cas9 genome-editing technology can be applied in barley to induce mutations at predefined positions.
- Mutations induced by CRISPR/Cas9 have mostly the character of single bp insertion or deletion and these mutations are heritable into the next generation.
- Different allelic variants of mutated *HvITPK1* showed distinguishable reactions to salinity stress during germination.
- Homozygous barley lines without original *Cas9* transgene were obtained over three generations.

The application of genome-editing techniques in crop biofortification is a feasible way for the production of nutritionally more valuable products. Developed barley lines serve as proof of concept that precise manipulation of the phytic acid biosynthetic pathway can result in lines with the altered ratio of phosphate form in the mature grains. Prepared homozygous experimental plant material can be subsequently utilized for testing of abiotic stress resistance. Functional validation of the *HvITPK1* gene confirmed its participation in the storage of phosphate in grains and also suggested possible interplay in regulation of the abiotic stress response. *In silico* analyses of *ITPK* sequences will further serve as the groundwork for the design of potential new targets using the CRISPR/Cas system.

7. References

Abe, F., Haque, E., Hisano, H., Tanaka, T., Kamiya, Y., Mikami, M., et al. (2019). Genome-Edited Triple-Recessive Mutation Alters Seed Dormancy in Wheat. *Cell Rep.* 28, 1362–1369. doi:10.1016/j.celrep.2019.06.090.

Abe, K., Araki, E., Suzuki, Y., Toki, S., and Saika, H. (2018). Production of high oleic/low linoleic rice by genome editing. *Plant Physiol. Biochem.* 131, 58–62. doi:https://doi.org/10.1016/j.plaphy.2018.04.033.

Abid, G., Silue, S., Muhovski, Y., Jacquemin, J. M., Toussaint, A., and Baudoin, J. P. (2009). Role of *myo*-inositol phosphate synthase and sucrose synthase genes in plant seed development. *Gene* 439, 1–10. doi:10.1016/j.gene.2009.03.007.

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., et al. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157. doi:10.1038/s41586-019-1711-4.

Barrangou, R., Boyaval, P., Moineau, S., Romero, D. A., and Horvath, P. (2007). CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* 315, 1709–1712. doi:10.1126/science.1138140.

Bhati, K. K., Aggarwal, S., Sharma, S., Mantri, S., Singh, S. P., Bhalla, S., et al. (2014). Differential expression of structural genes for the late phase of phytic acid biosynthesis in developing seeds of wheat (*Triticum aestivum* L.). *Plant Sci.* 224, 74–85. doi:10.1016/j.plantsci.2014.04.009.

Bieleski, R. L. (1973). Phosphate Pools, Phosphate Transport, and Phosphate Availability. *Annu. Rev. Plant Physiol.* 24, 225–252. doi:10.1146/annurev.pp.24.060173.001301.

Billard, V., Etienne, P., Jannin, L., Garnica, M., Cruz, F., Garcia-Mina, J. M., et al. (2014). Two Biostimulants Derived from *Algae* or Humic Acid Induce Similar Responses in the Mineral Content and Gene Expression of Winter Oilseed Rape (*Brassica napus* L.). *J. Plant Growth Regul.* 33, 305–316. doi:10.1007/s00344-013-9372-2.

Bilyeu, K. D., Zeng, P., Coello, P., Zhang, Z. J., Krishnan, H. B., Bailey, A., et al. (2008). Quantitative Conversion of Phytate to Inorganic Phosphorus in Soybean Seeds Expressing a Bacterial Phytase. *Plant Physiol.* 146, 468–477. doi:10.1104/pp.107.113480.

Bohn, L., Josefsen, L., Meyer, A. S., and Rasmussen, S. K. (2007). Quantitative Analysis of Phytate Globoids Isolated from Wheat Bran and Characterization of Their Sequential Dephosphorylation by Wheat Phytase. *J. Agric. Food Chem.* 55, 7547–7552. doi:10.1021/jf071191t.

Boonyaves, K., Wu, T.-Y., Gruissem, W., and Bhullar, N. K. (2017). Enhanced Grain Iron Levels in Rice Expressing an *IRON-REGULATED METAL TRANSPORTER, NICOTIANAMINE SYNTHASE*, and *FERRITIN* Gene Cassette. *Front. Plant Sci.* 8, 1–11. doi:10.3389/fpls.2017.00130.

Bouis, H. (2002). Symposium : Plant Breeding : A New Tool for Fighting Micronutrient Malnutrition Evaluating the Impact of Plant Biofortification on Human Nutrition 1. *J. Nutr.* 132, 491S-494S. doi:10.1093/jn/132.3.491S.

Brinch-Pedersen, H., Hatzack, F., Stöger, E., Arcalis, E., Pontopidan, K., and Holm, P. B. (2006). Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): Deposition pattern, thermostability, and phytate hydrolysis. *J. Agric. Food Chem.* 54, 4624–4632. doi:10.1021/jf0600152.

Brinch-Pedersen, H., Madsen, C. K., Dionisio, G., and Holm, P. B. (2012). High expression cereal phytase gene. 1–58. Available at: https://encrypted.google.com/patents/WO2012146597A1?cl=ru.

Brouns, S. J. J., Matthijs, J, J., Lundgren, M., Westra, E. R., Slijkhuis, R. J., Snijders, A. P., et al. (2008). Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science* 321, 960–964. doi:10.1126/science.1159689.

Budhagatapalli, N., Rutten, T., Gurushidze, M., Kumlehn, J., and Hensel, G. (2015). Targeted modification of gene function exploiting homology-directed repair of TALEN-mediated double-strand breaks in barley. *G3 Genes, Genomes, Genet.* 5, 1857–1863. doi:10.1534/g3.115.018762.

Butt, H., Eid, A., Ali, Z., Atia, M. A. M., Mokhtar, M. M., Hassan, N., et al. (2017). Efficient CRISPR/Cas9-mediated genome editing using a chimeric single-guide RNA molecule. *Front. Plant Sci.* 8, 1–8. doi:10.3389/fpls.2017.01441.

Carte, J., Wang, R., Li, H., Terns, R. M., and Terns, M. P. (2008). Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev.* 22, 3489–3496. doi:10.1101/gad.1742908.

Che, P., Zhao, Z., Glassman, K., Dolde, D., Hu, T. X., Jones, T. J., et al. (2016). Correction for Che et al., Elevated vitamin E content improves all- trans β-carotene accumulation and stability in biofortified sorghum. *Proc. Natl. Acad. Sci. U. S. A.* 113, E8209. doi:10.1073/pnas.1617908113.

Chiou, T.-J., and Lin, S.-I. (2011). Signaling network in sensing phosphate availability in plants. *Annu. Rev. Plant Biol.* 62, 185–206. doi:10.1146/annurev-arplant-042110-103849.

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., et al. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 756–761. doi:10.1534/genetics.110.120717.

Coelho, C. M. M., Tsai, S. M., and Vitorello, V. A. (2005). Dynamics of inositol phosphate pools (tris-, tetrakis- and pentakisphosphate) in relation to the rate of phytate synthesis during seed development in common bean (*Phaseolus vulgaris*). *J. Plant Physiol.* 162, 1–9. doi:10.1016/j.jplph.2004.06.013.

Connor, W. E. (2000). Importance of n-3 fatty acids in health and disease. *Am. J. Clin. Nutr.* 71 suppl, 171S-175S. doi:10.1093/ajcn/71.1.171s.

Cordell, D., Drangert, J. O., and White, S. (2009). The story of phosphorus: Global food security and food for thought. *Glob. Environ. Chang.* 19, 292–305. doi:10.1016/j.gloenvcha.2008.10.009.

Crespo-Herrera, L. A., Velu, G., and Singh, R. P. (2016). Quantitative trait loci mapping reveals pleiotropic effect for grain iron and zinc concentrations in wheat. *Ann. Appl. Biol.* 169, 27–35. doi:10.1111/aab.12276.

Daszkowska-Golec, A., Skubacz, A., Kurowska, M., Slota, M., Swiergolik, D., and Szarejko, I. (2019). "Methods for the Simple and Reliable Assessment of Barley Sensitivity to Abiotic Stresses During Early Development," in *Barley: Methods and Protocols*, ed. W. A. Harwood (Springer New York), 127–151. doi:10.1007/978-1-4939-8944-7_9.

Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J. K., et al. (2012). Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 335, 720–723. doi:10.1126/science.1215670.

Desai, M., Rangarajan, P., Donahue, J. L., Williams, S. P., Land, E. S., Mandal, M. K., et al. (2014). Two inositol hexakisphosphate kinases drive inositol pyrophosphate synthesis in plants. *Plant J.* 80, 642–653. doi:10.1111/tpj.12669.

Deshpande, P., Dapkekar, A., Oak, M. D., Paknikar, K. M., and Rajwade, J. M. (2017). Zinc complexed chitosan/TPP nanoparticles: A promising micronutrient nanocarrier suited for foliar application. *Carbohydr. Polym.* 165, 394–401. doi:10.1016/j.carbpol.2017.02.061.

Detterbeck, A., Pongrac, P., Rensch, S., Reuscher, S., Pečovnik, M., Vavpetič, P., et al. (2016). Spatially resolved analysis of variation in barley (*Hordeum vulgare*) grain micronutrient accumulation. *New Phytol.* 211, 1241–1254. doi:10.1111/nph.13987.

Di Tullo, P., Versini, A., Bueno, M., Le Hécho, I., Thiry, Y., Biron, P., et al. (2015). Stable isotope tracing: A powerful tool for selenium speciation and metabolic studies in non-hyperaccumulator plants (ryegrass *Lolium perenne* L.). *Anal. Bioanal. Chem.* 407, 9029–9042. doi:10.1007/s00216-015-9069-4.

Domergue, F., Abbadi, A., Zähringer, U., Moreau, H., and Heinz, E. (2005). *In vivo* characterization of the first acyl-CoA Delta6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. *Biochem. J.* 389, 483–490. doi:10.1042/BJ20050111.

Doolette, C. L., Read, T. L., Li, C., Scheckel, K. G., Donner, E., Kopittke, P. M., et al. (2018). Foliar application of zinc sulphate and zinc EDTA to wheat leaves: Differences in mobility, distribution, and speciation. *J. Exp. Bot.* 69, 4469–4481. doi:10.1093/jxb/ery236.

Du, H., Huang, M., Hu, J., and Li, J. (2016). Modification of the fatty acid composition in *Arabidopsis* and maize seeds using a stearoyl-acyl carrier protein desaturase-1 (*ZmSAD1*) gene. *BMC Plant Biol.* 16, 1–10. doi:10.1186/s12870-016-0827-z.

Ducsay, L., Ložek, O., Marček, M., Varényiová, M., Hozlár, P., and Lošák, T. (2016). Possibility of selenium biofortification of winter wheat grain. *Plant, Soil Environ.* 62, 379–383. doi:10.17221/324/2016-PSE.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* 19, 1349. doi:10.1093/nar/19.6.1349.

Fauser, F., Schiml, S., and Puchta, H. (2014). Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J.* 79, 348–359. doi:10.1111/tpj.12554.

Feeney, M., Kittelmann, M., Menassa, R., Hawes, C., and Frigerio, L. (2018). Protein Storage Vacuoles

Originate from Remodeled Preexisting Vacuoles in *Arabidopsis thaliana*. *Plant Physiol.* 177, 241–254. doi:10.1104/pp.18.00010.

Fernández, V., and Brown, P. H. (2013). From plant surface to plant metabolism: the uncertain fate of foliar-applied nutrients. *Front. Plant Sci.* 4, 1–5. doi:10.3389/fpls.2013.00289.

Fernández, V., and Eichert, T. (2009). Uptake of Hydrophilic Solutes Through Plant Leaves: Current State of Knowledge and Perspectives of Foliar Fertilization. *Crit. Rev. Plant Sci.* 28, 36–68. doi:10.1080/07352680902743069.

Field, R. J., and Bishop, N. G. (1988). Promotion of stomatal infiltration of glyphosate by an organosilicone surfactant reduces the critical rainfall period. *Pestic. Sci.* 24, 55–62. doi:10.1002/ps.2780240106.

Fileppi, M., Galasso, I., Tagliabue, G., Daminati, M. G., Campion, B., Doria, E., et al. (2010). Characterisation of structural genes involved in phytic acid biosynthesis in common bean (*Phaseolus vulgaris* L.). *Mol. Breed.* 25, 453–470. doi:10.1007/s11032-009-9344-1.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi:10.1038/35888.

Fitzpatrick, C. R., Copeland, J., Wang, P. W., Guttman, D. S., Kotanen, P. M., and Johnson, M. T. J. (2018). Assembly and ecological function of the root microbiome across angiosperm plant species. *Proc. Natl. Acad. Sci. U. S. A.* 115, E1157–E1165. doi:10.1073/pnas.1717617115.

Fleet, C. M., Yen, J. Y., Hill, E. A., and Gillaspy, G. E. (2018). Co-suppression of AtMIPS demonstrates cooperation of MIPS1, MIPS2 and MIPS3 in maintaining *myo*-inositol synthesis. *Plant Mol. Biol.* 97, 253–263. doi:10.1007/s11103-018-0737-6.

Frankel, A. D., Berg, J. M., and Pabo, C. O. (1987). Metal-dependent folding of a single zinc finger from transcription factor IIIA. *Proc. Natl. Acad. Sci. U. S. A.* 84, 4841–4845. doi:10.1073/pnas.84.14.4841.

Frye, C. A., Tang, D., and Innes, R. W. (2001). Negative regulation of defense responses in plants by a conserved MAPKK kinase. *Proc. Natl. Acad. Sci. U. S. A.* 98, 373–378. doi:10.1073/pnas.98.1.373.

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K., et al. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31, 822–826. doi:10.1038/nbt.2623.

Gasiunas, G., Barrangou, R., Horvath, P., and Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 109, 2579–2586. doi:10.1073/pnas.1208507109.

Gasparis, S., Kała, M., Przyborowski, M., Łyznik, L. A., Orczyk, W., and Nadolska-Orczyk, A. (2018). A simple and efficient CRISPR/Cas9 platform for induction of single and multiple, heritable mutations in barley (*Hordeum vulgare* L.). *Plant Methods* 14, 1–14. doi:10.1186/s13007-018-0382-8.

Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., et al. (2013). Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca^{2+} spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone. *New Phytol.* 198, 179–189. doi:10.1111/nph.12146.

George, T. S., Simpson, R. J., Gregory, P. J., and Richardson, A. E. (2007). Differential interaction of *Aspergillus niger* and *Peniophora lycii* phytases with soil particles affects the hydrolysis of inositol phosphates. *Soil Biol. Biochem.* 39, 793–803. doi:10.1016/j.soilbio.2006.09.029.

Gerth, K., Lin, F., Menzel, W., Krishnamoorthy, P., Stenzel, I., Heilmann, M., et al. (2017). Guilt by Association: A Phenotype-Based View of the Plant Phosphoinositide Network. *Annu. Rev. Plant Biol.* 68, 349–374. doi:10.1146/annurev-arplant-042916-041022.

Gibson, T. J., Postma, J. P. M., Brown, R. S., and Argos, P. (1988). A model for the tertiary structure of the 28 residue dna-binding motif ('zinc finger') common to many eukaryotic transcriptional regulatory proteins. *Protein Eng. Des. Sel.* 2, 209–218. doi:10.1093/protein/2.3.209.

Gil-Humanes, J., Wang, Y., Liang, Z., Shan, Q., Ozuna, C. V., Sánchez-León, S., et al. (2017). High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. *Plant J.* 89, 1251–1262. doi:10.1111/tpj.13446.

Gu, K., Yang, B., Tian, D., Wu, L., Wang, D., Sreekala, C., et al. (2005). *R* gene expression induced by a type-III effector triggers disease resistance in rice. *Nature* 435, 1122–1125. doi:10.1038/nature03630.

Guo, B., Jin, Y., Wussler, C., Blancaflor, E. B., Motes, C. M., and Versaw, W. K. (2008). Functional analysis of the Arabidopsis PHT4 family of intracellular phosphate transporters. *New Phytol.* 177, 889–898. doi:10.1111/j.1469-8137.2007.02331.x.

Hale, C. R., Zhao, P., Olson, S., Duff, M. O., Graveley, B. R., Wells, L., et al. (2009). RNA-Guided RNA Cleavage by a CRISPR RNA-Cas Protein Complex. *Cell* 139, 945–956. doi:10.1016/j.cell.2009.07.040.

Hamada, H., Liu, Y., Nagira, Y., Miki, R., Taoka, N., and Imai, R. (2018). Biolistic-delivery-based transient CRISPR/Cas9 expression enables *in planta* genome editing in wheat. *Sci. Rep.* 8, 1–7. doi:10.1038/s41598-018-32714-6.

Harwood, W. A. (2014). "A Protocol for High-Throughput *Agrobacterium*-Mediated Barley Transformation," in *Cereal Genomics: Methods and Protocols*, eds. R. J. Henry and A. Furtado (Totowa, NJ: Humana Press), 251–260. doi:10.1007/978-1-62703-715-0_20.

Hatzack, F., Hübel, F., Zhang, W., Hansen, P. E., and Rasmussen, S. K. (2001). Inositol phosphates from barley low-phytate grain mutants analysed by metal-dye detection HPLC and NMR. *Biochem. J.* 354, 473–480. doi:10.1042/0264-6021:3540473.

Heler, R., Samai, P., Modell, J. W., Weiner, C., Goldberg, G. W., Bikard, D., et al. (2015). Cas9 specifies functional viral targets during CRISPR-Cas adaptation. *Nature* 519, 199–202. doi:10.1038/nature14245.

Hensel, G., Mueller, S., Pouramini, P., Pillen, K., and Kumlehn, J. (2018a). Manipulation of flowering time in cultivated barley by ectopic expression of wild barley FT1 allele. in *IN VITRO CELLULAR*

& DEVELOPMENTAL BIOLOGY-PLANT (233 SPRING ST, NEW YORK, NY 10013 USA: SPRINGER), S88.

Hensel, G., Pouramini, P., Hiekel, S., Reuter, P., Baier, S., and Kumlehn, J. (2018b). Generation of new barley mutant alleles of LIPOXYGENASE 1 using CRISPR RNA/Cas9-endonuclease technology. in *IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY-PLANT* (233 SPRING ST, NEW YORK, NY 10013 USA: SPRINGER), S87–S88.

Hirano, T., and Sato, M. H. (2019). Diverse Physiological Functions of FAB1 and Phosphatidylinositol 3,5-Bisphosphate in Plants. *Front. Plant Sci.* 10, 1–8. doi:10.3389/fpls.2019.00274.

Holme, I. B., Dionisio, G., Brinch-Pedersen, H., Wendt, T., Madsen, C. K., Vincze, E., et al. (2012). Cisgenic barley with improved phytase activity. *Plant Biotechnol. J.* 10, 237–247. doi:10.1111/j.1467-7652.2011.00660.x.

Holme, I. B., Wendt, T., Gil-Humanes, J., Deleuran, L. C., Starker, C. G., Voytas, D. F., et al. (2017). Evaluation of the mature grain phytase candidate *HvPAPhy_a* gene in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 and TALENs. *Plant Mol. Biol.* 95, 111–121. doi:10.1007/s11103-017-0640-6.

Holubová, K., Hensel, G., Vojta, P., Tarkowski, P., and Gene, H. (2018). Modification of Barley Plant Productivity Through Regulation of Cytokinin Content by Reverse-Genetics Approaches Preparation of Constructs for Silencing. *Front. Plant Sci.* 9, 1–18. doi:10.3389/fpls.2018.01676.

Howells, R. M., Craze, M., Bowden, S., and Wallington, E. J. (2018). Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. *BMC Plant Biol.* 18, 1–11. doi:10.1186/s12870-018-1433-z.

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., et al. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nat. Commun.* 9, 1–13. doi:10.1038/s41467-018-05122-7.

Huang, X., and Salt, D. E. (2016). Plant Ionomics: From Elemental Profiling to Environmental Adaptation. *Mol. Plant* 9, 787–797. doi:10.1016/j.molp.2016.05.003.

Huang, Y., Xu, P. H., Hou, B. Z., and Shen, Y. Y. (2019). Strawberry tonoplast transporter, FaVPT1, controls phosphate accumulation and fruit quality. *Plant Cell Environ.* 42, 2715–2729. doi:10.1111/pce.13598.

Hussain, S., Rengel, Z., Mohammadi, S. A., Ebadi-Segherloo, A., and Maqsood, M. A. (2016). Mapping QTL associated with remobilization of zinc from vegetative tissues into grains of barley (*Hordeum vulgare*). *Plant Soil* 399, 193–208. doi:10.1007/s11104-015-2684-1.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., and Nakatura, A. (1987). Nucleotide Sequence of the *iap* Gene, Responsible for Alkaline Phosphatase Isoenzyme Conversion in *Escherichia coli*, and Identification of the Gene Product. *J. Bacteriol.* 169, 5429–5433.

Jang, G., Lee, S., Um, T. Y., Chang, S. H., Lee, H. Y., Chung, P. J., et al. (2016). Genetic chimerism of CRISPR/Cas9-mediated rice mutants. *Plant Biotechnol. Rep.* 10, 425–435. doi:10.1007/s11816-016-0414-7.

Jeon, J. S., Lee, S., Jung, K. H., Jun, S. H., Jeong, D. H., Lee, J., et al. (2000). T-DNA insertional mutagenesis for functional genomics in rice. *Plant J.* 22, 561–570. doi:10.1046/j.1365-313X.2000.00767.x.

Jiang, L., Phillips, T. E., Hamm, C. A., Drozdowicz, Y. M., Rea, P. A., Maeshima, M., et al. (2001). The protein storage vacuole: A unique compound organelle. *J. Cell Biol.* 155, 991–1002. doi:10.1083/jcb.200107012.

Jiang, W. Z., Henry, I. M., Lynagh, P. G., Comai, L., Cahoon, E. B., and Weeks, D. P. (2017). Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. *Plant Biotechnol. J.* 15, 648–657. doi:10.1111/pbi.12663.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., and Weeks, D. P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Res.* 41, 1–12. doi:10.1093/nar/gkt780.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A Programmable Dual-RNA – Guided DNA Endonuslease in Adaptive Bacterial Immunity. *Science* 337, 816–821. doi:10.1126/science.1225829.

Johnson-Beebout, S. E., Goloran, J. B., Rubianes, F. H. C., Jacob, J. D. C., and Castillo, O. B. (2016). Zn uptake behavior of rice genotypes and its implication on grain Zn biofortification. *Sci. Rep.* 6, 38301. doi:10.1038/srep38301.

Johnson, A. A. T., Kyriacou, B., Callahan, D. L., Carruthers, L., Stangoulis, J., Lombi, E., et al. (2011). Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS One* 6, e24476. doi:10.1371/journal.pone.0024476.

Kalendar, R., Grob, T., Regina, M., Suoniemi, A., and Schulman, A. (1999). IRAP and REMAP: Two new retrotransposon-based DNA fingerprinting techniques. *Theor. Appl. Genet.* 98, 704–711. doi:10.1007/s001220051124.

Kałużewicz, A., Bosiacki, M., and Spiżewski, T. (2018). Influence of biostimulants on the content of macro- and micronutrients in broccoli plants exposed to drought stress. *J. Elem.* 23, 287–297. doi:10.5601/jelem.2017.22.2.1450.

Kapusi, E., Corcuera-gómez, M., Melnik, S., and Stoger, E. (2017). Heritable Genomic Fragment Deletions and Small Indels in the Putative *ENGase* Gene Induced by CRISPR/Cas9 in Barley. *Front. Plant Sci.* 8, 1–11. doi:10.3389/fpls.2017.00540.

Kim, D., Alptekin, B., and Budak, H. (2018). CRISPR/Cas9 genome editing in wheat. *Funct. Integr. Genomics* 18, 31–41. doi:10.1007/s10142-017-0572-x.

Kim, M. J., Yang, S. W., Mao, H.-Z., Veena, S. P., Yin, J.-L., and Chua, N.-H. (2014). Gene silencing of *Sugar-dependent 1* (*JcSDP1*), encoding a patatin-domain triacylglycerol lipase, enhances seed oil accumulation in *Jatropha curcas*. *Biotechnol. Biofuels* 7, 36. doi:10.1186/1754-6834-7-36.

Kim, Y. G., Cha, J., and Chandrasegaran, S. (1996). Hybrid restriction enzymes: Zinc finger fusions to FokI cleavage domain. *Proc. Natl. Acad. Sci. U. S. A.* 93, 1156–1160. doi:10.1073/pnas.93.3.1156.

Kleinstiver, B. P., Pattanayak, V., Prew, M. S., Tsai, S. Q., Nguyen, N. T., Zheng, Z., et al. (2016). High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 529, 490–495. doi:10.1038/nature16526.

Koonin, E. V, Makarova, K. S., and Zhang, F. (2017). Diversity, classification and evolution of CRISPR-Cas systems. *Curr. Opin. Microbiol.* 37, 67–78. doi:10.1016/j.mib.2017.05.008.

Koprna, R., De Diego, N., Dundálková, L., and Spíchal, L. (2016). Use of cytokinins as agrochemicals. *Bioorganic Med. Chem.* 24, 484–492. doi:10.1016/j.bmc.2015.12.022.

Kumar, N., Galli, M., Ordon, J., Stuttmann, J., Kogel, K. H., and Imani, J. (2018). Further analysis of barley MORC1 using a highly efficient RNA-guided Cas9 gene-editing system. *Plant Biotechnol. J.* 16, 1892–1903. doi:10.1111/pbi.12924.

Kuo, H. F., Hsu, Y. Y., Lin, W. C., Chen, K. Y., Munnik, T., Brearley, C. A., et al. (2018). *Arabidopsis* inositol phosphate kinases IPK1 and ITPK1 constitute a metabolic pathway in maintaining phosphate homeostasis. *Plant J.* 95, 613–630. doi:10.1111/tpj.13974.

Lackey, K. H., Pope, P. M., and Johnson, M. D. (2003). Expression of 1L-Myoinositol-1-Phosphate Synthase in Organelles. *Plant Physiol.* 132, 2240–2247. doi:10.1104/pp.103.020610.

Larson, S. R., and Raboy, V. (1999). Linkage mapping of maize and barley *myo*-inositol 1-phosphate synthase DNA sequences: correspondence with a *low phytic acid* mutation. *Theor. Appl. Genet.* 99, 27–36. doi:10.1007/s001220051205.

Larson, S. R., Young, K. A., Cook, A., Blake, T. K., and Raboy, V. (1998). Linkage mapping of two mutations that reduce phytic acid content of barley grain. *Theor. Appl. Genet.* 97, 141–146. doi:10.1007/s001220050878.

Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Østergaard, L., Patron, N., et al. (2015). Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol.* 16, 1–13. doi:10.1186/s13059-015-0826-7.

Lee, S., Persson, D. P., Hansen, T. H., Husted, S., Schjoerring, J. K., Kim, Y. S., et al. (2011). Bio-available zinc in rice seeds is increased by activation tagging of *nicotianamine synthase*. *Plant Biotechnol. J.* 9, 865–873. doi:10.1111/j.1467-7652.2011.00606.x.

Li, C., Hao, M., Wang, W., Wang, H., Chen, F., Chu, W., et al. (2018a). An efficient CRISPR/Cas9 platform for rapidly generating simultaneous mutagenesis of multiple gene homoeologs in allotetraploid oilseed rape. *Front. Plant Sci.* 9, 1–9. doi:10.3389/fpls.2018.00442.

Li, C., Zong, Y., Wang, Y., Jin, S., Zhang, D., Song, Q., et al. (2018b). Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biol.* 19, 1–9. doi:10.1186/s13059-018-1443-z.

Li, M., Hensel, G., Mascher, M., Melzer, M., Budhagatapalli, N., Rutten, T., et al. (2019). Leaf Variegation and Impaired Chloroplast Development Caused by a Truncated CCT Domain Gene in *albostrians* Barley. *Plant Cell* 31, 1430–1445. doi:10.1105/tpc.19.00132.

Li, R., Liu, H. P., Hong, C. L., Dai, Z. X., Liu, J. W., Zhou, J., et al. (2017). Iodide and iodate effects on the growth and fruit quality of strawberry. *J. Sci. Food Agric.* 97, 230–235.

doi:10.1002/jsfa.7719.

Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., et al. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat. Commun.* 8, 1–5. doi:10.1038/ncomms14261.

Liu, J. C., Ockenden, I., Truax, M., and Lott, J. N. A. (2004). Phytic acid-phosphorus and other nutritionally important mineral nutrient elements in grains of wild-type and *low phytic acid* (lpa1–1) rice. *Seed Sci. Res.* 14, 109–116. doi:10.1079/SSR2004160.

Liu, J. F., Wang, X. F., Li, Q. L., Li, X., Zhang, G. Y., Li, M. G., et al. (2011). Biolistic transformation of cotton (*Gossypium hirsutum* L.) with the *phyA* gene from *Aspergillus ficuum*. *Plant Cell, Tissue Organ Cult.* 106, 207–214. doi:10.1007/s11240-010-9908-0.

Liu, J., Yang, L., Luan, M., Wang, Y., Zhang, C., Zhang, B., et al. (2015). A vacuolar phosphate transporter essential for phosphate homeostasis in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 112, E6571–E6578. doi:10.1073/pnas.1514598112.

Liu, Q., Segal, D. J., Ghiara, J. B., and Barbas, C. F. (1997). Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5525–5530. doi:10.1073/pnas.94.11.5525.

Liu, W., Xie, X., Ma, X., Li, J., Chen, J., and Liu, Y.-G. (2018). DSDecode: A Web-Based Tool for Decoding of Sequencing Chromatograms for Genotyping of Targeted Mutations. *Mol. Plant* 8, 1431–1433. doi:10.1016/j.molp.2015.05.009.

Loewus, F. A., and Murthy, P. P. N. (2000). *myo* -Inositol metabolism in plants. *Plant Sci.* 150, 1–19. doi:10.1016/S0168-9452(99)00150-8.

Long, X., Liu, Q., Chan, M., Wang, Q., and Sun, S. S. M. (2013). Metabolic engineering and profiling of rice with increased lysine. *Plant Biotechnol. J.* 11, 490–501. doi:10.1111/pbi.12037.

Longchamp, M., Angeli, N., and Castrec-Rouelle, M. (2016). Effects on the accumulation of calcium, magnesium, iron, manganese, copper and zinc of adding the two inorganic forms of selenium to solution cultures of *Zea mays*. *Plant Physiol. Biochem.* 98, 128–137. doi:10.1016/j.plaphy.2015.11.013.

Lopez-Bucio, J., Hernandez-Abreu, E., Sanchez-Calderon, L., Nieto-Jacobo, M. F., Simpson, J., and Herrera-Estrella, L. (2002). Phosphate Availability Alters Architecture and Causes Changes in Hormone Sensitivity in the Arabidopsis Root System. *Plant Physiol.* 129, 244–256. doi:10.1104/pp.010934.244.

Lott, J. N. a., Ockendena, I., Raboya, V., and Battena, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Sci. Res.* 10, 11–33. doi:10.1017/S0960258500000039.

Ma, D., Zuo, Y., Sun, D., Wang, C., and Guo, T. (2013). Characterization of the *TaMIPS* gene from winter wheat (*Triticum aestivum* L.) and changes in its expression pattern with phytic acid accumulation in seeds during grain filling. *J. Cereal Sci.* 57, 437–443. doi:10.1016/j.jcs.2013.01.012.

Ma, L., Zhu, F., Li, Z., Zhang, J., Li, X., Dong, J., et al. (2015a). TALEN-Based mutagenesis of lipoxygenase LOX3 enhances the storage tolerance of rice (*Oryza sativa*) Seeds. *PLoS One* 10, 1–16. doi:10.1371/journal.pone.0143877.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., et al. (2015b). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Mol. Plant* 8, 1274–1284. doi:10.1016/j.molp.2015.04.007.

Majumder, A. L., Chatterjee, A., Dastidar, K. G., and Majee, M. (2003). Diversification and evolution of L-*myo*-inositol 1-phosphate synthase. *FEBS Lett.* 553, 3–10. doi:10.1016/S0014-5793(03)00974-8.

Meuwissen, T. H. E., Hayes, B. J., and Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics* 157, 1819–1829. doi:11290733.

Mitsuhashi, N., Kondo, M., Nakaune, S., Ohnishi, M., Hayashi, M., Hara-Nishimura, I., et al. (2008). Localization of *myo*-inositol-1-phosphate synthase to the endosperm in developing seeds of *Arabidopsis*. *J. Exp. Bot.* 59, 3069–3076. doi:10.1093/jxb/ern161.

Morgante, M., and Olivieri, A. M. (1993). PCR-amplified microsatellites as markers in plant genetics. *Plant J.* 3, 175–182. doi:10.1111/j.1365-313x.1993.tb00020.x.

Murovec, J., Pirc, Ž., and Yang, B. (2017). New variants of CRISPR RNA-guided genome editing enzymes. *Plant Biotechnol. J.* 15, 917–926. doi:10.1111/pbi.12736.

Nuñez, J. K., Lee, A. S. Y., Engelman, A., and Doudna, J. A. (2015). Integrase-mediated spacer acquisition during CRISPR-Cas adaptive immunity. *Nature* 519, 193–198. doi:10.1038/nature14237.

O'Malley, R. C., and Ecker, J. R. (2010). Linking genotype to phenotype using the Arabidopsis unimutant collection. *Plant J.* 61, 928–940. doi:10.1111/j.1365-313X.2010.04119.x.

Ohlrogge, J. B., and Jaworski, J. G. (1997). Regulation of fatty acid synthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48, 109–136. doi:1040-2519/97/0601-0109.

Okada, A., Arndell, T., Borisjuk, N., Sharma, N., Watson-Haigh, N. S., Tucker, E. J., et al. (2019). CRISPR/Cas9-mediated knockout of *Ms1* enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. *Plant Biotechnol. J.* 17, 1905–1913. doi:10.1111/pbi.13106.

Ozseyhan, M. E., Kang, J., Mu, X., and Lu, C. (2018). Mutagenesis of the *FAE1* genes significantly changes fatty acid composition in seeds of *Camelina sativa*. *Plant Physiol. Biochem.* 123, 1–7. doi:10.1016/j.plaphy.2017.11.021.

Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., et al. (2005). Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat Biotech* 23, 482–487. doi:10.1038/nbt1082.

Pant, B. D., Buhtz, A., Kehr, J., and Scheible, W. R. (2008). MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J.* 53, 731–738. doi:10.1111/j.1365-313X.2007.03363.x.

Panzeri, D., Cassani, E., Doria, E., Tagliabue, G., Forti, L., Campion, B., et al. (2011). A defective ABC transporter of the MRP family, responsible for the bean *lpa1* mutation, affects the regulation of the phytic acid pathway, reduces seed *myo*-inositol and alters ABA sensitivity. *New Phytol.* 191, 70–83. doi:10.1111/j.1469-8137.2011.03666.x.

Patel, T., and Saraf, M. (2017). Biosynthesis of phytohormones from novel rhizobacterial isolates and their in vitro plant growth-promoting efficacy. *J. Plant Interact.* 12, 480–487. doi:10.1080/17429145.2017.1392625.

Paterson, A. H., Lander, E. S., Hewitt, J. D., Peterson, S., Lincoln, S. E., and Tanksley, S. D. (1988). Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature* 335, 721–726. doi:10.1038/335721a0.

Pathak, B., Zhao, S., Manoharan, M., and Srivastava, V. (2019). Dual-targeting by CRISPR/Cas9 leads to efficient point mutagenesis but only rare targeted deletions in the rice genome. *3 Biotech* 9, 1–12. doi:10.1007/s13205-019-1690-z.

Paul, J. Y., Khanna, H., Kleidon, J., Hoang, P., Geijskes, J., Daniells, J., et al. (2017). Golden bananas in the field: elevated fruit pro-vitamin A from the expression of a single banana transgene. *Plant Biotechnol. J.* 15, 520–532. doi:10.1111/pbi.12650.

Peng, R. H., Yao, Q. H., Xiong, A. S., Cheng, Z. M., and Li, Y. (2006). Codon-modifications and an endoplasmic reticulum-targeting sequence additively enhance expression of an *Aspergillus phytase* gene in transgenic canola. *Plant Cell Rep.* 25, 124–132. doi:10.1007/s00299-005-0036-y.

Pii, Y., Cesco, S., and Mimmo, T. (2015). Shoot ionome to predict the synergism and antagonism between nutrients as affected by substrate and physiological status. *Plant Physiol. Biochem.* 94, 48–56. doi:10.1016/j.plaphy.2015.05.002.

Popko, M., Michalak, I., Wilk, R., Gramza, M., Chojnacka, K., and Górecki, H. (2018). Effect of the new plant growth biostimulants based on amino acids on yield and grain quality of winter wheat. *Molecules* 23, 1–13. doi:10.3390/molecules23020470.

Puchta, H., Dujon, B., and Hohn, B. (1996). Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. *Proc. Natl. Acad. Sci. U. S. A.* 93, 5055–5060. doi:10.1073/pnas.93.10.5055.

Puchta, H., and Hohn, B. (1991). The mechanism of extrachromosomal homologous DNA recombination in plant cells. *Mol. Gen. Genet.* 230, 1–7. doi:10.1007/BF00290641.

Raboy, V., Gerbasi, P. F., Young, K. A., Stoneberg, S. D., Pickett, S. G., Bauman, A. T., et al. (2000). Origin and seed phenotype of maize *low phytic acid 1-1* and *low phytic acid 2-1*. *Plant Physiol.* 124, 355–368. doi:10.1104/pp.124.1.355.

Raghothama, K. G., and Karthikeyan, A. S. (2005). Phosphate acquisition. *Plant Soil* 274, 37–49. doi:10.1007/s11104-004-2005-6.

Regvar, M., Eichert, D., Kaulich, B., Gianoncelli, A., Pongrac, P., Vogel-Mikuš, K., et al. (2011). New insights into globoids of protein storage vacuoles in wheat aleurone using synchrotron soft X-ray microscopy. *J. Exp. Bot.* 62, 3929–3939. doi:10.1093/jxb/err090.

Rey, M. D., Martín, A. C., Smedley, M., Hayta, S., Harwood, W., Shaw, P., et al. (2018). Magnesium increases homoeologous crossover frequency during meiosis in *ZIP4* (*Ph1* gene) mutant wheat-wild relative hybrids. *Front. Plant Sci.* 9, 1–12. doi:10.3389/fpls.2018.00509.

Rohini, S., Aswani, R., Kannan, M., Sylas, V. P., and Radhakrishnan, E. K. (2018). Culturable Endophytic Bacteria of Ginger Rhizome and their Remarkable Multi-trait Plant Growth-Promoting Features. *Curr. Microbiol.* 75, 505–511. doi:10.1007/s00284-017-1410-z.

Ruiz-Lopez, N., Haslam, R. P., Napier, J. A., and Sayanova, O. (2014). Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. *Plant J.* 77, 198–208. doi:10.1111/tpj.12378.

Ryan, M. H., Kaur, P., Nazeri, N. K., Clode, P. L., Keeble-Gagnère, G., Doolette, A. L., et al. (2019). Globular structures in roots accumulate phosphorus to extremely high concentrations following phosphorus addition. *Plant Cell Environ.* 42, 1987–2002. doi:10.1111/pce.13531.

Saifullah, Javed, H., Naeem, A., Rengel, Z., and Dahlawi, S. (2016). Timing of foliar Zn application plays a vital role in minimizing Cd accumulation in wheat. *Environ. Sci. Pollut. Res.* 23, 16432–16439. doi:10.1007/s11356-016-6822-y.

Sample, E. C., Soper, R. J., and Racz, G. J. (1980). "Reactions of Phosphate Fertilizers in Soils," in *The Role of Phosphorus in Agriculture*, eds. F. E. Khasawneh, E. C. Sample, and E. J. Kamprath (Madison: American Society of Agronomy), 263–310. doi:10.2134/1980.roleofphosphorus.c12.

Sánchez-León, S., Gil-Humanes, J., Ozuna, C. V, Giménez, M. J., Sousa, C., Voytas, D. F., et al. (2018). Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol. J.* 16, 902–910. doi:10.1111/pbi.12837.

Sayanova, O., Ruiz-Lopez, N., Haslam, R. P., and Napier, J. A. (2012). The role of Δ6-desaturase acyl-carrier specificity in the efficient synthesis of long-chain polyunsaturated fatty acids in transgenic plants. *Plant Biotechnol. J.* 10, 195–206. doi:10.1111/j.1467-7652.2011.00653.x.

Schiml, S., Fauser, F., and Puchta, H. (2014). The CRISPR/Cas system can be used as nuclease for *in planta* gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. *Plant J.* 80, 1139–1150. doi:10.1111/tpj.12704.

Scholey, D., Burton, E., Morgan, N., Sanni, C., Madsen, C. K., Dionisio, G., et al. (2017). P and Ca digestibility is increased in broiler diets supplemented with the high-phytase HIGHPHY wheat. *Animal* 11, 1457–1463. doi:10.1017/S1751731117000544.

Shaikh, S., and Saraf, M. (2017). Biofortification of *Triticum aestivum* through the inoculation of zinc solubilizing plant growth promoting rhizobacteria in field experiment. *Biocatal. Agric. Biotechnol.* 9, 120–126. doi:10.1016/j.bcab.2016.12.008.

Shan, Q., Wang, Y., Li, J., Zhang, Y., Chen, K., Liang, Z., et al. (2013). Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* 31, 686–688. doi:doi:10.1038/nbt.2650.

Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., et al. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat. Methods* 11, 399–402.

doi:10.1038/nmeth.2857.

Shi, J., Hongyu, W., Wu, Y., Hazebroek, J., Meeley, R. B., and Ertl, D. S. (2003). The Maize Low-Phytic Acid Mutant *lpa2* Is Caused by Mutation in an Inositol Phosphate Kinase Gene. *Plant Physiol.* 131, 507–515. doi:10.1104/pp.014258.

Shi, J., Wang, H., Schellin, K., Li, B., Faller, M., Stoop, J. M., et al. (2007). Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. *Nat. Biotechnol.* 25, 930–937. doi:10.1038/nbt1322.

Sikdar, M. S. I., Bowra, S., Schmidt, D., Dionisio, G., Holm, P. B., and Vincze, E. (2016). Targeted modification of storage protein content resulting in improved amino acid composition of barley grain. *Transgenic Res.* 25, 19–31. doi:10.1007/s11248-015-9911-7.

Singh, M., Kumar, M., Albertsen, M. C., Young, J. K., and Cigan, A. M. (2018). Concurrent modifications in the three homeologs of *Ms45* gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). *Plant Mol. Biol.* 97, 371–383. doi:10.1007/s11103-018-0749-2.

Singh, S. P., Keller, B., Gruissem, W., and Bhullar, N. K. (2017). Rice *NICOTIANAMINE SYNTHASE 2* expression improves dietary iron and zinc levels in wheat. *Theor. Appl. Genet.* 130, 283–292. doi:10.1007/s00122-016-2808-x.

Slamet-Loedin, I. H., Johnson-Beebout, S. E., Impa, S., and Tsakirpaloglou, N. (2015). Enriching rice with Zn and Fe while minimizing Cd risk. *Front. Plant Sci.* 6, 1–9. doi:10.3389/fpls.2015.00121.

Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. a., Yan, W. X., and Zhang, F. (2015). Rationally engineered Cas9 nucleases with improved specificity. *Science* 351, 84–88. doi:10.1126/science.aad5227.

Srivastava, S., Upadhyay, M. K., Srivastava, A. K., Abdelrahman, M., Suprasanna, P., and Tran, L. S. P. (2018). Cellular and subcellular phosphate transport machinery in plants. *Int. J. Mol. Sci.* 19, 1–15. doi:10.3390/ijms19071914.

Stacey, M. G., Cahoon, R. E., Nguyen, H. T., Cui, Y., Sato, S., Nguyen, C. T., et al. (2016). Identification of Homogentisate Dioxygenase as a Target for Vitamin E Biofortification in Oilseeds. *Plant Physiol.* 172, 1506–1518. doi:10.1104/pp.16.00941.

Sternberg, S. H., Haurwitz, R. E., and Doudna, J. A. (2012). Mechanism of substrate selection by a highly specific CRISPR endoribonuclease. *RNA* 18, 661–672. doi:10.1261/rna.030882.111.

Stiles, A., Qian, X., Shears, S., and Grabau, E. (2008). Metabolic and Signaling Properties of an *Itpk* Gene Family in *Glycine max*. *FEBS Lett.* 582, 1853–1858. doi:10.1016/j.febslet.2008.04.054.

Sugio, A., Yang, B., Zhu, T., and White, F. F. (2007). Two type III effector genes of *Xanthomonas oryzae* pv. *oryzae* control the induction of the host genes *OsTFIIAγ1* and *OsTFX1* during bacterial blight of rice. *Proc. Natl. Acad. Sci. U. S. A.* 104, 10720–10725. doi:10.1073/pnas.0701742104.

Sun, Y., Thompson, M., Lin, G., Butler, H., Gao, Z., Thornburgh, S., et al. (2007). Inositol 1,3,4,5,6-pentakisphosphate 2-kinase from maize: Molecular and biochemical characterization. *Plant*

Physiol. 144, 1278–1291. doi:10.1104/pp.107.095455.

Suzuki, M., Tanaka, K., Kuwano, M., and Yoshida, K. T. (2007). Expression pattern of inositol phosphate-related enzymes in rice (*Oryza sativa* L.): Implications for the phytic acid biosynthetic pathway. *Gene* 405, 55–64. doi:10.1016/j.gene.2007.09.006.

Sweetman, D., Johnson, S., Caddick, S. E. K., Hanke, D. E., and Brearley, C. A. (2006). Characterization of an *Arabidopsis* inositol 1,3,4,5,6-pentakisphosphate 2-kinase (AtIPK1). *Biochem. J.* 394, 95–103. doi:10.1042/BJ20051331.

Tanaka, K., Yoshida, T., and Kasai, Z. (1974). Radioautographic demonstration of the accumulation site of phytic acid in rice and wheat grains. *Plant Cell Physiol.* 15, 147–151. doi:10.1093/oxfordjournals.pcp.a074974.

Tang, X., Liu, G., Zhou, J., Ren, Q., You, Q., Tian, L., et al. (2018). A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. *Genome Biol.* 19, 1–13. doi:10.1186/s13059-018-1458-5.

Tang, Z., Chen, S. Q., Chen, A., He, B., Zhou, Y., Chai, G., et al. (2019). CasPDB: an integrated and annotated database for Cas proteins from bacteria and archaea. *Database* 2019, baz093. doi:10.1093/database/baz093.

Toda, E., Koiso, N., Takebayashi, A., Ichikawa, M., Kiba, T., Osakabe, K., et al. (2019). An efficient DNA- and selectable-marker-free genome-editing system using zygotes in rice. *Nat. Plants* 5, 363–368. doi:10.1038/s41477-019-0386-z.

Turner, B. L., Papházy, M. J., Haygarth, P. M., and McKelvie, I. D. (2002). Inositol phosphates in the environment. *Philos. Trans. R. Soc. B Biol. Sci.* 357, 449–469. doi:10.1098/rstb.2001.0837.

Vaculova, K., Balounova, M., Sedlackova, I., Kvasnicka, F., Mikulikova, R., Belakova, S., et al. (2011). *METODIKA PREBREEDINGU JEČMENE JARNÍHO S DIFERENCOVANÝM OBSAHEM PŘIROZENÝCH ŠKODLIVÝCH LÁTEK V ZRNĚ PRO ŠLECHTĚNÍ ODRŮD NESLADOVNICKÉHO TYPU*. 1st ed. Kromeriz: Agrotest fyto, s.r.o.

Versaw, W. K., and Harrison, M. J. (2002). A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. *Plant Cell* 14, 1751–1766. doi:10.1105/tpc.002220.

Vrana, K. E., Churchill, M. E., Tullius, T. D., and Brown, D. D. (1988). Mapping functional regions of transcription factor TFIIIA. *Mol. Cell. Biol.* 8, 1684–1696. doi:10.1128/mcb.8.4.1684.

Wang, C., Yue, W., Ying, Y., Wang, S., Secco, D., Liu, Y., et al. (2015). Rice SPX-Major Facility Superfamily3, a Vacuolar Phosphate Efflux Transporter, Is Involved in Maintaining Phosphate Homeostasis in Rice. *Plant Physiol.* 169, 2822–2831. doi:10.1104/pp.15.01005.

Wang, M., Lu, Y., Botella, J. R., Mao, Y., Hua, K., and Zhu, J. kang (2017). Gene Targeting by Homology-Directed Repair in Rice Using a Geminivirus-Based CRISPR/Cas9 System. *Mol. Plant* 10, 1007–1010. doi:10.1016/j.molp.2017.03.002.

Wang, W., Pan, Q., He, F., Akhunova, A., Chao, S., Trick, H., et al. (2018a). Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. *Cris. J.* 1, 65–74.

doi:10.1089/crispr.2017.0010.

Wang, W., Simmonds, J., Pan, Q., Davidson, D., He, F., Battal, A., et al. (2018b). Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of *TaGW2* homoeologues to grain size and weight in wheat. *Theor. Appl. Genet.* 131, 2463–2475. doi:10.1007/s00122-018-3166-7.

Wang, X., Tu, M., Wang, D., Liu, J., Li, Y., Li, Z., et al. (2018c). CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. *Plant Biotechnol. J.* 16, 844–855. doi:10.1111/pbi.12832.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C., et al. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947–951. doi:10.1038/nbt.2969.

Wang, Y. H., Zou, C. Q., Mirza, Z., Li, H., Zhang, Z. Z., Li, D. P., et al. (2016). Cost of agronomic biofortification of wheat with zinc in China. *Agron. Sustain. Dev.* 36, 1–7. doi:10.1007/s13593-016-0382-x.

Watanabe, K., Breier, U., Hensel, G., Kumlehn, J., Schubert, I., and Reiss, B. (2016). Stable gene replacement in barley by targeted double-strand break induction. *J. Exp. Bot.* 67, 1433–1445. doi:10.1093/jxb/erv537.

Wei, Y., Shohag, M. J. I., and Yang, X. (2012). Biofortification and Bioavailability of Rice Grain Zinc as Affected by Different Forms of Foliar Zinc Fertilization. *PLoS One* 7, 1–10. doi:10.1371/journal.pone.0045428.

Wild, R., Gerasimaite, R., Jung, J. Y., Truffault, V., Pavlovic, I., Schmidt, A., et al. (2016). Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains. *Science* 352, 986–990. doi:10.1126/science.aad9858.

Wilson, J. H., Berget, P. B., and Pipas, J. M. (1982). Somatic cells efficiently join unrelated DNA segments end-to-end. *Mol. Cell. Biol.* 2, 1258–1269. doi:10.1128/mcb.2.10.1258.

Xie, K., Minkenberg, B., and Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci. U. S. A.* 112, 3570–3575. doi:10.1073/pnas.1420294112.

Xie, K., and Yang, Y. (2013). RNA-Guided genome editing in plants using a CRISPR-Cas system. *Mol. Plant* 6, 1975–1983. doi:10.1093/mp/sst119.

Xu, C., and Shanklin, J. (2016). Triacylglycerol Metabolism, Function, and Accumulation in Plant Vegetative Tissues. *Annu. Rev. Plant Biol.* 67, 179–206. doi:10.1146/annurev-arplant-043015-111641.

Xu, L., Zhao, H., Wan, R., Liu, Y., Xu, Z., Tian, W., et al. (2019). Identification of vacuolar phosphate efflux transporters in land plants. *Nat. Plants* 5, 84–94. doi:10.1038/s41477-018-0334-3.

Xue, Y. F., Xia, H. Y., McGrath, S. P., Shewry, P. R., and Zhao, F. J. (2015). Distribution of the stable isotopes ^{57}Fe and ^{68}Zn in grain tissues of various wheat lines differing in their phytate content. *Plant Soil* 396, 73–83. doi:10.1007/s11104-015-2582-6.

Yamunarani, R., Govind, G., Ramegowda, V., Vokkaliga Thammegowda, H., and Ambarahalli Guligowda, S. (2016). Genetic diversity for grain Zn concentration in finger millet genotypes: Potential for improving human Zn nutrition. *Crop J.* 4, 229–234. doi:10.1016/j.cj.2015.12.001.

Yang, Q., Zhang, C., Chan, M., Zhao, D., Chen, J., Wang, Q., et al. (2016). Biofortification of rice with the essential amino acid lysine: molecular characterization, nutritional evaluation, and field performance. *J. Exp. Bot.* 67, 4285–4296. doi:10.1093/jxb/erw209.

Yang, Q., Zhong, X., Li, Q., Lan, J., Tang, H., Qi, P., et al. (2020). Mutation of the D-hordein gene by RNA-guided Cas9 targeted editing reducing the grain size and changing grain compositions in barley. *Food Chem.* 311, 125892. doi:10.1016/j.foodchem.2019.125892.

Ye, X., and Beyer, P. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* 287, 303–305. doi:10.1126/science.287.5451.303.

Young, J., Zastrow-Hayes, G., Deschamps, S., Svitashev, S., Zaremba, M., Acharya, A., et al. (2019). CRISPR-Cas9 Editing in Maize: Systematic Evaluation of Off-target Activity and Its Relevance in Crop Improvement. *Sci. Rep.* 9, 1–11. doi:10.1038/s41598-019-43141-6.

Zhang, W., Liu, D., Li, C., Cui, Z., Chen, X., Russell, Y., et al. (2015). Zinc accumulation and remobilization in winter wheat as affected by phosphorus application. *F. Crop. Res.* 184, 155–161. doi:10.1016/j.fcr.2015.10.002.

Zhang, Y., Bai, Y., Wu, G., Zou, S., Chen, Y., Gao, C., et al. (2017). Simultaneous modification of three homoeologs of TaEDR1 by genome editing enhances powdery mildew resistance in wheat. *Plant J.* 91, 714–724. doi:10.1111/tpj.13599.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., and Capell, T. (2008). Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. *Proc. Natl. Acad. Sci. U. S. A.* 105, 18232–18237. doi:10.1073/pnas.0809737105.

Zhu, J., Lau, K., Puschmann, R., Harmel, R. K., Zhang, Y., Pries, V., et al. (2019). Two bifunctional inositol pyrophosphate kinases/phosphatases control plant phosphate homeostasis. *eLife* 8, 1–25. doi:10.7554/eLife.43582.

Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., et al. (2017). Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat. Biotechnol.* 35, 438–440. doi:10.1038/nbt.3811.

8. Supplements I, II

I. Vlčko T., Ohnoutková L. (2019). Recent advances and perspectives in crop biofortification. *Biologia Plantarum* 63:586–593. doi: 10.32615/bp.2019.056. IF: 1,384

II. Vlčko T., Ohnoutková L. (2020). Allelic Variants of CRISPR/Cas9 Induced Mutation in an Inositol Trisphosphate 5/6 Kinase Gene Manifest Different Phenotypes in Barley. *Plants* 9:1–20. doi: 10.3390/plants9020195. IF: 2,632

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Zelenka, J., Mrkvicová, E., Šťastník O., Jarošová M., Vlčko T., Ohnoutková L. (**2017**). Effect of Low- and High Phytase Barley on Phosphorus Retention and Bone Mineral Composition in Broilers. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 65 (2): 577–581.

Kupcikova, L., Lichovnikova, M., Anderle, V., Vlcko, T., Ohnoutkova, L., Svidrnoch, M., Maier, V., Hampel D. (**2017**) Pre-caecal digestible phosphorus in maize and wheat for broiler chickens, *British Poultry Science*, 58: 6, 712-717, DOI: 10.1080/00071668.2017.1370536. IF 1.096

Cejnar, P., Ohnoutkova, L., Ripl, J., Vlcko, T., Kundu, J.K. (**2018**) Two mutations in the truncated Rep gene RBR domain delayed the Wheat dwarf virus infection in transgenic barley plants. *Journal of Integrative Agriculture*, 17 (11): 2492-2500, DOI: 10.1016/S2095-3119(18)62000-3. IF: 1.042

Rehorova, K., Viktorova, J., Macuchova, B., Vlcko, T., Stankova, L., Jelenova, N., Ohnoutkova,L., Macek, T. (**2018**) Limen, Non-Toxic Recombinant Plant Defensin and Its Effect against Pathogenic Yeast and Fungi. *Journal of Pharmacy and Pharmacology*, 6, 945-955, DOI: 10.17265/2328-2150/2018.11.001.

Viktorova, j., Klcova, B., Rehorova, K., Vlcko, T., Stankova, L., Jelenova, N., Cejnar, P., Kundu, J.K., Ohnoutkova, L., Macek, T. (**2019**) Recombinant expression of osmotin in barley improves stress resistance and food safety during adverse growing conditions. *PLOS One*, e0212718, DOI: 10.1371/journal.pone.0212718. IF: 2.766

Kopecny, D., Vlcko, T. (**2020**) Editorial: Plant biotechnology: Green for Good V 2019. *New Biotechnology*, 57, 1-3, DOI: 10.1016/j.nbt.2020.01.004. IF: 3.739

Book chapter:

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REVIEW

Recent advances and perspectives in crop biofortification

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Abstract

The increasing world population and limited amount of land area appropriate for intensive agriculture necessitate high-yield cultivars. The focus is on the enrichment of existing crops deficient in nutrients, which is also called biofortification. Microelements, vitamins, and fatty acids belong to most important traits being subjected to biofortification. Biofortification strategies can be divided on fertilization-based strategy, which is characterized by direct application of nutrients or plant growth promoting substances on plants, and biotechnological strategy, which involves molecular biology techniques in order to enhance transport, production, and accumulation of nutrients. Recent advances in plant biotechnology, such as genome-editing, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9), and transcription activator-like effector nuclease (TALEN), as well as an extensive study of genetic diversity, are acceptable approaches to the development of biofortified crops.

Additional key words: gene-editing, programmable nucleases, RNA interference.

Introduction

Crop biofortification is the process of plant seed enrichment with nutritionally important substances (Bouis 2002). Attempts to biofortify crops have been conducted in order to meet demands of the mineral and vitamins for humans in populations under conditions of limited food sources. Prominent crops that have been subjected to biofortification efforts in recent years are: wheat (Singh *et al*. 2017), barley (Sikdar *et al*. 2016), maize (Zhu *et al*. 2008, Du *et al*. 2016), rice (Johnson *et al*. 2011, Lee *et al*. 2011, Ma *et al*. 2015, Yang *et al*. 2016), sorghum (Che *et al*. 2016), and banana (Paul *et al*. 2017); all serving as staple food sources in large areas of the world. Microelements, vitamins, and fatty acids (FAs) constitute the main groups of substances that were subjected to biofortification (Table 1). With regard to the current state of technology and knowledge, biofortification of crops can be achieved in two ways: *1*) fertilization-based and *2*) biotechnological approaches (Fig. 1). Fertilization-based approaches cover an area from direct fertilization by nutrients to stimulate better plant growth and interaction with soil microorganisms, whereas biotechnological approaches rely on application of molecular biology techniques and recombinant technology. The aim of this review is to analyze the advances in methods used for crop biofortification, which have been developed in recent years, and to shed light on the means of applying the current techniques.

Fertilization based approaches

These approaches precede biotechnological because of simplicity of practical application. Application of fertilizers belongs to first attempts that have been adapted for the

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Abbreviations: AA - amino acids; Cas9 - CRISPR-associated 9; CRISPR - clustered regularly interspaced short palindromic repeats; dapA - dihydrodipicolinate synthase; FA - fatty acid; FAD - fatty acid dehydrogenase; FAE - fatty acid elongase; GS-MAS - marker-assisted selection based on genomic selection; HGO - homogentisate dioxygenase; LKR - lysine ketoglutarate reductase; lysC - lysine feedback-insensitive aspartate kinase; LOX - lipoxygenase; MAS - marker-assisted selection; NAS - nicotianamine synthase; QTL - quantitative trait locus; RNAi - RNA interference; SAD1 - stearoyl-acyl carrier protein desaturase-1; SDH - saccharopine dehydrogenase; TALEN - transcription activator-like effector nuclease.

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Table 1. Biofortified traits in important crops.

Trait	Crop	Technique	Reference
Micro-, macro-elements			
Zn and Fe	*Triticum aestivum*	over-expression of *Osnas2*	Singh *et al*. 2017
Zn	*Triticum aestivum*	P fertilization	Zhang *et al*. 2015
Zn, Mg, Mn	*Triticum aestivum*	Se fertilization	Boldrin *et al*. 2016
Zn, Fe, N,P,K	*Triticum aestivum*	inoculation by rhizobacteria	Shaikh and Saraf 2017
Zn	*Hordeum vulgare*	over-expression of *Hvzip7*	Tiong et al. 2014
Zn	*Hordeum vulgare*	Zn fertilization	Uddin et al. 2014
Zn and Fe	*Oryza sativa*	over-expression of *Osnas1 and Osnas2*	Johnson *et al*. 2011
Zn	*Oryza sativa*	over-expression of *Osnas2*	Lee *et al*. 2011
Zn	*Oryza sativa*	over-expression of *Osnas2*	Moreno-Moyano *et al*. 2016
Fe	*Oryza sativa*	over-expression of *Atirt1, Atnas1, Pvfer*	Boonyaves *et al*. 2017
N, S, K, P	*Brassica napus*	biostimulant application	Billard *et al*. 2014
I	*Lactuca sativa*	KI and KIO_3 foliar application	Lawson *et al.* 2016
Vitamins and antioxidants			
Carotenoids	*Zea mays*	over-expression of *Zmpsy1, Pacrtl, Gllycb, Glbch, ParacrtW*	Zhu *et al*. 2008
Carotenoids	*Musa acuminata*	*Mtpsy2a, Zmpsy1, Pacrtl*	Paul *et al*. 2017
Vitamin B_9	*Oryza sativa*	over-expression of *adcs, gtpchi, fbp*	Blancquaert et al. 2015
Vitamin B_1	*Allium cepa*	arbuscular-mycorrhizal fungi	Rozpadek *et al*. 2016
β-carotene Vitamin E	*Sorghum bicolor*	over-expression of *psy1, crt1, At-dxs, pmi, hggt*	Che *et al*. 2016
Aminoacids			
Amino acids	*Hordeum vulgare*	silencing Chordein	Sikdar *et al*. 2016
Lysine	*Oryza sativa*	over-expression of *dapA* and *lysC* and silencing *lkr* and *sdh*	Yang *et al*. 2016
Fatty acids			
Fatty acids	*Oryza sativa*	mutagenesis in *Oslox3*	Ma *et al*. 2015
ω3 polyunsaturated fatty acids	*Camelina sativa*	over-expression of *pse1, TcΔ5, OtΔ6, Piw3, PsΔ12, Otelo5, EhΔ4*	Ruiz-Lopez *et al*. 2014

agricultural practice in order to enhance crop production. Conventional biofortification strategies mostly focusing on microelement enhancement are based on the application of elements as a constituent part of fertilizers applied: onto the leaves (foliar application); directly into the soil; or into the nutrient solution in the case of hydroponic cultures. The indisputable advantage of the conventional approach is the simplicity of the procedure in terms of the technical requirements, and it also obviates the legislative obstacles of genetically modified organism (GMO) techniques, especially in Europe. Interestingly, modern alternative approaches cover the application of biostimulants in promoting plant growth or the enhancement of a plant root system interaction with the soil bacteria and arbuscular-mycorrhizal fungi.

Foliar and soil fertilization: Foliar fertilization is based on the application of nutrients directly on the plants leaves. Absorption occur either *via* cuticular or *via* stomatal pathway (Fernández and Eichert 2009, Fernández and Brown 2013). Nutrient absorption *via* stomata can be further enhanced by using surfactants (Field and Bishop 1988). Recently, nutrients in the form of microparticles (< 4 μm) were launched on the market. These are expected to have improved ability to be absorbed and utilized in the plant tissues. Kaiser *et al*. (2014) reported that application with surfactant improves absorption of microparticles by stomatal pathway. Mobility of compounds within the plant tissues is of great importance, because it improves utilization of nutrient applied. Fageria *et al*. (2009) concluded that there existed large differences among plants species in ability to remobilize different nutrients. Ali *et al*. (2017) revealed that selenate is a more mobile form of selenium in the soil than selenite; therefore, it is a more appropriate form of selenium for root uptake. One study utilizing stable isotopes of selenium showed that selenite is metabolized in the root into both high- and low-molecular mass compounds of low mobility for transport to the leaves whereas selenate is accumulated in the leaves (Di Tullo *et al*. 2015). Importantly, results showed that

BIOFORTIFICATION		
FERTILIZATION-BASED STRATEGIES	DIRECT APPLICATION OF NUTRIENT	Application of nutrients onto leaves or directly into substrate (soil, hydroponic culture, *etc.*).
	ENHANCEMENT OF GROWTH	Treatment of plant with biostimulants.
	SUPPORT OF ROOT↔MICROORGANISM INTERACTION	Treatment of seeds with beneficial microorganisms.
BIOTECHNOLOGICAL STRATEGIES	IDENTIFICATION AND INTRODUCING OF NEW TRAITS	Comparison of contrasting genomes (Genome wide association study). Crossing and selection of traits (Marker-assisted selection).
	MODIFICATION OF TRANSPORT AND METABOLIC PATHWAYS	Plant *Agrobacterium*-mediated or biolistic transformation. Enhancement of transport or biosynthesis of desirable substance. Restriction of metabolization and degradation of desirable substance. Metabolomic analysis of intermediates. Anther cultures for transgene stabilization.
	CREATION OF BIOSYNTHETIC PATHWAYS *DE NOVO*	

Fig 1. Biofortification strategies.

the form and concentration of the microelement is crucial to achieve optimal biofortification without negative effects. For instance, IO_3^- applied to strawberries cause nitrate accumulation, thus reducing food quality (Li *et al*. 2017). Similarly, as in the case of iodine, the form and concentration of selenium applied onto plants is critical although it seems to be species-dependent. Application of selenate at a concentration of 5 μM results in increased accumulations of magnesium, zinc, and manganese in maize, whereas the combined application of selenite and selenate cause a fall off cation accumulations. Additionally, selenite alone induces a reduction in the production of plant biomass (Longchamp *et al*. 2016). In contrast, application of a selenite/selenate mixture at a concentration of 5 μM exhibits a positive effect on the root and stalk production in wheat (Boldrin *et al*. 2016). Overall, effectiveness of fertilization based strategy as a mean of biofortification have to be considered when it comes to quantification of costs. Interestingly, Wang *et al*. (2016) evaluated the foliar application of Zn combined with pesticide and concluded that this approach is economically cost-effective and sustainable. However, particularly in the case of zinc, results of the field experiment demonstrated that the enhancement of zinc requires optimal phosphate management in addition to zinc fertilization (Zhang *et al*. 2015).

Biostimulants and symbiotic microorganisms: An attractive approach of crop biofortification consists in the utilization of biostimulants – chemical substances possessing biological activity, which facilitates the uptake and storage of microelements or biomass production. Billard *et al*. (2014) tested in a hydroponic culture system two substances *AZAL5* and *HA7* on rapeseed that do not actually improve microelement uptake, but they induce root formation and so N, S, K, and P uptake. Also, an increased rate of Fe and Zn root-to-shoot translocation was observed in the treated plants. Substances derived from amino acids (AA) constitute an important group of biostimulants. Their foliar application can improve acquisition of microelements (Kałużewicz *et al*. 2018, Popko *et al*. 2018). Modification of sugar content can be strengthened using commercial biostimulants such as *Asahi SL* and *Kelpak SL* (Zarzecka and Gugała 2018). Another group of plant biostimulants constitute plant growth regulators and their derivatives that participate in various cell and morphological processes. Interestingly, an experimentally developed cytokinin derivative *2-Cl-3-MeOBAP* has been extensively tested in the field conditions and exhibits a positive effect on the number of productive tillers and improvement of grain filling leading to homogeneity of the spike (Koprna *et al*. 2016).

A promising approach for crop biofortification also lies in a better interaction of the root system and soil microorganisms. Soil bacteria and fungi are capable of the transformation of insoluble compounds into easily soluble

forms, which can be absorbed by plants. Moreover, it has been reported that endophytic bacteria are capable of plant growth enhancement even by means of plant hormone production (Rohini *et al*. 2017). Hence, improvements of the nutritional characteristics of arable crops could be achieved by refining interactions between plants and soil microorganisms. Higher biomass production, an abundance of vitamin B1 and its analogs, and increased content of organic acids have all been achieved by the inoculation of plant seeds with the arbuscular-mycorrhizal fungus *Rhizopagus irregularis* (Rozpadek *et al*. 2016). Similarly, the zinc solubilizing bacterium *Exiguobacterium aurantiacum* has been described and used to induce up to a 6-fold elevation in zinc content (Shaikh and Saraf 2017).

Biotechnological approaches

Biotechnology approaches utilize promptly achievements of scientific progress. Description of metabolic pathways and their regulation enables scientists to start thinking about modification or altering such pathways in order to increase content of desirable substances or minerals. Further, development of whole new biosynthetic pathways in host organisms entered the new possibilities of biotechnological approaches. Biotechnological strategy covers classical breeding techniques supplemented by marker assisted selection (MAS) and advanced methods of genetic engineering [gene over-expression, silencing by RNA interference (RNAi) technology, or the direct knocking-out of genes using the tools of genome editing]. Especially advanced biotechnological approaches depend on a good grasp of the basic research, *e.g.*, genes and enzymes involved in the synthesis, transport, and accumulation of the substances studied. Further, knowledge of gene sequences, regulatory pathways, cell signaling, enzyme biochemistry, and the phenotypic consequences of genetic manipulation are a precondition for biotechnological manipulation. In the case of heterologous expression, the choice of appropriate promoters and sequence optimization for a given organism need to be considered before any realization of the transformation experiment. Afterwards, the arrangement of diverse genetic sources (*i.e*., alternative genes, promoters, and terminators that might be exploitable for the biofortification) is of great importance.

Conventional biotechnology approach: Conventional biotechnology that utilizes MAS has become a part of breeding process of staple crops. It depends on identification of traits of interest that can be introduced into new elite cultivars. The differential comparison of staple crops, differences in the content of studied elements or substances by bioinformatic, transcriptomic, proteomic, and various analytical methods are capable of generating data relevant for the evaluation of new traits responsible for production and accumulation of desired substances. Initial efforts made in the field of biotechnology started with utilization of MAS (Paterson *et al*. 1988). Marker assisted selection could be considered as a reliable and robust technique that facilitates breeding and selection of suitable genotypes. It is utilized routinely in conventional breeding programs. Additionally, to identify traits with minor effect, MAS based on genomic selection (GS-MAS) could be exploited, and markers derived from such traits can be used later in selection (Meuwissen *et al*. 2001).
Methods mentioned above were used for enhancement of microelement content in mature seeds as microelements constitute a substantial part of the nutritional value of the plant seed. Once absorbed, the microelements are transported into vegetative tissues. Lately, microelements are transported into the developing seeds, where they can be also remobilized from other plant tissues. Identification of those traits responsible for the remobilization of microelements from the vegetative to the generative tissues is crucial. Hussain *et al*. (2016) mapped quantitative trait locus (QTL) in barley associated with Zn remobilization from the leaves to grains. The study revealed that there is a significant variability in plant ability to translocate microelements to grains, for instance barley cv. Sahara is able to remobilize almost 40 % of Zn. Similarly, Crespo-Herrera *et al*. (2016) identified QTL at wheat chromosome 4BS, which is responsible for one-fifth of Zn content variability in grains. Metal tolerance proteins, which are predominantly expressed in the cells of the aleurone layer, are engaged in the regulation of elemental deposition. Generally, the accumulation of microelements in the grain is affected by genotype. It has been proven that distinct rice genotypes can differ in their mean Zn deposition and if either translocation from the leaves or transport from the roots prevails (Johnson-Beebout *et al*. 2016).

Modification of pathways: A breaking point of biotechnology was discovery and application of *Agrobacterium*-mediated plant transformation (Schell and Van Montagu 1977). Lately, biolistic transformation was introduced as a suitable technique (Sanford 1990) and together with the *Agrobacterium*-mediated transformation became the most expanded techniques of plant genetic manipulation. Genetic modifications can be exploited for tailoring plant cell transport and synthetic or metabolizing pathways in order to increase microelement, vitamin, or FA content.

Initially, single or multiple gene over-expressions were used to modulate biochemical pathways. In plants, complex-forming organic molecules, such as nicotianamine and citrate, play a role in the translocation and transport of microelements. Therefore, research is focused on enhancement of microelement transport *via* increased production of microelement binding molecules such as nicotianamine. Both, non-specifically induced expression of *nicotianamine synthase* (*NAS*) in rice (Lee *et al*. 2011) and specifically over-expressed *OsNAS* or *OsNAS2* in the phosphate poor endosperm of rice (Johnson *et al*. 2011) and wheat (Singh *et al*. 2017) result in a considerable increase of Zn content. In the case of localized expression in developing grains, concomitant Fe enrichment in grains of rice and wheat is also observed (Johnson *et al*. 2011, Singh *et al*. 2017). Additionally, in order to improve Fe content in grains, over-expression of

NAS is coupled with over-expression of iron transporters and ferritin (Boonyaves *et al*. 2017).

Extensive research has been conducted on the topic of seed FA composition. Unfortunately, over-production of enzymes associated with transport or synthesis of desired substance is occasionally accompanied with negative phenotypic manifestations. For instance, Braybrook *et al*. (2006) reported that FA biofortification through constitutive expression of genes from the biosynthetic pathway result in some cases in growth and developmental anomalies. Maximization of FA content in seeds could not only lead to negative phenotypic manifestations but also to reaching the physiological limits of the organism. One solution could be the conversion of saturated into unsaturated FAs. Therefore, Du *et al*. (2016) employed RNAi technology to silence the *stearoyl-acyl carrier protein desaturase*-1 (*SAD1*), the enzyme that catalyzes conversion of the stearoyl-acyl carrier protein (ACP) to oleoyl-ACP, which is a precursor of polyunsaturated FAs. A lowered activity of SAD1 results in an increased amount of stearic acid and other long FAs with a concomitant decrease in oleic acid content. Kim *et al*. (2014) suggested that enhanced triacylglycerol accumulation in seeds could be achieved by restriction of its catabolism. Silencing sugar dependent 1 lipase, participating in the catabolism of FA, caused up to a 30 % higher content of total seed storage lipids along with a 7 % decrease in protein content. Quality of FA is enhanced by seed-specific silencing *FA dehydrogenase 2* (*FAD*2) and/or a *FA elongase 1* (*FAE1*). Especially, concomitant depression of *FAD2* and *FAE1* leads into an extreme ratio of different C18 unsaturated FAs, thus improving seed nutritional quality (Shi *et al*. 2017). Biofortification of desirable substance, such as AAs or vitamins, might be achieved *via* restriction of metabolizing pathways rather than boosting production by gene over-expression. Hence, new techniques that allow a more precise manipulation with genome and gene transcriptions, are adapted. Aminoacids, especially essential, such as lysine, methionine, threonine, and phenylalanine, have also been subjects of biofortification. Sikdar *et al*. (2016) used RNAi technology to reduce a storage C-hordein protein by up to 97 %. Concomitantly, a drop in C-hordein content is balanced by an increase in B-hordein. Noticeably, a decrease in C-hordein positively affects the content of the essential AAs threonine, methionine, and leucine. Thus, RNAi technology could assist a successful development of low hordein lines and enrichment of essential compounds.

Aside from gene transcription silencing, modification of biochemical pathways can be alternatively performed *via* new mutagenesis by using programmable nucleases, such as transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) (Jinek *et al*. 2012), that enables precise genomic alterations. Classical mutagenesis still has its place among other techniques. Development of a mutant by fast neutron irradiation was evidence for mutagenesis. Increased content of lipid-soluble antioxidants, such as vitamin E, could be achieved by single knock-out of *homogentisate dioxygenase* (*HGO1*). A mutation in this gene also causes tolerance to herbicides targeting homogentisate metabolism (Stacey *et al*. 2016). However, usage of programmable nucleases has several unique advantages such as a predicted mutated locus that does not have to be mapped, avoiding work with a mutant population, and ability to create mutations in homozygous constitution in the T_0 generation. The presence of these drawbacks in classical mutagenesis predicts a bright future for using a molecular biology tool such as CRISPR/Cas9 or TALEN. For example, a critical qualitative parameter for grain evaluation is the stability of unsaturated FAs after harvest. Degradation of FAs as a consequence of oxidation occurs to a large extent. Lipoxygenases, which catalyze dioxygenation, contribute significantly to the degradation of FAs. Hydroperoxide, as a product of the reaction, constitutes an important factor in the durability and viability of seeds. Using TALEN, Ma *et al*. (2015) developed a rice mutant in *lipoxygena*se *lox3*. Knock-out of this gene reduces FA oxidation in long-term stored rice grains and has no unfavorable effect on the performance of its agronomic traits. An indisputable advantage of programmable nucleases appears when crops with polyploid genomes are subjected to genetic manipulation. Efficiency of CRISPR/Cas9 nuclease was demonstrated on the allohexaploid *Camelina sativa* containing three alleles of *FA elongase 1* genes *FAE1*, when all three alleles are knocked-out in homozygous constitution in a single generation resulting in an almost total restriction of production of very long-chain FAs (Ozseyhan *et al*. 2018). Similarly, the precision of genome editing by Cas9 nuclease was also demonstrated producing knock-out in the *FAD2* gene in *C. sativa* (Jiang *et al*. 2017) or *OsFAD2-1* gene in rice (Abe *et al*. 2018) leading to alteration of the content of oleic acid in the mutant lines. For instance in *C. sativa*, the content of oleic acid rises from 16 to 50 %, and production of monounsaturated FA rises by 44 %.

***De novo* pathway development:** Wide knowledge of metabolic processes is a precondition for development of plants with engineered synthetic pathways. Such a strategy can be considered as advanced pathway modification. For sustainable production of a specific substance, key enzymes have to be identified. *De novo* created synthetic pathway requires, apart from key enzymes, also basal precursors of the desired substance, whose production is expected. Such a premise was recently integrated into development of transgenic sorghum. Che *et al*. (2016) prepared transgenic sorghum over-expressing *β-carotene synthesizing enzymes*, in a manner similar to that in golden rice (Paine *et al*. 2005). In addition they inserted a gene coding for 1-deoxyxylulose 5-phosphate synthase, a rate-limiting enzyme in the methylerythritol phosphate pathway, thereby increasing the availability of a precursor for carotenoid biosynthesis. Alterations in the amounts of intermediates and the restriction of their further metabolism may occur when new biosynthetic pathways are being constructed *in planta*. Such issues need to be considered when designing strategies for production of ω3-long-chain polyunsaturated FA in model plant species. (Qi *et al*. 2004)

and *Camelina sativa* (Ruiz-Lopez *et al*. 2014). Production of polyunsaturated FAs in above mentioned works was achieved by over-expression of 5 or 7 transgenes for eicosapentaenoic acid (20:5, n-3) and docosahexaenoic acid (22:6, n-3), respectively. Biosynthesis is streamlined by introducing an acyl CoA-dependent Δ6-desaturase from *Ostrococcus tauri* (Domergue *et al*. 2005) that enables accumulation of eicosapentaenoic acid and prevents undesirable accumulation of C18 biosynthetic intermediates (Sayanova *et al*. 2012, Ruiz-Lopez *et al*. 2013) thus depicting the importance of characterization of metabolome and a need of detailed metabolic evaluation of intermediates and their changes as a result of transgene integration. Similar obstacles have to be solved when lysine biofortified rice is prepared (Long *et al*. 2013, Yang *et al*. 2016). A transgenic line producing up to 25-fold higher content of lysine than the wild type was developed by boosting its synthesis *via DAPA* and *LYSC* over-expression and by crucial down-regulation of lysine metabolizing enzymes lysine ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) and thus catabolism of accumulated lysine into 2-oxoadipate. Notably, agronomic evaluations of plants suggested that significantly increased content of lysine only slightly affects plant height (Yang *et al*. 2016).

In almost all cases, novel traits produced by the biotechnological approaches discussed in this review will need to be crossed into high-yielding cultivars. However, such a process might be complicated by selecting an appropriate background genotype. The genetic background of the recipient cultivar should be carefully chosen in order to obtain the desired phenotypic manifestation. For instance, a better phenotypic manifestation is achieved when *Oryza sativa* gene *NAS* in the donor rice cv. Nipponbare (subspecies *japonica*) is introduced into a rice cultivars from the subspecies *japonica* rather than into those from subspecies *indica* (Moreno-Moyano *et al*. 2016). Therefore, selection of a suitable genetic background of the acceptor cultivar, which has none (or a minor) impact on the manifestation of the trait, is of importance. Moreover, the homozygous make up of a target trait is often required in the breeding process. Notably, production of doubled-haploids could reduce the time needed for stabilization of the transgene in the genome, and then could be used in the functional validation of the gene and subsequent crossing (Shen *et al*. 2015).

Interestingly, concerning practical testing of vitamin A content in feed showed that an increased uptake of carotenoids has a positive effect on the tested subjects. Nogareda *et al*. (2016) proved not only that broilers fed with transgenic maize producing increased amount of carotenoids are able to acquire carotenoids better than from additives, but that the carotenoid diet delays the reproductive cycle of the protozoan parasite *E. tenella*, the cause of coccidiosis. Vitamin A biofortified rice was assessed as a food additive in the diet of Ethiopian children. If consumed as a staple part of the diet, the consummation of this rice was an effective source of vitamin A. There were no cases of hypervitaminosis noted in the group consuming the biofortified rice whereas hypervitaminosis cases were in the artificially produced food additive group (Gannon *et al*. 2014).

Concluding remarks

Fertilization-based and biotechnological ways of plant biofortification are feasible and capable of increasing the content of important substances in plants. These advanced techniques, such as the use of microparticles for the foliar application of nutrients, the development of sophisticated molecular biology tools for precise genome manipulation, or the introduction of complete metabolic pathways, provide effective biofortification strategies. These approaches, which are proving attractive to farmers, have undergone substantial progress. The decision on which approach to use will depend on the legislative, financial, and practical options available to the producer. New molecular biology techniques, such as TALEN or CRISPR/Cas9 have been promptly implemented and can be expected to play an important role in production of biofortified crops in the future.

References

Abe, K., Araki, E., Suzuki, Y., Toki, S., Saika, H.: Production of high oleic/low linoleic rice by genome editing. - Plant Physiol. Biochem. **131**: 58-62, 2018.

Ali, F., Peng, Q., Wang, D., Cui, Z., Huang, J., Fu, D., Liang, D.: Effects of selenite and selenate application on distribution and transformation of selenium fractions in soil and its bioavailability for wheat (*Triticum aestivum* L.). - Environ. Sci. Pollut. Res. **24**: 8315-8325, 2017.

Billard, V., Etienne, P., Jannin, L., Garnica, M., Cruz, F., Garcia-Mina, J.M., Yvin, J.C., Ourry, A.: Two biostimulants derived from algae or humic acid induce similar responses in the mineral content and gene expression of winter oilseed rape (*Brassica napus* L.). - J. Plant Growth Regul. **33**: 305-316, 2014.

Blancquaert, D., Van Daele, J., Strobbe, S., Kiekens, F., Storozhenko, S., De Steur, H., Gellynck, X., Lambert W., Stove Ch., Van Der Straeten, D.: Improving folate (vitamin B9) stability in biofortified rice through metabolic engineering. - Nat. Biotech. **33**: 1076–1078, 2015.

Boldrin, P.F., De Figueiredo, M.A., Yang, Y., Luo, H., Giri, S., Hart, J.J., Faquin, V., Guilherme, L.R.G., Thannhauser, T.W., Li, L.: Selenium promotes sulfur accumulation and plant growth in wheat (*Triticum aestivum*). - Physiol. Plant. **158**: 80-91, 2016.

Boonyaves, K., Wu, T.Y., Gruissem, W., Bhullar, N.K.: Enhanced grain iron levels in rice expressing an iron-regulated metal transporter, nicotianamine synthase, and ferritin gene cassette. - Front. Plant Sci. **8**: 1-11, 2017.

Bouis, H.E.: Plant breeding: a new tool for fighting micronutrient malnutrition. - J. Nutr. **132**: 491S-494S, 2002.

Braybrook, S.A., Stone, S.L., Park, S., Bui, A.Q., Le, B.H., Fischer, R.L., Goldberg, R.B., Harada, J.J.: Genes directly regulated by leafy cotyledon2 provide insight into the control of embryo maturation and somatic embryogenesis. - Proc. nat. Acad. Sci. USA **103**: 3468-3473, 2006.

Che, P., Zhao, Z., Glassman, K., Dolde, D., Hu, T.X., Jones, T.J., Obukosia, S., Wambugu, F., Che, P., Zhao, Z., Glassman, K., Dolde, D., Hu, T.X., Jones, T.J., Fred, D.: Elevated vitamin

E content improves all-*trans* β-carotene accumulation and stability in biofortified sorghum. - Proc. nat. Acad. Sci. USA **113**: 11040-11045, 2016.

Crespo-Herrera, L.A., Velu, G., Singh, R.P.: Quantitative trait loci mapping reveals pleiotropic effect for grain iron and zinc concentrations in wheat. - Ann. appl. Biol. **169**: 27-35, 2016.

Di Tullo, P., Versini, A., Bueno, M., Le Hécho, I., Thiry ,Y., Biron, P., Castrec-Rouelle, M., Pannier, F.: Stable isotope tracing: A powerful tool for selenium speciation and metabolic studies in non-hyperaccumulator plants (ryegrass *Lolium perenne* L.). - Anal. bioanal. Chem. **407**: 9029-9042, 2015.

Domergue, F., Abbadi, A., Zähringer, U., Moreau, H., Heinz, E.: *In vivo* characterization of the first acyl-CoA delta6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. - Biochem. J. **389**: 483-490, 2005.

Du, H., Huang, M., Hu, J., Li, J.: Modification of the fatty acid composition in *Arabidopsis* and maize seeds using a stearoyl-acyl carrier protein desaturase-1 (*ZmSAD1*) gene. - BMC Plant Biol. **16**: 137, 2016.

Fageria, N.K., Filho, M.P.B., Moreira, A., Guimarães, C.M.: Foliar fertilization of crop plants. - J. Plant Nutr. **32**: 1044-1064, 2009.

Fernández, V., Brown, P.H.: From plant surface to plant metabolism: the uncertain fate of foliar-applied nutrients. - Front. Plant Sci. **4**: 1-5, 2013.

Fernández, V., Eichert, T.: Uptake of hydrophilic solutes through plant leaves: current state of knowledge and perspectives of foliar fertilization. - Crit. Rev. Plant Sci. **28**: 36-68, 2009.

Field, R.J., Bishop, N.G.: Promotion of stomatal infiltration of glyphosate by an organosilicone surfactant reduces the critical rainfall period. - Pestic. Sci. **24** :55-62, 1988.

Gannon, B., Kaliwile, C., Arscott, S.A., Schmaelzle, S., Chileshe, J., Kalungwana, N., Mosonda, M., Pixley, K., Masi, C., Tanumihardjo, S.A.: Biofortified orange maize is as efficacious as a vitamin A supplement in Zambian children even in the presence of high liver reserves of vitamin A: a community-based, randomized placebo-controlled trial. - Amer. J. clin. Nutr. **100**: 1541-1550, 2014.

Hussain, S., Rengel, Z., Mohammadi, S.A., Ebadi-Segherloo, A., Maqsood, M.A.: Mapping QTL associated with remobilization of zinc from vegetative tissues into grains of barley (*Hordeum vulgare*). - Plant Soil **399**: 193-208, 2016.

Jiang, W.Z., Henry, I.M., Lynagh, P.G., Comai, L., Cahoon, E.B., Weeks, D.P.: Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using *CRISPR/Cas9* gene editing. - Plant Biotechnol. J. **15**: 648-657, 2017.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J., Charpentier, E.: A programmable dual-RNA – guided DNA endonuclease in adaptive bacterial immunity. - Science **337**: 816-822, 2012.

Johnson, A.A.T., Kyriacou, B., Callahan, D.L., Carruthers, L., Stangoulis, J., Lombi, E., Tester, M.: Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. - Plos ONE **6**: e24476, 2011.

Johnson-Beebout, S.E., Goloran, J.B., Rubianes, F.H.C., Jacob, J.D.C., Castillo, O.B.: Zn uptake behavior of rice genotypes and its implication on grain Zn biofortification. - Sci. Rep. **6**: 38301, 2016.

Kaiser, H.: Stomatal uptake of mineral particles from a sprayed suspension containing an organosilicone surfactant. - J. Plant Nutr. Soil Sci. **177**: 6, 869-874, 2014.

Kałużewicz, A., Bosiacki, M., Spiżewski, T.: Influence of biostimulants on the content of macro- and micronutrients in broccoli plants exposed to drought stress. - J. Elements **23**: 287-297, 2018.

Kim, M.J., Yang, S.W., Mao, H.Z., Veena, S.P., Yin, J.L., Chua, N.H.: Gene silencing of sugar-dependent 1 (*JcSDP1*), encoding a patatin-domain triacylglycerol lipase, enhances seed oil accumulation in *Jatropha curcas*. - Biotechnol. Biofuels **7**: 36, 2014.

Koprna, R., De Diego, N., Dundálková, L., Spíchal, L.: Use of cytokinins as agrochemicals. - Bioorganic med. Chem. **24**: 484-492, 2016.

Lee, S., Persson, D.P., Hansen, T.H., Husted, S., Schjoerring, J.K., Kim, Y.S., Jeon, U.S., Kim, Y.K., Kakei, Y., Masuda, H., Nishizawa, N.K., An, G.: Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. - Plant Biotechnol. J. **9**: 865-873, 2011.

Li, R., Liu, H.P., Hong, C.L., Dai, Z.X., Liu, J.W., Zhou, J., Hu, C.Q., Weng, H.X.: Iodide and iodate effects on the growth and fruit quality of strawberry. - J. Sci. Food Agr. **97**: 230-235, 2017.

Long, X., Liu, Q., Chan, M., Wang, Q., Sun, S.S.M.: Metabolic engineering and profiling of rice with increased lysine. - Plant Biotechnol. J. **11**: 490-501, 2013.

Longchamp, M., Angeli, N., Castrec-Rouelle, M.: Effects on the accumulation of calcium, magnesium, iron, manganese, copper and zinc of adding the two inorganic forms of selenium to solution cultures of *Zea mays*. - Plant Physiol. Biochem. **98**: 128-137, 2016.

Ma, L., Zhu, F., Li, Z., Zhang, J., Li, X., Dong, J., Wang, T.: *TALEN*-based mutagenesis of lipoxygenase LOX3 enhances the storage tolerance of rice (*Oryza sativa*) seeds. - Plos ONE **10**: e0143877, 2015.

Meuwissen, T.H.E., Hayes, B.J., Goddard, M.E.: Prediction of total genetic value using genome-wide dense marker maps. - Genetics **157**: 1819-1829, 2001.

Moreno-Moyano, L.T., Bonneau, J.P., Sánchez-Palacios, J.T., Tohme, J., Johnson, A.A.T.: Association of increased grain iron and zinc concentrations with agro-morphological traits of biofortified rice. - Front. Plant Sci. **7**: 1463, 2016.

Nogareda, C., Moreno, J.A., Angulo, E., Sandmann, G., Portero, M., Capell, T., Zhu, C., Christou, P.: Carotenoid-enriched transgenic corn delivers bioavailable carotenoids to poultry and protects them against coccidiosis. - Plant Biotechnol. J. **14**: 160-168, 2016.

Ozseyhan, M.E., Kang, J., Mu, X., Lu, C.: Mutagenesis of the *FAE1* genes significantly changes fatty acid composition in seeds of *Camelina sativa*. - Plant Physiol. Biochem. **123**: 1-7, 2018.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L., Drake, R.: Improving the nutritional value of Golden rice through increased pro-vitamin A content. - Nat. Biotech. **23**: 482-487, 2005.

Paterson, A.H., Lander, E.S., Hewitt, J.D., Peterson, S., Lincoln, S.E., Tanksley, S.D.: Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. - Nature **335**: 721-726, 1988.

Paul, J.Y., Khanna, H., Kleidon, J., Hoang, P., Geijskes, J., Daniells, J., Zaplin, E., Rosenberg, Y., James, A., Mlalazi, B., Deo, P., Arinaitwe, G., Namanya, P., Becker, D., Tindamanyire, J., Tushemereirwe, W., Harding, R., Dale, J.: Golden bananas in the field: elevated fruit pro-vitamin A from the expression of a single banana transgene. - Plant Biotechnol. J. **15**: 520-532, 2017.

Popko, M., Michalak, I., Wilk, R., Gramza, M., Chojnacka, K., Górecki, H.: Effect of the new plant growth biostimulants based on amino acids on yield and grain quality of winter

wheat. - Molecules **23**: 470, 2018.
Qi, B., Fraser, T., Mugford, S., Dobson, G., Sayanova, O., Butler, J., Napier, J.A., Stobart, A.K., Lazarus, C.M.: Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. - Nat. Biotech. **22**: 739-745, 2004.
Rohini, S., Aswani, R., Kannan, M., Sylas, V.P., Radhakrishnan, E.K.: Culturable endophytic bacteria of ginger rhizome and their remarkable multi-trait plant growth-promoting features. - Curr. Microbiol. **75**: 505-511, 2017.
Rozpadek, P., Rapała-Kozik, M., Wezowicz, K., Grandin, A., Karlsson, S., Wazny, R., Anielska, T., Turnau, K.: Arbuscular mycorrhiza improves yield and nutritional properties of onion (*Allium cepa*). - Plant Physiol. Biochem. **107**: 264-272, 2016.
Ruiz-Lopez, N., Haslam, R.P., Napier, J.A., Sayanova, O.: Successful high-level accumulation of fish oil omega-3 long-chain polyunsaturated fatty acids in a transgenic oilseed crop. - Plant J. **77**: 198-208, 2014.
Ruiz-Lopez, N., Haslam, R.P., Usher, S.L., Napier, J.A., Sayanova, O.: Reconstitution of EPA and DHA biosynthesis in *Arabidopsis*: iterative metabolic engineering for the synthesis of n-3 LC-PUFAs in transgenic plants. - Metabol. Eng. **17**: 30-41, 2013.
Sanford, J.C.: Biolistic plant transformation. - Physiol. Plant. **79**: 206-209, 1990.
Sayanova, O., Ruiz-Lopez, N., Haslam, R.P., Napier, J.A.: The role of Δ6-desaturase acyl-carrier specificity in the efficient synthesis of long-chain polyunsaturated fatty acids in transgenic plants. - Plant Biotechnol. J. **10**: 195-206, 2012.
Schell, J., Van Montagu, M.: Transfer, maintenance, and expression of bacterial Ti-plasmid DNA in plant cells transformed with *A. tumefaciens*. - Brookhaven Symp. Biol. **29**: 36-49, 1977.
Shaikh, S., Saraf, M.: Biofortification of *Triticum aestivum* through the inoculation of zinc solubilizing plant growth promoting rhizobacteria in field experiment. - Biocatal. agr. Biotechnol. **9**: 120-126, 2017.
Shen, Y., Pan, G., Lübberstedt, T.: Haploid strategies for functional validation of plant genes. - Trends Biotechnol. **33**: 611-620, 2015.
Shi, J., Lang, C., Wang, F., Wu, X., Liu, R., Zheng, T., Zhang, D., Chen, J., Wu, G.: Depressed expression of *FAE1* and *FAD2* genes modifies fatty acid profiles and storage compounds accumulation in *Brassica napus* seeds. - Plant Sci. **263**: 177-182, 2017.
Sikdar, M.S.I., Bowra, S., Schmidt, D., Dionisio, G., Holm, P.B., Vincze, E.: Targeted modification of storage protein content resulting in improved amino acid composition of barley grain. - Transgenic Res. **25**: 19-31, 2016.
Singh, S.P., Keller, B., Gruissem, W., Bhullar, N.K.: Rice nicotianamine synthase 2 expression improves dietary iron and zinc levels in wheat. - Theor. appl. Genet. **130**: 283-292, 2017.
Stacey, M.G., Cahoon, R.E., Nguyen, H.T., Cui, Y., Sato, S., Nguyen, C.T., Phoka, N., Clark, K.M., Liang, Y., Forrester, J., Batek, J., Do, P.T., Sleper, D.A., Clemente, T.E., Cahoon, E.B., Stacey, G.: Identification of homogentisate dioxygenase as a target for vitamin e biofortification in oilseeds. - Plant Physiol. **172**: 1506-1518, 2016.
Tiong, J., Mcdonald, G.K., Genc, Y., Pedas, P., Hayes, J.E., Toubia, J., Langridge, P., Huang, C.Y.: HvZIP7 mediates zinc accumulation in barley (Hordeum vulgare) at moderately high zinc supply. - New Phytol. **201**: 131–143, 2014.
Uddin, M.N., Kaczmarczyk, A., Vincze, E.: Effects of Zn fertilization on hordein transcripts at early developmental stage of barley grain and correlation with increased Zn concentration in the mature grain. - PloS ONE **9**: e108546, 2014.
Wang, Y.H., Zou, C.Q., Mirza, Z., Li, H., Zhang, Z.Z., Li, D.P., Xu, C.L., Zhou, X. Bin, Shi, X.J., Xie, D.T., He, X.H., Zhang, Y.Q.: Cost of agronomic biofortification of wheat with zinc in China. - Agron. sustain. Dev. **36**: 44, 2016.
Yang, Q., Zhang, C., Chan, M., Zhao, D., Chen, J., Wang, Q., Li, Q., Yu, H., Gu, M., Sun, SS., Liu, Q.: Biofortification of rice with the essential amino acid lysine: molecular characterization, nutritional evaluation, and field performance. - J. exp. Bot. **67**: 4285-4296, 2016.
Zarzecka, K., Gugała, M.: The effect of herbicides and soil tillage systems on the content of polyphenols in potato tubers. - Polish J. environ. Stud. **64**: 82-87, 2018.
Zhang, W., Liu, D., Li, C., Cui, Z., Chen, X., Russell, Y., Zou, C.: Zinc accumulation and remobilization in winter wheat as affected by phosphorus application. - Field Crops Res. **184**: 155-161, 2015.
Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P., Capell, T.: Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathway in maize. - Proc. nat. Acad. Sci. USA **105**: 18232-18237, 2008.

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Allelic Variants of CRISPR/Cas9 Induced Mutation in an Inositol Trisphosphate 5/6 Kinase Gene Manifest Different Phenotypes in Barley

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Abstract: Inositol trisphosphate 5/6 kinases (ITPK) constitute a small group of enzymes participating in the sequential phosphorylation of inositol phosphate to inositol hexakisphosphate (IP6), which is a major storage form of phosphate in cereal grains. The development of lines with reduced IP6 content could enhance phosphate and mineral bioavailability. Moreover, plant *ITPK*s participate in abiotic stress signaling. To elucidate the role of *HvITPK1* in IP6 synthesis and stress signaling, a barley *itpk1* mutant was created using programmable nuclease Cas9. Homozygous single bp insertion and deletion mutant lines were obtained. The mutants contained altered levels of phosphate in the mature grains, ranging from 65% to 174% of the wild type (WT) content. Homozygous mutant lines were tested for their response to salinity during germination. Interestingly, insertion mutant lines revealed a higher tolerance to salinity stress than deletion mutants. Mature embryos of an insertion mutant *itpk1-2* and deletion mutant *itpk1-33* were cultivated in vitro on MS medium supplemented with NaCl at 50, 100, and 200 mM. While both mutants grew less well than WT on no or low salt concentrations, the *itpk1-2* mutant was affected less than the WT and *itpk33* when grown on the highest NaCl concentration. The expression of all *ITPK*s was induced in roots in response to salt stress. In shoots, the differential effect of high salt on *IPTK* expression in the two *iptk1* mutants was consistent with their different sensitivities to salt stress. The results extend the evidence for the involvement of *ITPK* genes in phosphate storage and abiotic stress signaling.

Keywords: CRISPR; barley mutant; abiotic stress; salinity; phosphate; phytic acid

1. Introduction

Barley (*Hordeum vulgare*) a member of the *Poaceae* family was one of the first domesticated crops. Barley belongs among the most important crops globally, being used primarily as animal feed, food, and especially in the brewing industry. One of the drawbacks of the barley-based feeding mixture is poor utilization of phosphate that is present mainly in the organic form of IP6, commonly known as phytic acid (PA). PA is the main storage form of phosphate in cereal grains [1]. Naturally, plant seeds produce phytases, which are a group of phosphatases that hydrolyse PA during germination to make phosphate available for the young seedling. Regarding livestock nutrition, PA is considered an anti-nutrient because of its low digestibility by non-ruminants such as poultry or pigs. Currently, feeding mixtures are supplemented with inorganic phosphate, mostly as calcium phosphate, in order to improve their poor phosphate bioavailability. A more advanced option for supplementing the feeding mixture is the addition of purified microbial phytase that aids PA degradation. Unfortunately, these forms of supplementation are relatively expensive.

There are two pathways of PA synthesis in plants, lipid independent and lipid dependent, which produce PA through sequential phosphorylation of *myo*-inositol or phosphatidylinositol, respectively [2]. The lipid-independent pathway was proposed to be involved mainly in phosphate storage during grain development [3]. On the other hand, the lipid-dependent pathway via phosphatidylinositol intermediates produces molecules active in cell signaling [4]. Disruption of the PA biosynthetic pathway led to decreased production of PA that was accompanied by increased levels of phosphate in the mature seeds of pea [5] or rice [6,7]. Interestingly, an intermediate in PA synthesis, inositol triphosphate (IP3), is an important cell signaling molecule playing a role as a secondary messenger [8,9]. Two approaches have evolved to cope with the issue of poor grain phosphate utilization. The earlier approach involved the development of low-phytic acid (*lpa*) lines using classical mutagenesis [5,10–15]. These lines carried mutations in genes encoding enzymes involved in PA biosynthesis, mostly inositol phosphate kinases or *myo*-inositol phosphate synthases. Feeding trials with poultry [16], swine [17,18], sheep [19], and rats [20] confirmed that *lpa* barley lines had better nutritional value, with increased mineral and phosphate digestibility. It was observed in previous studies with several *lpa* mutants that these plants have impaired seed or plant performance [21]. The affected phenotype included reduced germination rate [22] and yield [23] with some seed sterility [24]. These findings indicated that phytic acid and its biosynthetic intermediates are of considerable importance for plant metabolism and development. In the light of the performance of different *lpa* mutants grown at five locations over two seasons, Raboy et al. [25] concluded that out of six known *lpa* traits in barley only a few affected grain yields significantly. Moreover, a moderate reduction of PA content (mutant *lpa2-1*) had no effect on yield. Biotechnological approaches have included the development of transgenic lines over-expressing plant or microbial phytases, usually under seed-specific promoters [26–28]. The increased phytase activity in mature grains resulted in improved phosphate level and the phytase over-expressing lines successfully competed with *lpa* mutant lines for increased phosphate digestibility.

*ITPK*s are not only proposed to be involved in the "Pi storage" pathway, but they were also reported to play a role in the response to abiotic stress [29,30]. Drought and salinity represent important abiotic stresses, which can critically influence sustainable yield. Plants perceive abiotic stress via multiple pathways to cope with inhospitable environmental conditions. Plant hormones are essential in signaling during adverse growth and environmental conditions. Drought and salinity stress signaling pathways share a unique signal leading to the accumulation of the plant hormone - abscisic acid (ABA) [31]. Elucidating the crosstalk between stress and hormone signaling pathways is necessary for a better understanding of the processes by which plants respond to abiotic stress. *myo*-Inositol is an important biomolecule in plant metabolism, forming conjugates with plant hormones and acting as a substrate for phosphate accumulation, while isomeric inositols and their O-methyl esters participate in seed desiccation and salt tolerance [32]. Regulation of InsP synthesis by ABA signaling is supported by a report by Aggarwal et al. [33], who showed that ABA controls the expression of PA synthesis genes. There is growing evidence for the involvement of plant ITPKs in abiotic stress signaling. For example, Du et al. [29] reported that a rice *Ositpk2* mutant accumulated significantly less osmolytes, such as proline or soluble sugars, under drought stress conditions than did the wild type. Furthermore, Niu et al. [34] suggested that *OsITL1*, which encodes an ITPK, might be a negative regulator of osmotic stress signaling. Both *OsITPK2* and *OsITL1* are orthologues of barley *HvITPK2*, an intron-containing member of the *ITPK* group. As reported for *GaITPK2* [30], the promoter region of *HvITPK1* contains binding motifs for ABA responsive element and two drought-inducible MYB transcription factors, suggesting its involvement in abiotic stress signaling.

Currently, programmable nucleases have become a widespread biotechnological tool that enables precise genome manipulation. Initially, a transcription activator-like effector was fused with *Fok*I endonuclease to induce breaks in double-stranded DNA [35] and lately, TALE-nuclease (TALEN) fusion was used to create mutations at pre-designed genomic sites of *adh1* in *Arabidopsis thaliana* thus confirming its usability in plants [36]. When a revolutionary clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system was introduced to the scientific community [37], it instigated a new epoch of genetic engineering. The Cas9 system

was quickly adapted for plant genetic modifications [38]. Jiang et al. [39] and Shan et al. [40] demonstrated that Cas9 can be used to create mutations in dicot as well as monocot plant species. The simplicity of the design along with effectiveness in creating mutations constitute the main assets of the Cas9 system. Unlike TALEN, the Cas9 system can be easily used to target several genomic loci simultaneously by introducing multiple single guide RNAs (sgRNA) thus increasing the mutagenic potential of Cas9. Diverse versions of an expression vector have been proposed from using multiple sgRNA cassettes each with its own U6/U3 promoter [41] to chimeric tRNA/sgRNA constructs, which are processed in the cell [42]. Unfortunately, the Cas9 system possesses an undesirable feature, the so-called off-target activity, by which DNA is cleaved at remote sites similar to the designed protospacer. However, off-targeting can be substantially reduced by the application of paired Cas9-based nickases, which increase specificity and reduce the risk of off-target mutations [43]. Additionally, efforts have been made to increase the specificity and efficacy of Cas9 by altering its amino acid sequence [44,45] or sgRNA sequence [46].

Once the desired mutant has been produced by CRISPR/Cas9, it may be appropriate to separate the mutation from the *Cas9* transgene in order to reduce the risk of introducing off-target mutations in subsequent generations or for application in breeding programs, for which transgenic plants are not desirable. In plants, the most economically relevant approaches are simple segregation of the mutation in the progenitor lines or isolation of the desired mutation by crossing. For barley, the old cultivar Golden Promise has been used for the development of most transgenic lines [47], requiring the transmission of the mutation into elite cultivars if practical application was planned. Interestingly, promising research has been published describing the development of transgene-free mutant plants through transient expression without the need for exacting in vitro cultures [48,49], but the efficiency of such methods remains low and optimization of the process is needed.

In this work, nuclease Cas9 was used to induce mutations in *ITPK1* in barley. Primary transformants were screened for the desired mutation and its heritability was verified in the next generation. The presence of potential off-target sites was also investigated. Furthermore, the impact of the introduced mutation on phosphate content in mature barley grain and its effect on abiotic stress tolerance were examined.

2. Results and Discussion

2.1. Target Gene Analysis

In the present study, genome-edited barley plants were developed using the CRISPR/Cas9 system [37] adapted and optimized for use in monocot plants [41] in order to prepare barley *lpa* mutants. Not all the genes of the PA synthetic pathway can be considered as ideal targets for silencing. Several mutants in the PA synthesis pathway were described to exhibit weaker phenotype than WT line. For instance, mutants in the genes of an early phase of PA synthesis such as the *myo*-inositol phosphate synthase gene (*MIPS*) exhibited a reduced germination rate [15]. This is consistent with MIPS, which is critical for inositol metabolism [50], having a crucial role in several physiological processes in plant growth and seed development. On the other side, mutation of genes from the late phase of PA synthesis might also have deleterious effects on plant growth, as exemplified by an *A. thaliana* null mutant of *IPK*1, which was not viable [24]. Bhati et al. [51] identified four *ITPK* genes in wheat, whose expression peaked at different stages of grain development suggesting their involvement in phosphate storage. Therefore, genes in the middle part of the pathway were regarded as potential targets to knock-out. Barley *ITPK*s were chosen as a group of candidate targets because a maize *ITPK* gene had been already reported to be also responsible for the *lpa* phenotype if mutated [52]. The barley *ITPK* homologues were identified and analyzed *in silico*. There are six *ITPK* genes in the barley genome that can be classified into two groups. There are three intronless genes (HORVU7Hr1G033170, HORVU1Hr1G050760, and HORVU1HrG077420), while the other genes (HORVU4Hr1G065840, HORVU5Hr1G079750, and HORVU4Hr1G009540) consist of ten or twelve exons (Figure 1A). The nomenclature of the barley *ITPK*s was adopted from the rice nomenclature. The majority of barley genes have a corresponding rice ortholog, except one. Phylogenetic analysis

revealed that the barley genome contains three intronless genes in comparison to only two intronless genes in rice. Analogously, rice has four intron-containing genes while there are only three in barley. Close homology between *HvITPK4* and *HvITPK1* suggests that duplication of this gene occurred in barley, although this did not occur in rice. In order to closely investigate the role of the barley *HvITPK*1 gene in PA synthesis and abiotic stress signaling, *HvITPK1* was selected as a target gene for knock-out. *HvITPK1* is an intronless gene coding for a protein consisting of 345 amino acids (AA). The protospacer sequence was designed to maximize Cas9 activity [53] and designed at the beginning of the cds. The secondary structure of the sgRNA was verified using Mfold online tool (http://unafold.rna.albany.edu/?q=mfold), (Figure S1). Cas9 nuclease was directed 181-bp downstream of the *IPTK* start codon (Figure S2A). A multiple sequence alignment of *ITPK* coding sequences was made to identify putative off-target sites in homologous *ITPK* genes, showing varying degrees of nucleotide identity, from only 5 mismatches to 13 mismatches within the protospacer sequence (Figure 1B).

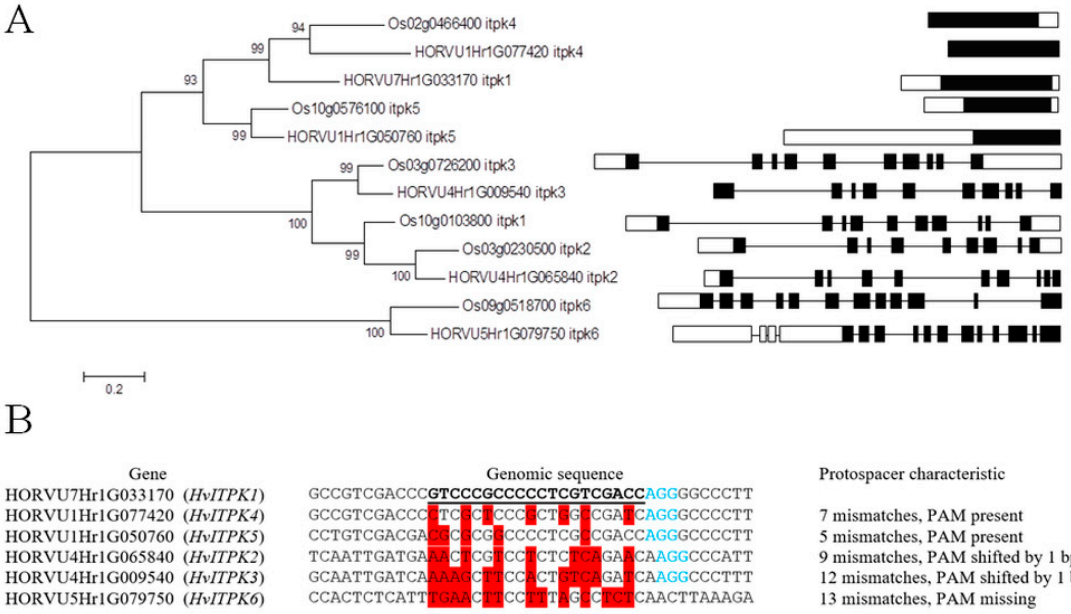
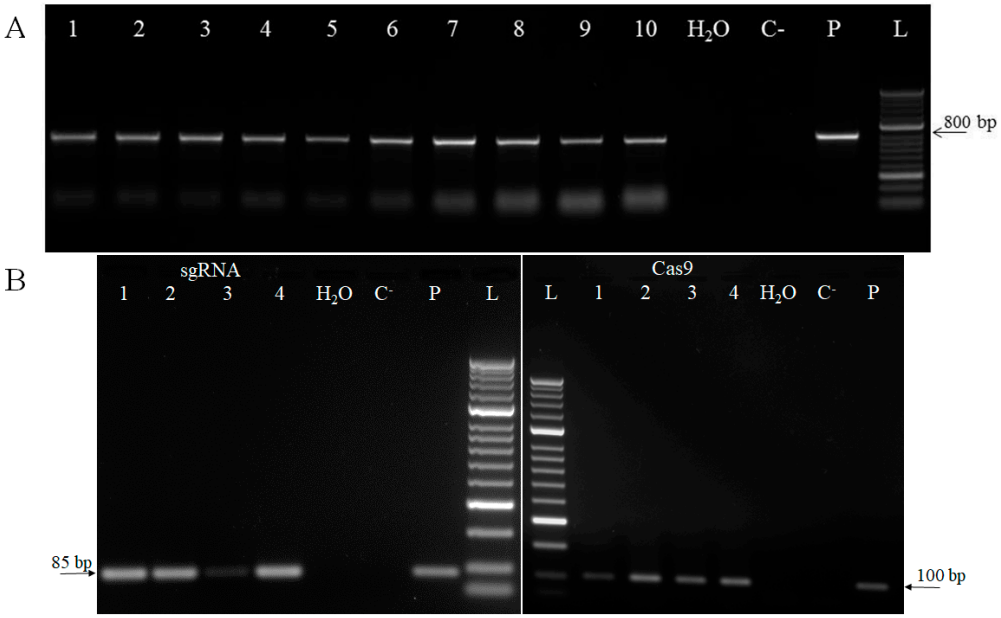


Figure 1. Analysis of barley *ITPK* genes. (**A**) Phylogenetic tree of barley and rice ITPK proteins. The phylogenetic tree was built using amino acid sequences. The scheme of *ITPK* genes illustrates exons (black), introns (joining), and untranslated regions (white). (**B**) Alignment of protospacer sequences within barley *ITPK* genes. Mismatches within the protospacer sequence are highlighted in red, the PAM sequence is shown in blue font.

2.2. Barley Transformation

In total, 100 immature barley embryos were used for *Agrobacterium*-mediated transformation with construct pYLCRISPR/Cas9P$_{ubi}$-H. The Golden Promise cultivar, which was used in this study, was selected due to its high capacity to regenerate in tissue culture, which is an essential condition for successful plant transformation [54]. After in vitro cultivation, selection and regeneration, 17 green plants were obtained. Based on the PCR screening of the primary regenerants (Figure 2A), the *Cas9* transgene was detected in 13 plants (independent transgenic events), accounting for transformation efficiency of 13%, which is lower than the average transformation efficiency of 25% reported for this protocol [55]. The expression of the transgenes was verified at the RNA level. Transcripts of both *Cas9* and *protospacer-sgRNA* were detected in the T0 generation of the transgenic plants (Figure 2B).



C

2A	CCCGTCCCGCCCCCTCGTCG-ACCAGGGGCCCTTCC	(WT)
	CCCGTCCCGCCCCCTCGTCGTACCAGGGGCCCTTCC	(+1 bp)
2B	CCCGTCCCGCCCCCTCGTCG-ACCAGGGGCCCTTCC	(WT)
	CCCGTCCCGCCCCCTCGTCGTACCAGGGGCCCTTCC	(+1 bp)
3A	CCCGTCCCGCCCCCTCGTC--ACCAGGGGCCCTTCC	(-1 bp)
	CCCGTCCCGCCCCCTCGTCGAACCAGGGGCCCTTCC	(+1 bp)
3B	CCCGTCCCGCCCCCTCGTC--ACCAGGGGCCCTTCC	(-1 bp)
	CCCGTCCCGCCCCCTCGTCGAACCAGGGGCCCTTCC	(+1 bp)
3C	CCCGTCCCGCCCCCTCGTC--ACCAGGGGCCCTTCC	(-1 bp)
	CCCGTCCCGCCCCCTCGTCGAACCAGGGGCCCTTCC	(+1 bp)
3D	CCCGTCCCGCCCCCTCGTC--ACCAGGGGCCCTTCC	(-1 bp)
	CCCGTCCCGCCCCCTCGTCGAACCAGGGGCCCTTCC	(+1 bp)
WT	**CCCGTCCCGCCCCCTCGTCG-ACCAGGGGCCCTTCC**	

Figure 2. Characterization of primary regenerants at DNA and RNA level. (**A**) Detection of *Cas9* transgene in samples of genomic DNA of T0 plants. L – DNA ladder (HyperLadder 50-bp, Bioline); P – plasmid; C–wild-type plant; 1–10 –samples of T0 plants. (**B**) Detection of sgRNA (left) and Cas9 (right) transcripts in T0 transgenic plants. L – DNA ladder (HyperLadder 50-bp, Bioline); P – plasmid, C- – wild-type plant, 1–4 – cDNA samples of T0 plants, sgRNA transcript product size 85-bp; *Cas9* transcript product size 100-bp. (**C**) Identified mutations in the T0 generation. Labels 2A, 2B, 3A, 3B, 3C, and 3D correspond to independent transgenic events, WT–wild type.

All 13 T0 transgenic plants were characterized at the genomic level. The target genomic locus amplified by PCR and then Sanger sequenced, indicating genetic modification at the target site in 6 plants, corresponding to 46% editing efficiency. In comparison, a similar efficiency was described by Holme et al. [56], while Kapusi et al. [57] described a higher efficiency at 78%. Lawrenson et al. [58] obtained a lower editing efficiency in the first described barley genome editing experiment, with 23% and 10% in primary transformants. Two types of mutations were identified in the transgenic plants, which contained a 1-bp deletion or insertion (Figure 2C). In 7 T0 transgenic plants, no mutation was detected despite the presence of Cas9 and protospacer-sgRNA transcripts. In the mutant plants, four were bi-allelic, containing an insertion of adenine and a 1-bp deletion concurrently. Additionally, two plants were heterozygous for a substitution at the target site. For monocots, the majority of mutations induced by Cas9 are reported to be single bp indels [41,59–61]. It was previously described that nucleotide insertions into the cleavage site are predominantly A or T [41]. In contrast, Sánchez-León et al. [62] reported for wheat that the majority of induced mutations were multiple bp deletions.

Moreover, deletions of several hundred bp have also been reported [56], suggesting that the nature of Cas9-induced mutations cannot be simply predicted. Cas9 has also been reported to produce chimeric plants [63]. Therefore, three mutant and two non-mutant T0 plants, which were characterized in the first screening, were additionally selected to evaluate chimerism within primary regenerants. For the analysis, total DNA from five different leaves from each of five selected plants was extracted and the target locus sequenced (Supplementary File S1). No mutation was detected in any of the non-mutant T0 plants. On the other hand, the same bi-allelic mutant constitution was confirmed in additional sequencing of five leaves from plants 3B and 3C, so that these two lines could be classified as uniform mutants. However, plant 2A, which was characterized as a mutant in the initial screening, was not confirmed after additional sequencing, suggesting that it was a chimera. It can be assumed that the mutations detected in plants 3B and 3C occurred early in the callus development. Hence, the biallelic mutant plant labelled 3B was selected for the evaluation of the transmission of the mutation into the next generation. Bi-allelic mutants 3B, 3C, and 3D showed the same mutations in the target locus, 1-bp deletion and insertion, which both caused a shift in the open reading frame. Deletion of G resulted in an occurrence of a premature stop codon in mRNA leading to truncated protein product (139-AA), meanwhile, insertion of A resulted in a prolonged transcript coding for a protein consisting of 368-AA.

In total, 34 T1 plants, which were the progeny of the T0 3B plant, were analyzed for the genetic constitution of the target locus. In the T1 generation, three genetic variants were observed: bi-allelic mutants and deletion or insertion homozygous mutants (Figure S2B). The bi-allelic mutants accounted for 21 lines, whereas the homozygous 1-bp insertion was present in five plants and the deletion in four lines. Possible off-target sites among barley *ITPK* homologues of *HvITPK1* were investigated. Since *HvITPK3* had more than half mismatching nucleotides within the protospacer and a 1-bp shifted PAM and *HvITPK6*, besides containing substantial mismatches lacked PAM completely, these two genes were excluded from the evaluation of off-target sites. No putative off-target mutation at the predicted sites within other *ITPK* genes was identified in the primary regenerants, as well as in the T1 and T2 generations of plant 3B. Interestingly, transgene-free homozygous mutant barley plants were detected in the T2 generation. Hence, the progeny of plant 3B was used in subsequent experiments.

2.3. Phosphate Analysis

A phylogenetic analysis of rice and barley *ITPK* genes revealed considerable similarity between the two groups of genes. In a recent study, it was reported that a loss of function in *OsITPK6*, a gene from the PA synthetic pathway, could result in a reduction of PA content in mature grains [7]. Hence, it is tempting to presume that the other *ITPK* members can be targeted for knock-outs to alter PA content. A substantial reduction of phytate level in mature grains could potentially be achieved via manipulation of its synthetic pathway without a detrimental effect on plant performance. As it was shown that a reduction of phytate resulted in a proportional increase in inorganic phosphate levels in grains [64,65], the phosphate content was measured in mature grain of the iptk1 mutants. This analysis revealed that the mutations in *HvITPK1* had a diverse effect (Figure 3) and, unexpectedly, did not result in an overall increase of phosphate content in all tested lines. The highest increase in phosphate content, of 74%, was detected in the homozygous deletion mutant *itpk1-14*. In comparison to WT, a majority of the analyzed samples showed only similar or even lower levels of phosphate. Remarkably, a comparison of a biallelic mutant with two homozygous mutant variants showed that these groups generally differed in phosphate content. The homozygous insertion mutants generally contained lower phosphate content in comparison to WT, this decrease reaching up to 35% in line *itpk1-17*. The biallelic mutant lines contained comparable or slightly higher phosphate levels than WT, while the homozygous deletion mutation affected phosphate content the most diversely. Within three tested lines, line *itpk1-33* showed no difference in the phosphate content, while the remaining two lines differed significantly: a decrease by 17% was observed in line *itpk1-27* and a substantial increase by 74% in line *itpk1-14*. Inconsistency in phosphate levels among the studied mutant lines,

especially the homozygous lines, could result from incomplete penetrance of the phenotype if the truncated or extended proteins produced from the mutated transcripts retain residual activity. The incomplete penetrance effect could be also associated with a relative position of a mutation within an open reading frame. This was reported recently for chemically (EMS)- and CRISPR-induced barley mutants, which demonstrated more severe phenotype if a mutation was located closer to N terminus [60].

Additionally, there is evidence confirming that mutation in a gene from the PA synthesis pathway affects the expression of the other PA synthesis members. For instance, Kim and Tai [66] showed that *A. thaliana lpa* mutants contained significant changes in gene expression of PA synthetic kinases during seed development. In comparison, Nagy et al. [67] emphasized that mutation in *Atabcc5*, which is a vacuolar PA transporter, resulted in almost doubled expression of some *ITPK* genes, while knock out of a *Phaseolus vulgaris* ABC transporter of PA resulted in a relative decrease in expression of *ITPK* genes [68]. ITPKs can differ substantially in their enzyme kinetic parameters and could phosphorylate both InsP3 and InsP4 [69]. Differential expression profile of wheat *ITPK*s during the grain development [51] suggests that ITPK enzymes are being developmentally -regulated during grain maturation. Considering the different kinetic parameters of ITPK enzymes with their differential expression profile during the grain development, it is tempting to suggest that differential expression of *ITPK* genes is part of a regulatory mechanism to control PA synthesis. The soya ortholog of *HvITPK1* was characterized as a key gene directing the flux of the inositol phosphate (InsP) pool to PA biosynthesis [70] and *TaITPK4*, which is characterized by an increased level of expression at the beginning of seed development [51], shows a high level of similarity with barley *HvITPK1*. It could be presumed that *HvITPK1* might have a similar function in barley by enhancing PA synthesis during early grain development. Hence, the varying level of phosphate among the homozygous mutant and bi-allelic lines, could be explained by impaired PA synthesis potentially coupled with residual protein activity of the HvITPK1 mutant enzymes.

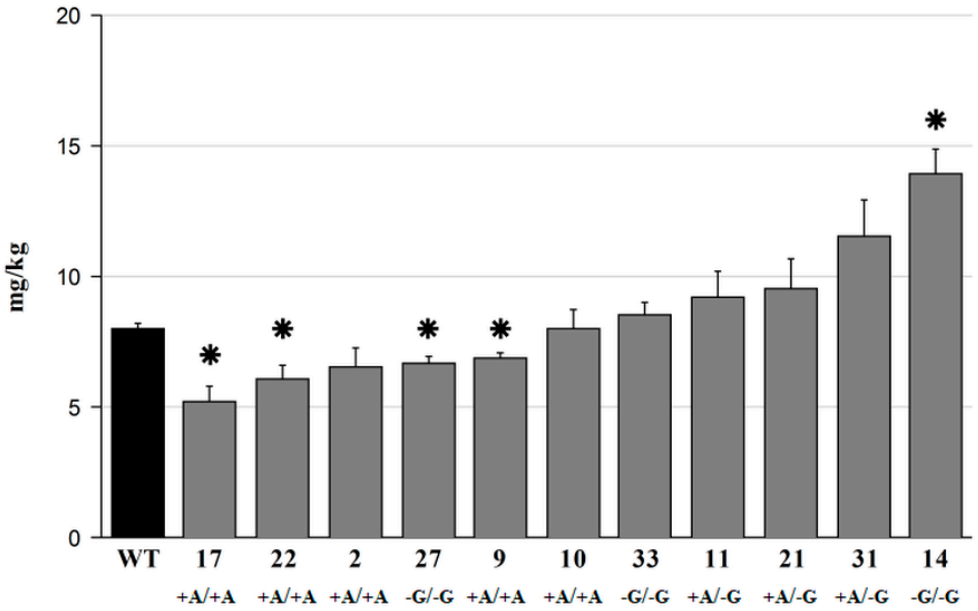


Figure 3. Mature grain phosphate content in *itpk1* mutants. Black column – wild-type plant, grey columns–T2 generation *itpk1* mutant lines. Allelic constitution at the target site for each of the selected parental T1 3B mutant lines shown below. Data represent means (n = 3 ± SD). The asterisks represent significant differences in the phosphate content at $p < 0.05$ compared to WT based on the Aspin-Welsch unequal-variance t-test.

2.4. Abiotic Stress Assay

In view of above-mentioned evidence for a role for ITPKs in abiotic stress responses, *itpk1* mutants were tested for their phenotypic performance under salinity stress in vivo and *in vitro*. Grains of five homozygous *itpk1* mutants, three insertion and two deletion lines, were germinated on moist filter paper in a growth chamber at 16/8 day/night regime. Under control conditions without salt, all tested lines including WT reached a germination rate of over 90% by the fourth day (Figure 4A). At two days, the two deletion mutants *itpk1-27* and *itpk1-33* and one insertion mutant *itpk1-10* showed a significantly slower germination rate than WT. However, by day three there was little difference between the lines with over 80% of the deletion mutant grain having germinated. Differences in germination rate between the mutant lines were more pronounced on treatment with 100 mM NaCl (Figure 4B). The insertion mutants exhibited similar or even more rapid germination than under control conditions, with *itpk1-22* reaching 100% germination on day 4. On day 3, the three insertion mutants had a higher germination percentage than the WT. In contrast, the germination of the two deletion mutants (*iptk1-33* and *iptk1-27*) was significantly delayed compared with the WT and insertion lines and whereas 90% of *iptk1-33* grain had germinated by day 5, almost 40% of *iptk1-27* grain had still not germinated by this time. The most visible differences in germination rate between the two clusters of homozygous mutant lines were observed after treatment with 200 mM NaCl (Figure 4C). Germination of all lines was notably slower. Two insertion lines, *iptk1-22* and *iptk1-10*, achieved a higher germination rate than the WT, whereas germination of the deletion mutants was significantly less efficient than that of the WT. Application of 300 mM NaCl resulted in total inhibition of germination in WT and *itpk1* mutants (data not shown).

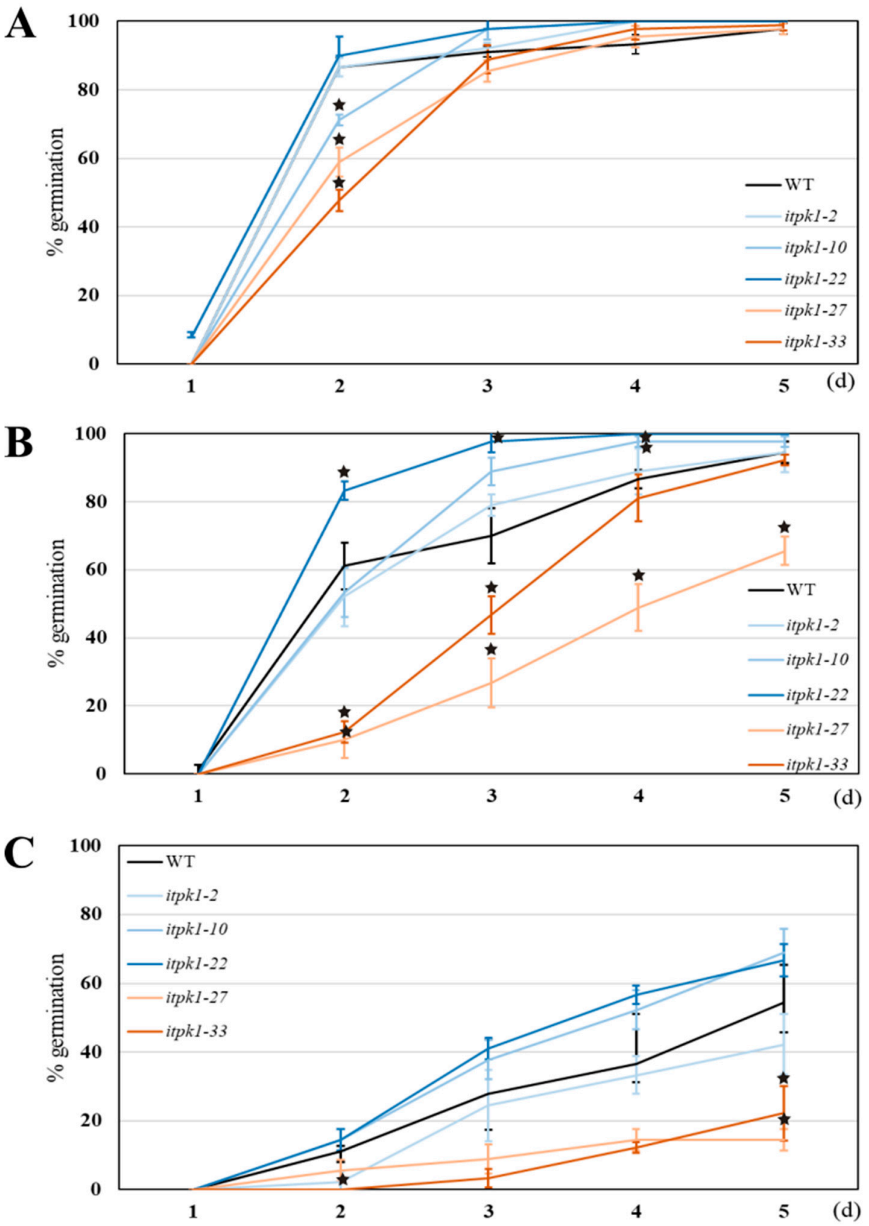


Figure 4. The effect of NaCl on germination of barley *itpk1* mutants. (**A**) Control conditions without NaCl. (**B**) Grains germinated on 100 mM NaCl. (**C**) Grains germinated on 200 mM NaCl. Results for homozygous insertion mutants are displayed with differently shaded blue lines, deletion mutants with orange lines and the WT with a black line. Data represent means (n = 3 ± SD). The asterisks represent significant differences in germination percentage at $p < 0.05$ compared to WT based on the Aspin-Welsch unequal-variance t-test.

The effect of salt stress on shoot growth for the *iptk1* mutants was evaluated by growing excised mature embryos on cultivation medium in vitro (Figure 5). In order to evaluate the phenotype of two homozygous mutant genotypes, the insertion mutant (+A) *itpk1-2* and deletion mutant (-G) *itpk1-33*, for which there was sufficient grain, were selected. As shown above in Figure 3, the phosphate content in mature grain of these lines did not differ significantly from that of WT. On MS medium without salt, there were differences in shoot growth between WT and the mutants. At the lower salt concentrations, 50 and 100 mM NaCl, WT plants also showed better growth than both mutants, out of which *itpk1-33* seemed to be more affected by salt stress than was *itpk1-2*. This was in contrast to

the highest salinity stress conditions (200 mM), at which the growth of all lines was severely inhibited, but *itpk1-2* showed significantly better growth than the WT and *iptk1-33*. Generally, the barley cultivar Golden Promise exhibits tolerance to salinity stress [71]. Thus, although mutant plants exhibited slower growth at 50 and 100 mM NaCl compared to WT, *itpk1-2* showed significantly better growth at the highest NaCl concentration, which supports a potential role for barley *HvITPK1* in salinity stress signaling. Involvement of an intronless member of the *ITPK* group in stress signaling is consistent with the report by Marathe et al. [30], who performed transcription profiling and heterologous expression of the soya ortholog of *HvITPK1* in *E. coli* and showed that this gene is important for response to dehydration and salinity by acting as a stress regulator. In contrast, germination of all *A. thaliana lpa* mutants, including *iptk1*, was more sensitive to salt, osmotic, and oxidative stress than the wild type [66]. In the current study, comparison of both assays showed that there were clear phenotypic differences between mutant lines cultivated under salt stress. These differences were conditioned by the type of induced mutation. Shoot growth was impaired relative to WT in both allelic mutant variants under control and lower salt concentration conditions, while at the highest salt concentration the homozygous insertion mutant *itpk1-2* out-performed WT and *itpk1-33* consistent with its enhanced salt tolerance. This observation was further supported by the germination assay, where the three homozygous insertion lines (*itpk1-2*, *itpk1-10*, and *itpk1-22*) showed higher germination rate than two deletion lines (*itpk1-27* and *itpk1-33*).

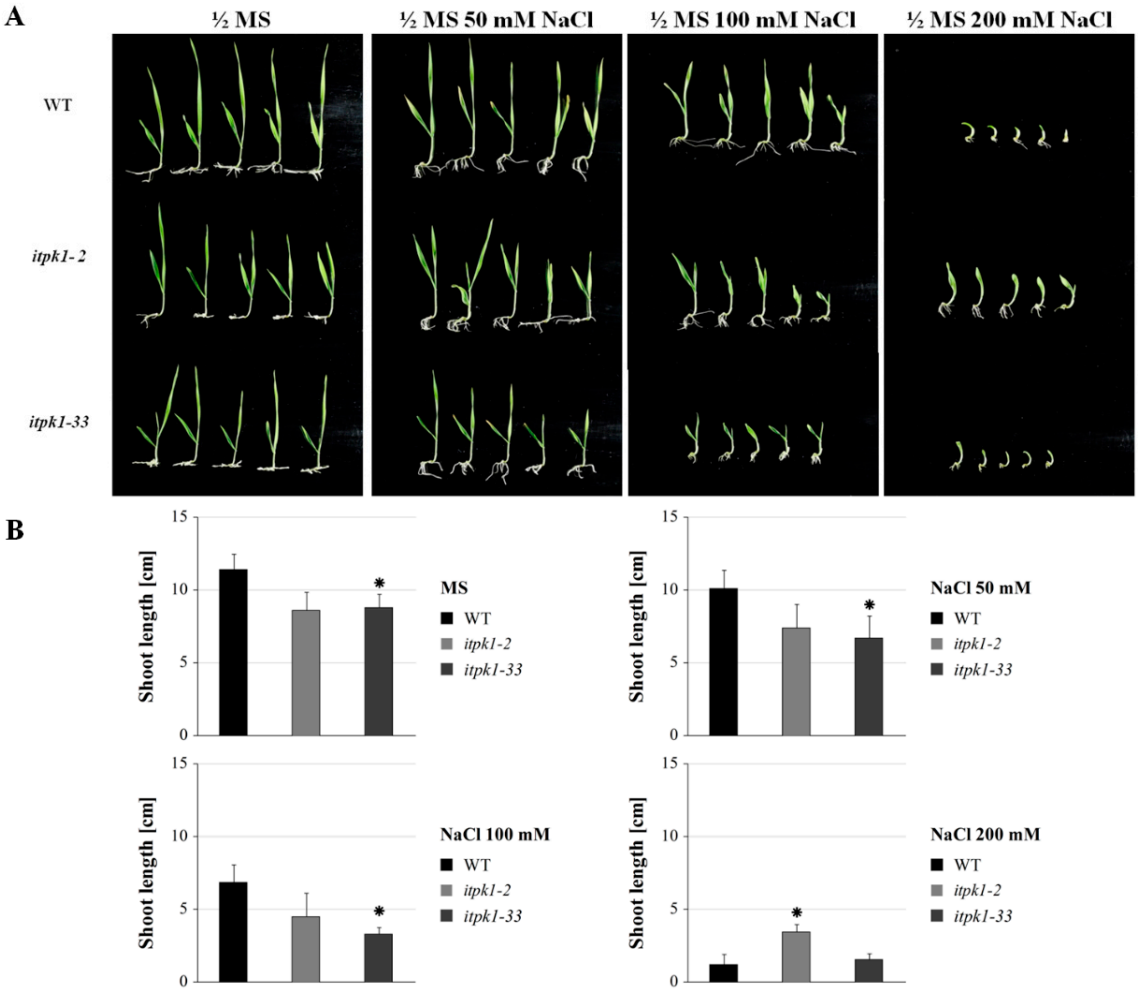


Figure 5. The effect of NaCl on in vitro cultivated barley *itpk1* mutants. (**A**) Barley seedlings from *itpk1-2*, *itpk1-33* and WT groups under control and increasing salt concentration conditions. (**B**) Shoot length after 10-day in vitro cultivation at control and salt stress conditions. The values are the means

± SD (n = 5). The asterisks represent significant differences in the shoot length at $p < 0.005$ compared to WT based on the t-test.

2.5. Transcription Profiling of ITPK Genes under Salinity Stress

An important role for inositol phosphate kinases during abiotic stress signaling is supported by the regulation of their expression by ABA [33], a potent abiotic stress signaling hormone. It is possible that enzymes from the initial phase of InsP synthesis might also participate in responses to abiotic stress. MIPS catalyses the initial step of PA synthesis, the conversion of glucose-6-phosphate to *myo*-inositol-3-phosphate. Kaur et al. [72] showed that heterologous expression of *CaMIPS2*, which is an early dehydration-responsive gene in *Cicer arietinum*, in *A. thaliana* led to higher tolerance to salinity and dehydration. In chickpea, the promoter region of *CaMIPS2* contains the CRT/DRE *cis*-regulatory element, which was predicted to play a key role in the regulation of exogenous ABA- or stress-induced expression [72]. Remarkably, the promoter region of *HvITPK1* contains ten copies of this element suggesting that expression of this gene is likely to be under regulation by ABA. Previously, Du et al. [29] demonstrated that knock-out or over-expression of *OsITPK2 (HvITPK2* ortholog*)* can affect the expression of some of the homologous genes. In the present study, transcript profiling performed on WT and the two *itpk1* (*itpk1-2* and *itpk1-33*) mutants grown on half MS medium with or without supplementation with 200 mM NaCl revealed an altered expression pattern of *ITPK* genes in the mutants in comparison to WT (Figure 6). Under control conditions, expression of *HvITPK1* was higher in shoots than in roots of WT. However, the expression of *HvITPK1* increased in roots, but dropped significantly in shoots under salt stress in WT, suggesting that *HvITPK1* plays a role in abiotic stress signaling. There was weak expression of *HvITPK1* in the leaves of both mutants relative to WT. Moreover, for the *itpk1-33* mutant, but not for *iptk1-2*, a drop in *HvITPK1* expression under salt was observed in shoots as for WT. In contrast, no significant increase in *HvITPK1* expression was noticed in roots of both mutants on salt treatment. It was reported that mutations in a gene open reading frame can result in the suppression of its transcription and translation, although, the residual mRNA level is not predictive of residual protein level [73,74].

Gene expression analysis of other *ITPK* genes in roots of WT and both mutants showed that expression of all *ITPK*s was generally increased in response to salt stress. Particularly, the expression of *HvITPK5* and *HvITPK2* was significantly increased in the roots of the mutants and WT. The expression of *HvITPK3* only was not altered in roots of WT in response to salinity, while there was a significant increase in both mutants. In contrast, the expression in shoots of WT and mutants varied. Expression of the remaining two intronless genes, *HvITPK5* and *HvITPK4*, was lower than that of the intron-containing genes. Actually, *HvITPK4,* which is the closest homolog of *HvITPK1*, showed very low or no expression in roots and shoots under control conditions and its expression was induced in the response to salt stress. In shoots of WT, there was an increase in the expression of *HvITPK5* under salt treatment, but this did not occur in the mutants. Previously, *OsITPK2* was characterized as a critical regulator of inositol phosphate-mediated signaling, which was induced by drought, salinity, or abscisic acid [29]. In the present work, a substantial downregulation of its barley ortholog by salinity was noticed in shoots of WT and also in *itpk1-33*. In shoots of WT, there was also a significant decrease in the relative expression level of *HvITPK3* in response to salt. A similar decrease in *HvITPK3* expression was observed in the *itpk1-33* mutant, although this was not statistically significant. In comparison, a decrease in expression of *HvITPK3* and *HvITPK2* after salt treatment was not evident in *itpk1-2*, where the expression increased slightly or was unchanged for *HvITPK3* and *HvITPK2*, respectively. Notably, the differential expression profiles of *HvITPK2* and *HvITPK3* in shoots in response to salt between the two *itpk1* mutants correlate with their different salt sensitivities. The data obtained in this study suggested that i) maintenance of *HvITPK1* expression in shoots of *itpk1-2* mutant might be related to the altered function of its protein product, possibly resulting in higher tolerance of young seedlings to salinity and ii) disruption of one *ITPK* gene can affect the expression of the other members of the *ITPK* group, at least in shoots.

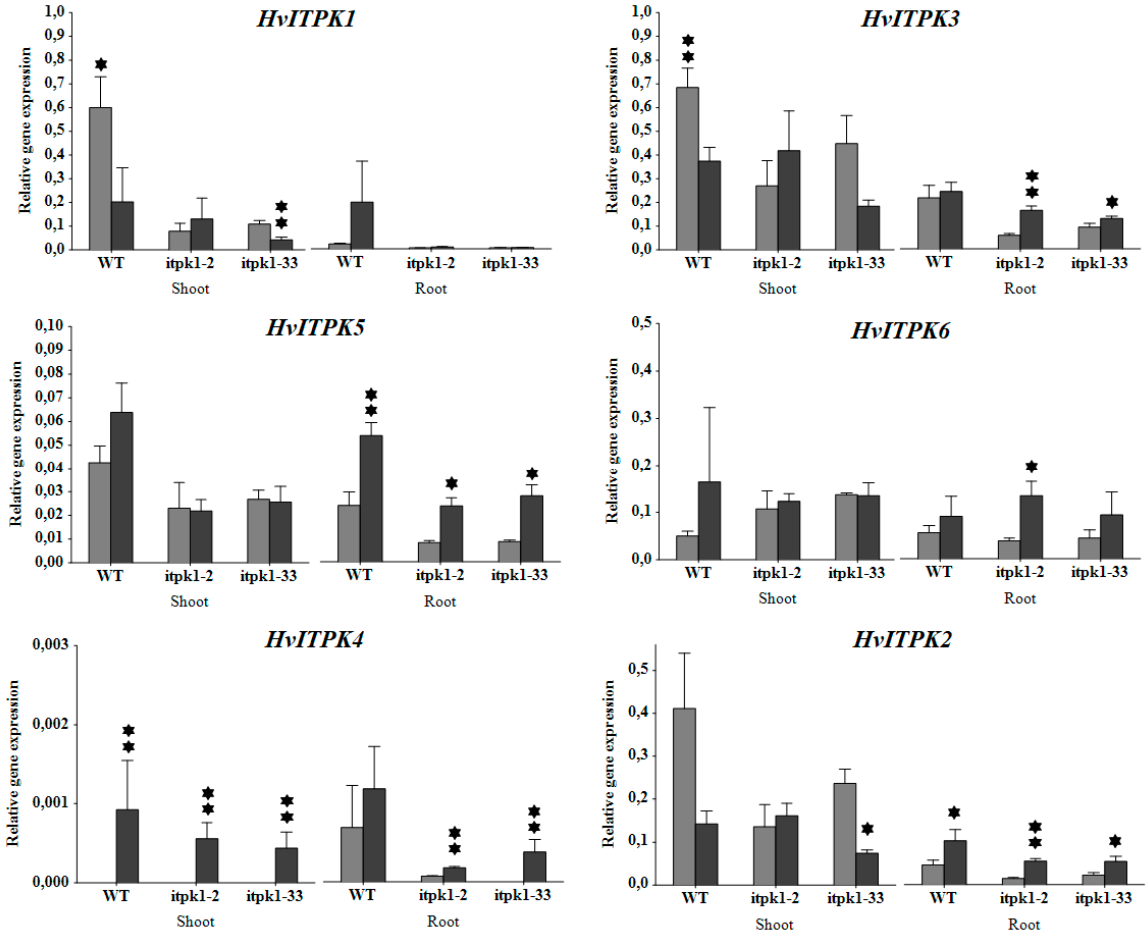


Figure 6. Relative transcript abundance of *ITPK* genes. Light grey – control condition (MS medium), black – salinity stress (200 mM NaCl). The values are means ± SD (n = 3). The asterisks denote significant differences in gene expression between control and stress conditions, for * at $p < 0.05$ and ** $p < 0.01$ based on the Aspin-Welsch unequal-variance t-test.

Taken together, factors such as residual enzyme activity, diverse rate of expression of mutated genes, and varying levels of compensation by altered expression of paralogs, could have an effect on the total phosphate content in the mature barley grains in *itpk1* barley mutants. In a recent study, it was pointed out that different mutations in a gene open reading frame can result in the different phenotype and influence gene function [74]. Due to the diverse effect of the two mutations on the phenotypic manifestation in the studied lines, it was not possible to determine the exact role of *HvITPK1* in abiotic stress signaling. Nevertheless, the observations support the thesis that both homozygous allelic variants have a negative effect on shoot growth under control and weak salinity stress (50 and 100 mM NaCl) conditions. However, at 200 mM NaCl, insertion mutation most likely conditioned salt tolerance in *itpk1-2*. The presence of multiple regulatory and binding elements in the promoter region of *HvITPK1* implies that it may have an important function during abiotic stress signaling. Hence, the potential involvement of barley *ITPK* genes during early responses to drought and salinity stress warrants further investigation. Two allelic variations were studied in this study. Phenotypic differences conditioned by the two alleles suggested that the mutation in *HvITPK1* could affect protein functionality, and in the case of the insertion mutation, this altered function may have enhanced salt tolerance.

3. Materials and Methods

3.1. Design and Cloning of Protospacer

Barley inositol trisphosphate 5/6-kinase (HORVU7Hr1G033170, here referred to as *HvITPK1*) was chosen as a target gene. The protein-coding sequence (cds) of *ZmIPK* [52] was used to blast the barley genomic DNA database (EnsemblPlants) to identify homologous barley *ITPK* genes and rice orthologs. MEGA 6.06 software was used for multiple sequence alignment of all barley *ITPK* cds. Neighbour-joining phylogenetic tree was constructed with protein sequences of barley and rice ITPKs using Clustal W and MEGA 6.06 with 1000 bootstrap replicates. Prior to protospacer design, the target locus in the donor plant material, barley cultivar Golden Promise, was PCR-amplified and Sanger sequenced, on the basis of which the protospacer sequence was designed. The protospacer was sub-cloned into pYLsgRNA-OsU6 and then the cassette containing sgRNA under the small U6 promoter was cloned into the expression vector pYLCRISPR/Cas9Pubi-H according to the protocol by Ma et al. [41]. Correct integration of the sgRNA cassette was verified by digestion with *Mlu*I, and by Sanger sequencing, (Figure S3). The vector harbouring sgRNA with protospacer sequence was transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation. Standard bacterial inoculums with OD_{600} of 1.0 were prepared and stored at -80 °C.

3.2. Barley Transformation

The barley model cultivar Golden Promise was used for transformation according to the protocol by Harwood [55]. Barley plants were grown in the greenhouse with a 12-h photoperiod and 15 °C for 10–12 weeks. For the identification of transgenic plants, genomic DNA from young leaves was extracted according to Edwards et al. [75] and analyzed for the presence of Cas9 transgene by polymerase chain reaction (PCR). The genotyping primers are listed in Table S1. For the PCR reaction, a premix REDTaq® ReadyMixTM PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA) was used. The reaction was started with 5 min denaturation at 95 °C, followed by 40 cycles of 30 s denaturation, 30 s annealing at 58 °C and 1 min elongation at 72 °C. The PCR products were separated on a 1% agarose gel stained with ethidium bromide.

3.3. Detection of the Cas9 and sgRNA Transcripts

Total RNA was extracted from young leaf tissue of T0 plants using a Total RNAqueous Kit (Thermo Fisher, Vilnius, Lithuania) and treated with Turbo DNase according to the manufacturer's protocol. The concentration of RNA was assessed spectrophotometrically (DeNovix Spectrophotometer DX-1), 1 μg of RNA was reverse transcribed using poly-T primers and RevertAid H minus Reverse Transcriptase (ThermoFisher). The cDNA was diluted ten times with nuclease-free water and amplified with specific primers for Cas9 and sgRNA transcripts, listed in Table S1. In the case of sgRNA, the forward primer was derived from the protospacer sequence and the reverse primer was located in the guide RNA sequence. PCR conditions were the same as used for genotyping the T0 plants, except for an annealing temperature of 60 °C for 20 s and a shorter extension time of 30 s.

3.4. Genotyping of Primary Regenerants and their Progeny

Plant material was collected two weeks after the transfer of regenerated plants into pots. Genomic DNA was extracted from young leaves of the transgenic plants as described [75] with two biological replicates, two different leaves, per plant. To evaluate the induction of target and off-target mutations, primer pairs for amplification of target and off-target loci in the closest homologues of *HvITPK1* within the group of *ITPK* kinases were designed, verified and used for genotyping. All the primers used for genotyping of target and off-target loci are listed in Table S1. The target and off-target loci in T0 transgenic plants were PCR-amplified and the PCR products were Sanger sequenced. For decoding and evaluation of the sequencing chromatograms, DSDecode online tool [76] was used. For the analysis of heritability of the target mutations and induction of off-target mutations in the

next generation, the progeny of the bi-allelic mutant line 3B was genotyped in the T1 and T2 generations.

3.5. Seed Phosphate Analysis

For phosphate analysis in mature grain, progenitor lines originating from the bi-allelic mutant plant T0 3B were selected. Mature barley grains (T2 generation) of eleven mutant T1 3B lines were harvested and homogenized to a fine flour. Lines containing a homozygous 1-bp insertion were 17, 22, 2, 9, and 10; lines carrying a homozygous 1-bp deletion were 27, 33, 14. The remaining lines 11, 21, and 31 were progeny of bi-allelic mutants. Phosphate analysis was done according to the method of Vaculova et al. [77]. Briefly, 50 mg of homogenized sample was mixed with 0.5 mL of 0.4 M HCl and incubated overnight at 4 °C. Then, the samples were centrifuged 10 min at 6000 g and the supernatant was used for analysis. Phosphate analysis was based on the colorimetric assessment of the complex formed from the reaction of Chen's reagent [78] with phosphate. Absorbance was measured at 822 nm after two-hour incubation with Chen's reagent and the phosphate concentration was determined from a calibration curve. Technical and biological measurements were made in triplicate. The mean value with standard deviation was plotted on a graph.

3.6. Abiotic Stress Experiments

3.6.1. Seedling Growth Assay

The homozygous T2 mutant lines, deletion mutant *itpk1-2* and insertion mutant *itpk1-33* were selected for in vitro testing of their response to salinity stress. Mature grains of WT as a control and *itpk1* mutants were soaked in sterile water overnight, the embryos dissected and collected in a tube with fresh sterile water. The embryos were sterilized for 1 min in 1% sodium hypochlorite solution with agitation and then washed twice in sterile water. Solid half MS medium with 10 g/L sucrose, Phytoagar (Duchefa) 6 g/L, with the pH adjusted to 5.8 was prepared in flasks. The media were supplemented with NaCl at 50, 100, or 200 mM. Pure half MS medium without NaCl supplement was prepared as a control. The sterilized embryos were placed on the surface of the medium, five embryos of each mutant line and WT per treatment. The flasks with embryos were placed in a growth cabinet and cultivated at 24 °C and a day/night regime of 18/6 h. After two weeks, the phenotype of the seedlings was evaluated by measuring the height of the seedlings.

3.6.2. Germination Assay

Homozygous T2 mutant lines, deletion mutants *itpk1-2*, *itpk1-10*, *itpk1-22*, and insertion mutants *itpk1-33* and *itpk1-27*, were selected for in vivo testing of their response to salinity stress during germination according to the published protocol [79] with a few adjustments. Grains were sown on 0, 100 or 200 mM NaCl at 30 per plate in three replicates per treatment for each mutant line and WT. Grains were placed on moist filter paper in Petri dishes and kept in the dark at 4 °C for three days. Then, the plates were transferred into the growth chamber under a 16/8 h day-night regime and a constant 23 °C. Germination was assessed daily for 5 days. Grains were considered germinated when the primary root reached a length of approximately 0.5 cm.

3.7. Expression Profiling of Barley ITPK Genes

Total RNA from young seedlings of WT and homozygous mutant lines *itpk1-2* and *itpk1-33* cultivated in vitro on pure half MS medium and medium supplemented with 200 mM NaCl was extracted and 500 ng of total RNA was reverse transcribed as described above. Total RNA was extracted from roots and leaves separately. The cDNA samples were diluted five times with nuclease-free water. Three biological replicates, where each replicate was represented by one plant, were prepared. *ITPK* transcript levels were determined by qRT-PCR using the primers listed in Table S1. The reaction mixture comprised SsoAdvanced™ Universal SYBR® Green Supermix (BioRad, Hercules, CA, USA), half of the reaction volume, 0.4 μL of each 10 μM primer solution, 1 μL of diluted

standard or cDNA sample, and water to 10 µL. CFX96 Touch™ Real-Time PCR Detection System (BioRad) was used for the qPCR analysis. Cycling conditions were initial denaturation at 95 °C for 3 min followed by 40 cycles of 10 s denaturation at 95 °C and 30 s of annealing and amplification step at 60 °C. Melting analysis of the PCR products was performed afterwards to assess the specificity of the primers. PCR products were then separated in agarose gel to further confirm the product specificity (Figure S4). The dilution series of standards for each *ITPK* gene and Elongation factor 1-α (*EF1-* α), Actin2 (*ACT*), and Ubiquitin (*UBI*), which were used as the reference genes, were prepared as follows: The standards were PCR-amplified from freshly prepared cDNA from non-transgenic barley, the PCR products were purified using paramagnetic beads (Agencourt® AMPure XP, Beckman Coulter) and subsequently diluted to concentrations ranging from 10^{-3} – 10^{-10}-fold purified product. The standards were run in technical duplicate and mean Cq values were used to create the standard curves. The amplification efficiency was calculated from the slope of the standard curve, (Figure S5). The plant samples were run in technical duplicate. For evaluation of gene expression, the ΔCt method was used applying the mean Ct value from three reference genes to normalize gene expression.

3.8. Statistical Analysis

Statistical analyses were performed using NCSS 9 software. The experimental data are presented with the standard deviations based on three to five replicates. For the phosphate measurements, each mutant line was compared with WT. The Aspin-Welsch unequal-variance t-test was used to test significant differences ($p < 0.05$) in phosphate content and germination percentage. For shoot length, the Student's t-test was employed. The values were the means of five replicates. The significant differences in the shoot length compared to WT were set at $p < 0.005$. Differential gene expression was analyzed using the Aspin-Welsch unequal-variance t-test.

4. Conclusions

In summary, barley homozygous mutants for *HvITPK1* gene were developed by CRISPR/Cas9. Analysis of mutant progenies confirmed stable heritability of the mutation as well as expected segregation associated with the development of transgene-free homozygous mutant lines. Phenotypic differences between homozygous insertion and deletion lines were observed; particularly in the phosphate content of mature grains and in reaction to salinity stress during germination. Additionally, the differences were confirmed at the RNA level by transcript profiling of the *ITPK* genes in the mutant lines in response to salt stress. The conducted experiments indicated that *HvITPK1* participate in processes related to PA synthesis and salinity stress response, thus confirming *HvITPK1* as a functional gene.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1. Secondary structure of Protospacer-sgRNA. Figure S2. Characterization of target genomic sequence in *HvITPK1*. Figure S3. Digestion of expression vector by *Mlu*I. Figure S4. Verification of qRT-PCR primers specificity. Figure S5. Calibration curves for internal control and *HvITPK* genes used in qRT-PCR. Table S1. List of primers used for genotyping and qRT-PCR. File S1. Genotyping of T0 plants.

Author Contributions: T.V.–conceptualization, investigation, and writing. L.O.–supervision, funding acquisition, final approval of the manuscript. Both authors read and approved the final manuscript.

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References

1. Cosgrove, D. Inositolhexakisphosphates. In *Inositol Phosphates: Their Chemistry, Biochemistry and Physiology*; Cosgrove, D., Ed.; Elsevier Scientific Publishing Company, New York, NY, USA, 1980; pp. 26–43. ISBN 0444418741.
2. Raboy, V. Forward genetics studies of seed phytic acid. *Isr. J. Plant Sci.* **2007**, *55*, 171–181, doi:10.1560/IJPS.55.2.171.
3. Suzuki, M.; Tanaka, K.; Kuwano, M.; Yoshida, K.T. Expression pattern of inositol phosphate-related enzymes in rice (*Oryza sativa* L.): Implications for the phytic acid biosynthetic pathway. *Gene* **2007**, *405*, 55–64, doi:10.1016/j.gene.2007.09.006.
4. Meijer, H.J.G.; Divecha, N.; Ende, H. Van Den; Musgrave, A.; Munnik, T. Hyperosmotic stress induces rapid synthesis of phosphatidyl-D-inositol 3,5-bisphosphate in plant cells. *Planta* **1999**, *208*, 294–298, doi:10.2135/cropsci2011.05.0285.
5. Warkentin, T.D.; Delgerjav, O.; Arganosa, G.; Rehman, A.U.; Bett, K.E.; Anbessa, Y.; Rossnagel, B.; Raboy, V. Development and characterization of low-phytate pea. *Crop Sci.* **2012**, *52*, 74–78, doi:10.2135/cropsci2011.05.0285.
6. Sakai, H.; Iwai, T.; Matsubara, C.; Usui, Y.; Okamura, M. A decrease in phytic acid content substantially affects the distribution of mineral elements within rice seeds. *Plant Sci.* **2015**, *238*, 170–177, doi:10.1016/j.plantsci.2015.06.006.
7. Jiang, M.; Liu, Y.; Liu, Y.; Tan, Y.; Huang, J.; Shu, Q. Mutation of inositol 1,3,4-trisphosphate 5/6-kinase6 impairs plant growth and phytic acid synthesis in rice. *Plants* **2019**, *8*, 114, 1–12, doi:10.3390/plants8050114.
8. Harada, A.; Sakai, T.; Okada, K. phot1 and phot2 mediate blue light-induced transient increases in cytosolic Ca2+ differently in *Arabidopsis* leaves. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 8583–8588, doi:10.1073/pnas.1336802100.
9. Franklin-Tong, V.E.; Drøbak, B.K.; Allan, A.C.; Watkins, P.A.C.; Trewavas, A.J. Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. *Plant Cell* **1996**, *8*, 1305–1321, doi:10.1007/s00122-007-0621-2.
10. Yuan, F.-J.; Zhao, H.-J.; Ren, X.-L.; Zhu, S.-L.; Fu, X.-J.; Shu, Q.-Y. Generation and characterization of two novel low phytate mutations in soybean (*Glycine max* L. Merr.). *Theor. Appl. Genet.* **2007**, *115*, 945–957, doi:10.1007/s00122-007-0621-2.
11. Wilcox, J.R.; Premachandra, G.S.; Young, K.A.; Raboy, V. Isolation of high seed inorganic P, low-phytate soybean mutants. *Crop Sci.* **2000**, *40*, 1601–1605, doi:10.2135/cropsci2000.4061601x.
12. Larson, S.R.; Raboy, V. Linkage mapping of maize and barley myo-inositol 1-phosphate synthase DNA sequences: Correspondence with a low phytic acid mutation. *Theor. Appl. Genet.* **1999**, *99*, 27–36, doi:10.1007/s001220051205.
13. Larson, S.R.; Rutger, J.N.; Young, K.A.; Raboy, V. Isolation and Genetic Mapping of a Non-Lethal Rice (*Oryza sativa* L.) Low Phytic Acid Mutation. *Crop Sci.* **2000**, *40*, 1397–1405, doi:10.2135/cropsci2000.4051397x.
14. Guttieri, M.; Bowen, D.; Dorsch, J.A.; Raboy, V.; Souza, E. Identification and characterization of a low phytic acid wheat. *Crop Sci.* **2004**, *44*, 418–424, doi:10.1007/s001220050878.
15. Larson, S.R.; Young, K.A.; Cook, A.; Blake, T.K.; Raboy, V. Linkage mapping of two mutations that reduce phytic acid content of barley grain. *Theor. Appl. Genet.* **1998**, *97*, 141–146, doi:10.1007/s001220050878.
16. Jang, D.A.; Fadel, J.G.; Klasing, K.C.; Mireles, A.J.; Ernst, R.A.; Young, K.A.; Cook, A.; Raboy, V. Evaluation of low-phytate corn and barley on broiler chick performance. *Poult. Sci.* **2003**, *82*, 1914–1924, doi:10.1093/ps/82.12.1914.
17. Veum, T.L.; Ledoux, D.R.; Raboy, V. Low-phytate barley cultivars improve the utilization of phosphorus, calcium, nitrogen, energy, and dry matter in diets fed to young swine. *J. Anim. Sci.* **2007**, *85*, 961–971, doi:10.2527/jas.2006-453.
18. Thacker, P.A.; Rossnagel, B.G.; Raboy, V. Phosphorus digestibility in low-phytate barley fed to finishing pigs. *Can. J. Anim. Sci.* **2003**, *83*, 101–104, doi:10.1080/00039420310001656686.
19. Leytem, A.B.; Taylor, J.B.; Raboy, V.; Plumstead, P.W. Dietary low-phytate mutant-M 955 barley grain alters phytate degradation and mineral digestion in sheep fed high-grain diets. *Anim. Feed Sci. Technol.* **2007**, *138*, 13–28, doi:10.1016/j.anifeedsci.2006.11.005.
20. Poulsen, H.D.; Johansen, K.S.; Hatzack, F.; Boisen, S.; Rasmussen, S.K. Nutritional value of low-phytate barley evaluated in rats. *Acta Agric. Scand. Sect. A Anim. Sci.* **2001**, *51*, 53–58,

doi:10.1080/090647001300004790a.

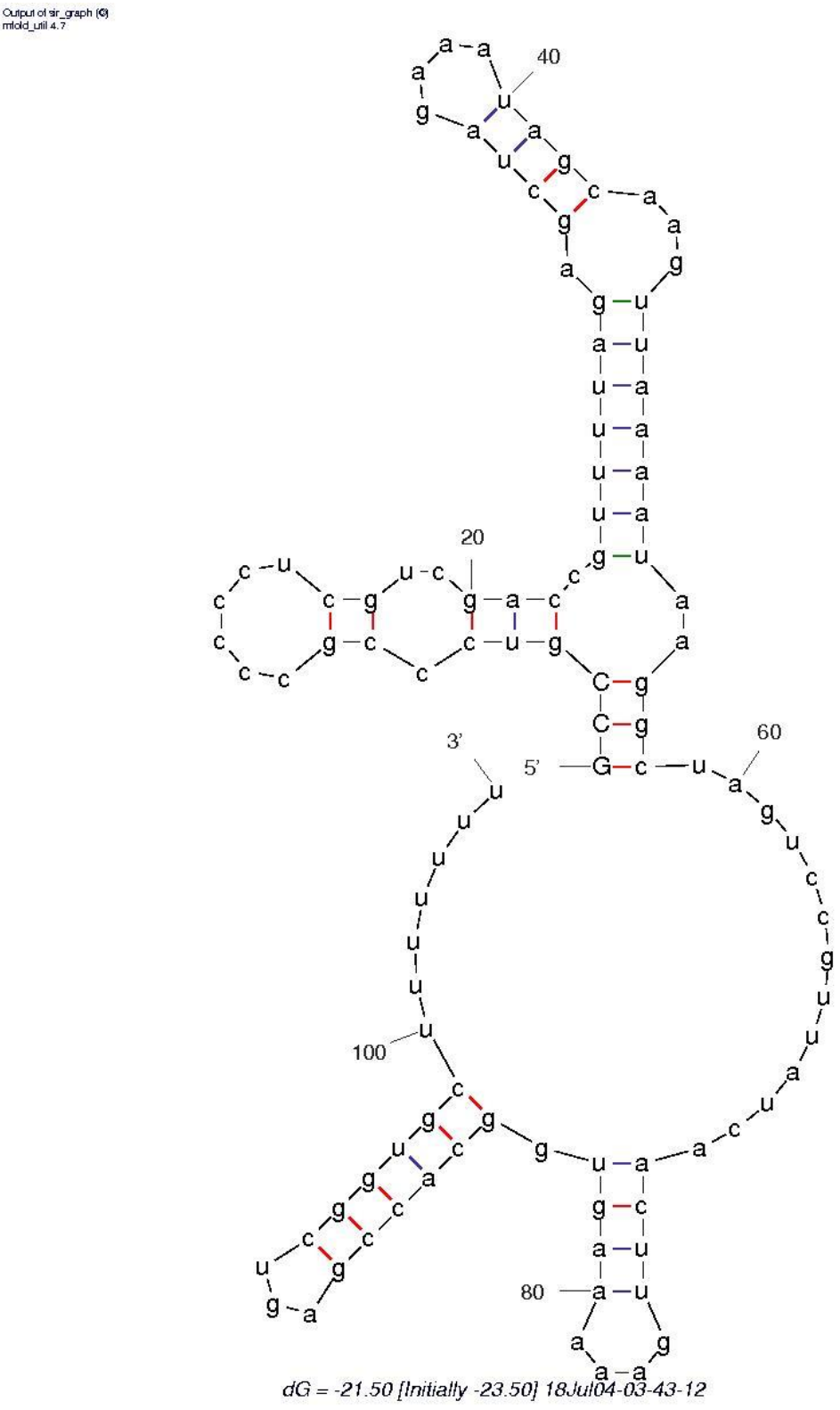
21. Raboy, V. Approaches and challenges to engineering seed phytate and total phosphorus. *Plant Sci.* **2009**, *177*, 281–296, doi:10.1016/j.plantsci.2009.06.012.
22. Pilu, R.; Panzeri, D.; Gavazzi, G.; Rasmussen, S.K.; Consonni, G.; Nielsen, E. Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (lpa241). *Theor. Appl. Genet.* **2003**, *107*, 980–987, doi:10.1007/s00122-003-1316-y.
23. Bregitzer, P.; Raboy, V. Effects of four independent low-phytate mutations in barley (*Hordeum vulgare* L.) on seed phosphorus characteristics and malting quality. *Cereal Chem.* **2006**, *83*, 460–464, doi:10.1094/CC-83-0460.
24. Kuo, H.; Chang, T.; Chiang, S.; Wang, W.; Charng, Y.; Chiou, T. *Arabidopsis* inositol pentakisphosphate 2-kinase, *AtIPK1*, is required for growth and modulates phosphate homeostasis at the transcriptional level. *Plant J.* **2014**, *80*, 503–515, doi:10.1111/tpj.12650.
25. Raboy, V.; Peterson, K.; Jackson, C.; Marshall, J.M.; Hu, G.; Saneoka, H.; Bregitzer, P. A substantial fraction of barley (*Hordeum vulgare* L.) low phytic acid mutations have little or no effect on yield across diverse production environments. *Plants* **2015**, *4*, 225–239, doi:10.3390/plants4020225.
26. Brinch-Pedersen, H.; Hatzack, F.; Stöger, E.; Arcalis, E.; Pontopidan, K.; Holm, P.B. Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): Deposition pattern, thermostability, and phytate hydrolysis. *J. Agric. Food Chem.* **2006**, *54*, 4624–4632, doi:10.1021/jf0600152.
27. Abid, N.; Khatoon, A.; Maqbool, A.; Irfan, M.; Bashir, A.; Asif, I.; Shahid, M.; Saeed, A.; Brinch-Pedersen, H.; Malik, K.A. Transgenic expression of phytase in wheat endosperm increases bioavailability of iron and zinc in grains. *Transgenic Res.* **2017**, *26*, 109–122, doi:10.1007/s11248-016-9983-z.
28. Holme, I.B.; Dionisio, G.; Brinch-Pedersen, H.; Wendt, T.; Madsen, C.K.; Vincze, E.; Holm, P.B. Cisgenic barley with improved phytase activity. *Plant Biotechnol. J.* **2012**, *10*, 237–247, doi:10.1111/j.1467-7652.2011.00660.x.
29. Du, H.; Liu, L.; You, L.; Yang, M.; Yubing, H.; Li, X.; Xiong, L. Characterization of an inositol 1,3,4-trisphosphate 5/6-kinase gene that is essential for drought and salt stress responses in rice. *Plant Mol. Biol.* **2011**, *77*, 547–563, doi:10.1007/s11103-011-9830-9.
30. Marathe, A.; Krishnan, V.; Vinutha, T.; Dahuja, A.; Jolly, M.; Sachdev, A. Exploring the role of Inositol 1,3,4-trisphosphate 5/6 kinase-2 (*GmITPK2*) as a dehydration and salinity stress regulator in *Glycine max* (L.) Merr. through heterologous expression in *E. Coli*. *Plant Physiol. Biochem.* **2018**, *123*, 331–341, doi:10.1016/j.plaphy.2017.12.026.
31. Zhu, J. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **2002**, *53*, 247–273, doi:10.1146/annurev.arplant.53.091401.143329.Salt.
32. Loewus, F.A.; Murthy, P.P.N. *Myo*-Inositol metabolism in plants. *Plant Sci.* **2000**, *150*, 1–19.
33. Aggarwal, S.; Shukla, V.; Bhati, K.K.; Kaur, M.; Sharma, S.; Singh, A.; Mantri, S.; Pandey, A.K. Hormonal regulation and expression profiles of wheat genes involved during phytic acid biosynthesis pathway. *Plants* **2015**, *4*, 298–319, doi:10.3390/plants4020298.
34. Niu, X.; Chen, Æ.Q.; Wang, Æ.X. *OsITL1* gene encoding an inositol 1,3,4-trisphosphate 5/6-kinase is a negative regulator of osmotic stress signaling. *Biotechnol. Lett.* **2008**, *30*, 1687–1692, doi:10.1007/s10529-008-9730-5.
35. Christian, M.; Cermak, T.; Doyle, E.L.; Schmidt, C.; Zhang, F.; Hummel, A.; Bogdanove, A.J.; Voytas, D.F. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* **2010**, *186*, 756–761, doi:10.1534/genetics.110.120717.
36. Cermak, T.; Doyle, E.L.; Christian, M.; Wang, L.; Zhang, Y.; Schmidt, C.; Baller, J.A.; Somia, N.V.; Bogdanove, A.J.; Voytas, D.F. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res.* **2011**, *39*, e82:1–e82:11, doi:10.1093/nar/gkr218.
37. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA —Guided DNA endonuclease in adaptive bacterial immunity. *Science* **2012**, *337*, 816–822, doi:10.1126/science.1225829.
38. Nekrasov, V.; Staskawicz, B.; Weigel, D.; Jones, D.J.; Kamoun, S. Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* **2013**, *31*, 691–693, doi:10.1038/nbt.2654.
39. Jiang, W.; Zhou, H.; Bi, H.; Fromm, M.; Yang, B.; Weeks, D.P. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice. *Nucleic Acids Res.* **2013**, *41*,

1–12, doi:10.1093/nar/gkt780.

40. Shan, Q.; Wang, Y.; Li, J.; Zhang, Y.; Chen, K.; Liang, Z.; Zhang, K.; Liu, J.; Xi, J.J.; Qiu, J.-L.; et al. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat. Biotechnol.* **2013**, *31*, 686–688, doi:10.1038/nbt.2650.
41. Ma, X.; Zhang, Q.; Zhu, Q.; Liu, W.; Chen, Y.; Qiu, R.; Wang, B.; Yang, Z.; Li, H.; Lin, Y.; et al. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicotplants. *Mol. Plant* **2015**, *8*, 1274–1284, doi:10.1016/j.molp.2015.04.007.
42. Xie, K.; Minkenberg, B.; Yang, Y. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 3570–3575, doi:10.1073/pnas.1420294112.
43. Fauser, F.; Schiml, S.; Puchta, H. Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant J.* **2014**, *79*, 348–359, doi:10.1111/tpj.12554.
44. Slaymaker, I.M.; Gao, L.; Zetsche, B.; Scott, D. a.; Yan, W.X.; Zhang, F. Rationally engineered Cas9 nucleases with improved specificity. *Science* **2015**, *351*, 84–88, doi:10.1126/science.aad5227.
45. Kleinstiver, B.P.; Pattanayak, V.; Prew, M.S.; Tsai, S.Q.; Nguyen, N.T.; Zheng, Z.; Keith Joung, J. High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **2016**, *529*, 490–495, doi:10.1038/nature16526.
46. Li, C.; Zong, Y.; Wang, Y.; Jin, S.; Zhang, D.; Song, Q.; Zhang, R. Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biol.* **2018**, *19*, 59, doi:10.1186/s13059-018-1443-z.
47. Bo, L.; Jia-jie, W.; Dao-lin, F. Constructing the barley model for genetic transformation in *Triticeae. J. Integr. Agric.* **2015**, *14*, 453–468, doi:10.1016/S2095-3119(14)60935-7.
48. Hamada, H.; Liu, Y.; Nagira, Y.; Miki, R.; Taoka, N.; Imai, R. Biolistic-delivery-based transient CRISPR/Cas9 expression enables *in planta* genome editing in wheat. *Sci. Rep.* **2018**, *8*, 1–7, doi:10.1038/s41598-018-32714-6.
49. Zhang, Y.; Liang, Z.; Zong, Y.; Wang, Y.; Liu, J.; Chen, K.; Qiu, J.-L.; Gao, C. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* **2016**, *7*, 1–8, doi:10.1038/ncomms12617.
50. Abid, G.; Silue, S.; Muhovski, Y.; Jacquemin, J.M.; Toussaint, A.; Baudoin, J.P. Role of myo-inositol phosphate synthase and sucrose synthase genes in plant seed development. *Gene* **2009**, *439*, 1–10 doi:10.1016/j.gene.2009.03.007.
51. Bhati, K.K.; Aggarwal, S.; Sharma, S.; Mantri, S.; Singh, S.P.; Bhalla, S.; Kaur, J.; Tiwari, S.; Roy, J.K.; Tuli, R.; et al. Differential expression of structural genes for the late phase of phytic acid biosynthesis in developing seeds of wheat (*Triticum aestivum* L.). *Plant Sci.* **2014**, *224*, 74–85, doi:10.1016/j.plantsci.2014.04.009.
52. Shi, J.; Hongyu, W.; Wu, Y.; Hazebroek, J.; Meeley, R.B.; Ertl, D.S. The maize low-phytic acid mutant lpa2 is caused by mutation in an inositol phosphate kinase gene. *Plant Physiol.* **2003**, *131*, 507–515, doi:10.1104/pp.014258.
53. Liang, G.; Zhang, H.; Lou, D.; Yu, D. Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. *Sci. Rep.* **2016**, *6*, 1–8, doi:10.1038/srep21451.
54. Serhantova, V.; Ehrenbergerova, J.; Ohnoutkova, L. Callus induction and regeneration efficiency of spring barley cultivars registered in the Czech Republic. *Plant, Soil Environ.* **2004**, *50*, 456–462, doi:10.17221/4058-pse.
55. Harwood, W.A. A protocol for high-throughput *Agrobacterium*-mediated barley transformation. In *Cereal Genomics: Methods and Protocols*; Henry, R.J., Furtado, A., Eds.; Humana Press: Totowa, NJ, USA, 2014; pp. 251–260. ISBN 978-1-62703-715-0.
56. Holme, I.B.; Wendt, T.; Gil, J.; Deleuran, L.C.; Starker, C.G.; Voytas, D.F.; Brinch-Pedersen, H. Evaluation of the mature grain phytase candidate HvPAPhy_a gene in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 and TALENs. *Plant Mol. Biol.* **2017**, *95*, 111–121, doi:10.1111/pbi.12636.
57. Kapusi, E.; Corcuera-Gómez, M.; Melnik, S.; Stoger, E. Heritable genomic fragment deletions and small indels in the putative ENGase gene induced by CRISPR/Cas9 in barley. *Front. Plant Sci.* **2017**, *8*, 1–11, doi:10.3389/fpls.2017.00540.

58. Lawrenson, T.; Shorinola, O.; Stacey, N.; Li, C.; Østergaard, L.; Patron, N.; Uauy, C.; Harwood, W. Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol.* **2015**, *16*, 1–13, doi:10.1186/s13059-015-0826-7.
59. Singh, M.; Kumar, M.; Albertsen, M.C.; Young, J.K.; Cigan, A.M. Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). *Plant Mol. Biol.* **2018**, *97*, 371–383, doi:10.1007/s11103-018-0749-2.
60. Li, M.; Hensel, G.; Mascher, M.; Melzer, M.; Budhagatapalli, N.; Rutten, T.; Himmelbach, A.; Beier, S.; Korzun, V.; Kumlehn, J.; et al. Leaf variegation and impaired chloroplast development caused by a truncated CCT domain gene in albostrians barley. *Plant Cell* **2019**, *31*, 1430–1445, doi:10.1105/tpc.19.00132.
61. Holubová, K.; Hensel, G.; Vojta, P.; Tarkowski, P.; Gene, H. Modification of Barley Plant Productivity Through Regulation of Cytokinin Content by Reverse–Genetics Approaches Preparation of Constructs for Silencing. *Front. Plant Sci.* **2018**, *9*, 1–18, doi:10.3389/fpls.2018.01676.
62. Sánchez-León, S.; Gil-Humanes, J.; Ozuna, C. V.; Sousa, C.; Voytas, D.F.; Barro, F. Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol. J.* **2018**, *16*, 902–910, doi:10.1111/pbi.12837.
63. Jang, G.; Lee, S.; Um, T.; Hyun Chang, S.; Yong Lee, H.; Chung, P.J.; Kim, J.-K.; Do Choi, Y. Genetic chimerism of CRISPR/Cas9-mediated rice mutants. *Plant Biotechnol. Rep.* **2016**, *10*, 1–11, doi:10.1007/s11816-016-0414-7.
64. Hatzack, F.; Johansen, K.S.; Rasmussen, S.K. Nutritionally relevant parameters in low-phytate barley (*Hordeum vulgare* L.) grain mutants. *J. Agric. Food Chem.* **2000**, *48*, 6074–6080, doi:10.1021/jf000669p.
65. Raboy, V.; Gerbasi, P.F.; Young, K.A.; Stoneberg, S.D.; Pickett, S.G.; Bauman, A.T.; Murthy, P.P.; Sheridan, W.F.; Ertl, D.S. Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. *Plant Physiol.* **2000**, *124*, 355–68, doi:10.1104/pp.124.1.355.
66. Kim, S.I.; Tai, T.H. Identification of genes necessary for wild-type levels of seed phytic acid in *Arabidopsis thaliana* using a reverse genetics approach. *Mol. Genet. Genomics* **2011**, *286*, 119–133, doi:10.1007/s00438-011-0631-2.
67. Nagy, R.; Grob, H.; Weder, B.; Green, P.; Klein, M.; Frelet-Barrand, A.; Schjoerring, J.K.; Brearley, C.; Martinoia, E. The *Arabidopsis* ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporterinvolved in guard cell signaling and phytate storage. *J. Biol. Chem.* **2009**, *284*, 33614–33622, doi:10.1074/jbc.M109.030247.
68. Panzeri, D.; Cassani, E.; Doria, E.; Tagliabue, G.; Forti, L.; Campion, B.; Bollini, R.; Brearley, C.A.; Pilu, R.; Nielsen, E.; et al. A defective ABC transporter of the MRP family, responsible for the bean *lpa1* mutation, affects the regulation of the phytic acid pathway, reduces seed *myo*-inositol and alters ABA sensitivity. *New Phytol.* **2011**, *191*, 70–83, doi:10.1111/j.1469-8137.2011.03666.x.
69. Stiles, A.; Qian, X.; Shears, S.; Grabau, E. Metabolic and signaling properties of an *Itpk* gene family in *Glycine max*. *FEBS Lett.* **2008**, *582*, 1853–1858, doi:10.1016/j.febslet.2008.04.054.
70. Marathe, A.; Krishnan, V.; Mahajan, M.M.; Thimmegowda, V.; Dahuja, A.; Jolly, M.; Praveen, S.; Sachdev, A. Characterization and molecular modeling of Inositol 1,3,4 tris phosphate 5/6 kinase-2 from *Glycine max* (L.) Merr.: Comprehending its evolutionary conservancy at functional level. *3 Biotech* **2018**, *8*, 50, doi:10.1007/s13205-017-1076-z.
71. Forster, B.P.; Pakniyat, H.; Macaulay, M.; Matheson, W.; Phillips, M.S.; Thomas, W.T.B.; Powell, W. Variation in the leaf sodium content of the *Hordeum vulgare* (Barley) cultivar Maythorpe and its derived mutant cv. Golden promise. *Heredity (Edinb).* **1994**, *73*, 249–253, doi:10.1038/hdy.1994.130.
72. Kaur, H.; Verma, P.; Petla, B.P.; Rao, V.; Saxena, S.C.; Majee, M. Ectopic expression of the ABA-inducible dehydration-responsive chickpea l-*myo*-inositol 1-phosphate synthase 2 (*CaMIPS2*) in *Arabidopsis* enhances tolerance to salinity and dehydration stress. *Planta* **2013**, *237*, 321–335, doi:10.1007/s00425-012-1781-0.
73. Smits, A.H.; Ziebell, F.; Joberty, G.; Zinn, N.; Mueller, W.F.; Clauder-Münster, S.; Eberhard, D.; Fälth Savitski, M.; Grandi, P.; Jakob, P.; et al. Biological plasticity rescues target activity in CRISPR knock outs. *Nat. Methods* **2019**, *16*, 1087–1093, doi:10.1038/s41592-019-0614-5.
74. Jiang, M.; Hu, H.; Kai, J.; Traw, M.B.; Yang, S.; Zhang, X. Different knockout genotypes of OsIAA23 in rice using CRISPR/Cas9 generating different phenotypes. *Plant Mol. Biol.* **2019**, *100*, 467–479, doi:10.1007/s11103-019-00871-5.
75. Edwards, K.; Johnstone, C.; Thompson, C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **1991**, *19*, 1349, doi:10.1093/nar/19.6.1349.

76. Liu, W.; Xie, X.; Ma, X.; Li, J.; Chen, J.; Liu, Y.-G. DSDecode: A Web-Based Tool for Decoding of Sequencing Chromatograms for Genotyping of Targeted Mutations. *Mol. Plant* **2018**, *8*, 1431–1433, doi:10.1016/j.molp.2015.05.009.
77. Vaculova, K.; Balounova, M.; Sedlackova, I.; Kvasnicka, F.; Mikulikova, R.; Belakova, S.; Benesova, K.; Pouch, M.; Ehrenbergerova, J. *Metodika Prebreedingu Ječmene Jarního s Diferencovaným Obsahem Přirozených Škodlivých Látek v Zrně Pro Šlechtění Odrůd Nesladovnického Typu*, 1st ed.; Agrotest Fyto, S.R.O.: Kromeriz, Czech Republic, 2011; pp. 12–15. ISBN 978-80-87555-00-2.
78. Chen, P.S.; Toribara, T.Y.; Warner, H. Microdetermination of Phosphorus. *Anal. Chem.* **1956**, *28*, 1756–1758.
79. Daszkowska-Golec, A.; Skubacz, A.; Kurowska, M.; Slota, M.; Swiergolik, D.; Szarejko, I. Methods for the Simple and Reliable Assessment of Barley Sensitivity to Abiotic Stresses During Early Development. In *Barley: Methods and Protocols*; Harwood, W.A., Ed.; Springer Science + Business Media, New York, NY, USA, 2019; pp. 127–151. ISBN 978-1-4939-8942-3.



Supplemental Figure S1 The secondary structure of sgRNA

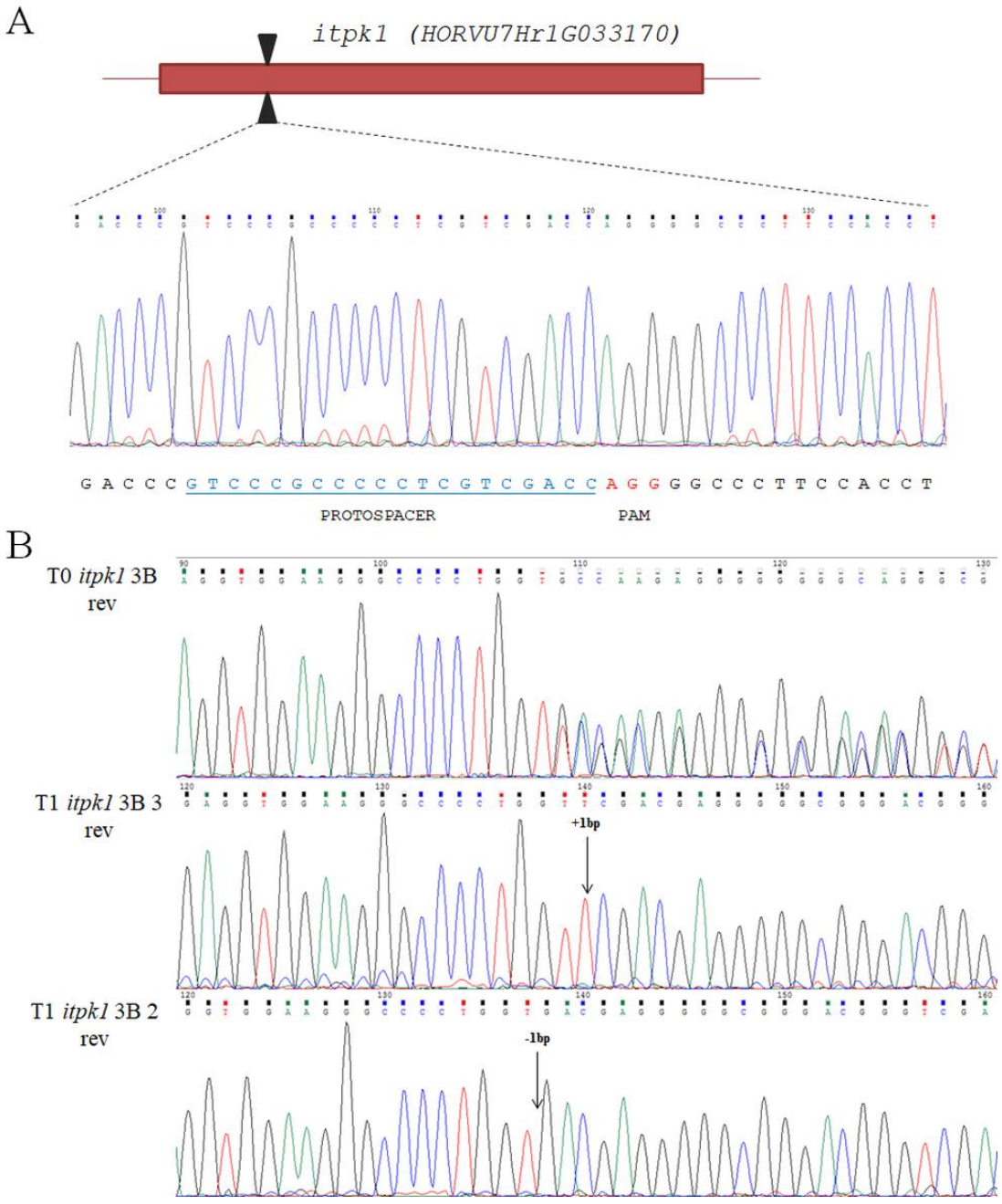


Figure S2. Characterization of target genomic sequence in *HvITPK1*.
A A scheme of *HvITPK1* gene with emphasized protospacer sequence. **B** Sequencing chromatograms from analysis of T0 and T1 3B plants.

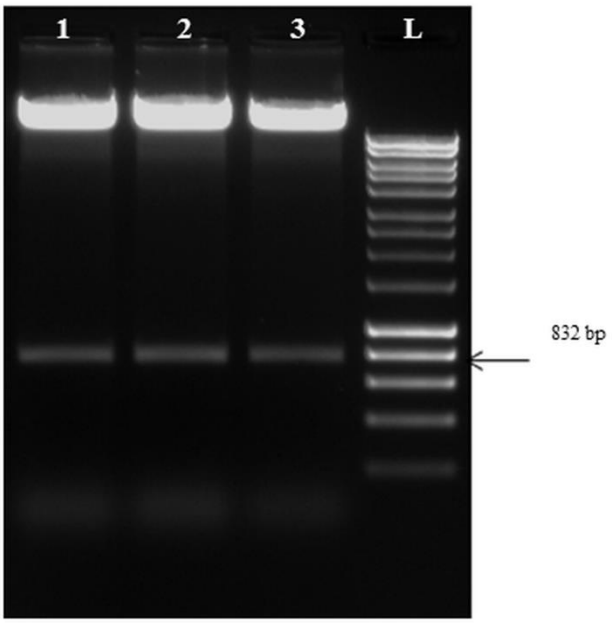


Figure S3. Diagnostic digestion of the expression vector with sgRNA cassette
L – DNA ladder (HyperLadder 50 bp, Bioline); 1 – 3 expression vector pYLCRISPR/Cas9Pubi-H harbouring the sgRNA cassette, expected size of cut cassette 832 bp

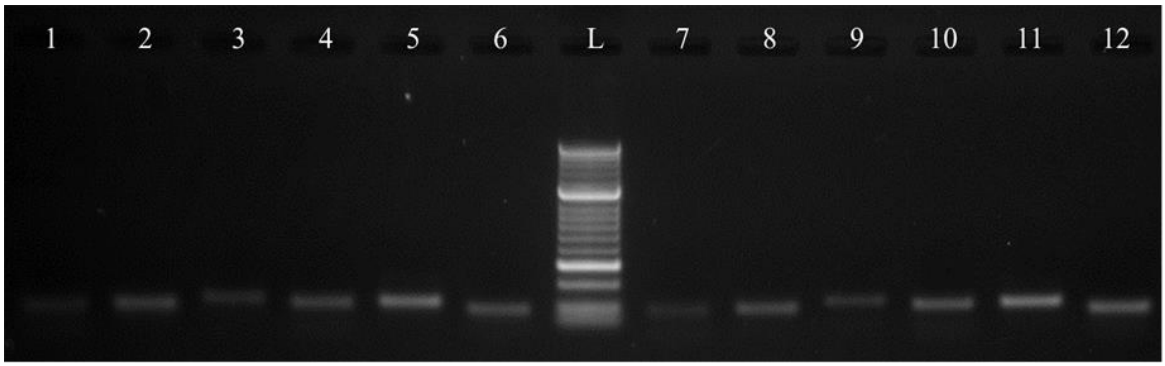


Figure S3. Evaluation of *ITPK* gene primer specificity
L – DNA ladder (HyperLadder 50 bp, Bioline); Lines 1 – 6 cDNA from WT, 7 – 12 cDNA from *Hvitpk1-2*. Lines 1, 7 - *HvITPK1*; 2, 8 – *HvITPK5*; 3, 9 – *HvITPK4*; 4, 10 – *HvITPK6*; 5, 11 – *HvITPK2*; 6, 12 – *HvITPK3*

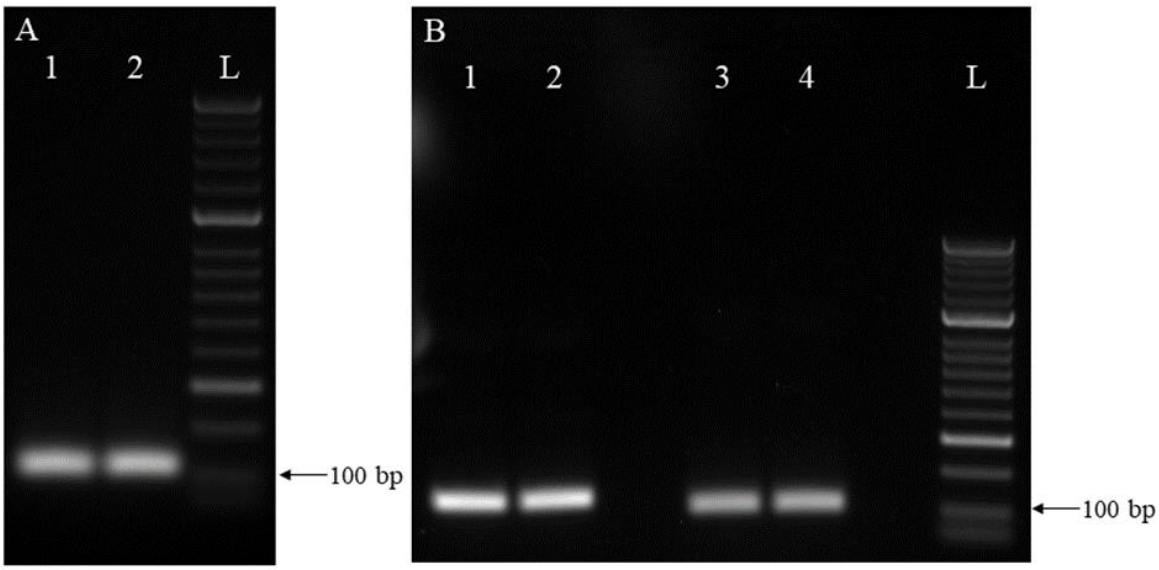
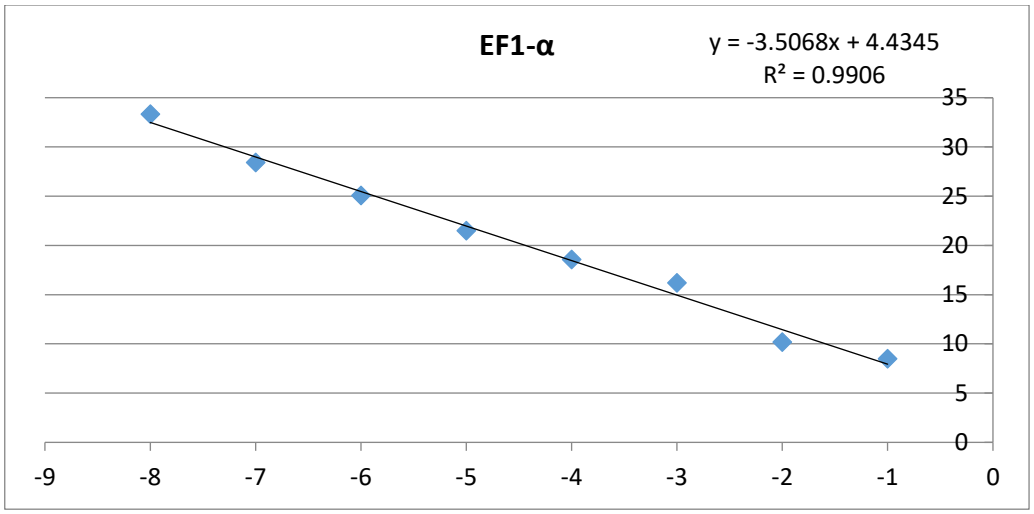
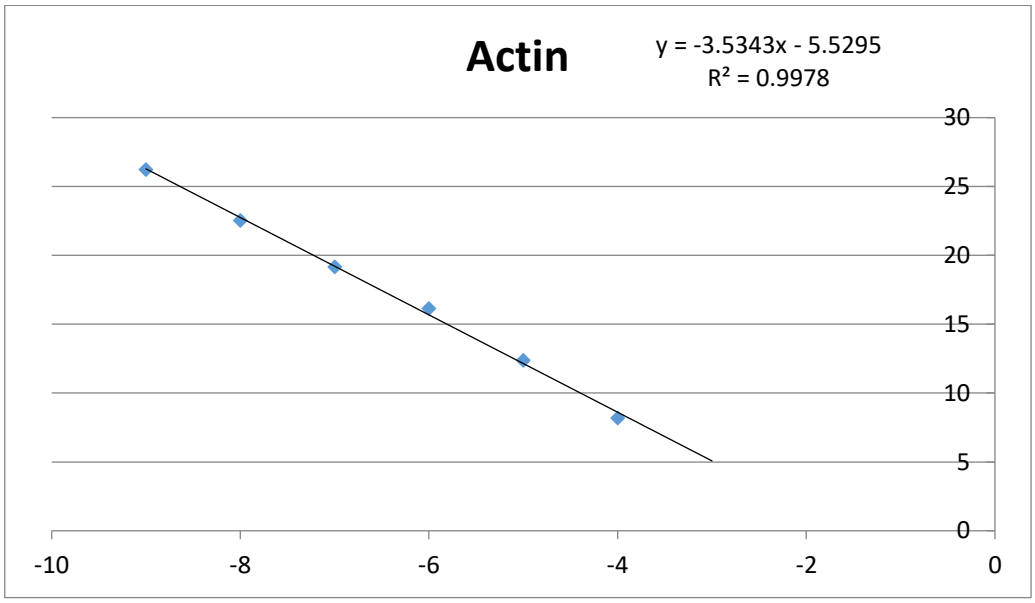
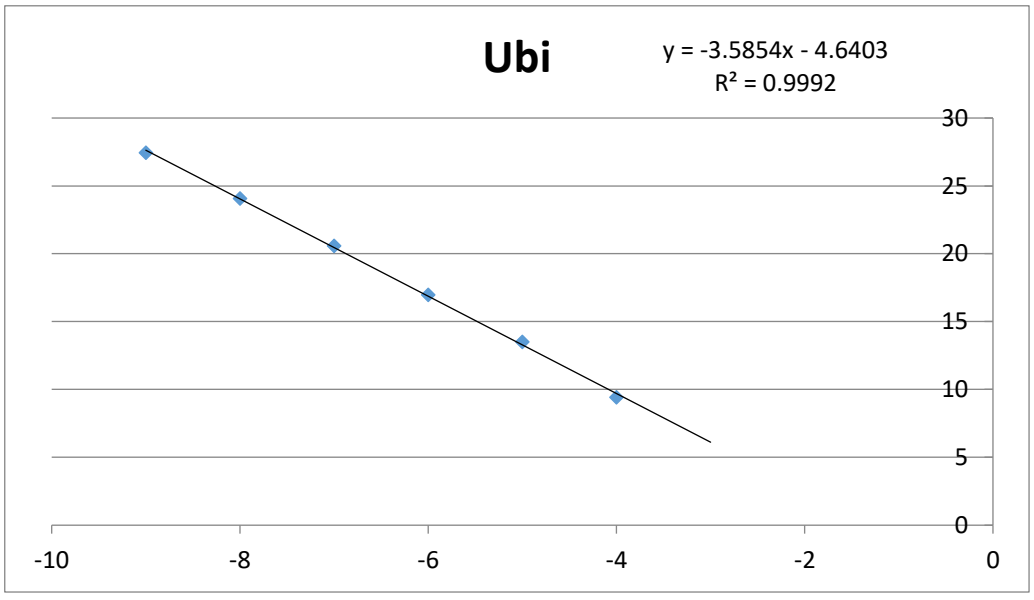
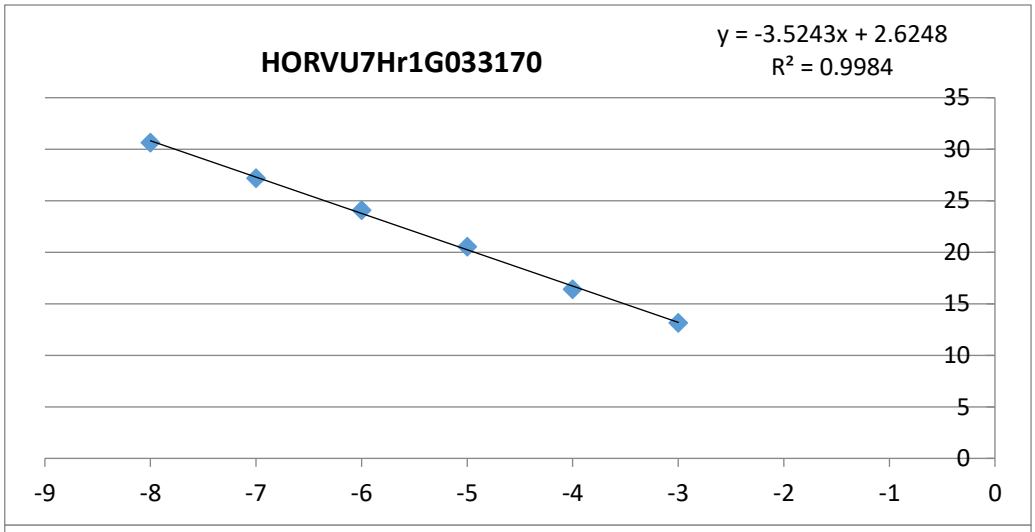


Figure S3. Evaluation of reference gene primers specificity
L – DNA ladder (HyperLadder 50 bp, Bioline); **A**) Line:1 – cDNA from WT; 2 – cDNA from *Hvitpk1-2* for EF1-α **B**) Line: 1 – cDNA from WT; 2 – cDNA from *Hvitpk1-2* for Actin2; 3 – cDNA from WT; 4 – cDNA from *Hvitpk1-2* for Ubiquitin


EF1-α
y = -3.5068x + 4.4345
R² = 0.9906
35
30
25
20
15
10
5
0
-9
-8
-7
-6
-5
-4
-3
-2
-1
0


Actin
y = -3.5343x - 5.5295
R² = 0.9978
30
25
20
15
10
5
0
-10
-8
-6
-4
-2
0


Ubi
y = -3.5854x - 4.6403
R² = 0.9992
30
25
20
15
10
5
0
-10
-8
-6
-4
-2
0


HORVU7Hr1G033170
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R² = 0.9984
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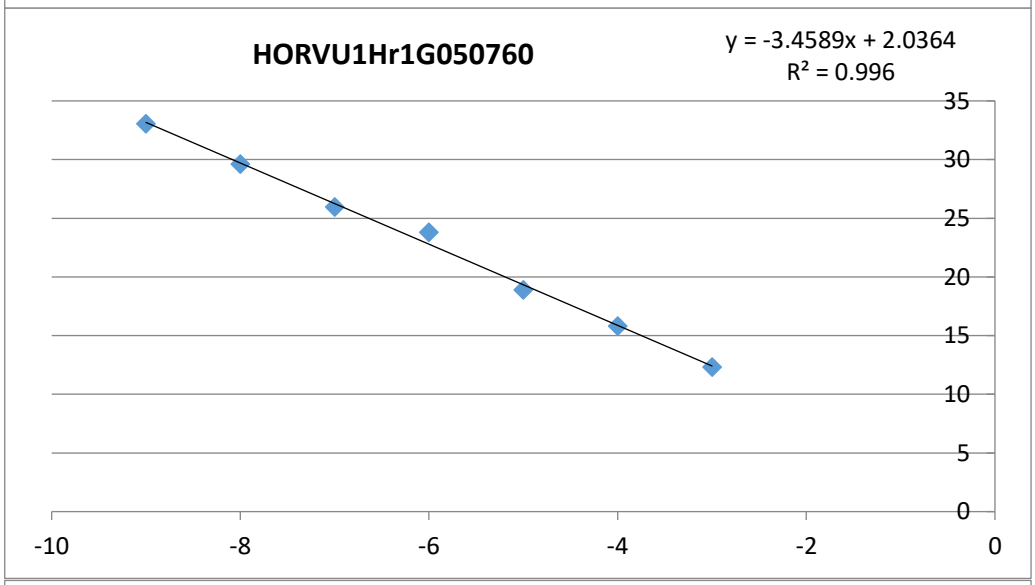
25
20
15
10
5
0
-9
-8
-7
-6
-5
-4
-3
-2
-1
0


HORVU1Hr1G050760
y = -3.4589x + 2.0364
R² = 0.996
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5
0
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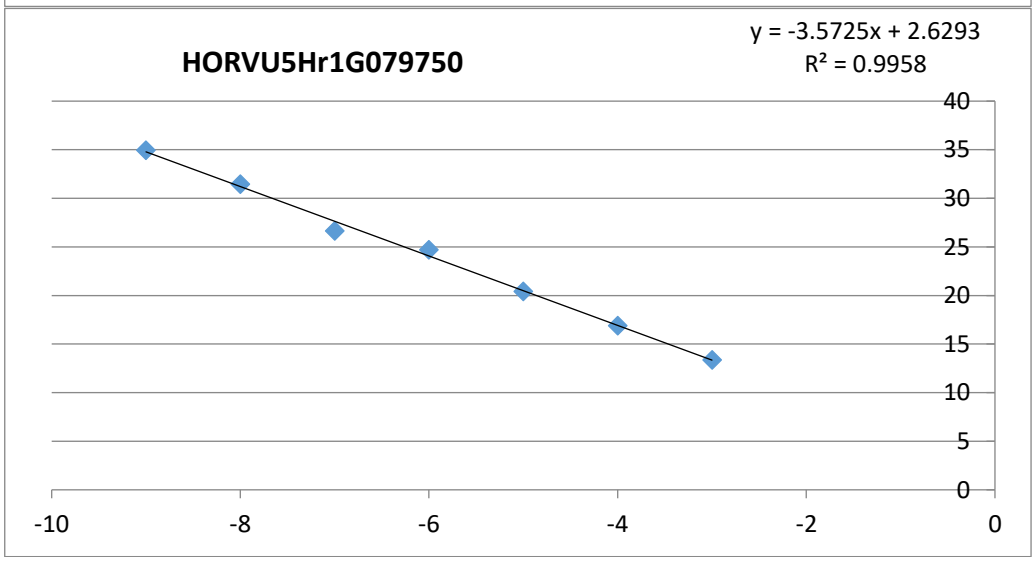
-8
-6
-4
-2
0


HORVU5Hr1G079750
y = -3.5725x + 2.6293
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-4
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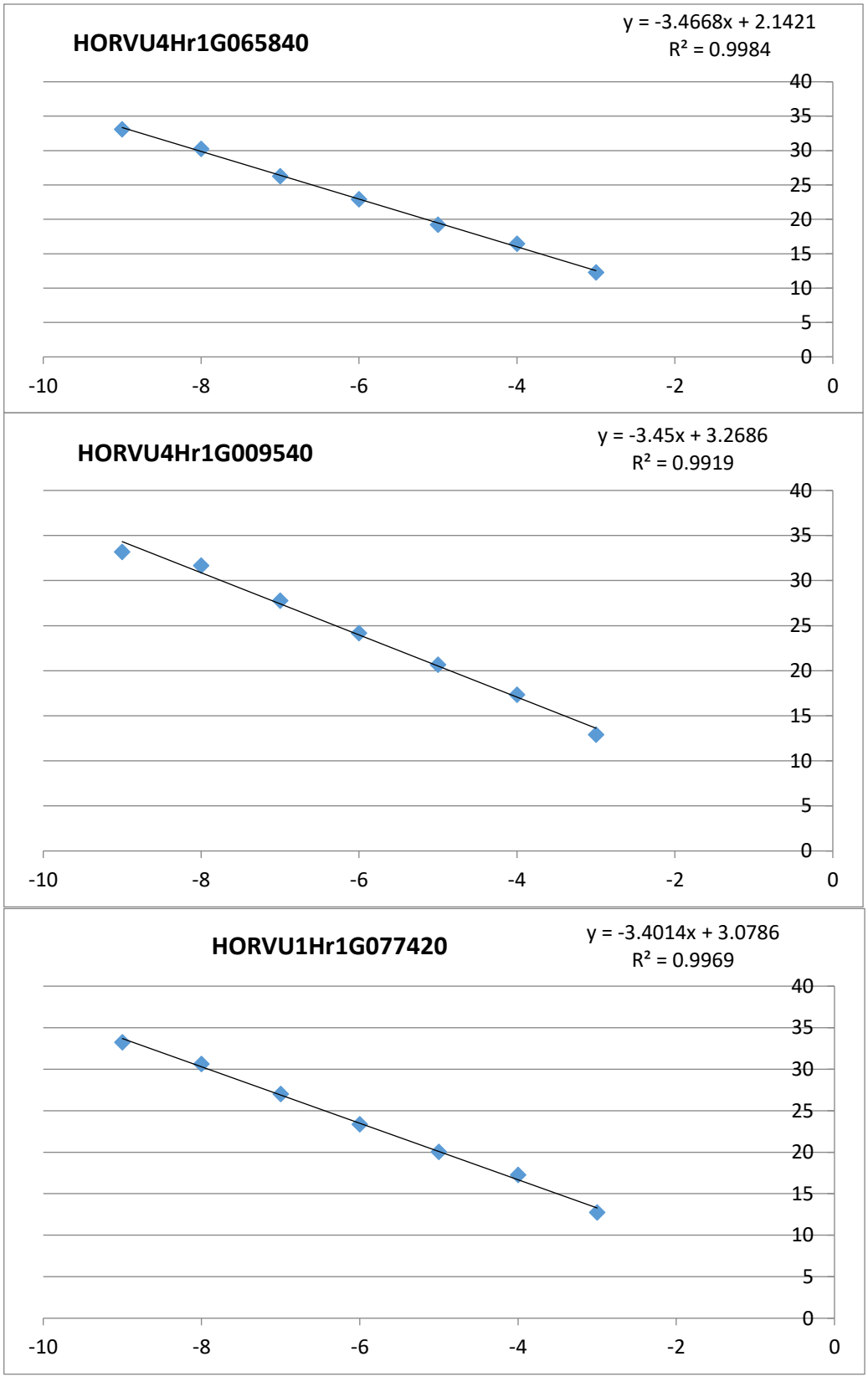

Supplemental Figure S5 Standard calibration curves for qRT-PCR

Gene		Sequence (5’ – 3’)	Amplicon [bp]
Cas9	*Genotyping*	TTCGCTACTGTTCGCAAGGT GGTGGATGAGAGTAGCGTCG	811
	RT-PCR	CGACGCTACTCTCATCCACC CTTTTTGGTGGCAGCAGGAC	100
sgRNA	*RT-PCR*	CCCCTCGTCGACCGTTTTAG CGACTCGGTGCCACTTTTTC	85
HORVU7Hr1G033170 *Hvitpk1*	*Sequencing*	ACGACCTCCTCACACCTACA GGAGACGACGTCGAGCATG	301
	qRT-PCR	TCTTCGGCGACATGATTCGT AACGTTGGAGGCTTCGGTAG	83
HORVU1Hr1G050760 *Hvitpk5*	*Sequencing*	GCCCAAGAAGCAGAACAGC GTGGTAGACGAGGGACATCTT	451
	qRT-PCR	TGCCTGGCTACGAGATTGTC CTCTGCTCCTCCTCCTCCTT	88
HORVU1Hr1G077420 *Hvitpk4*	*Sequencing*	TCAGTCGAGCGATCATGGTG TCCTTGTCCACCAGCCCTG	754
	qRT-PCR	TCCAAAGCAGGTGAGAGCAG GGCAAACGCTTGGAAGGATC	116
HORVU4Hr1G065840 *Hvitpk2*	*Sequencing*	GACCGAGTATTCAGGTGGCC TTACCTCCAGAATCCGCTGC	529
	qRT-PCR	GCTTCCTCCGAGACCACTTC ACCGGTCATTAGCTCCAAGC	110
HORVU5Hr1G079750 *Hvitpk6*	*qRT-PCR*	TTGAAGACCCTTCCAGTGGC CGTTTTGCAGCCTCTTCCAC	101
HORVU4Hr1G009540 *Hvitpk3*	*qRT-PCR*	ACCTTGCGGATTGTCATGGT ACCGCAGCTGGTATTGACAA	82
HORVU4Hr1G056830 *EF1-α*	*qRT-PCR*	GAAGATGATTCCCACCAAGC TGACACCAACAGCCACAGTT	107
HORVU4Hr1G008310 *Actin2*	*qRT-PCR*	TCGTGAGAAGATGACCCAGA CCGAGTCCAGCACAATACCT	122
HORVU3Hr1G080790 *Ubiquitin*	*qRT-PCR*	GCTTATCATGGCTTCAAAACGTA ACATATCCTCGCCAGAAGGAC	98

Table 1. List of primers used for genotyping and qRT-PCR.

PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Laboratory of Growth Regulators

Tomáš Vlčko

Summary of the Ph.D. Thesis

Biofortification of spring barley

P1527 Biology – 1501V019 Experimental biology

Supervisor

Prof. RNDr. Martin Fellner, Ph.D.

Olomouc

2020

This thesis was carried out in the Laboratory of Growth Regulators within the framework of internal Ph.D. Study of Experimental Biology, guaranteed by the Laboratory of Growth Regulators, Faculty of Science, Palacký University Olomouc between the years 2015-2020.

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The oral defence will take place on **15. 6. 2020** before the Commision for the Ph.D. thesis of the Study Program Experimental Biology, room SE-H, Šlechtitelů 27, Olomouc – Holice.

The Ph.D. thesis and expert reviews will be available 14 days before the defence in the Study Department of Faculty of Science (Mgr. M. Karásková), Palacký University, 17. Listopadu 12, Olomouc.

After the defence, the Ph.D. thesis will be stored in the Library of the Biological Departments of Faculty of Science, Palacký University, Šlechtitelů 27, Olomouc – Holice.

Prof. Ing. Miroslav Strnad, Csc. DSc.
Chairman of the Commision for the Ph.D. thesis,
Study Program Experimental Biology,
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Content

Introduction

Barley is annually sown on thousands of hectares and is the fourth most important cereal crop in the world. The majority of barley production is used as animal feed and smaller part in the production of malt used in the brewing of beer and in whiskey production. The most valuable part of the barley plant is its grains which are full of starch, vitamins, and microelements. Of these, phosphorus is an essential element with a high value for nutrition. It is also essential for plant growth and the second most limiting element to plant development. Therefore, in order to sustain high yield and especially in soils poor in nutrients, phosphorus is being added into the soil as a fertilizer to provide a sufficient amount of nutrients. However, global deposits of rock phosphate are not infinite. An alarming state was described by Cordell et al. (2009) who reported that the application of rock phosphate steeply increased in the second half of the twentieth century and further predicted the peak of production in a few decades. The remaining deposits could be depleted within this century. Requirements for agricultural production are high, regarding fuel consumption, application of fertilizers and technology, so that maximal effectiveness in the utilization of agricultural products is demanding. Maintenance or increase of yield under sub-optimal environmental conditions is of great importance. In this view, application of modern breeding techniques and biotechnology could assist in the breeding of modern cultivars.

Generally, agricultural production is energetically expensive. Additionally, cereals are very demanding crops that requires crop rotation and also rich fertilization for optimal yield. In an effort to achieve maximal effectiveness in the utilization of agricultural products for food and feed, it is of great interest to fully exploit their nutritional and energetic value. For instance, phosphate and microelement utilization from cereal grains depend strongly on the form in which phosphate and microelements are stored. Therefore, sophisticated approaches aiming at more efficient phosphate utilization are needed. The currently available tools of genome editing (GE) technology facilitate genetic manipulation. The major advantage is the ability to induce mutations at predefined sites in the genome by targeting one gene or even the whole gene family. In combination with available genome sequences of all major crops through free access databases, it has never been more realizable to identify an exact gene or gene family and elucidate its function.

In this work, application of clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein (Cas) system for development of mutant barley lines with altered phosphate content is described. Genes for inositol trisphosphate 5/6 kinases (ITPK) were selected as targets for GE. ITPKs play a role in sequential phosphorylation of *myo*-Inositol phosphates thus participating in the storage of phosphate in developing grains.

Aims and Scope

The scope of the work is the improvement of phosphate utilization in the barley-based feed. Since phosphate is stored in barley grains in an organic form of phytic acid, which is difficult to digest by non-ruminants, phosphate is not fully bio-available and a substantial part of the total content is undigested. Alteration of phosphate content in barley grains can improve phosphate utilization and increase the nutritional value of barley-based feed. GE techniques enable extraordinarily precise manipulation with genetic information including induction of mutations at predefined sites within the genome. Emphasis was placed on application modern techniques of GE in the preparation of biofortified barley with improved digestible phosphate content.

Aims of this thesis were

- •Evaluation of biofortification strategies focused on improved phosphate utilization
- Analysis of barley enzymes participating in phytic acid synthesis, selection of potential candidate genes and design and cloning of expression vector for genome-editing
- Preparation of transgenic plants expressing programmable nuclease Cas9, their genotypic characterization, identification of mutations
- Phenotypic characterization of mutant barley lines – phosphate content, germination rate

Materials and Methods

Chemicals

List of critical chemicals below, all the other chemicals used in the experiments were of molecular biology grade.

BsaI (cat. no. R0535S) and HF-*MluI* (cat. no. R3198S) (New England Biolab, Ipswich, MA, USA).
T4 DNA ligase (cat. no. M0202S, New England Biolab, Ipswich, MA, USA)
Platinum Pfx DNA polymerase (cat. no. 11708-013, Carlsbad, CA, USA)
MS medium (cat. no. M0221, Duchefa Biochemie, Haarlem, Netherland)
MS medium (cat. no., M0238, Duchefa Biochemie, Haarlem, Netherland)
Phyto agar (cat. no. P1003, Duchefa Biochemie, Haarlem, Netherland)
LB broth (cat. no. L1703, Duchefa Biochemie, Haarlem, Netherland)
Hygromycin B (cat. no. 10843555001 Roche, Merck, Mannheim, Germany)
Total RNAqueous Kit (cat. no. AM1912, Thermo Fisher, Vilnius, Lithuania)
Turbo DNA-freeTM Kit (cat. no. AM1907, Thermo Fisher, Vilnius, Lithuania)
RevertAid H minus Reverse Transcriptase (cat. no. EP0451, Thermo Fisher, Vilnius, Lithuania)
Oligonucleotides (Generi Biotech a.s.,Hradec Kralove, Czech Rep.)
ReadyMix^{TM} PCR Reaction Mix (cat. no. R2523, Sigma-Aldrich, St. Louis, MO, USA)
SsoAdvanced^{TM} Universal SYBR® Green Supermix (cat. no. 1725270, BioRad, Hercules, CA, USA)

Biological material

Spring barley (*Hordeum vulgare*) cultivar Golden Promise
Chemically competent *Escherichia coli* cells, strain DH5α
Electrocompetent *Agrobacterium tumafaciens* cells, strain AGL1

Equipment

Environmental Shaker-Incubator ES-20 (Biosan, Riga, Latvia)
Electroporator CelljecT Duo (Thermo Electron Corporation, Milford, MA, USA]
Flowbox HeraGuard (Thermo Scientific, Germany)
Centrifuge BR4i (Jouan, France)
Cultivation box Adaptis A1000 (Conviron, Winnipeg, MB, Canada)
Spectrophotometer DS-11 (DeNovix, Wilmington, Delaware, USA)
MM 400 mixer mill (Retsch, Haan, Germany)

Synergy Mx plate reader (BioTek Instruments, Winooski, VT, USA)

T100™ Thermal Cycler (Biorad, Singapore)

CFX96 Touch™ Real-Time PCR Detection System (BioRad, Singapore)

MEGA software version 6.06

A plasmid Editor (M. Wayne Davis)

Chromas version 2.6.6 (Technelysium, South Brisbane, Australia)

Methods

Design and cloning of protospacer

Barley inositol trisphosphate 5/6-kinase (HORVU7Hr1G033170 and HORVU1Hr1G050760, here referred to as HvITPK1 and HvITPK5 respectively) were chosen as target genes. The protein-coding sequence of *ZmIPK* was used to blast the barley genomic DNA database (EnsemblPlants) to identify homologous barley *ITPK* genes and rice or wheat orthologs. To evaluate phylogenetic relations between *ITPK* genes described in rice, wheat and barley orthologs, a phylogenetic tree was constructed using AA sequences due to its higher conservation across the plant species. The protospacers were designed based on the Golden Promise sequence. Protospacer sequences were sub-cloned into pYLsgRNA-OsU6 and then the U6 promoter-sgRNA cassette was cloned into the expression vector pYLCRISPR/Cas9Pubi-H according to the protocol described by Ma et al. (Ma et al., 2015). The expression vector was verified by digestion with *Mlu*I, and by Sanger sequencing. The vector harboring sgRNA with the protospacer sequence was transformed into Agrobacterium tumefaciens strain AGL.1 by electroporation.

Barley transformation

The barley cultivar Golden Promise was used for transformation according to the protocol by Harwood (2014). Two expression vectors coding Cas9 nuclease targeting either *HvITPK1* or *HvITPK5* were transformed independently. Immature barley embryos were used for *Agrobacterium*-mediated transformation. Tissue cultures were derived and callus cultures were selected on Hygromycin. Subsequently, regenerating plantlets were transferred onto regeneration medium in flasks. Regenerating plants were transferred into pots with soil substrate and grown in the greenhouse at 16°C with 16-h light photoperiod until maturity. For the identification of transgenic plants, genomic DNA from young leaves was extracted according to Edwards et al. (1991). DNA samples were used for analyses of the presence of Cas9 transgene by polymerase chain reaction (PCR) using a premix REDTaq® ReadyMix™ PCR Reaction Mix. The PCR products were separated in 1% agarose gel stained with ethidium bromide. Separated PCR products were evaluated in UV transilluminator.

Detection of the Cas9 and sgRNA transcripts

Transgenic plants with confirmed presence of Cas9 transgene were picked for the analysis of transgene expression. Total RNA was extracted from leaves using a Total RNAqueous Kit. Extracted RNA was treated with DNase. The concentration of RNA was assessed spectrophotometrically. 1 µg of RNA was used for reverse transcription using poly-T primers and RevertAid H minus Reverse Transcriptase. cDNA samples were diluted ten times with nuclease-free water. RT-PCR was performed using specific primers for Cas9 and sgRNA transcripts. PCR products were analyzed using electrophoresis as described in the section Barley transformation.

Genotyping of primary regenerants and their progeny.

To evaluate the induction of target and off-target mutations, primer pairs for amplification of the target and off-target loci in the closest homologs of *HvITPK1* within the group of *ITPK* were designed, verified and used for genotyping. The specificity of the primers was verified by gradient PCR for each primer pair. The optimal annealing temperature for each primer pair was selected. Then, the target and off-target loci in T0 transgenic plants were PCR-amplified. The PCR products were Sanger sequenced. Visual evaluation of sequencing files was conducted in the chromatogram viewer Chromas. For decoding and evaluation of the sequencing chromatograms, the DSDecode online tool (Liu et al., 2018) was used. For the analysis of heritability of the target mutations and induction of off-target mutations in the next generation, the progeny of the bi-allelic mutant plant 3B was genotyped in the T1 and T2 generations.

Seed phosphate analysis

For phosphate analysis in mature grains, progenitor lines originating from bi-allelic mutant plant T0 *itpk1* 3B were selected. T1 progeny of 3B plant was grown in greenhouse to maturity. Mature barley grains (T2 generation) of mutant T1 3B plants were harvested. 2 g of grains were homogenized to a fine flour. Phosphate analysis was done according to the method of Vaculova et al. (2011). Technical and biological measurements were made in triplicate. The mean values with standard deviations were plotted on a graph.

Abiotic stress experiments

The homozygous T2 mutant lines, deletion mutant *itpk1-2* and insertion mutant *itpk1-33* were selected for *in vitro* testing of their response to abiotic stress factors (NaCl and D-mannitol). Non-transgenic Golden Promise referred to as WT was used as a control. Mature grains of WT and *itpk1* mutants were surface sterilized and soaked in sterile water overnight in the dark. The embryos were dissected, sterilized for 1 min in 1% sodium hypochlorite solution with agitation and then washed twice in sterile water. Embryos were placed on the surface of solid half Murashige-Skoog medium with 10 g/L sucrose, Phytoagar 6 g/L, with the pH adjusted to 5.8 was prepared in flasks. The media were supplemented with NaCl (*Supplement II*) or D-mannitol in

concentrations 100, 200, and 300 mM. Five embryos of each mutant line and WT per treatment. The flasks with embryos were placed in a growing cabinet and cultivated at 24°C and a day/night regime of 18/6 hours. After two weeks, the phenotype of the seedlings was evaluated by measuring the height of the seedlings.

Germination assay

Grains of *itpk1-2*, *itpk1-33* mutants and WT were assayed for salinity stress during germination according to the protocol of Daszkowska-Golec et al. (2019). Grains were sown 30 per plate. Salt levels were determined at 100 and 200 mM.

Expression profiling of barley ITPK genes under salinity stress

Total RNA from young seedlings of WT and homozygous mutant lines *itpk1-2* and *itpk1-33* cultivated *in vitro* on pure half MS medium and medium supplemented with 200 mM NaCl was extracted as described above. Samples of leaves and roots which were additionally washed in deionized water to remove residues of medium, were immediately after collecting frozen in liquid nitrogen. Three biological samples per treatment were taken. Extracted RNA was treated with DNase. Then, the concentration of RNA was assessed spectrophotometrically. RNA was reverse transcribed as described above. cDNA samples were diluted with nuclease-free water. Diluted cDNA samples were used for qRT-PCR analysis. *ITPK* transcript levels were determined by qRT-PCR.

Survey of Results

Biofortification of staple crops is a complex process that requires substantial knowledge of plant physiology. Multiple plant species have been subjected to biofortification using various methods and sophisticated approaches as reviewed in *Supplement I*. Simple techniques characterized by direct application of nutrients evolved into the use of encapsulated nutrients in small particles that can be applied to the soil under the seed. Dramatic progress is also reviewed for biotechnological approaches. The portfolio of biotechnological techniques has expanded mainly by a group of GE systems, of which CRISPR/Cas is overwhelmingly superior. Precise and effective manipulation with gene sequences facilitates identification of interesting candidate genes, which can be eventually integrated into breeding programs directed to crop biofortification. On the other hand, developing de novo synthetic pathways will still rely on the preparation of classic transgene over-expression and cannot be easily circumvented. All things considered, GE technology shows great potential for preparing plant material with valuable traits as documented by many barley and wheat mutant lines that have been developed using the GE technology to date.

The CRISPR/Cas system was used to develop mutant barley plants in candidate *ITPK* genes (*Supplement II*). Initially, *in silico* analysis of *ITPK* genes revealed there are six members of the *ITPK* group in barley. Two *ITPK* genes, *HvITPK1* and *HvITPK5*, were selected as targets for knock-out. Plasmid vectors were prepared and used for *Agrobacterium*-mediated transformation. No transgenic plants were obtained in the transformation experiment with Cas9 designed for *HvITPK5*. On the other hand, transgenic plants were obtained for the *HvITPK1* gene.

Expression analysis confirmed the transcription of both CRISPR/Cas components Cas9 and sgRNA (*Supplement II*, Figure 2B). No detection of Cas9 at the protein level in transgenic plants was conducted. The presence of the Cas9 protein and, importantly, the activity of the Cas9 effector complex would be confirmed by the detection of mutation at a defined position in a target gene. Indeed, sequencing of target locus in transgenic plants revealed mutated allelic variants of the *HvITPK1* gene in several primary regenerants (*Supplement II*, Figure S2). Chimeric as well as uniform mutant plants were detected in primary regenerants. Sequencing of potential off-target sites among *ITPK* genes revealed no unintended mutation, proving the on-target editing of Cas9.

Analysis of progeny of selected bi-allelic mutant plant 3B revealed stable transmission of mutation into the next generation and segregation of mutant alleles, which resulted in the occurrence of two variants of homozygous mutant plants. These plants showed a homozygous state in single bp deletion or insertion. A substantial portion of progeny maintained the bi-allelic constitution.

Mutation in the *HvITPK1* gene did not result in over-all increased phosphate content, but the phosphate levels varied among the obtained progeny of mutant plants. Among tested mutant lines, those showing a substantial increase in mature grain phosphate content were obtained (*Supplement II*, Figure 3). Deletion and insertion mutant lines in *HvITPK1* differed phenotypically in reaction to abiotic stress during germination and seedling growth under salinity stress. For instance, the germination rate was more affected in *itpk1-33* under salt stress conditions. (*Supplement II*, Figure 4, 5).

Expression profiling revealed the up-regulation of most ITPK genes in roots in reaction to salinity stress, which further supported the hypothesis of involvement of ITPK genes in the regulation of reaction to salinity in young seedlings (Supplement II, Figure 6). Expression profile of *ITPK* genes in *itpk1-2* and *itpk1 33* varied between the two mutant lines under salinity stress conditions.

Extended *in silico* analysis of barley, rice and additionally wheat *ITPK* genes was performed. Protein coding sequences were mined from the Ensembl Plant database. It was confirmed that three intronless *ITPK* genes identified in barley were confirmed also in wheat, suggesting that divergence of ITPK groups between *Oryzoideae* and *Pooideae* subfamilies had occurred after these two subfamilies split phylogenetically.

Additionally, to study abiotic stress response in young seedlings more extensively, a similar experiment as described in *Supplement II* was performed. Here, abiotic stress in young seedlings was induced by adding D-mannitol into cultivation medium. D-mannitol affected seedling growth more with increasing concentration. There were no substantial differences between wild type and mutant lines. It can be assumed that both types of mutations have no visible impact on the phenotype of mutant lines in response to the form of abiotic stress induced by D-mannitol in young seedlings.

Moreover, to demonstrate phenotypic performance in more mutant lines, homozygous insertion mutant *itpk1-10* was included in both variants of abiotic stress, NaCl and D-mannitol, Figure 4. Following the published results (*Supplement II*), mutant line *itpk1-10* showed better growth at the highest salt concentration in comparison to WT. Treatment with D-mannitol induced stress caused no distinguishable phenotypic differences between the mutant and WT. Genotyping of T2 generation homozygous mutant lines revealed mutant lines in which Cas9 transgene was not detected. These lines were considered transgene-free.

Conclusion and Perspectives

This thesis focuses on phosphate biofortification of the spring barley cultivar Golden Promise. Modern techniques of GE were used to modify phytic acid biosynthesis in order to increase the phosphate content in mature barley grains. Several experiments were performed to further unravel the potential link between *ITPK* genes and reaction to abiotic stress.

The most important outcomes of the work are the following:

- An overview of the published literature on the topic of crop biofortification using modern breeding techniques including genome-editing technology that can be successfully implemented in the process of crop biofortification.
- Barley *ITPK* genes were phylogenetically clustered and ordered.
- It was verified that CRISPR/Cas9 genome-editing technology can be applied in barley to induce mutations at predefined positions.
- Mutations induced by CRISPR/Cas9 have mostly the character of single bp insertion or deletion and these mutations are heritable into the next generation.
- Different allelic variants of mutated *HvITPK1* showed distinguishable reactions to salinity stress during germination.
- Homozygous barley lines without original *Cas9* transgene were obtained over three generations.

The application of genome-editing techniques in crop biofortification is a feasible way for the production of nutritionally more valuable products. Developed barley lines serve as proof of concept that precise manipulation of the phytic acid biosynthetic pathway can result in lines with the altered ratio of phosphate form in the mature grains. Prepared homozygous experimental plant material can be subsequently utilized for testing of abiotic stress resistance. Functional validation of the *HvITPK1* gene confirmed its participation in the storage of phosphate in grains and also suggested possible interplay in regulation of the abiotic stress response. *In silico* analyses of *ITPK* sequences will further serve as the groundwork for the design of potential new targets using the CRISPR/Cas system.

List of publications

This thesis is based on the following papers, which are referred in the text by the corresponding roman numerals. The papers are enclosed in the Supplement section.

I. Vlčko T., Ohnoutková L. (2019). Recent advances and perspectives in crop biofortification. *Biologia Plantarum* 63:586–593. doi: 10.32615/bp.2019.056. IF: 1,384

As the first author, TV performed a literature review, evaluated literature sources and wrote the manuscript.

II. Vlčko T., Ohnoutková L. (2020). Allelic Variants of CRISPR/Cas9 Induced Mutation in an Inositol Trisphosphate 5/6 Kinase Gene Manifest Different Phenotypes in Barley. *Plants* 9:1–20. doi: 10.3390/plants9020195. IF: 2,632

As the first author, TV did design of the experiment, performed in silico analyses of gene sequences and protospacer design, cloning of the expression vector, plant transformation, genotyping and characterization of regenerated plants, characterization of phosphate content in mature grains, and abiotic stress assays. TV evaluated the data and wrote the manuscript.

List of co-authored publications

Zelenka, J., Mrkvicová, E., Šťastník O., Jarošová M., Vlčko T., Ohnoutková L. (**2017**). Effect of Low- and High Phytase Barley on Phosphorus Retention and Bone Mineral Composition in Broilers. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 65 (2): 577–581.

Kupcikova, L., Lichovnikova, M., Anderle, V., Vlcko, T., Ohnoutkova, L., Svidrnoch, M., Maier, V., Hampel D. (**2017**) Pre-caecal digestible phosphorus in maize and wheat for broiler chickens, *British Poultry Science*, 58:6, 712-717, DOI: 10.1080/00071668.2017.1370536, IF 1.096

Cejnar, P., Ohnoutkova, L., Ripl, J., Vlcko, T., Kundu, J.K. (**2018**) Two mutations in the truncated Rep gene RBR domain delayed the Wheat dwarf virus infection in transgenic barley plants. *Journal of Integrative Agriculture*, 17 (11), 2492-2500, DOI: 10.1016/S2095-3119(18)62000-3, IF: 1.042

Rehorova, K., Viktorova, J., Macuchova, B., Vlcko, T., Stankova, L., Jelenova, N., Ohnoutkova,L., Macek, T. (**2018**) Limen, Non-Toxic Recombinant Plant Defensin and Its Effect against Pathogenic Yeast and Fungi. *Journal of Pharmacy and Pharmacology*, 6, 945-955, DOI: 10.17265/2328-2150/2018.11.001

Viktorova, j., Klcova, B., Rehorova, K., Vlcko, T., Stankova, L., Jelenova, N., Cejnar, P., Kundu, J.K., Ohnoutkova, L., Macek, T. (**2019**) Recombinant expression of osmotin in barley improves

stress resistance and food safety during adverse growing conditions. *PLOS One*, e0212718, DOI: 10.1371/journal.pone.0212718, IF: 2.766

Kopecny, D., Vlcko, T. (**2020**) Meeting report: Plant biotechnology: Green for Good V 2019. *New Biotechnology*, 57, 1-3, DOI: 10.1016/j.nbt.2020.01.004, IF: 3.739

Book chapter:

Ohnoutkova, L., Vlcko, T., Ayalew, M. (**2019**) Barley Anther Culture in *Barley: Methods and Protocols*, Methods in Molecular Biology, vol. 1900, Humana Press. Editor: Wendy A. Harwood. DOI: 10.1007/978-1-4939-8944-7_4

Published abstracts

ESNA conference, Brno, Czech Rep. 25. – 29. Aug 2015
T. Vlcko, J. Vaskova, L. Ohnoutkova.GM barley for better phosphate utilization (poster)

13. dny studentů experimentální biologie rostlin (DSEBR), Brno, Czech Rep. 7. – 8. Sep 2015
T. Vlcko. Transgenosis for increased seed phytase activity (presentation)

Growth Regulators on the Way, Malá Morávka, Czech Rep. 3.- 5. Mar 2016
T. Vlcko. Biofortification of spring barley (presentation)

Young Scientist Meeting, Pulawy, Poland 31. May - 2. June 2016
T. Vlcko, J. Vajdak, J. Vaskova, L. Ohnoutkova. Phytase activity of transgenic barley SCLW-GP-PHYA at two locations in the Czech Republic (poster)

Plant Biology Europe, EPSO/FESPB 2016 Congress; Prague, Czech Rep. 26. – 30. Jun 2016
T. Vlcko, M. Hanakova, J. Vaskova, L. Ohnoutkova. Transgenic high phytase activity barley in new breeding technology (poster)

Chemistry and Biology of Phytohormones and Related Substances, Kouty nad Desnou, Czech Rep. 21. - 23. May 2017
T. Vlcko, L. Ohnoutkova. Programmable nucleases - the most effective tool of molecular genetics (presentation)

2nd iPLANTA Conference, RNAi: The Future of Crosstalk, Poznan, Poland 14. - 16. Feb 2018
T. Vlcko, P. Cejnar, J. Kumar, L. Ohnoutkova. Doubled-haploids of spring barley with partial wdv replication protein (poster)

3rd iPLANTA Conference, What Future for RNAi-Based Products: RNAi Modified Plants or Spray Products, Lisabon, 27. Feb – 1. Mar 2019
T. Vlcko, K.S. Singh, P. Cejnar, J.Kumar, L. Ohnoutkova. Innovative biotechnological strategies against WDV infection in wheat and barley (poster)

Plant Biotechnology: Green for Good V, Olomouc, Czech Rep. 10. – 13. Jun 2019
T. Vlcko, K.S. Singh, P. Cejnar, J.Kumar, L. Ohnoutkova. Biotechnological approaches for preparation of resistant wheat and barley varieties against WDV infection (poster)

Souhrn (in Czech)

Název práce: Biofortifikace jarního ječmene

Disertační práce je zaměřena na biofortifikaci, což představuje moderní přístup mající za cíl zvýšení nutriční hodnoty potravin, nejčastěji zvýšení obsahu živin, esenciálních aminokyselin, popřípadě mikro- či makro prvků. Ječmen setý (*Hordeum vulgare*) se řadí mezi nejdůležitější hospodářské plodiny. Ječmen je významné krmivo pro hospodářská zvířata, využíván je i jako potravina a pro výrobu sladu v pivovarském průmyslu. Fosfor je jeden z důležitých a hojně zastoupených prvků v ječmenných obilkách, který je rostlinou primárně přijímán ve formě fosfátu a je využíván v řadě biochemických reakcí na buněčné úrovni. U živočichů je fosfor nezbytný pro stavbu kostí, ve kterých je ho velká většina uložena. U obilnin je fosfor v zrnech převážně ukládán v organické formě – kyselině fytové, na kterou jsou vázány důležité kationty. Tyto fytátové komplexy jsou pro monogastrické živočichy primárně prase domácí, ale také drůbež nestravitelné.

Strategie pro zlepšení dostupnosti fosforu u rostlin, biofortifikace, jsou zaměřeny hlavně na změnu formy, ve které je fosfor přítomen v obilkách. V rostlinných buňkách jsou pro ukládání fosfátu využívány dvě biosyntetické dráhy. Narušením jednoho z důležitých kroků biosyntetické dráhy lze docílit snížení obsahu kyseliny fytové v obilce, které je doprovázeno zvýšením obsahu fosfátu. Pokroková technika editace genomu CRISPR/Cas byla využita k vytvoření mutace v genu *HvITPK1*, který se podílí na syntéze kyseliny fytové. Vytvořené mutantní rostliny byly charakterizovány na genomické úrovni. Byly získány rostliny nesoucí mutaci v bi-allelické konstituci, současně byla vytvořena jednobázová delece a inserce. V následující generaci byly získány homozygotní mutatní line. Ve zralých obilkách T2 generace byl analyzován obsah fosfátu. Byly identifikovány nejen linie se zvýšeným obsahem fosfátu v obilce, ale překvapivě také linie s nižším obsahem fosfátu.

Geny z biosyntetické dráhy kyseliny fytové se podílí na řízení odpovědi na abiotický stres, proto byla u vybraných linií v průběhu klíčení sledována odpověď na stres vyvolaný NaCl a D-mannitolem. Byl pozorován rozdílný fenotypový projev u tří vybraných *itpk1* mutantních linií v odpovědi na stres zasolením. Následně byl při stresových podmínkách hodnocen transkripční profil *ITPK* genů u *itpk1* mutantů. Rozdílný fenotyp *itpk1* mutantů byl navíc potvrzen odlišnou mírou transkripce *ITPK* genů v nadzemní části rostliny. Naopak v kořenech byla pozorována obecně zvýšená míra transkripce *ITPK* genů v odpovědi na stres, a to jak u mutantních linií, tak u kontrolní linie. V T3 generaci byly identifikovány linie, u kterých došlo k segregaci *Cas9* transgenu od *itpk1* mutace, a tímto způsobem byly přirozeně získány homozygotní mutantní netransgenní linie.

References

Cordell, D., Drangert, J. O., and White, S. (2009). The story of phosphorus: Global food security and food for thought. *Glob. Environ. Chang.* 19, 292–305. doi:10.1016/j.gloenvcha.2008.10.009.

Daszkowska-Golec, A., Skubacz, A., Kurowska, M., Slota, M., Swiergolik, D., and Szarejko, I. (2019). "Methods for the Simple and Reliable Assessment of Barley Sensitivity to Abiotic Stresses During Early Development," in *Barley: Methods and Protocols*, ed. W. A. Harwood (Springer New York), 127–151. doi:10.1007/978-1-4939-8944-7_9.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* 19, 1349. doi:10.1093/nar/19.6.1349.

Harwood, W. A. (2014). "A Protocol for High-Throughput Agrobacterium-Mediated Barley Transformation," in *Cereal Genomics: Methods and Protocols*, eds. R. J. Henry and A. Furtado (Totowa, NJ: Humana Press), 251–260. doi:10.1007/978-1-62703-715-0_20.

Liu, W., Xie, X., Ma, X., Li, J., Chen, J., and Liu, Y.-G. (2018). DSDecode: A Web-Based Tool for Decoding of Sequencing Chromatograms for Genotyping of Targeted Mutations. *Mol. Plant* 8, 1431–1433. doi:10.1016/j.molp.2015.05.009.

Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., et al. (2015). A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Mol. Plant* 8, 1274–1284. doi:10.1016/j.molp.2015.04.007.

Vaculova, K., Balounova, M., Sedlackova, I., Kvasnicka, F., Mikulikova, R., Belakova, S., et al. (2011). *METODIKA PREBREEDINGU JEČMENE JARNÍHO S DIFERENCOVANÝM OBSAHEM PŘIROZENÝCH ŠKODLIVÝCH LÁTEK V ZRNĚ PRO ŠLECHTĚNÍ ODRŮD NESLADOVNICKÉHO TYPU*. 1st ed. Kromeriz: Agrotest fyto, s.r.o.